METHODS IN MOLECULAR MEDICINE™

DNA Vaccines

Methods and Protocols

Edited by

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Humana Press

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Purification of Supercoiled Plasmid

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1. Introduction

Current technologies for the purification of supercoiled plasmids are limited. The use of cesium chloride gradients in the presence of ethidium bromide is time consuming, labor intensive, requires the use of known mutagens and is not conducive to large scale. As a result, first-generation high-performance liquid chromatography (HPLC) methods based on anion-exchange and size exclusion have been developed but are difficult to accommodate production at large scale and still result in compromised purity ***(1,2)***. The success of DNA vaccines in animal models and the initiation of human trials ***(3,4)*** has led to a need to increase the level of supercoiled plasmid purity as well as the methodology utilized to produce these plasmids at large scale. Several parameters of the purification process need to be addressed:

- The ability to prepare supercoiled plasmid at purity levels acceptable for clinical material.
- The ability to prepare clinical grade supercoiled plasmid that will be scalable in order to produce gram quantities of product.
- The ability to prepare clinical grade supercoiled plasmid in accordance with cGMP principles.
- The ability to develop validated assays to assess purity, yield, and contamination levels.

Challenges to the successful development of a purification process can be divided into biological and practical. The biological challenge arises from the spectrum of biomolecules that must be purified away from the supercoiled plasmid product (**Table 1**). Additionally, the spectrum of nucleic acid contaminants and plasmid isoforms within that spectrum, as shown in **Table 2**, must be removed. The removal of the relaxed DNA, DNA catenanes as well as endotoxins ***(5,6)*** are a particular problem requiring additional steps in the process.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

Table 1
Constituents in Crude Lysate

Plasmid DNA
Chromosomal DNA
RNA
Lipids
Endotoxin
Proteins
Carbohydrates

Table 2
Plasmid DNA Forms

Monomer supercoiled
Nicked
Linear
Dimers
Catenanes

The practical challenge arises because the purification process that is developed must produce highly pure product at high yield and must be reproducible, scalable and economical (*see* **Note 1**).

We describe a new purification process that has been used to generate clinical material using a proprietary non-porous polymer resin, PolyFlo ®, which uses principles of ion-pair reverse-phase chromatography to achieve separation based on size and charge density. The process can be performed using either acetonitrile (ACN) or ethanol (EtOH). Simultaneous removal of contaminating endotoxins, chromosomal DNA, RNA, proteins, and plasmid isoforms during purification is a unique advantage of this resin. This process meets the challenges for purity, yield, reproducibility, and scalability ***(1)***.

2. Materials

2.1. Crude Starting Material

The preparation of crude starting material from biomass is typically performed using acid/base extraction ***(7)***. This classic alkaline-lysis process provides material significantly reduced in protein, lipid and chromosomal DNA. Newer protocols have been adopted to improve the initial purity even further, including the use of temperature shift during fermentation ***(8)*** or the addition of a second acetate precipitation (NH_4Ac) to reduce the RNA burden ***(9)***. We describe two purification methods: an ACN process using starting material in which minimal efforts have been made to reduce the RNA burden, and ***(2)*** an EtOH process in which the starting material has been reduced for RNA by anion-exchange chromatography and/or diafiltration. The protocols described do not incorporate the use of RNase (*see* **Note 2**).

2.2. ACN Purification Materials

1. Glass borosilicate chromatography column packed with PolyFlo resin (*see* **Note 3**).
2. Starting material (ammonium acetate supernatant) (*see* **Note 4**).

3. 1.0 *M* TEAA (triethylamine acetate) pH 7.0.
4. 0.5 *M* KPO_4 pH 7.0.
5. 1.0 *M* TBAP (tetrabutylammonium phosphate); Aldrich Chemicals (Milwaukee, WI) no.# 26, 810-0; 1 *M* in H_2O).
6. 100% Acetonitrile (ACN, American Chemical Society (ACS) grade or equivalent).
7. TES (20 m*M* Tris-HCl pH 8.0, 1.0 m*M* EDTA, 5.0 m*M* NaCl).
8. Equilibration buffer: 0.1 *M* TEAA pH 7.2, 6% ACN.
9. Wash buffer I: TES, 5% ACN.
10. Wash buffer II: 0.1 *M* KPO_4, 2.0 m*M* TBAP, 5% ACN.
11. Wash buffer III: 0.1*M* KPO_4, 2.0 m*M* TBAP, 15% ACN.
12. Elution buffer: 0.1 *M* KPO_4, 2.0 m*M* TBAP, 25% ACN.
13. Sanitization buffer: 0.5 N NaOH.

2.3. Ethanol Purification Materials

1. Glass borosilicate chromatography column packed with PolyFlo resin.
2. RNA-reduced plasmid sample.
3. 0.5 *M* KPO_4 pH 7.0.
4. 1.0 *M* TBAP (tetrabutylammonium phosphate; Aldrich Chemicals # 26, 810-0; 1 *M* in H_2O).
5. Ethanol (EtOH, ACS grade or equivalent).
6. TES (0.02 *M* Tris-HCl pH 8.0, 1.0 m*M* EDTA, 5.0 m*M* NaCl).
7. Equilibration buffer: 0.1 *M* KPO_4 pH 7.0, 2.0 m*M* TBAP, 1% ethanol.
8. Wash buffer I: TES, 7% ethanol.
9. Wash buffer II: 0.1 *M* KPO_4, 2.0 m*M* TBAP, 5% ethanol.
10. Elution buffer: 0.1 *M* KPO_4, 2.0 m*M* TBAP, 25% ethanol.
11. Sanitization buffer: 0.5 N NaOH.

2.4. Post-Purification Materials

Millipore Pellicon II (Bedford, MA) or A/G Technology (Needham, MA) diafiltration/ultrafiltration technologies are applicable for buffer exchange and concentration.

3. Purification Protocols

A schematic of the two protocols is shown in **Fig. 1**.

3.1. ACN Protocol

1. Adjust to obtain a linear flow rate of 150 cm/h and equilibrate column in ≥3 column volumes of equilibration buffer.
2. Prepare sample by diluting 1/5 with TES and adjusting to 0.1 *M* TEAA using 1 *M* TEAA stock solution. Sample load should be no more than 0.5 mg/mL of resin (load concentration is based on total A_{260nm}).
3. Load sample and wash with equilibration buffer until the monitor returns to baseline (~2 column volumes). Collect wash.

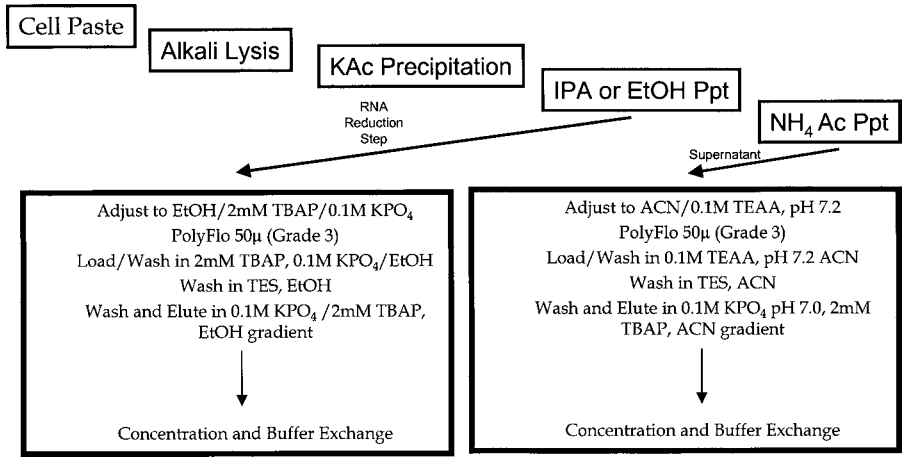

Fig.1. Schematic flow-chart of PolyFlo purification process.

4. Wash with ~3 column volumes of wash buffer I (TES, 5% ACN). Make sure monitor returns to baseline. Collect wash.
5. Wash with ~3 column volumes of wash buffer II (0.1 *M* KPO_4, 2.*0 mM* TBAP, 5% ACN).
6. Wash with ~3 column volumes of wash buffer III (0.1 *M* KPO_4, 2.0 m*M* TBAP, 15% ACN) until the monitor returns to baseline. Collect wash.
7. Elute product with a 10-column volumes linear gradient from 0.1 *M* KPO_4, 2.0 m*M* TBAP, 15% ACN to 0.1 *M* KPO_4, 2.0 m*M* TBAP, 25% ACN. Collect elution fractions. This is the purified product.
8. Clean column by running 2 column volumes of sanitization buffer (0.5 N NaOH). Turn pump off and let sit in 0.5 *N* NaOH for 1 h. Re-equilibrate column with ≥3 column volumes of equilibration buffer. Monitor pH to assure that all the NaOH has been removed.
9. Purified sample from step 8 may be processed through concentration and/or buffer exchange steps. It is recommended to diafilter against 0.5 *M* Na-acetate pH 7.8 to remove residual TBAP.

3.2. ACN Protocol Results

The results of the ACN protocol are shown in **Fig. 2**. No difference in the purity of the product is seen using starting material representing 1 g or 100 g of biomass. The RNA is eliminated. Despite the significant quantities of relaxed DNA, >50% of total plasmid, this contaminant is removed in the wash step. The final purity is >90%.

3.3. Ethanol Protocol

1. Adjust pump speed to obtain a linear flow rate of 150 cm/hr and equilibrate column in ≥ 3 column volumes of equilibration buffer.

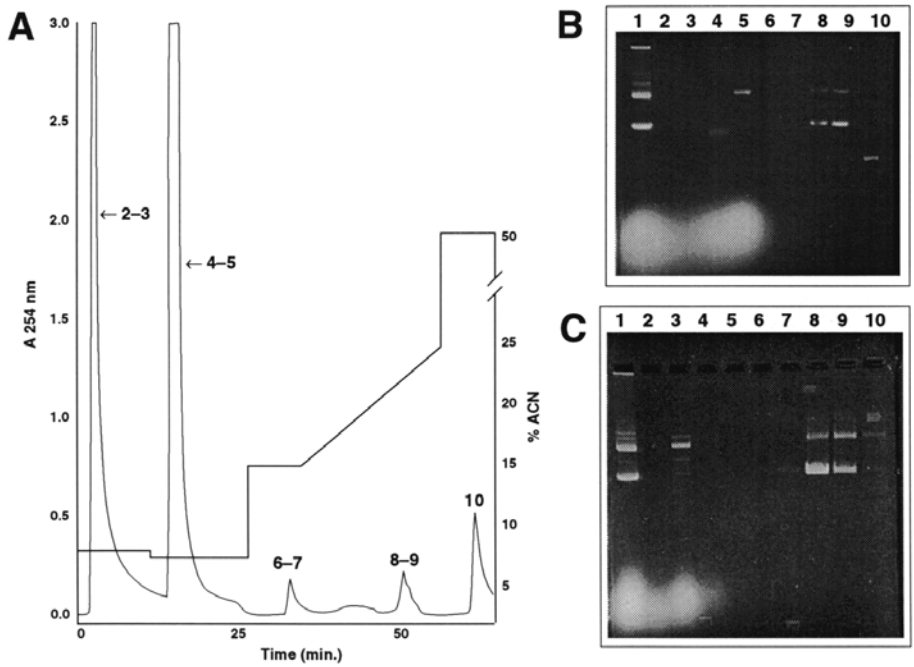

Fig. 2. PolyFlo chromatography of plasmid using ACN process. **(A)** Chromatographic tracing for application of nucleic acid sample extracted from 1.0 g *E. coli* cells. **(B,C)** 1% Agarose gel analysis of resolved peaks from 1.0 g biomass **(B)** and 100 g biomass **(C)** . Lane 1 = 1 µg nucleic acid sample; lanes 4 and 5 = TES and ACN wash; lanes 6 and 7 = 15% (v/v) ACN/TBAP wash; lanes 8 and 9 = 15-25% (v/v) ACN gradient; and lane 10 = 50% ACN strip. Reprinted from ***(1)***.

2. Prepare sample for loading by adjusting to 2 m*M* TBAP and 1% ethanol. Sample load should be no more than 0.5 mg/mL of resin (load concentration is based on total A_{260nm}). Sample should be <0.5 *M* NaCl.
3. Load sample and wash with equilibration buffer until the monitor returns to baseline. Collect flow through.
4. Wash with ~3 column volumes of wash buffer I (TES, 6% ethanol). Make sure monitor returns to baseline. Collect wash.
5. Wash with ~3 column volumes of wash buffer II (0.1 *M* KPO_4, 2.0 m*M* TBAP, 5% ethanol).
6. Elute product with a 10-column volumes linear gradient from 0.1 *M* KPO_4, 2.0 m*M* TBAP, 5% Ethanol to 0.1 *M* KPO_4, 2.0 m*M* TBAP, 25% ethanol. Collect elution fractions. This is the purified product.
7. Clean column by running 2 column volumes of sanitization buffer (0.5 N NaOH). Turn pump off and let sit in 0.5 N NaOH for 1 h. Re-equilibrate column with ≥3 column volumes of equilibration buffer. Monitor pH to assure that all the NaOH has been removed.

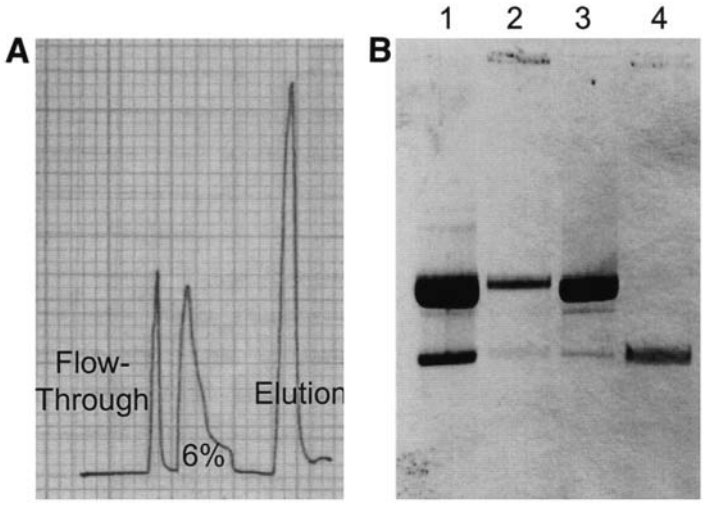

Fig. 3. PolyFlo Chromatography of plasmid using EtOH process. Crude lysate was reduced for RNA by diafiltration against 10–20 vol TES using a Millipore XL membrane (100,000 MWCO) prior to application onto a 1 × 4 cm PolyFlo column. **(A)** Chromatographic tracing at 254 nm; **(B)** 1% Agarose gel of resolved peaks. Lane 1 = starting material; lane 2 = 1% EtOH flow-through peak; lane 3 = 6% EtOH wash peak; and lane 4 = 5–25% EtOH gradient.

8. Purified sample from step 7 may be processed through concentration and/or buffer exchange steps. It is recommended to diafilter against 0.5 *M* Na-acetate pH 7.8 to remove residual TBAP.

3.4. Ethanol Protocol Results

The results of the Ethanol protocol are shown in **Fig. 3**. The residual RNA is eliminated. Despite the significant quantities of relaxed DNA, >60% of plasmid, this contaminant is removed in the wash step. The final purity is >95%.

4. Notes

4.1. Organic Solvent

The choice of organic solvent for chromatography is predicated on the amount of contaminating RNA. In general, if the RNA burden is less than 50%, the ethanol process may be employed. This can be accomplished through anion-exchange chromatography, diafiltration or RNase treatment. A rigorous test of the amount of contaminating RNA below which the ethanol process can be used has not been performed. If RNA reduction is achieved by RNase digestion, the sample must be diafiltered or dialyzed to remove excess ribonucleotides prior to PolyFlo chromatography.

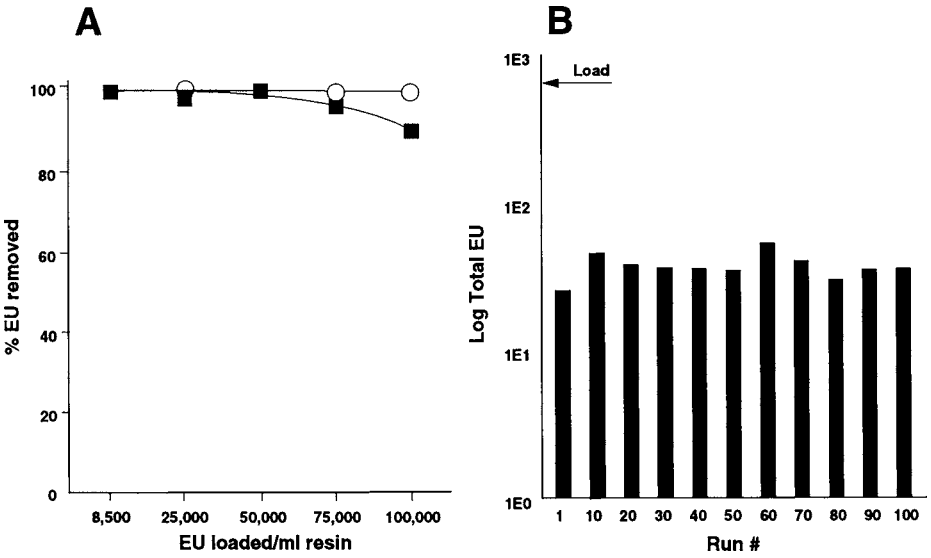

Fig. 4. Endotoxin binding to a PolyFlo column (1 × 4 cm). **(A)** Total endotoxin units (EU) were determined in the flow-through (solid squares) and gradient elution (open circles) after loading sample buffer was spiked with increasing levels of endotoxin. **(B)** Analysis of endotoxin levels in purified plasmid preparations at defined intervals during 100 consecutive applications of a single PolyFlo chromatography column. Reprinted from ***(1)***.

4.2. Endotoxin Removal

PolyFlo has an extremely hydrophobic surface. As such, significant quantities of endotoxin are removed as part of the purification process and in a reproducible manner (**Fig. 4**).

4.3. Specifications

No specifications for the purity of supercoiled plasmid, the levels of residual contaminants or even the methods for evaluating purity have been codified ***(10)***. While many methods can be used to analyze plasmid DNA, it is only recently that these methods have been applied to plasmid DNA as a potential pharmaceutical product ***(11)***. **Table 3** describes some of the target specifications and methods used within the industry.

4.4. Multiple Chromatography Runs

PolyFlo is a chemically inert polymer that withstands rigorous sanitization procedures which allows for multiple runs. One hundred consecutive applications of crude plasmid with no change in purity or contamination levels have

Table 3
Target Specifications

Parameter	Target specification	Testing method
Purity		
% Monomer supercoiled	>95	1% Agarose gel
Purity	1.8–2.0	A260:A280
Contaminants		
RNA	<1%	1% Agarose gel
Genomic DNA	<1%	Slot-blot hybridization
Endotoxin	<100EU/mg	LAL gel clot
Protein	Negative	SDS-PAGE

been documented ***(1)***. The resin can be sanitized to remove any residual nucleic acid, protein, lipid and endotoxin by exposure to 0.5 N NaOH alone or in combination with 0.1 *M* HCl.

4.5. Potential Interferences

PolyFlo resin is sensitive to detergents but not chaotropes or salts. The use of PEG in the lysis process does not affect PolyFlo performance ***(1)***. However, detergents such as SDS, in concentrations >0.005% prevent binding to the resin and should be avoided.

4.6. Process Optimization

As with all chromatographic procedures, there are several key steps that will affect results. Using PolyFlo in the chromatography of plasmids, the key elements are the organic solvent concentration in the loading buffer and the column wash steps. For example, if the load concentration of organic is too high, product will be lost in the flow-through fraction. If the load concentration is too low, then the RNA will bind and will not be eliminated in the flow-through. As a consequence, trace RNA levels may be seen throughout the chromatography. The same considerations can be applied to the wash concentration.

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2

Production of Plasmid DNA in Industrial Quantities According to cGMP Guidelines

Joachim Schorr, Peter Moritz, and Martin Schleef

1. Introduction

Within the last five years, the exponential growth of research activities on the development of genetic vaccination and gene therapy has made it necessary to develop an easy, cost-effective, industrial scale process for production of plasmid DNA (*see* **Note 1**). One main issue is that the process should conform to cGMP guidelines and be acceptable to the FDA or other national regulatory agencies. The cGMP environment should be implemented independently of the intended use of the DNA product.

A typical application would be the supply of genetic information that is missing within the cell, e.g., because of a genetic disease like cystic fibrosis (CF). In such cases the "therapy" has been performed by transferring liposome-plasmid DNA complexes to the lung epithelium to express the absent chloride channel gene (CFTR) for the restoration of the draining system of this tissue ***(1)***. The more preventive approach of "gene medicine" could involve vaccination using plasmid DNA either by subcutaneous or intramuscular injection ***(2–6)*** or other techniques (for an overview *see* **ref. *7***, and this volume). The expression of immunogenic epitopes can cause both humoral and CTL response ***(8,9)***, and Chapter 6.

In all cases, it is essential to be able to use a therapeutic agent (the "biologic," usually a plasmid DNA) free of any other materials. Such contamination includes components used in the isolation process and coming from the organism from which the plasmid is isolated, mainly residual proteins, RNA, and genomic DNA of the host cell. In this chapter, we describe the development of a pharmaceutical manufacturing process to isolate plasmid DNA start-

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

ing from a technology (Qiagen, Hilden, Germany) that has been shown over the past years to be a tool in research and development that fulfills the requirements described in **Subheading 1.** (*see* **Notes 2–4**).

1.1. The Host Cell Selection

A single appropriate host strain for all research work or industrial scale pharmaceutical manufacturing does not exist (*see* **Note 5**). An appropriate strain should be a clone derived from a host strain stock that is completely characterized and free of any contamination. It should be safe for the environment, for the isolated product, the exposed patients, and the employees doing the manufacturing work as well as health care personnel. Considerable experience in the field of molecular cloning and DNA techniques has been obtained with *Escherichia coli* (*E. coli*), and *E. coli* K12 fulfills the needs for a safe, well-characterized host strain for DNA production.

Systematic analysis of over 20 different *E. coli* substrains demonstrated that very large qualitative and quantitative differences exist among all the substrains tested. These differences mainly concern particularly the amount of plasmid DNA per gram of biomass and the plasmids isoform distribution.

Plasmid isoforms consist of supercoiled molecules, dimers, or concatemers or catenanes (chains of two or more plasmids), as well as linear or nicked plasmids. The observed differences in isoforms depend on the plasmid as well as on the host strain. This means that not only the genetic background of the host is responsible for the differences of the plasmid isoform distribution, but the plasmid itself also contributes to a certain extent.

1.2. Growth Conditions

Bacterial cultures for the purpose of plasmid isolation were performed in a batch mode, using culture bottle volumes of up to a maximum of 2 L. Studies were done to determine the growth medium and conditions for optimal bacterial growth and plasmid yields, resulting in optical density (O.D. 600) values of around 3–6 O.D. units in complex bacterial growth media. For purposes such as research grade plasmid preparations for cloning, sequencing, and transfection experiments, this procedure is adequate and the final analysis of the prepared DNA is usually an agarose gel electrophoresis, DNA quantification and identity test (restriction digestion).

These test criteria are not stringent enough for pharmaceutical purposes, and the procedure of the manufacturing had to be drastically modified. The first point to consider in the development of a pharmaceutical grade process is that the batch culture method has no type of online monitoring or regulation. The growth conditions are adjusted before inoculating the medium and left unchanged, usually for between 16–20 h. No pH monitoring or adjustment is

performed, oxygen and carbon source are also neither monitored nor regulated. Essential substrates are depleted and toxic products accumulate. Degraded cellular components, including plasmids, accumulate in overgrown cultures and cell death follows.

To overcome these problems for the isolation of recombinant proteins, high performance fermentation technology has been developed over the last few years.

Fermentation processes require different growth media than batch cultures. The possibility of monitoring the growth conditions allows for the introduction of essential media components before they are exhausted (feeding), and for the maintenance of a constant pH control and oxygen supplies. Besides the effects of such regulation, the culture process becomes more defined and the pharmaceutical requirements on documentation can be fulfilled. A further feature of the fermentation technology for the large-scale plasmid production, is the potential of high-density fermentation to yield large amounts of biomass. Experimental work on the composition of bacterial growth media for bottle cultures and fermentation demonstrates that the choice of fermentation conditions and growth media strongly influence the yields of plasmid that are obtained from *E. coli* cells. One main focus was put on the amount of plasmid per cell (copy number), that can be monitored on-line by capillary gel electrophoresis ***(10)***.

1.3. Downstream Processing

The isolation of a biomolecule from the bacterial culture (usually referred to as downstream processing or DSP) is performed to separate plasmid DNA from other undesired components present within the source of material. These undesirable components are genomic DNA, RNA, proteins, lipids, lipopolysaccharides (LPS) or endotoxins, components of the cell wall and intact bacteria (*see* **Note 6**). The alkaline lysis ***(11)*** was modified and is reproducibly performed in scales up to five liter bacterial culture (ultrapure 100 chromatography system, Qiagen). The most important feature of this technique is the formation of a complex of most of the undesired components mentioned above, which can easily be removed by centrifugation (research scale) or floating and filtration (research and industrial scale).

The resulting "cleared lysate" is applied to an industrial scale process chromatography column with anion exchange resin, to specifically bind the negatively charged plasmid DNA and (under appropriate buffer conditions) not to bind residual undesired components (e.g., protein, RNA, nucleotides, LPS). Such anion exchange chromatography is not limited in scale (compared to approaches such as gel filtration). Moreover, in the case of specific types of resin material, possessing dense, high surface charge, a one-step process can be used.

As an additional pharmaceutical requirement, a process for the complete, rapid removal of LPS molecules was developed (*see* **Subheading 2.5.**). Endotoxins such as *E. coli* LPS can have cytotoxic effects on mammalian cells in vitro and in vivo ***(12–15)*** and if present in large enough amounts in vivo can cause symptoms of toxic shock syndrome and activation of the complement cascade ***(16)***.

In our process development we focused on the use of only non-toxic substances, and in particular avoided any potentially carcinogenic or immunogenic reagents. Additionally, the environment was controlled and the resulting liquid waste was biodegradable.

1.4. Quality Assurance and Quality Control

When we began our work on DNA manufacturing, the only criteria for the quality of plasmid DNA were those of typical research work. Usually the quality for this research grade material was estimated using analytical gel electrophoresis, restriction enzyme digestion, and DNA sequence readings.

We therefore established a set of quality criteria ***(17)*** that is now well accepted by the scientific community. Relevant issues from this work were discussed at the Food and Drug Administration (FDA)/World Health Organization (WHO) conference on Nucleic Acid Vaccines, February 5–7, 1996 at the National Institute of Allergy and Infectious Diseases/National Institutes of Health (NIAID/NIH) (Bethesda, MD) ***(18)***. An overview of the regulatory aspects for design, manufacturing, quality assurance, and quality control of vaccination vectors are summarized in the WHO "Guidelines for Assuring the Quality of DNA Vaccines" (WHO Technical Report, Jan. 17, 1997).

The design of the production process focused on its acceptance by national and international authorities such as the FDA (Washington, DC), Medicines Control Agency (UK) and others, and had to fulfill the appropriate cGMP regulations (*see* **Note 7**).

2. Materials

2.1. Buffers

1. P1: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 100 µg/mL RNaseA.
2. P2: 200 m*M* NaOH, 1% (w/v) SDS.
3. P3: 3.0 *M* KAc, pH 5.5.
4. QBT: 750 m*M* NaCl, 50 m*M* MOPS, pH 7.0, 15% (v/v) isopropanol, 0.15% (v/v) Triton-X-100.
5. QC: 1.0 *M* NaCl, 50 m*M* MOPS, pH 7.0, 15% (v/v) isopropanol.
6. QN: 1.6 *M* NaCl, 50 m*M* MOPS, pH 7.0, 15% (v/v) isopropanol.

2.2. Transformation and Host Cells

Prepare competent cells such as *E. coli* K12 DH5α (Life Technologies, Eggenstein, Germany), DH10B (Life Technologies) or TG1 (# 6056; Deutsche

Sammlung von Mikroorganismen und Zell Culturen, Braunschweig, Germany), transform the plasmid DNA, and select recipients on agar plates containing the appropriate selection factor.

2.3. Fermentation

Cultivate cells using a suitable fermenter, such as a Biostate B bioreactor (B. Braun Biotech, Melsungen, Germany) with a working volume of 5 L. Use, e.g., the complex bacterial growth media LB with additional salt ***(19)***.

2.4. Cell Harvest

Cells can be harvested by batch centrifugation at 4600*g* for 15 min at 4°C.

1. Beckman J2-21 centrifuge with a JA-10E rotor.
2. 500 mL Polypropylene bottles (Nalgene, Rochester, NY).

2.5. The Anion Exchange Chromatography System

Perform anion exchange chromatography (Qiagen) to specifically bind double stranded DNA. Single stranded DNA, RNA, nucleotides, proteins, LPS. and other contaminants do not bind to the chromatographic resin under appropriate conditions.

1. For small-scale preparations (e.g., test runs using the produced biomass), 500 µg batches of DNA use the Qiagen EndoFree Plasmid Kit (Ref. #12362).
2. For larger scale preparations, use an anion-exchange chromatography column for the isolation of up to 100 mg plasmid DNA (e.g., ultrapure 100 column #11100, Qiagen) and LPS-free processing buffers (#11910, Qiagen).

3. Methods

The complete process of plasmid DNA production is performed under well-documented conditions and in the case of GMP manufacturing under controlled environmental conditions. The following examples of the process we use will give some insight into the steps performed (**Fig. 1**).

3.1. The Host Cell Selection

To obtain a pure and well-characterized production strain capable of high yields of DNA, the selection of an appropriate *E. coli* K12 plasmid host cell clone is essential. Besides good microbiological practices and the use of standard operating procedures (SOPs), a well established quality assurance and quality control system is of great relevance, since all further process steps depend on this.

1. Check the DNA received by the Qiagen DNA Production Facility for large-scale manufacturing for its identity first (size, restriction pattern, sequence), and if it is satisfactory, release it for further processing.
2. Transform the DNA to *E. coli* K12 host cells, and select individual colonies for further cultivation.

Fig. 1. Flow chart of a cGMP plasmid manufacturing procedure. "QA" indicates the types of quality assurance tests that must be performed at certain steps to ensure consistent quality and reproducibility, and to fulfill the needs of process documentation.

3. Use 3 mL of an overnight culture of cells for a small-scale plasmid isolation (QIAprep, Qiagen). In case of large numbers of clones, use an automated device for the isolation of DNA in a 96-well format (BioRobot 9600, Qiagen).
4. Identify appropriate cell clones by comparing them and selecting those with high plasmid yield and a proper plasmid isoform distribution for further production steps (**Fig. 2**).

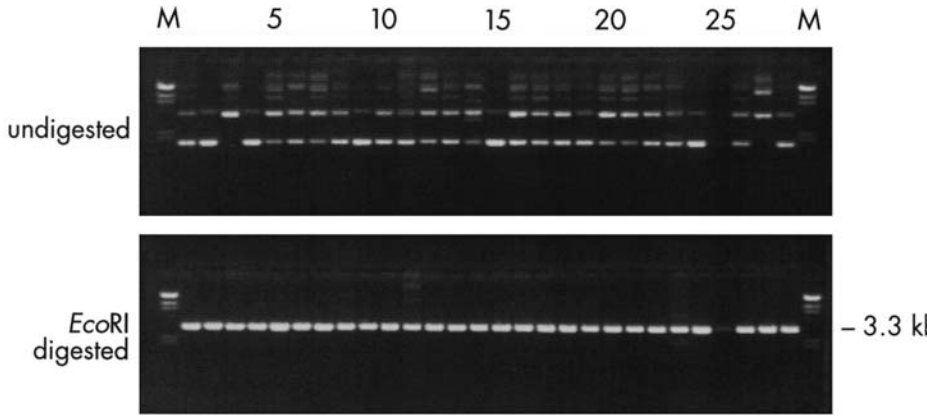

Fig. 2: Comparison of plasmid pUC21 DNA produced in different *E. coli* K12 host cells. The upper panel shows the undigested DNA with its different isoforms. The lower panel shows as a control the same amount of DNA after an *Eco*RI digestion. The molecular size marker (M) is *Hin*dIII digested λ-DNA. Gel: 1% (w/v) agarose in TAE, pH 8.0, run at 5V/cm and stained with ethidium bromide.

5. Further purify the selected clone by two single-colony passages and check it for identity and absence of microbiological contaminants. Use it subsequently for the inoculation of a culture to prepare a glycerol stock of between 100–500 vials. This stock is called Master Cell Bank (MCB); it is necessary to be able to reproducibly inoculate culture media from the MCB in the following process step and any future manufacturing run.
6. Perform an extensive quality assurance program to check the quality of this MCB. Test the identity, plasmid content, as well as absence of microbiological contaminants before proceeding with the following step. An important additional requirement is the complete sequencing of the DNA construct at this stage to exclude any difference to the original plasmid and to have a data backup for post-production sequencing.
7. Use vials of the MCB to inoculate a fresh culture to produce an equally large set of stocks (100–500 vials), which are required for the reproducible inoculation of the fermentation-precultures. This second glycerol stock is called Manufacturing Working Cell Bank (MWCB). Perform the same tests for Quality Assurance (QA) as with the MCB.

3.2. Fermentation

A fermentation process for *E. coli* cells carrying plasmids in a certain copy number must be well characterized, reproducible, easy to monitor and regulate; If possible it should run automatically. The MCB and MWCB described above are the backbones for any reproducibility. Further important issues are

the types of fermenter, regulation and growth medium used. Batches of 5–200 L are routinely run, and if required, further scaling-up is possible.

1. Use an appropriate amount of the MWCB to inoculate a pre-culture in *E. coli* growth medium.
2. Use the pre-culture to inoculate the fermenter for an overnight run at 37°C with controlled pH (7.5) at maximum aeration.
3. Harvest the cells by use of a flow-through centrifuge and determine the biomass content (wet and dry weights).

3.3. Lysis of Bacteria

To isolate the plasmid DNA from the *E. coli* cells, a modified alkaline lysis procedure ***(11)*** is used. This step is of critical importance to reduce contaminants such as protein, RNA, genomic DNA, and cell wall residues. Here we describe, as a pilot scale example, the approach of isolating up to 100 mg plasmid DNA starting from 60 g wet weight biomass (see also the protocol supplied with the Qiagen ultrapure100 kit).

1. Thoroughly resuspend 60 g biomass in 1000 mL buffer P1 in a 5-L glass bottle.
2. Add 1000 mL of buffer P2, mix the complete volume and incubate it at room temperature for 5 min.
3. Add 1000 mL of buffer P3 and mix it carefully.
4. Incubate the lysate for 30 min at room temperature to allow the flaky white precipitate of SDS, protein, genomic DNA, and cell residue to rise to the surface.
5. Carefully pump the lysate out of the bottle.
6. Filter the lysate through a QIAfilter™ unit (Qiagen), mixed with 1/10 volume of buffer ER (Qiagen), and collect the filtrate for subsequent chromatography.

3.4. Anion Exchange Chromatography

The anion exchange chromatography columns are loaded by pumping lysate with a peristaltic pump or preferably with a process chromatography system for better monitoring of the process.

1. Equilibrate the Qiagen ultrapure 100 column with 350 mL buffer QBT at a flow rate of 10 mL/min.
2. Load the column at a flow rate of approximately 4 mL/min overnight.
3. Wash the charged column with 3 L of LPS-free buffer QC at a flow rate of 20 mL/min.
4. Elute plasmid DNA with 400 mL LPS-free buffer QN at a flow rate of 3 mL/min.
5. Precipitate the DNA with 0.7 volumes of isopropanol at 4°C and centrifuge at 20,000*g* for 30 min in LPS-free centrifuge bottles.
6. Wash the DNA pellet with LPS-free 70% EtOH and rinse.
7. Dry the DNA pellet and resuspend it in the appropriate buffer system for further applications.

3.5. Quality Assurance

The following QA is performed within the manufacturing process as an in-process control (IPC).

1. Restriction Analysis: Digest the plasmid DNA to completion by use of different restriction enzymes, following the instructions of the suppliers. Use agarose gel electrophoresis to confirm that the total DNA size and molecular weight of fragments are consistent with those expected from the knowledge of the sequence.
2. Sequencing: Determine the complete nucleotide sequence of both DNA strands by DNA sequencing. Perform all steps following SOPs and document the data in a sequencing report.
3. Plasmid Stability: Monitor the presence or absence of a plasmid containing an antibiotic resistance marker by inoculating a defined amount of cells on both antibiotic and non-selective agar plates. If cells are not able to grow on selective plates, the percentage of clones growing on both media represents the "plasmid stability."
4. DNA Quality: In addition to the analyses of fragment identity and sequence, use spectrophotometric scans between 220–320 nm for the detection of salt and organic contamination ***(20)*** within the DNA. Inspect the appearance of a sample in an agarose gel electrophoresis. Important features are the isoform distribution (by agarose gel electrophoresis) and the DNA concentration. Also determine the content of RNA, genomic DNA and LPS by HPLC, Southern blot and the kinetic QCL test kit (BioWhittaker, Walkersville, MD) respectively.
5. DNA Quantity: Determine the DNA concentration by spectrophotometric analysis and calculation from its absorbance at 260 nm.

4. Notes

1. For large scale DNA production, we focused on the development of a technology for industrial-scale manufacturing of nucleic acids that combines cost effectiveness with the flexibility to install the system in every research laboratory (pilot scale) or GMP facility (industrial scale).
2. A major consideration in the development of this technology was to avoid time-consuming centrifugation and multiple chromatographic column runs. Centrifugation of large volumes to clear bacterial lysates can now be replaced by just one passage through a filtration unit that makes it possible to filter large volumes of bacterial lysate.
3. The process includes the establishment of Master Cell Banks and Master Working Cell Banks; fermentation and downstream processing are monitored at all stages by extensive in-process controls.
4. The three most important factors which need to be considered in the process development for plasmid DNA production are: selection of the optimal host strain, optimization of growth conditions, and the nucleic acid preparation method.

5. A large set of different *E. coli* host strains has been studied to identify strains producing large amounts of plasmid DNA per cell with the highest quality. Quality criteria for the selection of a host strain are the homogeneity of the plasmid DNA isolated from the host strain (>90% covalently closed circle), and the endotoxin content of the DNA purified from a specific host strain.
6. Endotoxins (LPS) are major contaminants of nucleic acids, especially plasmid DNA preparations. Due to their negatively charged phosphate groups, endotoxins tend to co-purify with nucleic acids. It has been demonstrated that LPS contamination of DNA has a direct influence on transfection efficiency into many types of cultured cells, and that different cells show variable sensitivity to this contamination ***(13)***.
7. The Qiagen procedure has been approved to produce DNA for human clinical Phase I studies in the UK ***(1)*** and other European countries, as well as in the United States by the FDA ***(21)***. A drug master file (DMF) for the clinical grade manufacturing process is filed with the FDA.

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3

Development and Characterization of Lyophilized DNA Vaccine Formulations

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1. Introduction

The potential applications of using plasmid DNA for immunization and other gene therapy approaches have been discussed in an increasing number of publications in the past few years. Injection of mouse muscle with naked DNA (plasmid DNA in saline) resulted in significant episomal expression from a number of encoded reporter genes such as firefly luciferase, chloramphenicol acetyltransferase, and β-galactosidase ***(1)***. DNA vaccination has been shown to induce neutralizing antibodies against the gene product, helper T-cell responses of the Th1 phenotype, and cytotoxic T lymphocyte responses ***(2)***. Vaccination with plasmid DNA stimulates immunogenicity and provides protection against various infectious diseases in pre-clinical animal models. Examples include hepatitis B in chimpanzees ***(3)***, bovine herpes virus in mice ***(4)***, influenza A virus in ferrets ***(5)***, human immunodeficiency virus in rhesus monkeys ***(6)***, *Mycobacterium tuberculosis* in mice ***(7,8)***, malaria in mice ***(9,10)***, and genital herpes simplex virus in guinea pigs ***(11)***. Recently, DNA vaccines for the protection against influenza (Merck Research Laboratories, Rahway, NJ), malaria (Vical Inc., San Diego, CA), and HIV (Apollon Inc., Philadelphia, PA), have entered phase I human clinical trials. Rapid progress has been made in the areas of adjuvants for DNA vaccines ***(12)***, route of immunization ***(13)***, industrial scale fermentation and pharmaceutical grade purification ***(14)***. One major interest in the commercial development of DNA vaccines, especially for developing countries, is to increase DNA vaccine stability at room

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

temperature, to reduce the requirement for costly cold storage, and to extend product shelf-life.

Freeze-drying, or lyophilization, has been used in pharmaceutical processes to prolong product stability, particularly for protein products ***(15,16)***. Freeze-drying is used for an attenuated virus vaccine against yellow fever ***(17)*** and a live rinderpest virus vaccine for cattle ***(18)***. The freeze-drying process can be divided into three successive stages: freezing, primary drying, and secondary drying. After freezing the product, the primary drying process involves lowering pressure and supplying heat for water vapor sublimation. During the secondary drying stage, the residual absorbed moisture evaporates from the dried material. In this chapter, we describe a lyophilized DNA vaccine formulation that provides acute protection during lyophilization and permits a full recovery of product activity.

1.1. Screening Buffer and pH

To screen excipients used in lyophilized vaccine DNA formulations, we evaluated buffers and pH using an in vivo reporter gene assay. Plasmid DNA VR1223 encoding a gene for luciferase ***(19,20)*** was formulated and injected intramuscularly into adult mouse rectus femoris muscle at 50 μg DNA in 50 μL volume. Injections were performed on 5 mice (10 muscles) for each formulation. Luciferase enzyme activity was measured 7 d post-injection. The results are shown in **Table 1**. There was no statistical difference in expression from any of the tested formulations by non-parametric Mann-Whitney rank sum test ($p < 0.05$). Either pH 6.0 or pH 7.0 was appropriate. Similarly, either phosphate-buffered saline (PBS) or citrate-buffered saline permitted gene expression. There were no adverse effects detected in injected mice.

1.2. Screening Lyoprotectants

Lyoprotectant, a required component in a lyophilized formulation, provides protection of biological molecules from freezing and drying processes and gives mechanical support to the finished product. To screen and select lyoprotectants, various sugars and polymers were added to the liquid formulation and tested in mouse muscles. The results are summarized in **Table 2**. Considerable variation in luciferase enzyme expression was noted among various concentrations of sugars or polymers, and among similar experiments performed by using different batches of mice. There was no statistical difference in expression from any of the tested lyoprotectants compared to PBS, pH 7.0, by non-parametric Mann-Whitney rank sum test ($p < 0.05$). However, a 2- to 3-fold enhancement was observed from formulations containing sugar lyoprotectant or sugar/polymer lyoprotectant. The sugars included trehalose, mannitol, lactose, sucrose, and sorbitol; and the polymers included polyvinyl pyrrolidone (PVP)

Table 1
Screening Buffers and pH Used in DNA Vaccine Formulations

Buffers and pH	Average ± SEM: ng of luciferase per muscle	Total number of muscles
0.9% NaCl	96.4 ± 17.5	10
0.9% NaCl, 10m*M* sodium phosphate, pH 7.0	228.2 ± 76.9	10
0.9% NaCl, 10 m*M* sodium citrate, pH 7.0	216.9 ± 37.6	10
0.45% NaCl, 10m*M* sodium citrate, pH 7.0	178.6 ± 67.9	10
0.45% NaCl, 10 m*M* sodium citrate, pH 6.0	137.9 ± 35.7	10
Lactated Ringer's buffer	82.5 ± 17.4	10

All formulations contained VR1223 plasmid DNA at 1 mg/mL concentration. Luciferase was extracted from mouse leg muscle and assayed 7 d post-injection. None of these values were significantly different from saline control by non-parametric Mann-Whitney rank sum test ($p < 0.05$).

K-30 and polyethylene glycol (PEG)-3350. No adverse effect on these mice was observed during the 7 d following injection of the test formulations.

Trehalose, PVP K-30, and PEG-3350 were chosen for further vaccine formulation development. Trehalose is a non-reducing disaccharide of glucose found in several organisms that are able to survive desiccation ***(21)***. Trehalose has been demonstrated to protect cells from freezing injury and is an effective drying protectant ***(22)***. Commercial formulations containing trehalose and PVP have shown good recovery of enzyme activity after freeze-drying and more than one year storage stability at 2–8°C ***(23)***.

1.3. Freeze-Drying Cycle Development

Next, we characterized two proposed formulations by freeze-drying microscopy ***(24)*** and examined the effect of lyophilization parameters on the finished freeze-dried product to determine a freeze-drying cycle. Sodium phosphate crystallizes during freezing and causes pH shifts ***(25)***. Sodium chloride has a rather low eutectic temperature and requires a rather low primary drying temperature ***(25)***. Therefore, sodium phosphate and sodium chloride were removed from the proposed lyophilized formulation, but both components were included later in the reconstituting reagent. Freeze-drying microscopic analysis was performed on two formulations, and the results indicated that the retention of cake-like structure in the dried region was observed below the collapse temperature. The collapse temperature for formulation A (VR1223 DNA [1 mg/mL]/12% trehalose [w/v]/2% PVP [w/v]) was –26°C. The collapse temperature for formulation B (VR 1223 DNA [1 mg/mL]/15% trehalose [w/v]/2% PVP [w/v]/2% PEG [w/v]) was –27°C. A complete loss of structure was observed above the collapse temperature (Dr. Michael Pikal, University of Connecticut, Storrs,

Table 2
Screening Lyoprotectants
by Using In Vivo Mouse Muscle Luciferase Assay

Lyoprotectants	Average ± SEM: ng of luciferase per muscle	Total number of muscles[a]	Percent of PBS control
PBS/pH 7.0	143.7 ± 23.4	50	100
PBS/4% trehalose	197.5 ± 47.1	20	137
PBS/12% trehalose	105.1 ± 53.7	10	73
PBS/15% trehalose	100.9 ± 52.7	10	70
PBS/18% trehalose	50.1 ± 19.8	10	35
PBS/12% mannitol	357.9 ± 111.5	10	249
PBS/15% mannitol	81.4 ± 31.6	10	57
PBS/20% mannitol	166.1 ± 58.6	10	116
PBS/12% lactose	49.9 ± 22.8	10	35
PBS/15% lactose	298.8 ± 105.6	10	208
PBS/20% lactose	162.2 ± 50.8	10	113
PBS/4% sucrose	211.6 ± 52.2	20	147
PBS/2% sorbitol	170.4 ± 43.0	20	119
PBS/2% PVP	84.7 ± 25.9	20	59
PBS/2% PEG	207.5 ± 38.9	20	144
PBS/4% trehalose/1% PEG/1% PVP	120.8 ± 54.1	10	84
PBS/4% mannitol/1% PEG/1% PVP	159.3 ± 80.8	10	111
PBS/4% lactose/2% PEG	167.8 ± 52.9	10	117
PBS/12% lactose/0.9% benzoyl alcohol	128.1 ± 40.5	10	89

All formulations contained VR1223 plasmid DNA at a concentration of 1 mg/mL. The pH of all formulations was 7.0

[a]Total number of muscles was from 1–5 experiments. None of the results were significantly different from PBS control by non-parametric Mann-Whitney rank sum test ($p < 0.05$).

CT, personal communication). Therefore, the primary drying temperature was maintained below the collapse temperature by adjusting the chamber pressure to 53 μm Hg. As the sublimation of ice was completed, the product temperature was increased to +35°C for 12 h during the secondary drying. This process resulted in drying of 1 mL of liquid DNA formulation within 2 d.

1.4. Physical Chemical Characterization

A summary of results from tests on the finished product is shown on **Table 3**. The lyophilized product is a white "cake" and partially detached from the glass vial. The cake contains approximately 2% water by weight. The lyophilized cake can be reconstituted with 1 mL of PBS, pH 7.0 ± 0.2. The lyophilized

Table 3
Physical-Chemical Test on Lyophilized and Reconstituted Vials

Physical-chemical tests	Results
General appearance	White cake, partially detached from glass vials
Moisture content	2%
Solubility	<1 min
Reconstituted appearance	Clear colorless odorless solution
pH	7.0 ± 0.1
Agarose gel /ethidium bromide staining	Identical to the liquid plasmid DNA standard
Quantitation of DNA	98 ± 2% recovery

VCL1102 plasmid DNA was lyophilized in formulation A (12% trehalose, 2% PVP) or formulation B (15% trehalose, 2% PVP, 2% PEG). The freeze-dried vial was reconstituted with 1 mL PBS, pH 7.0.

cake dissolves within 1 min and forms a clear, colorless, and odorless solution. There was no detectable loss of DNA determined by spectrophotometric absorption at 260 nm wavelength. Supercoiled plasmid DNA structure was retained without apparent degradation, as revealed by agarose gel electrophoresis with ethidium bromide staining.

1.5. In Vitro Bioassays of the Reconstituted Lyophilized DNA Formulation

Two plasmids, VCL1005 ***(26)*** and VCL1102 ***(27)***, were lyophilized and tested for in vitro potency. Both plasmids are currently being used in human anti-cancer clinical trials ***(26,27)***. The VCL1005 encoding human HLA-B7 gene was lyophilized in formulation A (12% trehalose/2% PVP), and reconstituted with 1 mL PBS, pH 7.0 ± 0.2. The reconstituted VCL1005 formulation was complexed with DMRIE/DOPE (1,2-dimyristyl-oxypropyl-3-dimethyl-hydroxyethyl ammonium bromide/dioleoylphosphatidylethanolamine) and used to transfect UM449 human melanoma tumor cells ***(28)***. **Table 4** indicates that the HLA-B7 is synthesized and expressed on the cell surface of UM449 at 48 h after transfection. The level of HLA-B7 expression was not significantly different from the expression of the non-lyophilized DNA control.

Plasmid VCL1102 encoding the gene for human IL-2 was also lyophilized in formulation A in addition to formulation B (15% trehalose/2% PVP/2% PEG). Freeze-dried vials were reconstituted with PBS, pH 7.0 ± 0.2 and complexed with DMRIE/DOPE, and both formulations were used to transfect UM449 cells. The culture supernatant was assayed for human IL-2 by ELISA ***(27)***. **Table 5** indicates that IL-2 is synthesized and secreted in cell culture

Table 4
Plasmid VCL1005 Lyophilized in Formulation A

Samples	Corrected mean fluorescence	S.D.	Number of repeats	Percent of control
Control, non-lyophilized VCL1005	32.71	0.16	2	100
Lyophilized VCL1005, reconstituted	34.65	4.17	2	106
Assay reference	35.52	0.81	2	N/A

VCL1005 was lyophilized in formulation A (12% trehalose, 2% PVP) and reconstituted in 1 mL PBS, pH 7.0. HLA-B7 expression was measured by FACS analysis by using an in vitro cell transfection assay.

Table 5
Plasmid VCL1102 Lyophilized in Fomulation A or B

Samples	Average corrected mean I.U./mL	S.D.	Number of repeats	Percent of control
Control, non-lyophilized VCL1102	1957	635	4	100
Lyophilized VCL1102 in formulation A	2508	710	4	128
Lyophilized VCL1102 in formulation B	1720	297	4	88
Assay reference	2485	524	4	N/A

Lyophilized VCL 1102 was reconstituted in 1 mL PBS, pH 7.0. Human IL-2 expression in the supernatant of UM449 cells after transfection was measured by ELISA. Formulation A is: 12% trehalose, 2% PVP. Formulation B is: 15% trehalose, 2% PVP, 2% PEG.

supernatants 48 h post-transfection. There is no significant difference in IL-2 expression between lyophilized VCL1102 and non-lyophilized control DNA.

1.6. In Vivo Bioassays of the Reconstituted Lyophilized DNA Formulation

A panel of lyophilized VR1223 plasmid DNA formulations were evaluated for luciferase expression in mice. Lyophilized formulations contained trehalose, PVP, and PEG lyoprotectants. Freeze-dried vials were reconstituted with PBS, pH 7.0 ± 0.2 and injected into mouse rectus femoris muscles. Luciferase activities were assayed at 7 d after injection. Results, as shown in **Table 6**, indicate that the level of muscle expression from reconstituted formulation A (12% trehalose/2% PVP) is similar to non-lyophilized DNA control. In addition, there is a statistical difference in expression between formulation A and formulation F (8% trehalose/2% PVP/2% PEG) by non-parametric Mann-Whitney rank sum test ($p < 0.05$). The reconstituted formulation A yields 3-fold higher expression than reconstituted formulation F. There was no detectable adverse side effect on mice for 7 d after injection.

Table 6
Plasmid VR1223 Lyophilized Formulations

Lyophilized formulations	Average ± SEM: ng of luciferase per muscle	Total number of muscles
Control, non-lyophilized VR1223 in PBS, pH 7.0	202.9 ± 54.1	10
Lyophilized VR1223 in formulation A*a*	265.9 ± 60.4	10
Lyophilized VR1223 in formulation B	184.5 ± 63.5	10
Lyophilized VR1223 in formulation C	172.7 ± 56.2	10
Lyophilized VR1223 in formulation D	136.0 ± 50.6	10
Lyophilized VR1223 in formulation E	131.4 ± 61.4	10
Lyophilized VR1223 in formulation F*a*	98.4 ± 41.9	10
Lyophilized VR1223 in formulation G	90.7 ± 21.1	10

Lyophilized formulations were reconstituted with 1 mL PBS, pH 7.0 and injected in mouse leg muscle. Luciferase activity was assayed 7 d after injection. Formulation A is: 12% trehalose, 2% PVP. Formulation B is: 15% trehalose, 2% PVP, 2% PEG. Formulation C is: 10% trehalose, 2% PVP, 2% PEG. Formulation D is: 12% trehalose, 2% PVP, 2% PEG. Formulation E is: 15% trehalose, 2% PVP. Formulation F is: 8% trehalose, 2% PVP, 2% PEG. Formulation G is: 10% trehalose, 2% PVP.

*a*The result with formulation A is significantly different from formulation F by non-parametric Mann-Whitney rank sum test ($p < 0.05$).

1.7. Conclusion and Perspectives of Lyophilized DNA Vaccines

The experimental work presented in this chapter describes development of a lyophilized naked DNA vaccine for potential use in human gene therapy. Freeze-dried live or attenuated viral vaccines for yellow fever and rinderpest diseases in cattle were previously reported ***(17,18)***. In this report, we evaluated various sugars as lyoprotectants, particularly the non-reducing disaccharides, such as trehalose, and water soluble polymers, such as PVP and PEG. A freeze-drying cycle for plasmid DNA formulation was developed based on freeze-drying microscopic analysis and an examination of the effect of lyophilization parameters. Typical finished cake was white and readily dissolved in PBS, pH 7.0. Recovery of full product activity was demonstrated by plasmid DNA gene expression from mammalian cell culture in vitro and from mouse muscle in vivo. Thus, the freeze-drying process did not introduce any adverse effect on the integrity and potency of plasmid DNA.

The results suggest that a lyophilized DNA vaccine formulation is commercially feasible. Stability, sterility, and toxicity studies are currently being pursued and are required prior to clinical development. The procedures described herein may be used to prepare a safe and effective lyophilized DNA vaccine with a shelf-life that is appropriate for clinical use.

2. Materials

2.1. Chemicals

All ingredients in these formulations are approved drug substances for injection.

1. Trehalose, mannitol, lactose, PVP K-30, PEG-3350, sorbitol, and sucrose are USP grade and from Spectrum Quality Products (New Brunswick, NJ).
2. USP grade sterile water for injection (SWFI) and sodium chloride (5%) in SWFI are from Baxter Healthcare (Round Lake, IL).
3. Sugar and buffer stock at concentrations of 20–40% are prepared in SWFI and filtered through 0.22 μm filters (Nalgene, Rochester, NY).
4. Plasmid DNA is replicated and isolated from *Escherichia coli* DH10B strain ***(29)***. Supercoiled plasmid is purified by column chromatography ***(30)***. Plasmid constructs are precipitated by ethanol ***(29)***, re-solubilized in SWFI, and stored at –20°C. Plasmid endotoxin levels are <30 endotoxin units/mL based on LAL gel clot assay (Associates of Cape Cod, Woods Hole, MA) and the spectrophotometric A_{260}/A_{280} ratios are between 1.8 and 2.0.

2.2. Containers and Equipment

1. The 5-mL glass vials (Type 1 glass), 13-mm gray butyl stoppers, aluminum crimp seals, and a crimper are obtained from West Co. (Lionville, PA).
2. A pilot freeze-dryer (Duro-stop MP) is available from FTS Kinetics (Stone Ridge, NY).
3. Karl Fisher water titration Aquastar apparatus is available from EM Science (Gibbstown, NJ).
4. A microplate luminometer, Dynatech Model ML250 is available from Analytical Luminescence Labs (San Diego, CA).
5. Statistical software such as SigmaStat version 2.0 is available from Jandel Scientific Software (San Rafael, CA).
6. FACS can/LYSIS II system is available from Becton Dickinson (Mountain View, CA).

2.3. Formulations and Filling Vials

Prepare formulations in a biological safety hood by mixing concentrated sterile stock solutions including desired plasmid DNA constructs, sugar, polymer, buffer, and 5% sodium chloride.

1. Prepare 0.2 *M* sodium phosphate by dissolving 3.86 g of sodium phosphate dibasic and 0.77 g of sodium phosphate monobasic in 100 g of SWFI. Prepare PBS, pH 7.0 ± 0.2 by mixing SWFI, 0.2 *M* sodium phosphate at pH 7.0 ± 0.2, and 5% sodium chloride (in SWFI) to achieve final concentrations of sodium phosphate at 10 mM and sodium chloride at 0.9%. Filter stock solutions through a 0.22 μm filter.
2. Wash and rinse glass vials and stoppers with SWFI. Bake glass vials at 225°C in a depyrogenation oven for at least 4 h. Autoclave glass vials and stoppers before formulation filling.
3. Dispense formulations in aliquots of 1 mL into 5-mL glass vials (Type 1 glass, West Co.).

2.4. Plasmid Constructs

1. Plasmid DNA VCL1005 ***(26)*** contains the Rous Sarcoma virus long terminal repeat (RSV-LTR) promoter/enhancer that drives the transcription and translation of the MHC class I human leukocyte antigen B7 (HLA-B7) gene and the chimpanzee β_2 microglobulin gene in a pUC19 backbone. Plasmid VCL1005 also possesses polyadenylation sequences from bovine growth hormone and kanamycin resistance gene as a bacterial selection marker.
2. Plasmid VCL1102 ***(27)*** contains the coding sequence for the human interleukin-2 (IL-2) gene that is downstream from the CMV immediate-early gene promoter/enhancer and 5' untranslated sequences in a pUC18 backbone.
3. Plasmid VR1223 ***(19,20)*** encodes the *Photinus pyralis* luciferase gene under the control of human CMV immediate-early promoter with intron A in a partially deleted pBR322. VR1223 contains a kanamycin-resistance sequence as a bacterial selection marker.

3. Methods

3.1. Freeze-Drying Methods

1. Determine the collapse temperature of the formulation by using freeze-drying microscopic analysis ***(24)*** (*see* **Note 1**).
2. Place the vials on the freeze-drier shelves at room temperature and subsequently equilibrate at –1°C for about 30 min.
3. Cool the shelves to –55°C and hold that temperature for 2 h.
4. Carry out primary drying at a product temperature of about –32°C, or 5°C below the collapse temperature.
5. Carry out secondary drying at 35°C. Complete the drying after adjusting the chamber pressure to between 55–120 μm Hg.
6. Insert the stoppers into the vials under vacuum in the freeze-dryer. Crimp-seal the freeze-dried vials and store them at 2–8°C.

3.3. Physical-Chemical Characterization

1. Visually inspect the lyophilized "cake" for the color, texture, and volume under a translucent light against a black background.
2. Analyze the moisture content using Karl Fisher water titration.
3. Reconstitute the freeze-dried cake with 1 mL of PBS, pH 7.0 ± 0.2 and visually inspect the solubility.
4. Estimate plasmid DNA recovery using agarose gel (0.8%) electrophoresis ***(29)*** alongside a control of non-lyophilized plasmid DNA standard for comparison. Determine plasmid DNA concentration and recovery by spectrophotometric absorption at 260 nm ***(29)***.

3.4. In Vivo Bioassays

1. Inject formulated plasmid VR1223 (50 μg) intramuscularly in the rectus femoris muscle of female 4- to 12-wk-old BALB/c mice (Harlan Sprague-Dawley) (*see* **Note 2**).

2. At 7 d post-injection, extract the luciferase from the entire quadriceps muscle and assay using an automated microplate luminometer ***(20)***.
3. Perform statistical comparisons using the non-parametric Mann-Whitney rank sum test.

3.5. In Vitro Bioassays

1. Reconstitute freeze-dried vials containing plasmid DNA VCL1005 with 1 mL PBS, pH 7.0 ± 0.2.
2. Complex the plasmid DNA (5 μg) with DMRIE/DOPE in 1:1 lipid mixture to yield a DNA/lipid mass ratio of 1:1 to enhance introduction of the DNA into cells ***(28)***.
3. Use the DNA/lipid mixture to transfect UM-449 human melanoma cells (*see* **Note 3**).
4. Harvest transfected cells after 48 h of incubation and subject them to immunofluorescent staining with HLA-B7 monoclonal antibody (hybridoma BB7.1, ATCC # HB56) and flow cytometric analysis ***(26)***.
5. Reconstitute freeze-dried VCL1102 with 1 mL PBS, pH 7.0 ± 0.2 to a final DNA concentration of 1 mg/mL.
6. Complex the plasmid DNA (30 μg) with 6 μg of DMRIE (as DMRIE/DOPE 1:1 lipid mixture) to yield a DNA/lipid mass ratio of 5:1. Dilute the DNA/lipid mixture to 2 μg DNA/mL and transfect IL-2 deficient UM449 cells ***(28)***.
7. Harvest the culture supernatant after 48 h and test in triplicate for the presence of IL-2 using an Enzyme Amplified Sensitivity Immunoassay kit (Medgenix Diagnostics, Fleurus, Belgium) ***(27)***.

4. Notes

1. The microscopic analysis of our freeze-dried Formulations A and B was performed at Dr. Michael Pikal's laboratories at the School of Pharmacy, University of Connecticut, Storrs, CT.
2. Animal care throughout the study is in compliance with the "Guide for the Use and Care of Laboratory Animals" (U.S. Department of Health and Human Services, National Institutes of Health, NIH Publication No. 86-23, revised 1985) as well as with local Institutional Animal Care and Use Committee requirements.
3. UM-449 human melanoma cells do not express HLA-B7 molecules and are deficient in expressing β_2-microglobulin. They were obtained as a gift from Mark Cameron, Alfred Chang, and Gary Nabel, University of Michigan, Ann Arbor, MI.

Acknowledgments

The authors would like to thank Research Production for providing DNA, Quality Control for analytical support, Francine Cornefert-Jensen and Pamela Strauch for technical support, and George Gray for continued guidance and support.

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4

Repeated Use of Qiagen Columns in Large-Scale Preparation of Plasmid DNA

Derek Gregory, Ricardo E. Tascon, and Douglas B. Lowrie

1. Introduction

The preparation of large amounts of high-purity intact plasmid DNA is a significant expense in DNA vaccine research. In our laboratory, mice are typically each immunized by injection of 100 μg on four occasions, so that an experiment with 50 mice requires 20 mg DNA as a minimum. The Qiagen tip-10,000 (Giga) column is intended to deliver up to 10 mg of high copy-number plasmid DNA from *Escherichia coli*. We have found that by repeatedly regenerating the column and reapplying the flowthrough volume of DNA extract, we can readily obtain 2–4-fold increased yields, up to 40 mg, from the standard 2.5 L broth culture.

2. Materials

1. Qiagen tip-10,000 Giga column kit (Cat. No. 12191; five columns with buffers per kit) is available from Qiagen Ltd., Crawley, West Sussex, UK.
2. Luria Berlini (LB) broth: Bacto-tryptone, 25 g; Bacto-yeast extract (Difco Laboratories, Surrey, UK) 12.5 g; NaCl 25 g; dissolve in 2.5 L distilled water, adjust the pH to 7.5 with NaOH, and sterilize by autoclaving. Dispense the medium by half-filling sterile conical flasks and add the selective antibiotic appropriate to the plasmid of interest.
3. LB agar plates: these are prepared as above but containing 15 g/L Bacto-agar and dispensed into sterile Petri dishes after addition of the antibiotic.

3. Methods

1. Streak out the transfected bacteria on two LB agar plates, one containing the recommended concentration of antibiotic and one containing double the concentration. Incubate the plates for 24 h at 37°C.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

Table 1
Sequential Yields of Plasmid DNA

	Pass 1	Pass 2	Pass 3	Pass 4	Pass 5	Total yield
Yield (mg)*a*	8.9	7.1	6.1	4.0	2.3	28.4
LPS content*b*	<0.05	<0.05	<0.005	<0.005	<0.0005	
Immunogenicity*c*	NT	1.6 ± 0.3	2.4 ± 0.2	3.5 ± 0.5	NT	

[a] pCMV4.65 ***(1)*** was recovered from *E. coli* lysate by passing the lysate through a Qiagen tip-10,000 column five times (Qiagen).

[b] Endotoxin units/microgram assayed by limulus endotoxin assay kit.

c Balb/c mice were injected intramuscularly with 2 × 50 μg DNA four times at 4-wk intervals, then killed after 2 wk. Splenocytes from two to three mice were pooled, cultured for 48 h with or without recombinant antigen (10 μg/mL), then supernatants were assayed for interferon-γ by enzyme-linked immunosorbent assay ***(1)***. Results are shown as ng/mL ± SD increment in the presence of antigen.

2. Select five or six typical colonies growing at the highest concentration of antibiotic and inoculate them separately into 10 ml LB broth and incubate the cultures for 8–10 h.
3. Make minipreps of plasmid DNA from these samples and select the culture showing the highest yield.
4. Inoculate 1.0 or 0.5 L volumes of LB broth (total 2.5 L) with 1.0 or 0.5 mL of the selected culture and incubate the cultures on an orbital flask shaker overnight at 37°C.
5. Prepare the bacterial lysate and pass it through the Qiagen column as described in the kit but retaining the flowthrough lysate.
6. After eluting the plasmid from the column according to the protocol, wash the column with 100 mL sterile distilled water and then with a 50 mL equilibration buffer. The column is now ready for reuse (*see* **Note 1**).
7. Rerun the flow-through lysate through the column, elute the retained plasmid, wash and reequilibrate the column as before.
8. Repeat this step a further three times (*see* **Note 2**). Typical yields are shown in **Table 1**.

4. Notes

1. The columns can be sealed with parafilm and stored at 4°C.
2. The column can be used for a further 2.5 L of culture (of the same plasmid) with somewhat reduced yield before the flow becomes too restricted.

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5

The Immunology of DNA Vaccines

Thomas Tüting, Jonathan Austyn, Walter J. Storkus, and Louis D. Falo Jr.

1. Introduction

The surprising observation that direct inoculation of an expression plasmid encoding a foreign protein into the skin of mice resulted in the induction of antibody responses, demonstrated that injection of "naked" DNA could result in antigen expression in an immunogenic form ***(1)***. This observation and the subsequent demonstration that intramuscular injections of plasmid DNA encoding influenza nucleoprotein could protect mice against a challenge with live influenza virus have opened up new avenues for vaccine development ***(2–3)***. Immunization with plasmid DNA has been shown to activate both humoral and cellular immune responses, including the generation of antigen-specific $CD8^+$ cytotoxic T cells as well as $CD4^+$ T helper cells ***(4)***. An increasing number of studies using experimental animal models have demonstrated that plasmid DNA immunization can promote effective immune responses against numerous viruses, including influenza, rabies, HIV, HBV, HCV, and HSV; several bacteria, including: *Mycobacterium tuberculosis*, *Mycoplasma pulmonis*, and *Borrelia burgdorferi*; as well as parasites, such as malaria and leishmania ***(4)***. Phase I clinical vaccine trials are currently being performed for HIV, HBV, and influenza virus. With the molecular identification of tumor antigens ***(5)***, there has also been increasing interest in the development of DNA-based immunization for cancer. Preclinical studies demonstrate that DNA-based immunizations targeting model tumor antigens such as chicken ovalbumin ***(6)***, β-galactosidase ***(7)***, or CEA ***(8)*** induce protective immune responses leading to rejection of a subsequent, normally lethal challenge with antigen-expressing tumor cells.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

Vaccines consisting of naked plasmid DNA have several potential advantages over alternative immunization approaches relying on the delivery of purified or recombinant proteins, or live attenuated or recombinant viruses. They offer the promise of a readily deliverable, molecularly defined reagent that results in antigen synthesis *in situ,* but that is neither infectious nor capable of replication. Importantly, both humoral and cell-mediated immune responses may be elicited against multiple defined antigens simultaneously. Furthermore, it may become possible to manipulate the nature of the resulting immune response through the co-delivery of genes encoding immunomodulating cytokines or costimulatory molecules. Genetic constructs can be modified, allowing for the removal or insertion of transmembrane domains, signal sequences, or other residues that affect the intracellular trafficking and subsequent processing of antigen. The sequence may also be modified by site-directed mutagenesis, permitting single amino-acid exchanges designed to enhance the antigenic potency of individual epitopes or to abolish unwanted physiologic effects of the wild-type protein. Importantly, plasmid DNA, encoding suitable antigens or immune modulators, can readily and economically be constructed and produced in large quantities with a high degree of purity and stability.

2. DNA Vaccines: Antigen Expression, Processing, and Presentation

It is now well established that injection of plasmid DNA through various methods and routes can induce both humoral and cell-mediated immune responses. Significant titers of neutralizing anti-viral antibodies and potent cytotoxic T lymphocyte (CTL) responses have been documented in a number of experimental animal models ***(4,9)***. Importantly, antigen-specific antibodies and CTL reactivity could be detected in rodents longer than 1 yr after immunization ***(9)***. These studies suggest that plasmid DNA immunization may promote long-lasting humoral and cellular immune responses qualitatively similar to live attenuated or recombinant viral vaccines without the safety hazards of inoculation of live virus.

2.1. Effects of Variations in Gene Delivery and Expression

Plasmid DNA immunization has been accomplished using epidermal, mucosal, intramuscular, and intravenous routes of administration ***(3)***. Striated muscle initially was considered to be the only tissue capable of efficiently taking up and expressing free plasmid DNA in aqueous solution ***(10–12)***. This in vivo gene transfer method was extensively studied by gene therapists as a simple way to deliver recombinant proteins such as Factor IX, growth hormone, or α-1-antitrypsin. Transfection of muscle fibers could readily be dem-

onstrated using reporter genes such as β-galactosidase or firefly luciferase. Indeed, β-galactosidase activity could be detected in up to 5% of myofibrils and luciferase activity was demonstrated up to 19 mo following intramuscular injection of plasmid ***(11)***. Analyses by PCR and Southern blotting revealed that the injected plasmid persisted episomally, presumably due to the low turnover of myocytes in vivo. The gene gun has been of particular interest as an alternative to intramuscular injection as the mode of delivery of plasmid DNA. The gene gun propels DNA-coated gold particles directly into the cytoplasm of cells in target tissue by means of electrical discharge or helium pulse ***(1,3,13,14)***. Expression of biolistically delivered DNA encoding β-galactosidase or luciferase was found to be transient in bombarded epidermis or liver, and low levels of long-term expression could be observed in the dermis ***(13)***.

Both direct injection of plasmid DNA in saline and particle-mediated delivery of plasmid DNA have now been extensively investigated as immunization methods. Particle-mediated delivery of DNA to the epidermis of mice using the gene gun required up to 5000 times less DNA than intramuscular or intradermal inoculation of DNA in aqueous solutions for the induction of immune responses ***(14)***. Following injection of plasmid DNA in saline, cells presumably take up DNA from extracellular spaces. In contrast, using the gene gun, DNA is delivered directly into the cell cytoplasm. These differences in gene delivery are believed to account for the fact that efficient transfection and induction of immune responses can reproducibly be achieved with nanogram quantities of plasmid DNA using the gene gun, whereas injection of plasmid DNA in saline usually requires 25–100 μg ***(15,16)***. It is important to note, however, that the transfection efficiency of the target tissue does not necessarily correlate with the efficiency of immunization. Indeed, one of the striking results of early studies examining different routes of DNA delivery was that intradermal injection of free plasmid DNA can elicit potent immune responses to the encoded antigen despite the relatively low efficiency of DNA uptake and expression in skin when compared to muscle ***(17)***.

Recent evidence suggests that the site and method of DNA delivery may affect the nature of the immune response induced against antigens encoded by plasmid DNA ***(16,18)***. The two most common targets for gene delivery, skin and muscle, are generally considered to have significant differences in immunocompetency. The skin and mucous membranes are the site where most foreign antigens are normally encountered, and these tissues have highly developed immune surveillance functions. The epidermis contains numerous bone-marrow-derived Langerhans cells (LC), which are professional antigen presenting cells specialized for the initiation of immune responses ***(19)***. Resident LC can efficiently take up and process foreign antigens. In addition, both keratinocytes and LC can secrete proinflammatory cytokines such as IL-1,

TNF-α, and GM-CSF in response to allergens, infection, or injury. This cytokine milieu can induce the maturation and migration of LC to regional lymph nodes, where they present antigen to T cells. In contrast, muscle is not generally considered to have a specialized immune system and it contains relatively few, if any, resident dendritic cells, macrophages, or lymphocytes.

Interestingly, intradermal or intramuscular injection of DNA in saline has been found to preferentially induce a Th1 bias of the immune response in mice, with the expansion of IFN-γ-producing $CD4^+$ T cells and the production of antibodies of the IgG2a isotype ***(16,20,21)***. Surprisingly, plasmid DNA immunization using the gene gun targeting skin or muscle ***(16,21)*** induces immune responses with a Th2 bias. There also appear to be differences in the dependency of the immune response on gene expression in the target tissue for intramuscular needle vs. epidermal gene gun DNA delivery ***(18)***. Excision of an injected muscle bundle within 10 min of DNA inoculation did not affect the magnitude or duration of antibody responses. In contrast, biopsy of the skin target site up to 24 h after gene gun bombardment completely abrogated the antibody response in the majority of mice. In these studies, gene gun delivery to the skin was performed with only 1 μg of plasmid DNA, whereas intramuscular injection utilized 100 μg of DNA in 50 μL of saline. The authors interpreted these results to suggest that gene gun-bombardment of skin resulted primarily in antigen expression by epidermal cells, which appeared to be required for the induction of immune responses. On the other hand, intra-muscular injection was thought to lead to the rapid movement of DNA or DNA-transfected cells out of the injected muscle, so that the immunostimulatory events leading to antibody production and CTL reactivity took place primarily in distal tissue.

Taken together, these studies demonstrate that variations in gene delivery can have important qualitative effects on the nature of the resulting immune response. At this point, the relative contribution of DNA delivery variables—including the target site, the method of delivery, and/or the quantity and adjuvant properties of DNA delivered—to the observed differences in the nature of the immune response remains unclear.

2.2. Processing and Presentation of Genetically Introduced Antigens

The precise mechanisms involved in the activation of immune responses to antigens expressed in vivo following injection of plasmid DNA have not yet been determined. In the past decade, important insights into the molecular nature of antigen processing, presentation, and recognition have shed new light on the induction of immune responses. The mechanism by which lymphocytes recognize antigen has been outlined in significant detail in a number of recent review articles ***(22–25)*** (illustrated in **Fig. 1**). $CD8^+$ cytolytic T lymphocytes

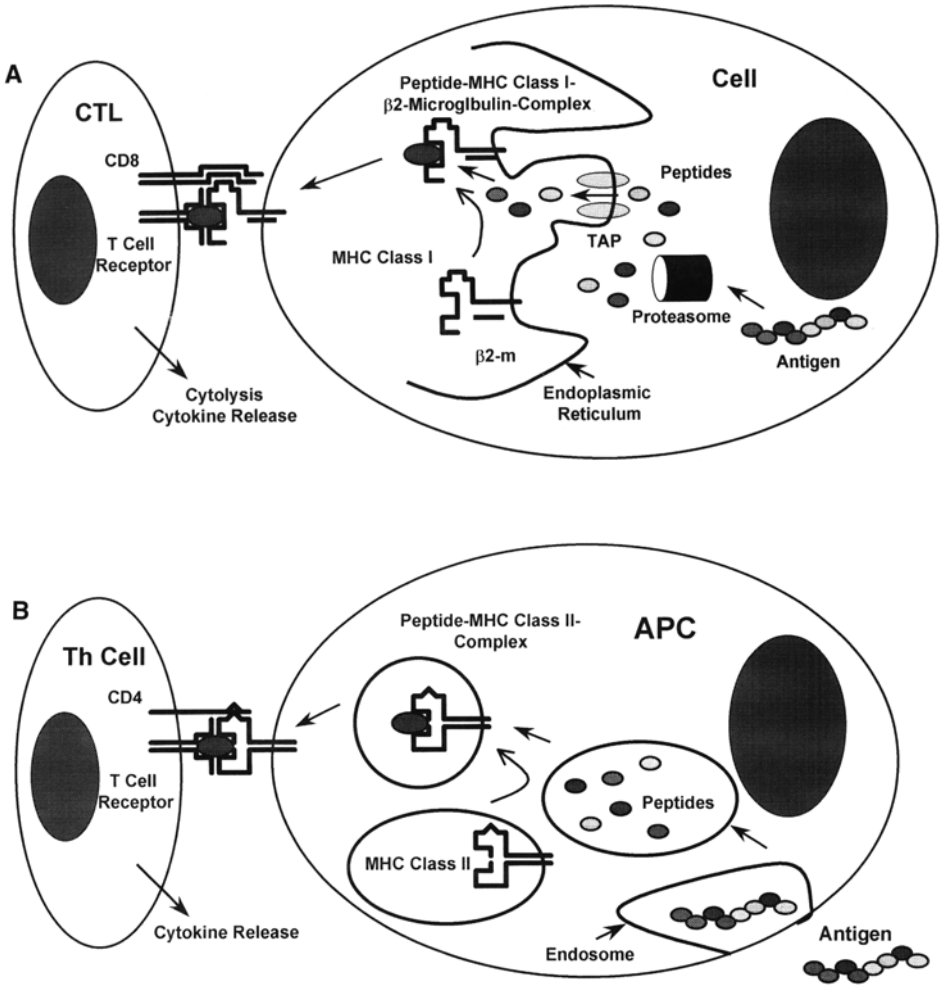

Fig. 1. MHC class I and class II restricted antigen-processing pathway.

recognize a non-covalent trimeric complex consisting of a major histocompatibility complex (MHC) class I heavy chain allele, β_2-microglobulin, and an 8–12 amino acid long peptide antigen. $CD4^+$ helper T lymphocytes, in contrast, recognize 12–25 amino acid long peptide antigens presented in the context of dimeric MHC class II molecules. Most of the peptides presented by MHC class I molecules are derived from endogenously synthesized protein antigens processed in the cytosol by multicatalytic proteasomes. The resulting short peptides are delivered into the endoplasmic reticulum by the transporter associated with antigen presentation (TAP). In the endoplasmic reticulum,

compatible peptides interact with nascent class I heavy chains and β_2-microglobulin-generating transport-competent trimeric complexes that are subsequently delivered to the cell surface where they become accessible to $CD8^+$ T cell scrutiny. In contrast, exogenous proteins taken up into endocytic compartments from the extracellular milieu are the major source of antigens presented in the context of MHC class II molecules. Internalized antigen is denatured by low pH and degraded by endosomal and lysosomal proteases, exposing peptide segments that bind to MHC class II molecules. The resulting class II–peptide complexes are transported to the membrane for detection by $CD4^+$ T cells. One result of this general segregation of processing pathways is that protein antigens introduced into the extracellular fluids (as by injection) typically do not gain access to the cytosol and are therefore generally excluded from the MHC class I processing pathway. This represents a theoretical hurdle for protein-based vaccine design and may explain why the elicitation of strong CTL responses with non-living protein antigens has generally been problematic. Several studies, however, have found that cell-associated antigens can elicit $CD8^+$ T-cell responses in vivo, and accumulating evidence suggests that some phagocytic cell types, such as macrophages or immature dendritic cells (DC), can take up exogenous proteins for class I-restricted presentation ***(24)***.

Once a protein antigen is denatured and/or digested, the ability of a given peptide to bind to, and be presented by, a given MHC class I or class II allele is determined by structural motifs within the peptide sequence. These motifs allow for sufficient compatibility between peptide amino acid side-chains and micropockets formed within the peptide-binding groove of the MHC molecule ***(25)***. The degree of intermolecular compatibility determines the affinity of peptide for an individual MHC molecule, the corresponding half-life of peptide–MHC complexes and, to a large degree, the likelihood that the peptide–MHC complex is immunogenic.

In addition to the recognition of peptide–MHC ligand by the T-cell receptor, T-cell priming requires the presentation of antigen by professional antigen-presenting cells (APCs) capable of providing "co-stimulation." Presentation of antigens to T cells in the absence of appropriate costimulatory signals can result in activation induced cell death by apoptosis ***(26)***. Dendritic cells are the most potent APCs identified to date ***(27,28)***. They are capable of capturing antigens in the periphery, and then migrating to T-cell-rich areas of secondary lymphoid organs, where they can efficiently stimulate naive or quiescent T cells. Upon encounter with antigen, DCs undergo a maturational process, which is associated with their capacity to activate T cells. This includes the upregulation of MHC, costimulatory, and adhesion molecule expression on their cell surface. Factors associated with the extraordinary capacity of dendritic cells to function as antigen-presenting cells are summarized in **Fig. 2**.

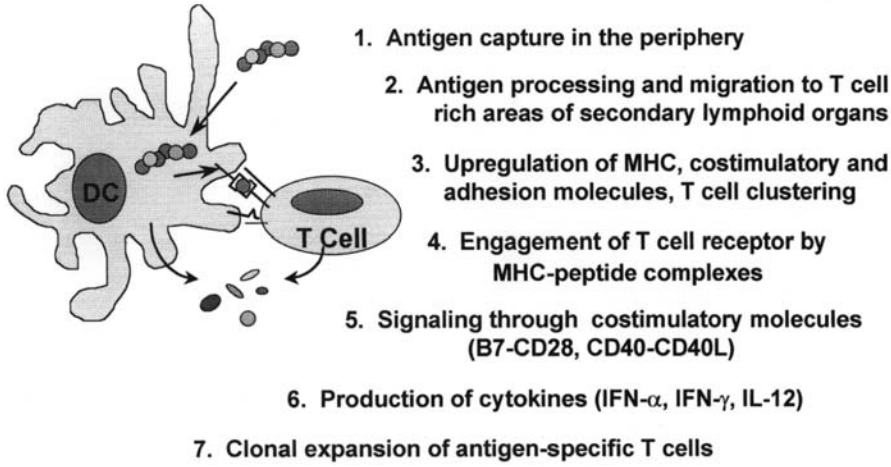

Fig. 2. Dendritic cells as professional antigen-presenting cells.

Because genetic immunization can elicit both humoral and cellular immune responses, the DNA-encoded antigen presumably gains access to both the MHC class I and class II antigen processing pathways, and is also capable of interacting with immunoglobulin molecules on the surface of B-cells. Initially, the efficient induction of $CD8^+$ CTL was considered to be a result of endogenous antigen synthesis in vivo, which presumably permitted antigen to access the class I restricted antigen processing pathway of transfected cells. However, the predominant transfected cell type following intramuscular DNA injection is the myocyte. Myocytes express only low levels of MHC class I molecules and lack expression of costimulatory molecules, such as B7.1 and B7.2. Presentation of antigens by muscle cells alone would be expected to cause either immunological ignorance or tolerance.

2.3. A Critical Role for Professional Antigen Presenting Cells

To determine the role of professional APCs in the induction of $CD8^+$ CTL responses following DNA immunization, several groups have performed studies using bone marrow-chimeric mice ***(29–32)***. They utilized the influenza nucleoprotein (NP) as a model antigen, since CTL epitopes have been defined for multiple strains (H-2^b, H-2^d, and H-2^k). Parent $\rightarrow F_1$ bone marrow chimeras were generated in which H-$2^{b \times d}$ (C57BL/6 × BALB/c) recipient mice were reconstituted with bone marrow from either H-2^b (C57BL/6) or H-2^d (BALB/c) animals. CTL responses following immunization with plasmid DNA encoding influenza NP via intramuscular or intradermal inoculations were restricted to the MHC haplotype of the donor bone marrow alone, and not to the other recipient MHC haplotype, which was also expressed by the recipient's myocytes

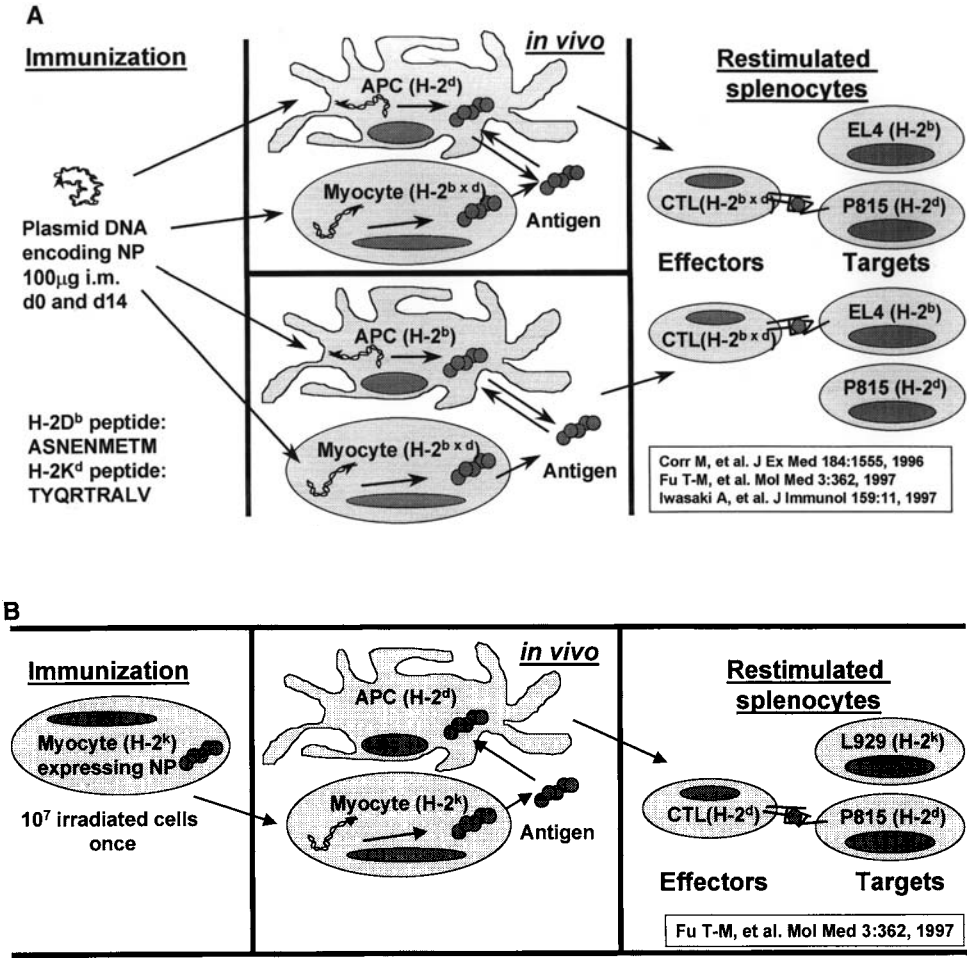

Fig. 3. Cross-priming in genetic immunization: immune induction requires antigen presenting by professional APCs.

(29–32) (illustrated in **Fig. 3A**). Thus, CTL could only be stimulated if antigen was presented in the context of MHC molecules on the bone marrow-derived professional antigen-presenting cells, ruling out the possibility that myocytes or keratinocytes were directly activating cytotoxic T cells. This view was further supported by transplantation experiments, in which NP-transfected myoblasts (H-2^k) were transplanted into F1 bone marrow chimeras consisting of H-$2^{k \times d}$ (C3H × DBA/2) recipient mice which had received bone marrow from H-2^d (DBA/2) donors *(33)*. Here, the resulting influenza nucleoprotein-spe-

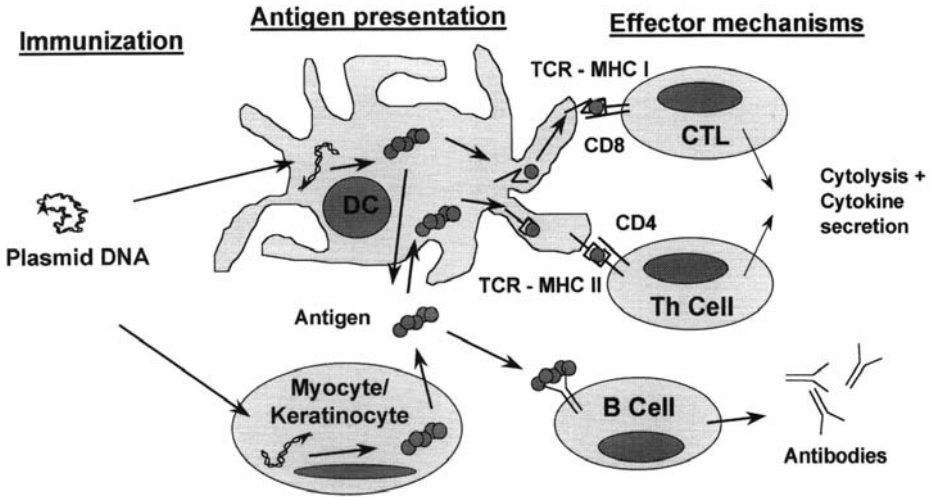

Fig. 4. Genetic immunization: mechanisms of antigen presentation.

cific CTL were restricted to the MHC haplotype of the bone marrow donors (H-2^d, *see* **Fig. 3B**). Thus, specific CTL responses could be induced as a result of the transfer of antigen from the transplanted nucleoprotein-expressing muscle cells (H-2^k), to a genetically disparate bone-marrow-derived APC (H-2^d) capable of presenting the NP-derived antigen in the context of its own MHC class I molecules (cross-priming).

Together, these data strongly suggest that the initiation of cell-mediated immune responses following plasmid DNA immunization requires the presentation of antigen by professional APCs. How professional APCs acquire antigen is much less clear. Professional APCs could acquire the antigen either through direct transfection and expression within the APC or via the uptake of exogenous antigen synthesized within other transfected cell types (illustrated in **Fig. 4**). Muscle transplantation studies described previously ***(18)*** suggest that antigen-expression by transplanted myoctes can be sufficient for T-cell activation ***(33)***. However, previously described studies demonstrate that the generation of an immune response after intramuscular injection in vivo does not require persistence of the transfected target muscle tissue, suggesting that the mechanism of immune induction after gene delivery in vivo is less clear ***(18)***. The same studies suggest a striking difference in dependency on gene expression in the target tissue, depending on the target site, since excision of gene gun targeted skin for as long as 24 h after delivery prevented immunization, whereas excision of transfected muscle as soon as 10 min after injection did not appear to diminish the immune response.

We have directly demonstrated that cutaneous DNA immunization using the gene gun results in the direct transfection of skin-derived dendritic cells in vivo *(34)*. These transfected APCs traffic to and localize in the draining lymph nodes, where they continue to express endogenously synthesized antigen in the T-cell-rich areas of draining lymph nodes. Subsequently, and consistent with these observations, Song et al. *(35)* have reported that splenic dendritic cells contained proviral DNA and expressed antigenic proteins after direct administration of antigen-encoding genes using retroviral vectors. Casares et al. *(36)* demonstrated that DC migrating out of DNA injected tissue appear to carry plasmid DNA and trigger immune responses to the transgene. However, whether transfection of APCs is critical for the efficient induction of immune responses following in vivo plasmid DNA immunization remains unclear. The relative contributions of direct transfection of APCs vs re-uptake and presentation of antigen synthesized in bystander cells remains to be determined. Clearly, very low numbers of antigen-presenting dendritic cells are sufficient for immune induction. Early studies using epidermal LC have demonstrated that injection of as few as 10 LC are sufficient for induction of allospecific immune responses *(37)*. Timares-Lebow et al. *(38)* have recently reported a direct comparison of the efficiency of transfected, adoptively transferred skin-derived fibroblast vs dendritic cell lines in generating immune responses against transgene encoded antigens. In this model, in which transfected cells are injected subcutqaneously into A/J mice, 100-fold less dendritic cells are required for immunization.

The presentation by APCs of endogenously synthesized antigen and exogenously acquired antigen is not mutually exclusive, and both mechanisms may be operating following in vivo gene delivery. Though the relative contribution of each mechanism to priming is unclear, it is not unlikely that antigen release from transfected non-APCs could potentiate or prolong the resulting immune response, especially when these transfected cells are targeted by the first wave of antigen-specific cytotoxic T cells. Evidence for immune-mediated destruction of muscle fibers following direct gene transfer with antigen-expressing plasmid DNA has indeed been found and support this hypothesis *(39)*.

2.4. Genetic Engineering of the Immune Response

There are several examples of attempts to modulate the immune response elicited by plasmid DNA immunization through the co-delivery and expression of antigen with other immunologically relevant molecules. For example, simultaneous delivery of plasmid DNA encoding GM-CSF enhances immune responses resulting from plasmid DNA immunization *(40,41)*. This is consistent with the involvement of DCs in the activation of the immune system since GM-CSF is important for the growth, differentiation, and maturation of den-

dritic cells, at least in vitro. In addition, several groups have recently shown the adjuvant effects of co-delivery of plasmids encoding immunostimulatory cytokines including IL-2, IL-7 or IL-12 ***(42–45)***. Adjuvant administration of plasmids encoding T-cell growth factors such as IL-2 and IL-7, and Th1-biasing cytokines such as IFN-α and IL-12, may be expected to selectively enhance the induction of cell-mediated immunity.

Another potential strategy for the enhancement of T-cell responses is the adjuvant administration of plasmids encoding costimulatory molecules such as B7.1 or B7.2, presumably to provide the second signal required for optimal T-cell activation ***(46,47)***. Co-delivery of costimulatory molecule genes for B7.1, and particularly for B7.2, have been shown to enhance the induction of cell-mediated immunity following intramuscular injection of plasmid-encoded antigen ***(41,45,48)***. These observations have led investigators to question whether expression of such costimulatory molecules by muscle cells endows them with the capability to directly stimulate T cells ***(49)***. Whether or not expression of a single co-stimulatory molecule alone can convert a muscle cell or other somatic cell type to a professional APC is unclear, but testable. The possibility of indirect effects of members of the B7 family should also be considered. Signaling via the B7-CD28 pathway prevents T-cell apoptosis ***(26)*** and may be expected to promote secondary expansion of primed CTLs. In addition, B7 family members also appear to induce cytokine production, particularly IFN-γ, by NK cells leading to enhanced immune responses (our own unpublished observations).

The observation that immunostimulatory DNA sequences (ISS) containing unmethylated CpG dinucleotide motifs in a particular base context can contribute to effective induction of an immune response is particularly relevant for DNA vaccines. Studies using β-galactosidase as a model antigen demonstrated that the Th1 bias of the immune response induced by plasmid DNA immunization, with the expansion of IFN-γ-producing $CD4^+$ T cells and cytotoxic T cells, was maximal when the plasmid DNA backbone contained such ISS ***(50)***. Concomitant injection of ISS-deficient plasmid DNA encoding the antigen with non-coding ISS-enriched plasmid DNA also induced a Th1-biased response, indicating that the ISS-enriched plasmid DNA exerts an adjuvant effect. Several groups have reported that bacterial DNA containing ISS is capable of activating NK cells and macrophages in vitro leading to the production of IFN-α/β, IFN-γ, TNF-α, IL-1, IL-6, IL-12, and IL-18 ***(50–56)***. Both the type I interferons and IL-12 are known to enhance the generation and increase the cytotoxicity of NK cells and $CD8^+$ CTL and to promote a T helper 1-like phenotype (characterized by high IFN-γ production) in the antigen-specific differentiation of naive $CD4^+$ T cells. Local IFN-γ production by NK cells is capable of activating macro-

phages, enhancing antigen uptake and presentation. Thus, injection of high doses of ISS-containing plasmid DNA activates innate immunity, and leads to the production of Th1-biasing cytokines within the skin or muscle, promoting cell-mediated immunity. Interestingly, the potent immunostimulatory effects of Freund's adjuvant may depend on the ISS-enriched mycobacterial DNA. Indeed, the use of plasmid DNA-containing ISS as an adjuvant can provide, in part, the immunostimulatory effects of Freund's adjuvant without the severe inflammatory and toxic side effects *(56)*. It has been reported that plasmid DNA immunization by biolistic cutaneous delivery induces immune responses with a Th2 bias *(16,21)*. This might reflect the fact that these gene gun immunizations use only 1/100th as much plasmid DNA compared to saline DNA immunization. This amount of plasmid DNA may not contain sufficient quantities of ISS to induce the specific cytokine milieu capable of promoting a Th1 immune response. We have observed that plasmids encoding the Th1-biasing cytokines IFN-α or IL-12 can shift the type of immune response elicited by cutaneous gene gun delivery towards a Th1 phenotype *(86)*.

In conclusion, by choosing an appropriate injection vehicle and desired route of delivery, and co-delivering plasmids encoding immunomodulating cytokines or costimulatory molecules, it may be possible to tailor the induction of the immune response in vivo towards effector mechanisms that are best suited for a given pathogen or tumor.

3. Dendritic Cells as Biological Adjuvants for DNA Immunization

The establishment of culture conditions that allow for the in vitro generation of large numbers of immunostimulatory DC from precursor populations in bone marrow and blood has stimulated significant interest in the use of DC as a biological adjuvant, particularly for cancer vaccines *(57–63)*. Mouse DC have been isolated from spleen, or grown from bone marrow cells cultured in GM-CSF and IL-4. Human DC have been grown from $CD34^+$ progenitors isolated from cord blood, bone marrow, or peripheral blood. In general, culture of these $CD34^+$ progenitors in GM-CSF and TNF-α induces growth and differentiation of DC, and yields can be increased by inclusion of c-kit ligand (stem cell factor [SCF]) and Flt-3 ligand, which expand the progenitor pool. DC can also be generated by culture of human blood monocytes in GM-CSF and IL-4 (monocyte-derived DC). Cultured murine bone marrow-derived DC, when adoptively transferred, can effectively present tumor antigen peptides to the immune system leading to the induction of prophylactic and therapeutic cell-mediated immunity against experimental murine tumors that express the same antigen *(64–68)*. Primary tumor antigen-specific T-cell responses could also efficiently be induced in vitro in a human model using cultured, peptide-pulsed, autolo-

gous DC as stimulators and peripheral blood leukocytes from healthy donors or cancer patients as responders ***(69–71)***. Clinical trials using cultured, peptide-pulsed DC that are adoptively transferred for the treatment of metastatic melanoma have already been initiated at the University of Pittsburgh and at other institutions. The insertion of cDNA encoding an entire tumor antigen into cultured DC is currently being explored by a number of laboratories as an alternative to the use of peptides for DC-based tumor immunotherapy ***(72–88)***. A major strength of such gene-based vaccines compared to peptide-based approaches is that their application does not require prior knowledge of the patient HLA-haplotype or of specific T-cell epitopes. In addition, they may promote both class I- and class II-restricted T-cell responses. The realization that the induction of immune responses following plasmid DNA immunization is likely to require antigen presentation by professional APCs provides an additional rationale for DC adoptive transfer strategies. Endogenous antigen synthesis within DC ensures direct access to the MHC class I antigen processing pathway. After homing to T-cell-rich areas of secondary lymphoid organs, DCs could present appropriate MHC class I-binding epitopes to naive T cells in the context of costimulation.

Vaccines consisting of murine bone marrow-derived DC ex vivo genetically modified to express tumor antigens using viral vectors ***(72,80–83)***, plasmid DNA ***(87)***, or RNA ***(92)***, are able to promote tumor antigen-specific T cell responses and prophylactic cell-mediated immunity against tumors in experimental mouse models. Of importance, vaccination with cultured DC that had been infected ex vivo with recombinant adenoviral vectors was shown to circumvent the problem of neutralizing antibodies, severely limiting the repeated direct administration of adenoviruses in vivo ***(78)***. Thus, cultured DC may be an ideal vehicle for the implementation of adenoviral vector-based strategies for immunization. Human cultured DC have also been transduced using retroviral vectors ***(74–77)***, adenoviral vectors ***(78,79)***, poxviruses ***(84)***, or plasmid DNA ***(85,86)***.

3.1. Retroviral Vectors

Retroviral vectors have been used to deliver genes to DC. The potential advantage of this approach is that the genes may be expressed throughout the life of the cell, and can be transmitted to the cell progeny. However, integration of retroviral vectors into the chromosomal DNA of target cells requires that the cells undergo cell division. Hence, there has been particular interest in the use of retroviral-mediated gene transfer into human DC grown from proliferating $CD34^+$ progenitors.

Murine bone marrow-derived DC can be retrovirally transduced by co-culture with ecotropic producer cell lines ***(72)*** or by centrifugal trans-

duction with retroviral supernatant on d 2,3, and 4 of the DC culture *(73)*. The use of ecotropic retroviral packaging cells or supernatant with a high titer is critical, since murine stem cells do not express the receptor for amphotropic retroviral envelope proteins. Adoptive transfer of DCs expressing a model tumor antigen can induce antigen specific immunity leading to the prophylactic and therapeutic antitumor immunity *(72)* (**Table 1**). Intratumoral injection of DC expressing IL-12 genes can mediate effective therapy of established tumors *(73)*.

Human DC developing in culture from bone marrow and cord blood $CD34^+$ progenitors have been transduced with mouse CD2 as a reporter gene *(74)*. The mature DC were shown to exhibit a normal, mature phenotype (e.g., CD40, CD80, CD83,CD86, p55, S100 expression) and function (allogeneic MLR, SEA superantigen, and tetanus toxoid recall responses). Average transduction efficiencies of 11.5% for cells grown from bone marrow progenitors, and 21.2% for cord blood progenitors, were reported for the $CD1a^+$ DC progeny. Importantly, the proportion of DC and other myeloid populations that developed in the retrovirally transduced cultures was similar to that in mock transduced cultures, indicating that retroviral transduction does not impose a bias on development of one cell type over another.

Tumor-associated genes have been expressed in human DC following retroviral-mediated transduction *(75,76)*. The MUC-1 gene, encoding a target antigen for breast carcinoma, was introduced into DC by centrifugal transduction grown from cord blood $CD34^+$ progenitors. A transduction efficiency of >13.5% was reported for the $CD1a^+$ progeny *(75)*. Importantly, the MUC-1 product was found to exhibit the same non-polarized membrane expression in DC as in tumor cells, and the DC were competent stimulators of allogeneic MLR responses. The melanoma-associated MART-1 gene has also been expressed in DC grown from $CD34^+$ progenitors derived from peripheral blood of patients with malignant melanoma *(76)*. Transduction efficiencies were reported to be higher following coculture of the developing cells with one packaging cell line (PG13; 22–28%) compared to another (PA317), and higher than when the cells were incubated with retroviral supernatants. The transduced DC stimulated the production of IFN-gamma from tumor-infiltrating lymphocyte cell lines, and induced antigen-specific CTL from autologous T cells, indicating that they were capable of antigen presentation. However, as pointed out *(74)*, retroviral infection and stable gene expression was not directly demonstrated in this study, and it is possible that antigen released by transduced cells could have been acquired and presented by nontransduced cells. Human monocyte-derived DC have also been retrovirally transduced with the reporter genes *LacZ* and CAT *(77)*. In view of the usual requirement for cell division for retroviral integration, it is interesting that a high trans-

duction efficiency of 42–60% was reported for $CD1a^+$ cells and that the gene products were still detectable after 20 d culture.

Interestingly, as previously described, splenic DC have been implicated in the induction of immunity following intramuscular injection of retroviral vectors encoding an HIV protein or ovalbumin in mice ***(35)***. DC isolated from the spleens of these mice were shown to contain provirus, to express retrovirally encoded proteins, to present ovalbumin to a specific T cell line, and to induce CTL specific for the HIV protein (i.e., as peptide-MHC complexes) after adoptive transfer to naive mice. However, it is unclear whether DC within the muscle were transduced prior to their migration into the spleen, or whether the retrovirus entered the blood after intramuscular injection and transduced DC within the spleen.

3.2. Adenoviral Vectors

Adenoviral vectors can be used for gene transfer in nondividing cells, but their utility may be limited by transient gene expression and immune responses against the virus itself. In one study ***(78)*** human monocyte-derived DC were infected with adenovirus (AdV) type 5 strains at a multiplicity of infection (MOI) of 100:1. Although only 1–5% of the cells were found to express early or late virus proteins, these cells were shown to induce virus-specific $CD4^+$ and $CD8^+$ T proliferative responses and CTL when they were cultured with naive T cells in restimulation assays. The apparently low infectivity of DC in this is broadly consistent with findings from another report in which an E1-deleted, replication-incompetent AdV type 5 vector was used to transfer genes to human monocyte-derived DC ***(79)*** (**Table 2**). To achieve gene expression in 95% or more of the cells, MOIs greater than 1000 were required. While a melanoma cell line was more readily transduced, these viral loads resulted in cytopathic effects that were not seen for DC; for example, >90% of the DC remained viable after exposure to virus at an MOI of 10,000 whereas 100% of the melanoma cells were killed. This report demonstrated that high level expression of reporter genes (*LacZ* and luciferase) IL-2, and IL-7 could be achieved by AdV-mediated transduction at these high MOIs. In contrast, a variety of physical methods for gene transfer, including DNA/liposome complexes, electroporation and calcium phosphate precipitation, were relatively inefficient or ineffective in the same study.

Adenoviral vectors have also been used to transduce immortalized murine DC lines or cultured bone marrow-derived DC with model tumor antigens such as β-galactosidase or ovalbumin ***(80–83)***. Adoptive transfer of transduced DC also promotes tumor-antigen-specific CTL responses and prophylactic and therapeutic anti-tumor immunity. However, none of the studies reported so far have employed tumor antigens similar to those found for human cancers, which

Table 1
Ex Vivo Gene Transduction of Dendritic Cells: Retroviral Transduction

Ref. *(72)*	
Vector:	Retroviral vector MFG, MLV LTR, in ecotropic CRE packaging cell line
Gene:	i) Beta-gal (*LacZ*) as model tumor antigen ii) Human HER-2/neu as control
Cell:	Murine bone marrow progenitors cultured in GM-CSF and IL-4
Mode:	Co-culture with packaging cell lines for the first 2 d of DC generation analysis and use on d 7
Findings:	Gene expression, antigen presentation in vitro induction of CTL and prophylactic/therapeutic antitumor immunity in vivo
Ref. *(73)*	
Vector:	Retroviral vector MFG, MLV, LTR, transient transfection of ecotropic BOSC23 or ecotropic CRE packaging cell lines
Gene:	i) Human CD80 ii) Murine IL-12
Cell:	Murine bone marrow progenitors cultured in GM-CSF and IL-4
Mode:	Infection at d 2, d 3, and d 4 of culture (3 cycles) by centrifugal transduction (2 h at 32°C) with supernatant from cell lines, analysis on d 7
Findings:	Gene expression, antitumor effect in vivo
Ref. *(74)*	
Vector:	Retroviral vector MFG, MLV LTR, in amphotropic CRIP packaging cell line
Gene:	Mouse CD2 reporter gene
Cell:	DC from human bone marrow and cord blood $CD34^+$ progenitors cultured in GM-CSF, TNF-α, SCF, and Flt-3 ligand
Mode:	Infection at d 3 of culture cells incubated with cell line plus polybrene, 24 g analysis at d 10–d 13
Findings:	Gene expression, normal DC phenotype and function
Ref. *(75)*	
Vector:	Retroviral vector MFG, MLV, LTR, in amphotropic BING packaging cell line
Gene:	i) Beta-gal (*LacZ*) reporter gene ii) Human MUC-1
Cell:	Human cord blood $CD34^+$ progenitors cultured in GM-CSF, TNF-α, IL-4 and SCF
Mode:	Infection at d 1 and d 2 of culture (2 cycles) cells incubated with supernatant from cell line, 2 h, analysis e.g., at d 14
Findings:	Gene expression, normal DC, phenotype and (increased) function

Table 1 *(continued)*

Ref. ***(76)***	
Vector:	Retroviral vector MFG, MLV, LTR, in amphotrophic PA317 or PG13 (gibbon ape leukemia virus envelope) packaging cell lines
Gene:	i) Murine CD80 ii) Human MART-1
Cell:	Human cord blood $CD34^+$ progenitors cultured in GM-CSF, TNF-α, and SCF
Mode:	Infection at d 1, d 2, and d 3 of culture (3 cycles) cells incubated with cell lines or supernatant from cell lines plus polybrene, 6 h, analysis on d 12
Findings:	Gene expression, Antigen presentation in vitro
Ref. ***(77)***	
Vector:	Retroviral plasmid MFG, MLV, LTR, in amphotropic CRIP packaging cell line
Gene:	Beta-gal (*LacZ*) reporter gene
Cell:	DC from human blood monocytes cultured in GM-CSF and IL-4
Mode:	Infection at approx d 7–d 8 of culture cells incubated with supernatant from cell line, 4 h; 3 cycles of infection on successive days
Findings:	Gene expression

all appear to derive from normal germ line-encoded genes. It will be critical to evaluate whether the inherent immunogenicity associated with adenoviral vectors will help or suppress the induction of immune responses to these "self" molecules, which may be considerably less immunogenic.

3.3. Naked DNA

Non-viral gene delivery methods have several important advantages of potential clinical interest:

1. More than one gene can readily be transfected simultaneously, allowing for cotransfection of genes encoding distinct tumor antigens and/or immunostimulatory cytokines.
2. Only the gene of interest is transcribed without immunological interference from viral proteins both in vitro and in vivo.
3. There is no risk of recombination associated with replication-deficient viral vectors.
4. Insertion of foreign DNA into the genome is less likely due to the transient nature of gene transfer.
5. The approach uses highly purified DNA, which can readily be produced in large quantities, and is very stable.

Table 2
Ex Vivo Gene Transduction of Dendritic Cells: Adenoviral Transduction

Ref. ***(79)***	
Vector:	Adenoviral vector, CMV promoter/enhancer, SV40 polyA termination signal (E1-deleted, replication-deficient, Ad-5 vector)
Gene:	i) Luciferase and beta-gal (*LacZ*) as reporter genes ii) IL-2, IL-7
Cell:	DC from human blood monocytes cultured in GM-CSF and IL-4
Mode:	Incubation with adenoviral vector in RPMI plus 2% human AB serum for 2 h on d 6; analysis after 24 h
Findings:	Gene expression
Ref. ***(80)***	
Vector:	Adenoviral vector, RSV promoter/enhancer, SV40 polyA termination signal (E1-deleted, replication-deficient, Ad-5 vector)
Gene:	i) Beta-gal (*LacZ*) as reporter genes ii) Ovalbumin
Cell:	Immortalized DC line JAWS II and Freshly isolated splenic DC
Mode:	Incubation with adenoviral vector; analysis after 48 h or injection d 0 and 7
Findings:	Gene expression, induction of CTL and anti-tumor immunity
Ref. ***(81)***	
Vector:	Adenoviral vector, CMV promoter/enhancer, SV40 polyA termination signal (E1-deleted, replication-deficient, Ad-5 vector)
Gene:	MART-1
Cell:	Murine bone marrow progenitors, cultured in GM-CSF and IL-4
Mode:	Incubation with adenoviral vector in RPMI plus 2% FCS for 2 h on d 6, i.v. injections d 0, 7, and 14
Findings:	Gene expression, induction of CTL and anti-tumor immunity
Ref. ***(82)***	
Vector:	Adenoviral vector, CMV promoter/enhancer, SV40 polyA termination signal (E1-deleted, replication-deficient, Ad-5 vector)
Gene:	i) Luciferase and beta-gal (*LacZ*) as reporter genes ii) Polyoma middle T antigen
Cell:	Murine bone marrow progenitors cultured in GM-CSF and IL-4
Mode:	Incubation with adenoviral vector for 2 h on d 6, analysis after 24 h or injection
Findings:	Gene expression, induction of CTL and anti-tumor immunity

Table 2 *(continued)*

Ref. *(83)*	
Vector:	Adenoviral vector, CMV promoter / enhancer, SV40 polyA termination signal (E1-/E3-deleted, replication-deficient, Ad-5 vector)
Gene:	Beta-gal (*LacZ*) as model tumor antigen
Cell:	Immortalized DC lineXS52 and murine bone marrow progenitors cultured in GM-CSF and IL-4
Mode:	Incubation with adenoviral vector for 2 h on d 6, anaylsis after 24 h or injection
Findings:	Gene expression, induction of CTL and anti-tumor immunity

Human monocyte-derived DC have been transduced with plasmid DNA encoding reporter genes and melanoma-associated human tyrosinase by a liposome-based transfection ***(85)*** (**Table 3**). Low level expression of the reporter genes (CAT, and *LacZ* encoding β-galactosidase) and tyrosinase was detected at the protein level. Furthermore, DC transfected with the tyrosinase gene were shown to cluster with a CTL line specific for a tyrosinase peptide-MHC class I peptide, and to induce secretion of TNFa by these antigen-specific T-cells, suggesting that the antigen expressing DC were capable of antigen presentation. We have obtained similar results with peripheral blood-derived DC transfected with plasmid DNA encoding melanoma antigens by particle bombardment using the gene gun ***(86)***. The use of particle-mediated gene transfer consistently allows the insertion of genes encoding antigens into DC without interference from viral proteins, albeit with considerably lower transduction efficiency when compared to viral vectors. Five different melanoma antigens were evaluated: MART-1/Melan-A, pmel 17/gp100, tyrosinase, MAGE-1, and MAGE-3. Our results suggest that a non-viral gene delivery system can also be used to present a given melanoma-associated antigen in an immunogenic format by gene-modified DC and promote the induction of primary melanoma-reactive CTL in vitro. The adoptive transfer of murine bone marrow-derived DC genetically engineered to express tumor antigens by particle-bombardment ***(87)*** was capable of inducing antigen-specific CTL and protective anti-tumor immunity.

In a comparative study, mouse splenic DC or macrophages were transduced with plasmid DNA encoding HSV-1 proteins ***(88)***. Following intramuscular injections of transduced cells into naive mice, the induction of anti-HSV immunity was assessed. It was found that mice injected twice at a weekly intervals with transduced DC were protected against a subsequent challenge with

Table 3
Ex Vivo Transduction of Dendritic Cells: Naked DNA

Ref. ***(85)***	
Vector:	i) Naked DNA, CMV promoter (Stratagene, Heidelberg, Germany) ii) cDNA eukaryotic expression vector (pCEP4; Invitrogen, Amstel, The Netherlands)
Gene:	i) Beta-gal (*LacZ*) and CAT reporter genes ii) Human tyrosinase
Cell:	DC from human blood monocytes cultured in GM-CSF and IL-4
Mode:	Transfection at d 7 of culture, cells incubated with DNA plus cationic liposomes (Lipofectin; Life Technology); 24 h analysis 2–5 d after transfection
Findings:	Gene expression, antigen presentation in vitro
Ref. ***(86)***	
Vector:	Naked DNA, CMV promoter (pCI; Promega, Madison, WI)
Gene:	i) Luciferase and GFP reporter genes ii) MART-1, gp100/pmel 17, tyrosinase, MAGE-1, MAGE-3
Cell:	DC from human blood monocytes cultured in GM-CSF and IL-4
Mode:	Cells biolistically transfected using the gene gun
Findings:	Gene expression, antigen presentation and induction of primary CTL in vitro
Ref. ***(87)***	
Vector:	Naked DNA, CMV promoter (pCI; Promega)
Gene:	i) Luciferase and GFP reporter genes ii) HPV16-E7, murine p53
Cell:	DC from murine bone marrow precursors cultured in GM-CSF and IL-4
Mode:	Cells biolistically transfected using the gene gun intravenously injections on d 0 and 7 and analysis at d 14
Findings:	Gene expression, induction of CTL and protective antitumor immunity in vivo
Ref. ***(88)***	
Vector:	Naked DNA, CMV promoter (pcDNAI; Invitrogen, San Diego, CA)
Gene:	HSV-1 gB or ICP-27
Cell:	Mouse primary spleen DC or macrophages
Mode:	Cells incubated with vector plus DOTAP, 3 h; i.m. injections on d 0 and 7 and analysis at d 14
Findings:	Gene expression, immunity in vivo

HSV-1. This enhanced immunity appeared to be associated mainly with an increased Th1 $CD4^+$ T cell response as demonstrated by the production of high titers of IgG2a but not IgG1 antibodies in vivo even after just one intramuscu-

Table 4
Ex Vivo Gene Transduction of Dendritic Cells: RNA

Ref. ***(92)***	
Vector:N/A	
Gene:	Ovalbumin, tumor antigens
Cell:	Mouse primary spleen DC
Mode:	Cells incubated with RNA plus DOTAP, 2–4 h
Findings:	Induction of CTL responses and immunity in vivo

lar injection. Furthermore, splenocytes from the vaccinated mice produced IFN-g and IL-2, but not IL-4, when they were restimulated in vitro. Enhancement of antigen-specific T-cell proliferative responses in vitro and DTH responses in vivo was also observed. The level of immunity induced by vaccination with transduced DC was not as high as that elicited by the virus itself, but was at least as potent, and in several assays considerably stronger than that induced following intramuscular injection of the vectors alone. Moreover, transduced macrophages were found to be ineffective at inducing immunity, despite the fact that these cells were shown to express vector-specific mRNA and protein.

3.4. RNA

Several reports indicate that mRNA can be used for in vivo genetic vaccination in mice. For example, virus-specific CTL were induced following immunization with liposome-encapsulated, in vitro synthesized, RNA encoding influenza nucleoprotein ***(89)***; antibodies were elicited following intramuscular injection of in vitro synthesized RNA encoding human carcinoembryonic antigen ***(90)***; and antibodies were produced after delivery of human α-1-antitrypsin mRNA by gene gun into the skin epidermis ***(91)***.

Mouse splenic DC have been pulsed with RNA in vitro and have been found to induce CTL in vitro and immunity in vivo ***(92)*** (**Table 4**). It was shown that DC pulsed with in vitro synthesized RNA for ovalbumin, or with total or polyA+ RNA from ovalbumin-expressing tumor cells, could stimulate antigen-specific CTL responses in vitro. Furthermore, mice vaccinated with DC pulsed with RNA from the latter source were found to be protected against a subsequent challenge with the tumor cells. In addition, vaccination of mice with DC that were pulsed with total or polyA+ RNA from a poorly immunogenic and highly metastatic melanoma cell line resulted in a dramatic reduction of lung metastases. The potential advantage of this approach is, for example, that it should be possible to obtain and amplify RNA from even a very small amount of tumor tissue for DC-based clinical immunotherapy.

4. Summary

Despite some uncertainties, it is clear from the studies outlined above that genes can be delivered to DC by a variety of means and expressed in functional (e.g., enzymes and cytokines) or immunogenic (e.g., viral and tumor antigens) forms. What is not yet clear are the relative efficiencies of these individual techniques for delivery and expression of defined genes. Systematic, comparative studies will be required using standardized reagents and clearly defined protocols. The immune responses resulting from the adoptive transfer of cultured DC gene-modified ex vivo using different viral and non-viral vector systems should be carefully characterized in animal models and compared to those induced by direct inoculation of plasmid DNA in vivo. Such studies are now underway in several laboratories and the first results should be forthcoming over the next year or two. The further evaluation of immunogenic viral vectors such as adenoviruses will also be important. These vectors can transduce DC very efficiently and have been effective in murine models in vivo. The expression of immunogenic viral proteins might severely limit their applicability or, conversely, might provide non-specific help for the induction of immune responses to the encoded antigen. With further improvements in gene transfer technology, strategies may also be developed to target the delivery of genes directly to DC in vivo, thus obviating the requirement for ex vivo transduction of the cells prior to vaccination.

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6

Methodology Used in DNA-Based Prophylactic and Therapeutic Immunization Against Hepatitis B Virus in Chimpanzees

Alfred M. Prince and Betsy Brotman

1. Introduction

1.2. Chimpanzee Welfare

Chimpanzees, because of their near-human nature, have special needs that must be met by those who carry out medical research with them. Perhaps the most important of these is the need for companionship. Chimpanzees kept alone become obviously depressed, and manifest stereotypic behavior such as compulsive rocking. We have followed a policy of keeping chimpanzees in groups of two or more, whenever possible. This has virtually prevented overt depressive symptomatology. This policy has not significantly affected studies on hepatitis B and C ***(1)***.

Additional measures to prevent depression and enrich the lives of these animals include providing a variety of food sources, including those that require considerable ingenuity to access; e.g., coconuts or palm nuts that can be opened only by the use of a "hammer and anvil," or two stones with jam or mustard held in a 50-mL disposable centrifuge tube in a pipe about 2 m outside of the cage which can be reachable with the use of a long thin twig. This "fishing" activity resembles closely the hunting for termites in the wild and is a favorite activity.

Chimpanzees, especially adults, are very physical, engaging in sometimes frightening displays. These are important for the animals' well-being and require that cages be large, ideally at least 20 ft long, and at least 12 ft high. Ropes and tires on chains are important play objects.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

The foregoing sources of enrichment are essential to the human conduct of experiments with chimpanzees.

2. Preliminary Studies of Immunogenicity

An important preliminary to carrying out studies on prophylaxis or immunotherapy in the chimpanzee model is to investigate the immunogenicity of hepatitis B surface antigen-encoding plasmids (HBsAg) in mice. This was done in a series of important studies between 1993–1996 ***(2–7)***. These studies used a variety of plasmid constructs, most commonly under the control of the cytomegalovirus (CMV) immediate early promoter. Doses of 100 μg/mouse given in two intramuscular sites appeared to be optimal. Pretreatment of muscle with cardiotoxin accelerated the antibody response but probably had no effect on its magnitude ***(2)***. Although a single injection of DNA produced a strong anti-HBs response, which did not diminish with time, a booster DNA injection resulted in a 10- to 200-fold further increase of titer ***(7)***.

In a study that was an important precursor of attempted immunotherapy in the chimpanzee model, Mancini et al. immunized mice transgenic for HBsAg with a single injection of 100 μg of plasmid DNA encoding HBsAg and Pre-S2 determinants ***(3)***. This resulted in a disappearance of circulating HBsAg and hepatitis B (HBV) messenger RNA in the liver, in the absence of any liver histopathology. Passive transfer experiments showed that the effect was modulated by T cells. Unfortunately, these dramatic results were not confirmed in trials with five other transgenic lines (F. Chisari, personal communication). The reasons for this discrepancy need to be further investigated.

Only a single study has investigated the immunogenicity of HBsAg-encoding plasmids in chimpanzees ***(8)***. Each animal was immunized at 0, 8, 16, and 27 wk, with one animal receiving 2 mg/dose and the second 400 μg. The animals were immunized with a Biojector needleless injection system for the priming dose and for the 8- and 27-wk boosters (Bioject, Seattle, WA). The 16 wk-booster was administered by needle and syringe after pretreatment of the injection site by injection of 25% sucrose. At 52 wk both animals received an additional boost with conventional yeast derived HBV vaccine. Anti-HBs responses were quantitated by three separate assays and expressed as mIU/mL by comparing it to the World Health Organization International Standard. This is an essential form of standardization if comparison of immunogenicity is to be made between different laboratories. The animal receiving 2 mg doses reached nearly 10,000 mIU/mL after the first booster and 190,000 mIU/mL one mo after the vaccine booster. The response in the animal receiving 400 μg doses was much lower, with no antibody detectable before the first boost, and transient low-level responses afer each booster. Unfortunately the design of the study did not permit a comparative evalua-

tion of the Biojector in comparison to needle injection into sucrose-pretreated muscle.

3. Prophylactic Immunization of Newborn Chimpanzee Against HBV

Only a single study has been done to evaluate the protective efficacy of DNA-based immunization ***(9)***. We chose to attempt to protect newborn chimpanzees against a challenge with HBV. The rationale is that, in Asia, about 40% of transmissions of HBV leading to carrier-state infections originate in infected mothers, with transmission occuring mostly during the first day of life. Thus, in Asia it is essential that HBV immunization be given on the day of birth. The design of this study raised the methodological question of whether the babies should be taken from their mothers and raised by human surrogates. Because many chimpanzees who are not raised by chimpanzee mothers have difficulty resocializing with chimpanzees and later becoming good mothers themselves, we chose to return the chimps to their mothers immediately after each immunization and after challenge. There was a very small risk that the babies could infect their mothers after challenge. This did not occur. Both mothers and babies are undoubtedly happy with our decision.

The plasmid used in this study encoded HBsAg and the Pre-S2 determinant under the control of the CMV promoter ***(6)***. On the day of birth and at 6 and 24 wk each of the newborn chimpanzees received 1.0 mg divided into four sites intramuscularly. Blood samples (1–2 mL) were obtained at 2 wk intervals. The animals were challenged at 33 wk with 100 chimpanzee infectious doses (CID50) of our standard challenge stock of HBV subtype adw. This stock has been titrated in chimpanzees more that 10 yr ago and has since been stored in aliquots at –70°C.

Anti-HBs responses were weak, peaking at 20–30 mIU/mL, and transient. At the time of challenge, little or no anti-HBs was detectable. Nevertheless, by standard criteria, the animals remained uninfected after challenge developing no HBsAg or antibody to the core protein (anti-HBc). An unimmunized control developed both of these markers. After challenge, both immunized animals made anamnestic anti-HBs responses. These were shown to be result from minimal post-challenge replication of HBV, demonstrable only by polymerase chain reaction (PCR).

We concluded from this study that non-sterilizing, but protective, immunity had been achieved. We now need to determine whether this can be achieved when the challenge is also done on the day of birth. This would mimic the requirements of the human situation.

4. DNA Canary Pox-Based Immunotherapy of Chronic HBV Infection

The extraordinary results reported by Mancini et al. ***(3)*** stimulated us to attempt immunotherapy of the one chronic carrier available in our colony. This animal has

been chronically infected since 1985. We used the following constructs in this attempt.

4.1. Plasmid Constructs

Plasmid pJW-So, which encodes HBsAg, but not Pres-1 and PreS2, was used for the first DNA-based immunization. Both plasmids were purified by double banding in CsCl-ethidium bromide gradients . This plasmid utilized the vector pJW4303 ***(10)***, which was kindly provided to us by Dr. James Arthos. This expression system is based on the CMV immediate early promoter element for transcriptional control and contains a TPA-mimic leader sequence of 23 amino acids that is cloned in frame with the amino terminus of expressed proteins by means of a unique NheI site. The vector provides a bovine growth hormone polyadenylation signal for messenger RNA expression. High-level secretion of HBsAg by this vector was observed after transfection of COS-1 cells: 33 ng/mL of supernatant by quantitative Ausria, 3 d after transfection.

The HBV sequence was obtained by PCR from a human plasma, subtype ayw, with an upstream primer that provided a *Nhe*I site, and hybridized to the second amino acid codon of the HBsAg sequence open reading frame at the amino end. The first amino acid of the HBsAg sequence was omitted. At the carboxyl end of the HBsAg gene, a 3' primer generating a *Bgl*II site and including the TAA stop codon was used. PJW4303-So was constructed by directionally cloning the product of this PCR at the *Nhe*I and *Bam*H1 sites of pJW4303.

The vector pCDNA III, obtained from Invitrogen (Carlsbad, CA) employs the CMV promoter of pJW4303 as well as the 3' BGH polyadenylation site but lacks the TPA leader element. The pCDNAIII-So clone was constructed by PCR from the HBsAg gene as a *Hin*dIII/*Bgl*II fragment. The fragment was cloned into pCDNA at unique *Hin*dIII/*Bam*HI. This clone was used for the second DNA immunization, as it was not clear which of the foregoing plasmids was more immunogenic. Transfection of pCDNA II into Cos-1 cells did not reveal secretion of HBsAg into the supernatant.

4.2. Canary Pox Construct

The canary pox construct ALVAC HBV L;M (vCP 157, Virogenetics Inc., Rensselaer, NY) encodes HBsAg, PreS-1, and PreS-2. This construct uses as a vector the ALVAC vaccine strain of canary pox ***(11)***. ALVAC HBV L;M contains two expression cassettes, which encode two different forms of the surface antigen of the ayw strain of HBV: the L form (HBsAg, Pre-S1, and Pre-S2), and the M form (HBsAg and Pre-S2). The canary pox vector was used for the final boosts.

4.3. Immunization

Two milligrams of pJW-So plasmid DNA was given in four intramuscular sites at wk 0. Four weeks later, the animal was similarly injected with 2.0 mg pCDNA III-So. On weeks 15 and 28, the animal was boosted with the recombinant canarypox vector ALVAC HBV L;M 4×10^8 PFU in four sites intramuscularly and the same quantity intravenously. The canary pox vector was used for the final boosts, as it has been shown that recombinant pox virus vectors give superior anamnestic responses, compared with DNA alone *(12)*.

4.4. Results

Quantitative PCR revealed a 1000-fold drop in viral load in plasma beginning 1 wk after the ALVAC booster. The levels remained undetectable by quantitative PCR for 18 wk; however, borderline quantities of HBV DNA were detected during this time by nested PCR, at least 70 wk of follow-up to date. HBsAg declined about fourfold after the ALVAC booster but rapidly returned to normal levels. No abnormalities in transaminase levels were seen.

5. Discussion

Our results indicate that DNA-based immunization can protect even newborn chimpanzees against overt infection with HBV. Future experiments will be needed to determine whether this protection also will extend to virus challenge at the time of birth. This would be required for protection of newborn infants from maternally derived infections.

Protection against HBV infections is usually related to levels of anti-HBs, 10 mIU/mL being considered to be a protective level of antibody *(8)*. In our study, the newborn animals made transient anti-HBs responses. Little or no antibody was present at the time of challenge. It is likely that the protection observed depended on cytotoxic T cells, although this could not be evaluated due to the small size of the animals.

The attempt to control viremia in a chronically infected animal by DNA- and ALVAC-based immunotherapy was encouraging, at least 70 wk of follow up.

Acknowledgments

The generosity of Marion Perkus (at Virogenetics, Troy, NY) and Pasteur Merieux Connaught, UK, in providing the ALVAC vector is gratefully acknowledged. The pJW-So and pCDNA III-So plamids were prepared by Dr. Chuck Tackney.

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7

Intramuscular and Intradermal Injection of DNA Vaccines in Mice and Primates

Heather L. Davis

1. Introduction

There are several different ways to administer plasmid DNA vaccines ***(1)***. Those most commonly used include intramuscular (i.m.) injection or intradermal (i.d.) injection of pure plasmid DNA ("naked" DNA), or biolistic introduction of DNA-coated gold particles into the epidermis with a "gene gun." It is also possible to deliver naked or liposome-formulated DNA to mucosal surfaces.

The chief advantages of the injection methods are that they do not require any specialized equipment and they are easy to perform and generally efficacious. However, they do cause pain in non-anesthetized animals. In contrast, the gene gun approach requires special preparation to coat the DNA onto the gold particles, which must then be stored under dry conditions to prevent the DNA from detaching, and specialized equipment (the Accell gene gun itself is available from Bio-Rad, Hercules, CA). However, the gene gun is painless and it allows immune responses to be induced with very small quantities of DNA, apparently because of the high efficiency of transfection with direct delivery of the DNA into cells.

It is not completely clear which is the best method for DNA-based immunization, because of very few comparative studies using the same DNA vaccine in the same animal model. In addition, numerous other factors, including the skill of the investigator can greatly influence the outcome of the transfection procedure ***(2)***. Immunization of mice is generally effective with i.m. injection of plasmid DNA (not taking into account differences in doses). In this case a single administration of DNA encoding most antigens can induce strong

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

immune responses that last for the life of the animal ***(3)***. In contrast, the gene gun usually requires repeated administrations for sustained immune responses in mice. Intradermal injection of plasmid DNA can work as well as i.m. injection, but this depends heavily on the skill of the investigator.

For larger animals, i.m. injection of a plasmid DNA vaccine can also be highly effective with a single administration, for example, in dogs (Henry Baker, personal communication). In other cases, repeated i.m. immunizations are required to attain and maintain high antibody titers, as we have found with the chimpanzee ***(4)***, making this approach more comparable to the i.d. and gene gun approaches. Somewhat better results can be obtained for i.m. injection of DNA vaccines in larger animals using a needleless injection system such as the Biojector® (Bioject Inc., Portland, OR), which gives better distribution of the injected substance ***(5)***. For some large species, intradermal injection may work better than i.m., as seems to be the case for the *Aotus* monkey ***(6)***.

In summary, it appears that the optimal DNA delivery method depends on the animal species, the antigen being expressed, and the protective correlates for that disease. Thus, for a given antigen and animal species, the best route of administration will probably have to be determined empirically. Nevertheless, the experience gained by others can be used as a guide to choose the best method of immunization for a given animal model.

The following describes are described methods to administer naked DNA by i.m. or i.d. injection to rodents and non-human primates.

2. Materials

2.1. Injection with Needle and Syringe

1. Appropriate anesthesia or tranquilizer for animal (e.g., halothane [Halocarbon Laboratories, River Edge, NJ] or Metafane® [Janssen Pharmaceuticals, North York, ON, Canada] for mice, ketamine for primates) (*see* **Note 1**).
2. Razor, preferably electric, to shave skin over injection site (*see* **Note 2**).
3. U-100 insulin syringe (100, 50 or 30 units = 1, 0.5, or 0.3 mL, respectively) with attached 29G1/2 needle (Becton Dickinson, Franklin Lakes, NJ) or tuberculin syringe fitted with 28G needle (Becton Dickinson) (*see* **Note 3**).
4. Polyethylene tubing (PE 20, i.d. = 0.38 mm).
5. Plasmid DNA vaccine dissolved in endotoxin-free saline (Sigma, St. Louis, MO) at appropriate concentration (e.g., 0.1 mg/mL to deliver 10 µg in 100 µL, or 1 mg/mL for delivery of 100 µg in same volume) (*see* **Notes 4** and **5**).

2.2. Injection with Biojector®

1. Appropriate anesthesia for animal (e.g., halothane or Metafane for mice).
2. Razor, preferably electric, to shave skin over injection site (*see* **Note 2**).
3. Biojector needleless injection system with syringes and CO_2 cartridges (Bioject Inc.) (*see* **Note 6**).

3. Methods

3.1. I.M. Injection of Mice with Needle and Syringe

1. Prepare needle by placing a piece of polyethylene tubing over the needle and cutting it carefully to a length such that only 2–3 mm of needle protrudes (basically just the beveled portion should protrude). This serves as a guide for how deep the needle should be inserted into the muscle.
2. Draw DNA solution into the syringe and ensure there are no air bubbles. For a bilateral injection, you will need 100 µL for each mouse (*see* **Note 5**).
3. Anesthetize mice (*see* **Notes 1** and **7**).
4. Shave the fur over the anterior, lateral and medial surfaces of the leg from above the knee to the ankle.
5. Position the mouse on its back, anchor one foot to the table with your thumb (of the opposite hand than you hold the syringe) and use your index finger of the same hand to stretch the body and anchor the abdomen. Make sure the anterior tibial ridge (bony ridge running vertically between the knee and ankle) is facing directly up—if the leg is rotated you will have a poor injection and poor results.
6. We have found the best muscle to inject is the tibialis anterior (TA) muscle (*see* **Notes 8** and **9**). Insert the needle at a 45° angle into the TA about 3 mm lateral (to the outside) of the anterior tibial tuberosity (a bump on the tibial ridge about halfway between the knee and the ankle). Make sure the syringe is aligned with the body axis and not angled to either side (in which case the DNA may end up in the interstitial space). The needle should be inserted until the PE tubing rests against and slightly depresses the skin, but don't press so hard that the skin is deeply indented by the PE tubing. Slowly inject (i.e., over 5–10 s) 50 µL of the DNA solution (*see* **Note 10**) without changing the pressure of the PE tubing on the leg (this takes some practice), hold the needle in place for a few seconds, then carefully withdraw the needle. A small drop of liquid may form over the injection site. For bilateral injection, repeat in the same manner for the opposite leg (*see* **Note 9**).

3.2. I.M. Injection of Primates with Needle and Syringe

1. Shave the area over the injection site (the deltoid, biceps, and quadriceps have been used successfully) and wipe with an alcohol swab.
2. Draw the desired volume of DNA (usually between 200 µL and 1 mL) into the syringe.
3. Insert the needle completely into the muscle belly at a 45° angle and inject slowly, withdrawing the needle slowly as you do so (but it should still be in the muscle belly when all DNA has been injected).

3.3. I.M. Injection with Biojector®

1. Anesthetize or tranquilize the animal if necessary and shave the area over the injection site (the deltoid, biceps, and quadriceps have been used successfully). Wipe the site with an alcohol swab.
2. Draw the desired volume of DNA (usually between 200 µL and 1 mL) into the appropriate size of syringe (*see* **Note 6**) using the detachable needle provided for this purpose. Detach the needle and discard.

3. Make sure the CO_2 cartridge in the Biojector injection device has enough pressure (there is an indicator for this), then insert the syringe into the device.
4. Press the end of the syringe firmly against the skin, making sure that it is perpendicular to the surface of the skin, and pull the trigger.

3.4. I.D. Injection

1. Anesthetize or tranquilize the animal if necessary and shave the area over the injection site. In mice, the skin over the anterior abdominal wall or the lower back are frequently used; in primates, the skin over the back or the limbs may be used.
2. Draw DNA solution into the syringe and ensure there are no air bubbles. For each site, the maximum volume that you can comfortably inject is 20–25 µL. Most i.d. injections are given at several (i.e., 2–8) sites.
3. Stretch the skin taut between thumb and index finger and with the syringe almost parallel to the skin, insert the needle with bevel up into the skin just until the bevel is completely within the skin (about 3–4 mm). Slowly rotate the needle 90° so that the bevel is now facing sideways. Slowly inject the DNA solution. Once the desired volume has been injected (it will form a blip on the skin), rotate the needle 90° to the bevel-up position and slowly withdraw (*see* **Note 11**).

4. Notes

1. Mice should be anesthetized since, when awake, they may contract their muscles and squeeze the DNA solution out. Larger animals may best be tranquilized to avoid their struggling or biting during injection.
2. Shaving is essential for good visualization of landmarks when choosing the injection site), and when the injection is in process. Regular veterinary or dog-grooming clippers are suitable for large animals; however, they are a bit large for mice, especially if the teeth are widely spaced. The electric razors that barbers use to trim sideburns are ideal for mice, as they have a small head with closely spaced teeth, and are often rechargeable, so there is no interference with the cord. Manual razors are cumbersome and slow to use and easily cut the delicate skin of a mouse.
3. The insulin syringes, by virtue of their fused needle, have virtually no dead space. They are preferable to the tuberculin syringes, which have about 100 µL of dead space (wasting DNA), and often develop air bubbles, which can make accurate volume delivery difficult. The most accurate volumes are possible with the 0.3 mL insulin syringe, but this has to be reloaded more frequently than the 1 mL syringe. Make sure you have U-100 insulin syringes so that the units will correspond to volume (1 unit = 10 µL). Some countries do not use U-100 insulin and you may not be able to get these syringes at your pharmacy. In this case you may use U-60 syringes (1 unit = 16.7 µL) but you will have to calculate the correct number of units for the desired volume and mark it on the side with a fine-tip marker. Since it is best to have the bevel pointing down and the number scale up to see it, you may have to discard many insulin syringes where the needle has been fused on in the wrong orientation.

4. Most investigators familiar with in vitro transfection methods routinely dissolve their DNA in TE. This could be used for i.d. injection, but should not be used for i.m. injection, since EDTA is a chelating agent and can kill muscle fibers whose function is calcium-dependent. Some investigators simple dissolve the plasmid DNA in water; however, this can lead to relaxing of the DNA from the preferred super-coiled state to a relaxed state. Physiological saline (0.9% NaCl) is fine as long as the solution will not be kept for long as the pH may change. Phosphate-buffered saline may also be used. The maximum concentration possible is about 10 mg/mL.
5. The effective dose of DNA vaccine delivered by i.m. injection to a mouse is usually 10–100 μg. This may be injected unilaterally or divided into two equal parts and injected bilaterally; the latter is preferred because it can compensate for one bad injection. In either event, each TA muscle should be injected with 50 μL. Thus, administration of a 100 μg dose unilaterally would be 50 μL at 2 mg/mL, or bilaterally, a total of 100 μL at 1 mg/mL.
6. The address for Bioject Inc. is 7620 SW Bridgeport Rd., Portland, OR 97224 USA, Tel: (503) 639-7221, FAX: (503) 620-6431. The apparatus costs about $1000 (US). Syringes are single use only and must be purchased separately. CO_2 cartridges provide the propulsion to depress the syringe plunger, and each will do for about 10 injections. Syringes come in various sizes (1–7) which refer to the aperture size and thus the depth of penetration of the liquid. A #1 has the smallest aperture and most superficial delivery, a #7 is largest and deepest. The Bioject device is too powerful for use on mice (even with the #1), but the #1 syringe can be used for i.m. injection of rats. In larger animals, higher numbers may be required for i.m. injection (e.g., #2 for Aotus monkey, #3 for Rhesus macaque, #4–7 for chimpanzee, depending on the site). A too-low number for the species will give a subcutaneous injection, which is not desired. Bioject provides a guide for which syringe size to use for i.m. injection of humans of different size and body weights. This, at best, can be only a guide for other animals which may have a very different thickness of skin or connective tissue surrounding the muscle. The only way to know for sure is to perform a dissection after a trial, or, to avoid having to kill the animal, to take an X-ray after injection of a radio-opaque substance. It is *also* possible to give an i.d. injection with the Biojector using a special spacer and syringe #1.
7. It is optimal to use 6–8-wk-old mice (weight 19–21 g) as they are large enough to inject the 50-μL volume into the TA muscle. Much older mice may give poorer immune responses. For a given antigen, different strains of mice will respond differently, thus the best strain to use may have to be determined empirically, although BALB/c mice seem to respond well to most antigens. Females may give better immune responses than males, and are nicer to work with as they are less aggressive and can easily be group housed.
8. Many people inject DNA vaccines into the quadriceps muscle of the mouse because this is the one muscle that every non-anatomist knows, and there is the misconception that it comprises a large muscle and therefore impossible to miss.

In fact, the quadriceps is four separate muscles with a common tendon of insertion, so there is a risk of injecting between two of these muscle bellies into the interstitial space, which would give a very poor immune response. In addition, the skin of the mouse is loose and fatty over the thigh (it looks like their legs start at the knee), and this increases the risk of injecting the DNA subcutaneously, which will also give poor results. We have obtained best results with the TA muscle, which is the fleshy muscle belly between the knee and the ankle and to the outside of the anterior tibial ridge (if you pull your toes and ankle up, you can feel this muscle contract on yourself). It is larger than any of the individual bellies of the quadriceps, and is immediately below the skin of the leg, which is taut and thin, allowing one to actually see the muscle belly enlarging as you inject the DNA.

9. It is strongly recommended that you first practice your injection using a colored liquid (e.g., India ink or trypan blue). Do this on freshly killed mice and after each injection remove the skin and dissect the muscle out to see how well you did. All of the colored substance should be in the TA, the belly of which will be uniformly colored. If it is under the skin and in the space under the TA muscle, it is not a good injection. Many people inject one side of the body better than the other. To determine if this is the case for you, inject several left and right TA muscles with 10 µg of a luciferase reporter gene construct in a 50 µL volume and assay the muscles 3–5 d later for luciferase activity. With that dose, we routinely measure luciferase activity at 3 d in the TA muscle of about 500,000 relative light units per second per milligram protein ***(2)***. One hint is to use your thumb to push the plunger on the syringe as that will give you much better control than using your index finger. Another hint: if you accidentally pull the needle out after it was inserted but before you injected, try to reinsert the needle into the same hole, otherwise the DNA you inject will come out of this hole. This is another reason for shaving the fur off, as you will never find the hole otherwise.
10. We have found that a volume of 50 µL is ideal for injection of the TA as this allows distribution of the DNA throughout the entire muscle without causing excessive damage. Larger volumes will cause damage and have excessive pressure buildup, which then cause DNA leakage. Smaller volumes can give more variable results.
11. With an i.d. injection, the blip should persist in the skin for at least several minutes. If it disappears quickly or never forms, you have given a subcutaneous injection, which is not likely to work.

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8

Veterinary DNA Vaccines

Sylvia van Drunen Littel-van den Hurk, Ralph P. Braun, and Lorne A. Babiuk

1. Introduction

1.1. Desired Characteristics of Veterinary DNA Vaccines

Vaccination has relied, in general, on two technologies for the production of antigenic material suitable for the generation of a protective immune response; live infectious agents and inactivated or subunit vaccine preparations. Live infectious agents generally are most efficacious, but there is always a risk factor when using this type of vaccine. The major advantage of inactivated and subunit preparations is their safety; however, limited efficacy and duration of immunity and/or high cost may limit the usefulness of these types of vaccines. DNA vaccines represent a new and potentially powerful approach to the development of subunit vaccines.

Veterinary vaccines have their own set of desired characteristics. In addition to the obvious requirement for efficacy, vaccines for veterinary use have to be relatively inexpensive, stable under field conditions and easy to administer. Where meat quality is an issue, as for food-producing animals, the method of delivery is important in that it is undesirable to have needle tracts or vaccine residues in the relevant tissues. This is one of the reasons why the currently preferred route of injection is subcutaneous or intradermal, rather than intramuscular. Furthermore, from a management viewpoint, it is desirable to immunize once and at an early age. Finally, multivalent vaccines are preferred, in order to reduce expenses incurred by vaccination and handling.

Several characteristics of plasmid DNA make it an excellent vector for delivery of genes encoding protective antigens to animals. These include great

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

ease and speed of production, easy quality control, non- integration of the DNA and lack of immunogenicity of the vector itself. In addition, there is evidence that, by using DNA immunization, it may be possible to immunize at an early age with one dose of a multi-component vaccine, and induce long-lived immunity. Thus, over the lifetime of the animal, the expenses incurred by vaccination and treatment could be significantly lower.

1.2. Considerations for Development of Veterinary DNA Vaccines

The primary targets for DNA immunization in livestock animals are infectious agents such as viruses, bacteria, and parasites. The use of DNA immunization to combat autoimmune disease or tumors is not of great relevance for livestock animals because of their short life spans, but may be important for companion animals. To develop an appropriate vaccine it is crucial to understand the pathogenesis of the particular agents. In general, immune responses generated by vaccines are designed to replicate responses found in animals during natural infection. One major advantage of DNA immunization is that this technology is extremely versatile in the types of responses that can be generated, and thus can be more readily adapted to specific situations.

An important initial consideration for DNA immunization is plasmid delivery. Because the site and method of delivery will affect the type of immune response, an appropriate procedure must be chosen that balances the practical aspects of vaccine delivery to large animals, with the desire to generate the most protective response possible. Intramuscular injection, although popular in many animal models, may be undesirable for livestock because of the potential effects on meat quality. Delivery of plasmid into the epithelium (skin or mucosal surfaces) is considered to have the most promise because of the immunocompetence of these tissues. Additionally, because these tissues are the sites of entry by most pathogens, immunization at these sites is expected to be more effective.

Several methods may be used to modulate the type of response generated by DNA immunization. Experiments in which different forms of the antigen are expressed by the plasmid demonstrate that cell-associated antigens (either cytoplasmic or membrane bound) may bias a response more towards a cell-mediated response in comparison to secreted antigens. Secreted antigens, however, can still generate cell-mediated responses, and under certain conditions, show enhanced abilities to generate humoral responses.The method of delivery, and the co-expression of cytokine or immunomodulatory products, may also affect the type of response. We have found that the types of responses produced vary with the antigen, animal species, and route of immunization. Thus, each plasmid must be evaluated separately for its ability to induce the desired responses in the target species and most importantly to protect against challenge from the infectious agent.

Although many antigens have been found to work well when administered by polynucleotide immunization, there are genes that do not function effectively in producing an immune response. When targeting vaccines for specific diseases the choice of antigens may be limited; thus certain genes may need to be re-engineered. Genes from bacteria or RNA viruses are normally not transcribed in the nucleus of animal cells and thus may either contain inappropriate sequences, or lack sequences important for proper functioning in the complex sequence of events involved in RNA transcription, maturation and transport. The presence of a 5' intron such as that commonly found with the human cytomegalovirus immediate early promoter, in our experience, is crucial for the expression of genes from bacteria and RNA viruses. Further adaptations of genes such as altering codon bias, or removing inappropriate post-transcriptional modification sites may also be employed to get stronger expression of functional proteins. Proteins may also be modified to enhance their antigenicity. Targeting proteins to specific cell populations, formation of large particles or chimeras, or co-expression of other genes may enhance immunogenicity of proteins, but these are likely to be specific for each protein and this emphasizes the importance of testing each plasmid separately.

The experimental designs further described in this chapter are primarily based on our experience using a ruminant (sheep, cattle) model. In order to reduce expenses, preliminary screening of a DNA vaccine candidate is conducted in the mouse model to insure that the constructs function in vivo. Once the immunogenicity of the plasmid-expressed proteins has been confirmed, it is generally possible to test the efficacy of a veterinary DNA vaccine using the natural or a very similar host as the animal model. We have used both sheep and cattle as models to test induction of immunity and protection against respiratory disease in cattle. The immune responses observed in outbred animals are generally not as homogeneous as those observed in mice, so larger numbers are required to obtain statistically significant results. In many respects, the methods can be readily adapted to other species such as pigs, goats, horses, and companion animals. Generally, the assays used to analyze the immune responses induced by DNA immunization of large animals are similar to those used in the mouse model, although there are some limitations with respect to availability of reagents. However, one of the most important considerations for using plasmid DNA as a veterinary vaccine, is that the methods and routes of delivery of plasmid DNA developed for the mouse model generally need to be adapted for use in target species. Following a general introduction on the methods and protocols used, specific details are described for immunizing animals with plasmids encoding a protective antigen of bovine herpesvirus-1 (BHV-1), glycoprotein D (gD).

2. Materials

2.1. Construction, Production, and Purification of Plasmids

Use standard molecular biology techniques as described by Sambrook et al. ***(1)***.

2.2. In Vitro Analysis of Protein Production

1. Dulbecco's Minimal Essential Medium (DMEM) and Opti-MEM are available from Gibco-BRL (Grand Island, NY).
2. Fetal bovine serum (FBS) is available from Sigma Chemical Co. (St Louis, MO).
3. Lipofectin is available from Gibco-BRL.

2.3. Delivery of Plasmids

1. Special equipment: the Helios Gene-gun system is available from Bio-Rad Laboratories (Hercules, CA); Minitome cryosectioner is available from Damon/IEC Division (Needham Heights, MA); fluorescent microscope.
2. 18- and 27-Gage needles and tuberculin syringes are available from Becton Dickinson (Franklin Lakes, NJ).
3. A plasmid expressing a green fluorescent protein (GFP) is available from Quantum Biotechnologies (Laval, Québec, Canada).
4. Phosphate-buffered saline (PBS): 0.01 *M* Na_2HPO_4/KH_2PO_4, 0.15 *M* NaCl/KCl, pH 7.3 (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 per liter). Store at room temperature, discard if solution becomes cloudy or precipitates.

2.4. Humoral Immunity

1. Special equipment: an enzyme-linked immunosorbent assay (ELISA) reader is available from Bio-Rad Laboratories (Mississauga, Ontario, Canada).
2. Polystyrene microtiter plates (Immulon 2) are available from Dynatech Laboratories (Chantilly, VA).
3. Coating buffer: 0.01 *M* $Na_2CO_3/NaHCO_3$, pH 9.6 for ELISA; 0.05 *M* $Na_2CO_3/NaHCO_3$, pH 9.8 for B cell ELISPOT. Store at room temperature for up to 2 mo.
4. PBST: PBS with 0.05% Tween-20. Make this fresh for each experiment.
5. Antibodies: appropriate alkaline phosphatase-conjugated secondary antibodies or biotinylated secondary antibody and streptavidin-alkaline phosphatase; available from various companies such as Kirkegaard and Perry Laboratories (Gaithersburg, MD), Zymed (South San Francisco, CA), and Biocan Scientific (Mississauga, Ontario, Canada). Store at 4°C or –20°C.
6. Substrate *p*-nitrophenyl phosphate (PNPP) is available from Sigma Chemical Co.: dilute 100 × PNPP stock in PNPP buffer; 100 × stock: 1 g PNPP in 10 mL 1% diethanolamine buffer. Store at –20°C.
7. Diethanolamine buffer, 1% (10 mL diethanolamine, 990 mL ddH_2O, 1 mL 500 m*M* $MgCl_2$, pH to 9.8 with conc. HCl). Store at 4°C.
8. Stop solution: 0.3 *M* ethylenediaminetetraacetic acid (EDTA) (111.6 g Na_2 EDTA, 13 g NaOH, 800 mL ddH_2O, pH 8.0; make up to 1 L). Store at room temperature.

9. Nitrocellulose plates are available from Millipore (Bedford, MA), or Polyfiltronics Inc. (Rockland, MA).
10. Complete medium for sheep; AIM V (Gibco-BRL), supplemented with 2% FBS (Sigma), 50 μg/mL gentamycin and 5×10^{-5} m*M* 2-mercaptoethanol: for cattle; MEM (Gibco-BRL), supplemented with 10% FBS (Sigma), 2 m*M* L-glutamine, 50 μg/mL gentamycin and 5×10^{-5} m*M* 2-mercaptoethanol. Store at 4°C for up to 6 mo.
11. ELISPOT substrate: SIGMA*FAST*™ (Sigma) 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) tablets. Dissolve one tablet in 10 mL ddH_2O, make fresh.

2.5. Cellular Immunity

1. Special equipment: A Coulter counter is available from Coulter Electronics Inc. (Hialeah, FL) or use a hemocytometer, Cell harvester (available from Skatron Inc., Sterling, VA) and Stereoscope (Olympus S2 series or other); benchtop centrifuge (CS–6R; Beckman).
2. Vacutainer tubes with EDTA(K3) additive, or no additives are available from Beckton Dickinson.
3. Citrate buffer (0.05 *M*) (28.8 g dextrose, 44 g Na-citrate, 16 g citric acid, anhydr., per L). Store at 4°C.
4. PBS/EDTA: PBS with 0.1% Na_2EDTA. Store at room temperature.
5. Isotonic 60% Percoll: Percoll is available from Pharmacia (Mississauga, Ontario, Canada); make Percoll isotonic by adding one part 10X PBS to 9 parts of Percoll; then make 60% by diluting 6 parts Percoll with 4 parts PBS. Store at 4°C.
6. AIM V (Gibco-BRL), supplemented with 2% FBS (Sigma), 50 μg/mL gentamycin and 5×10^{-5} m*M* 2- mercaptoethanol. Store at 4°C for up to 6 mo.
7. Ficoll-Paque PLUS is available from Pharmacia. Store at 4°C.
8. Hanks' Balanced Salt Solution (HBSS): 0.001 *M* Na_2HPO_4/KH_2PO_4, 0.15 *M* NaCl/KCl, 0.001 *M* $CaCl_2$, 0.001 *M* $MgSO_4$, 0.1% dextrose (8 g NaCl, 0.4 g KCl, 0.14 g $CaCl_2$, 0.2 g $MgSO_4$ in 45 mL ddH_2O + 0.06 g Na_2HPO_4 anhydr., 0.06 g KH_2PO_4, 1 g dextrose in 45 mL ddH_2O, mix slowly and check pH 7.3, make up to 1 L). Store at 4°C.
9. MEM (Gibco-BRL), supplemented with 10% FBS (Sigma), 2 m*M* L-glutamine, 50 μg/mL gentamycin and 5×10^{-5} m*M* 2-mercaptoethanol. Store at 4°C for up to 6 mo.
10. Dexamethasone is available from Sigma.
11. 96-well round-bottom and 24-well flat bottom plates (Costar) are available from Fisher Scientific (Nepean, Ontario, Canada).
12. [*methyl*-^{3}H]Thymidine is available from Amersham (Oakville, Ontario, Canada); dilute in AIM V (sheep) or MEM (cattle) to 20 μCi per milliliter and store at 4°C.
13. Filtermats are available from Skatron Inc. (Sterling, VA).
14. Antibodies: rabbit anti-bovine IFNγ (non-commercial), which also cross-reacts with sheep IFNγ.
15. Nitrocellulose plates, antibodies, ELISPOT substrate.

2.6. Challenge and Protection

Special equipment: ULTRA-NEB™ 99 nebulizer is available from DeVilbiss (Barrie, Ontario, Canada).

3. Methods

3.1. Construction, Production and Purification of Plasmids

Plasmids that will direct the expression of the desired gene in animal systems are constructed using standard molecular biology techniques *(1)*. A popular promoter, and one we have most commonly used, is the human cytomegalovirus (HCMV) immediate early promoter that also has a 5' intron, designated intron A. We have also used the promoter from the Rous sarcoma virus (RSV) long terminal repeat, which is a reasonably strong promoter, but which induces lower levels of protein expression in vitro than the HCMV promoter. Genes are inserted into restriction sites that follow the promoter region, and the inserted sequences must contain the start and stop codons of the protein. After the inserted gene the plasmid contains 3' sequences, such as those derived from the bovine growth hormone gene, which include the polyadenylation signal.

1. Obtain genes by restriction digests of other plasmids, or from fragments generated by polymerase chain reaction (PCR) using proofreading polymerases to reduce potential sequence modifications.
2. Cut vector at an appropriate site, and ligate the vector and fragment together by DNA ligase.
3. Transform competent *Escherichia coli* with the ligation mixture.
4. Grow overnight on antibiotic-containing agar plates to select for transformed bacteria.
5. Grow overnight cultures from single colonies, purify the plasmids and characterize by restriction digestion.
6. Verify the construct by in vitro expression of the protein.
7. Other modifications to the genes may be made before or after insertion of the genes into the expression plasmid, with confirmation by in vitro expression and/or sequencing of the relevant regions of the plasmid.
8. To purify large quantities of plasmid, grow overnight bacterial cultures and collect bacteria by centrifugation (*see* **Note 1**). Plasmid may be purified from bacterial pellets by using ion exchange resins such as those obtained from Qiagen (Chatsworth, CA), or by cesium chloride centrifugation *(1)*.
9. Assess plasmid purity by agarose gel electrophoresis *(1)* and by determining the ratio of the absorbance at 260 nm over 280 nm, which should be at least 1.8. Restriction digests are done to verify the identity of the plasmid.

3.2. In Vitro Analysis of Protein Production

Before using plasmid constructs in vivo, protein production is confirmed in vitro by transient transfection assays. A number of different cell lines and transfection agents may be used with satisfactory results. Routinely, we use COS-7 cells. The transfection efficiency in other cells, such as Madin Darby bovine kidney (MDBK) cells, is generally found to be lower than in COS-7 cells.

1. Grow COS-7 cells to 50% confluency in DMEM supplemented with 10% fetal bovine serum (FBS).
2. For a 35-mm tissue culture plate, dilute 20 µg of lipofectin to 100 µL in Opti-MEM and leave at room temperature for 30 min.
3. Dilute the DNA to 2 µg/100 µL of Opti-MEM, mix with 20 µg of lipofectin in 100 µL of Opti-MEM and incubate at room temperature for 15 min (*see* **Note 2**).
4. Wash cells with Opti-MEM, dilute the 200 µL of lipofectin-DNA complex to 1 mL with Opti-MEM and add dropwise to the cells.
5. After 5 h, remove Opti-MEM solution and add fresh DMEM containing 10% FBS to the cells.
6. One to three days later, harvest the transfected cells and supernatant medium and determine the levels of foreign protein production in the cells and/or supernatant medium, depending on whether you are dealing with cell-associated or secreted proteins. This may be done by immunofluorescence ***(2)***, by immunoprecipitation after radio-labeling the cells ***(3)***, by Western blotting ***(4,5)***, or by ELISA ***(6)***. Secreted proteins may have to be concentrated by immunoprecipitation before analysis by Western blotting. The percentage of transfected cells may be determined by FACS analysis ***(2)***.

3.3. Delivery of Plasmids

For all types of delivery, animals are awake and may be restrained in a chute for cattle, with a halter if required, or by an attendant for sheep. Hair may be removed by clippers to assist vaccine delivery. Vaccinating into sites that are infected by pathogens, or share draining lymph nodes with the site of infection, is expected to be the best approach to induce protection.

3.3.1. Intramuscular (IM) Injection

1. Make up plasmid (typically 500 µg) in 2 mL of saline.
2. Using a 3-mL syringe and a 1 1/2 in. 18-gauge needle, inject plasmid into one site in either the gluteal or longisimus dorsal muscle in cattle. We have not used intramuscular injections in sheep (*see* **Note 3**).

3.3.2. Intradermal (ID) Injection

1. Adjust plasmid concentration to 1 mg/mL in saline.
2. Using a 1-mL syringe and 1/2 in. 27-gage needle, inject plasmid into 5 sites (100 µL per site) in the skin on the outside of the ear or the leg of sheep or cattle (*see* **Note 4**).

3.3.3. Gene Gun Delivery

Delivery of plasmid by a gene gun may be desirable because of the lower amounts of DNA that are used. Gold particles are coated with the desired plasmid, and particles are "shot" into the cells of the skin. Use of marker genes (i.e., chloroamphenicol acetyltransferase, β-galactosidase, GFP) is recommended to determine the best conditions for delivery of plasmid. For large animals, sites with little hair, such as around the tail, axillary regions of the legs, or in the mouth, may be chosen. Other sites can be used but may require clipping and/or shaving. Further hair removal can be achieved by using an electric shaver or a blade razor with soap. Some reddening of the skin has at times been found after delivery of gold particles, but the animals have not shown any discomfort (*see* **Note 5**).

We have recently begun to use the Helios gene-gun system (Bio-Rad) that uses a helium discharge to deliver gold particles. To determine the appropriate firing conditions we use a plasmid expressing a GFP to coat the gold particles, and then use a variety of firing conditions, skin treatments, and sites to deliver the DNA-coated gold (*see* **Note 5**).

1. Coat gold particles (1.6 μm) with plasmid as described by the manufacturer.
2. Prepare site(s) by clipping and/or shaving, if required, and fire gold particles coated with GFP plasmid. Firing pressures for the mouse abdomen are between 150–200 psi, and for sheep and cattle, firing pressures of 300–400 psi have been used.
3. Twenty-four hours after delivering plasmid, take skin samples and fix in formalin for 2 h, then wash with PBS and freeze in 30% sucrose at –70°C.
4. Section the frozen samples and view them with a fluorescent microscope, to determine the region and level of gene expression.

3.4. Humoral Immunity

Humoral or antibody responses can be measured in a number of ways. One of the most common methods for detection of antibody responses is ELISA. Although ELISAs are typically used to quantitate antibody levels in the serum, they can also be used with other samples such as nasal secretions. Isotype and subclass of antibodies may also be determined by ELISA, and this can be an important indicator of the type of response generated by the vaccine. Single antibody-secreting cells can be detected using B-cell ELISPOT assays. These assays can help localize the plasma cells secreting antibodies and characterize these antibodies. Neutralization assays, which measure the ability of antibodies to block or neutralize the infectious agent are an important assessment of the functionality of the antibody response. Since neutralization assays are very specific for different pathogens, we do not describe protocols here.

3.4.1. ELISA Protocol for Sheep and Cattle Immunized with Plasmid-Encoding BHV-1 gD

Many different types of ELISAs have been described *(6)*. We use the following general procedure. Polystyrene plates are coated with the appropriate antigen and incubated with serially diluted ovine or bovine sera. Affinity-purified alkaline phosphatase (AP)-conjugated or horseradish peroxidase (HRPO)-conjugated secondary antibodies against total IgG are used as the detecting antibody. The reaction is visualized with PNPP or 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate *[6]*) (ABTS). Antibody isotypes and subclasses are determined in an indirect ELISA using glycoprotein D (gD)-coated plates and isotype- or subclass-specific monoclonal antibodies (*see* **Note 6**).

1. Coat polystyrene microtiter plates with 200 µL of gD (0.25 µg/mL) per well in coating buffer and incubate plates at room temperature overnight. Glycoprotein D is an affinity-purified preparation.
2. Wash the plates 4 times with PBST.
3. Incubate plates for 2 h at room temperature with 200 µL per well of ovine/bovine sera serially diluted in PBST.
4. Wash the plates 4 times with PBST.
5. Incubate plates with 200 µL per well of affinity-purified alkaline phosphatase (AP)-conjugated goat anti-ovine/bovine IgG at a dilution of 1:7,500.
6. Wash the plates 4 times with PBST.
7. Add 150 µL per well of substrate PNPP.
8. After 1 h, add 50 µL per well of 0.3 *M* EDTA to stop the reaction.
9. Read reaction with an ELISA reader at 405/490 nm.

3.4.2. B cell ELISPOT Assay Specific for BHV-1 gD

The presence of antibody secreting B cells may be determined in B-cell ELISPOT *(7)* assays using isolated cells from tissues such as bone marrow, Peyers patches or lymph nodes where plasma cells are typically found. Cells are incubated on nitrocellulose plates that have been coated with the desired antigen. Antibodies secreted from these cells bind to the antigen on the plate. A biotinylated secondary antibody, followed by a streptavidin AP conjugate (or an AP-conjugated secondary antibody) are then added and the antibodies are visualized using a substrate for AP that forms insoluble complexes. These complexes appear as "spots" on the plate, which represent single antibody secreting B cells. **Steps 1–3** are done under sterile conditions.

1. Coat nitrocellulose plate with 150 µL of antigen (4–5 µg/mL gD, 10 µg/mL BHV-1) per well in coating buffer overnight at 4°C. Wells coated with a nonspecific antigen should also be included.

2. Wash plate 3 times with sterile PBST, 3 times with sterile PBS, and then block with 150 µL/well of complete medium at room temperature for 1–2 h (or 30 min at 37°C) prior to plating cells.
3. Obtain a single-cell suspension from your tissue of choice and dilute to 1 and 0.5×10^7 cells/mL in complete medium. Discard blocking solution from the plate and add duplicate or triplicate samples of cells in 100 µL. Incubate the plate on a level surface in a humidified CO_2 incubator at 37°C, for 6–12 h, without disturbing it.
4. Wash the plate 6 times with PBST to remove all cells. Add secondary antibodies specific for different isotypes or IgG subclasses. Make antibody solutions up in PBST containing 1% FBS (or BSA) usually at 1:6000 to 1:8000. Incubate for 1–2 h at room temperature or longer at 4°C. For biotinylated antibodies go to **step 5**, for alkaline phosphatase conjugated antibodies proceed to **step 6**.
5. Wash plate 3 times with PBST. Add streptavidin alkaline phosphatase conjugate in PBST at 1:1000. Incubate at room temperature for 1–2 h.
6. Wash plate 4 times with PBST, and once with PBS. Add the NBT/BCIP substrate, wait for spots to appear (1/4—1h) and wash with ddH_2O before background is too strong. Let the surface dry and then count the spots with a stereoscope.

3.5. Cellular Immunity

A second component of the immune response is the cellular immune response, which is crucial for control of most infectious diseases. One method to measure the cellular response to vaccination is lymphoproliferation. Co-culture of isolated lymphocytes with antigen causes lymphocytes specific for the antigen to proliferate, signifying a response to the vaccine. The second method described here is the measurement of cytokines produced by stimulated T lymphocytes. Specific types of cytokines are secreted in response to infectious agents and vaccines, and the cytokine ELISPOT is able to identify and quantitate the cytokine secretion from T lymphocytes (*see* **Note 7**).

3.5.1. Proliferation Assays for Sheep and Cattle Immunized with Plasmid Encoding BHV-1 gD

In order to detect activated T cells, antigen-specific proliferation assays are performed. In mice, lymphoproliferation is usually measured in splenocytes. However, in sheep and cattle, this assay is generally performed with mononuclear cells isolated from peripheral blood (PBMCs), which is collected in vacutainer tubes containing an anti-coagulant. The PBMCs are isolated and purified from blood by gradient centrifugation, washed, counted and cultured in the presence or absence of antigen. This may be crude or purified antigen;

however, it is important to do a dose titration. After 3 d in culture, the cells are pulsed with [*methyl*- ^{3}H]thymidine. The cells are harvested 18 h later and thymidine uptake is measured by scintillation counting. Proliferative responses are calculated as the means of triplicate wells and expressed as stimulation index or increased cpm over medium control. Depending on the species, different assay conditions are favored. The entire assay is carried out under sterile conditions.

3.5.1.1. Isolation of PBMCs from Whole Ovine Blood

1. Transfer blood (20–40 mL from vacutainer tubes with EDTA[K3] additive) into 50-mL polypropylene centrifuge tubes.
2. Centrifuge at 1500*g* for 20 min at 10°C in a benchtop centrifuge with brake turned off.
3. Remove the buffy-coat layer (white layer of cells, approximately two-thirds from the top) and mix with PBS/EDTA to a total volume of 10 mL using a pre-wet pasteur pipet, then layer over 5 mL isotonic 60% Percoll in a 15-mL polypropylene centrifuge tube.
4. Centrifuge at 2500*g* for 20 min at 20°C with brake off.
5. Remove the mononuclear cell band (white layer in the middle) with a pre-wet pasteur pipet and transfer into another 15 mL centrifuge tube. There should be four distinct layers: erythrocytes and granulocytes (bottom), next Percoll, then lymphocytes, monocytes and platelets forming a distinct white band at the interface between Percoll and PBS.
6. Wash 2 times with 10 mL of PBS/EDTA and 2 times with PBS, and centrifuge cells for 7 min at 250*g*.
7. Resuspend last cell pellet in 10 mL of AIM V containing 2% FBS and count cells with a hemocytometer or Coulter counter.
8. Dilute cells in AIM V containing 2% FBS to obtain 3.5 x 10^6 cells/mL for proliferation and 6×10^6 cells/mL for ELISPOT assay (*see* **Note 8**).

3.5.1.2. Isolation of PBMCs from Whole Bovine Blood

1. Transfer blood (40–50 mL from vacutainer tubes prefilled with 1/10 volume citrate buffer) into 50 mL polypropylene centrifuge tubes.
2. Centrifuge at 1000*g* for 30 min at 20°C with the brake turned off.
3. Remove the buffy coat layer in approx 5 mL, mix it with 5 mL HBSS and layer over 5 mL Ficoll-Paque in a 15-mL polypropylene centrifuge tube.
4. Centrifuge at 1500*g* for 45 min at 20°C in a benchtop centrifuge with the brake off.
5. Remove the mononuclear cell band (white layer in the middle) and transfer it into another 15 mL centrifuge tube.
6. Wash 3 times with 10 mL of HBSS and centrifuge cells for 8 min at 250*g*.
7. Resuspend the last cell pellet in 10 mL of MEM containing 10% FBS and count the cells with a hemocytometer or Coulter counter.
8. Dilute the cells in MEM containing 10% FBS to obtain 3.5×10^6 cells/mL for proliferation and 6×10^6 cells/mL for ELISPOT assay (*see* **Note 8**).

3.5.1.3. Proliferation Assay

1. Prepare the appropriate antigens for stimulation: BHV-1 gD at 0.1 and 1 μg/mL or UV-irradiated BHV-1 at 5×10^5 and 5×10^4 plaque-forming units/mL. To round-bottom 96-well plates add 100 μL of antigen per well. Control wells contain medium only. Dilute the antigens in appropriate medium (AIM V/2% FBS for sheep and MEM/10% FBS for cattle; *see* **Note 9**). Plates may be frozen at –20°C.
2. Prepare a suspension of 3.5×10^6 cells/mL PBMCs, as described in Subheading 3.5.1.1., step 8, and in Subheading 3.5.1.2., step 8.
3. To the antigen-containing plates add 100 μL of cell suspension per well (3.5×10^5 cells). Each sample is tested in triplicate.
4. Incubate in a humidified CO_2 incubator at 37°C for 3 d.
5. Add 20 μL (0.4 μCi) of [*methyl*-^{3}H] thymidine per well and incubate ON (18 h) at 37°C in a humidified CO_2 incubator.
6. Cells may be frozen at this time until they are ready for processing. This freeze-thaw lyses the cells very effectively.
7. Harvest the cells on filtermats using a semi-automatic cell harvester and count the incorporation of [*methyl*- ^{3}H] thymidine in a scintillation counter.
8. Calculate the stimulation index (SI) as counts per minute in the presence of antigen/counts per min in the absence of antigen, where an SI of >2 is considered positive. Alternatively, the level of proliferation may be expressed as increased counts above those obtained with the medium control.

3.5.2. Cytokine ELISPOT for Sheep and Cattle Immunized with Plasmid Encoding BHV-1 gD

ELISPOT assays are used to measure the production of cytokines from activated T cells. Presently, we routinely measure ovine and bovine IFNγ, but this assay may be used to measure any cytokine provided that the appropriate antibodies are available. PBMCs are isolated as described for the proliferation assay, cultured for 24–48 h in the presence and absence of the appropriate antigen, washed and resuspended to the appropriate concentration in culture medium (*see* **Note 9**). Nitrocellulose plates are coated with an IFNγ-specific monoclonal antibody. Different dilutions of PBMCs are added to triplicate wells, such that cytokine secreted from the PBMCs may bind to the antibody coated on the wells. After an overnight incubation, the plates are washed and incubated with IFNγ-specific rabbit serum, followed by a biotinylated secondary antibody and streptavidin- alkaline phosphatase. Substrate consisting of BCIP and NBT is used to visualize the spots, each of which represent single IFN-γ secreting cells. The number of ELISPOTS from antigen-stimulated cells are compared to those produced by unstimulated cells.

Day 1:

1. Isolate PBMCs from ovine or bovine blood.
2. Pre-incubate 6×10^6 cells in 1 mL in the absence or presence of gD (0.5 μg/mL) in 24-well plates for 24–48 h at 37°C in a humidified CO_2 incubator. Set up triplicate wells for each sample.

3. Coat 96-well nitrocellulose plates over night at 4°C with bovine IFNγ-specific antibody at a 1/400 dilution in coating buffer.

DAY 2:

1. Wash nitrocellulose plate 3 times with sterile PBST.
2. Block with AIM V/2% FBS (sheep) or MEM/10% FBS (cattle) for 2 h at 37°C in a humidified CO_2 incubator.
3. Harvest cells, centrifuge for 7–8 min at 250*g*, then count and dilute them to 1×10^7, 5×10^6, and 1×10^6/mL.
4. Wash nitrocellulose plate 3 times with sterile PBST and add 100 µL of cells to triplicate wells.
5. Incubate over night at 37°C on a level shelf in a humidified CO_2 incubator.

DAY 3:

1. Wash the plates 2 times in cold PBST, 1 time in ddH_2O, and 1 time in PBST (allow extra time for the first wash to effectively lyse the cells).
2. Add rabbit anti-bovine IFNγ at a 1:100 dilution in PBST-0.1% BSA.
3. Incubate for 2–4 h at room temperature and wash 3 times in PBST.
4. Add biotinylated rat anti-rabbit IgG at a 1:1000 dilution in PBST-0.1% BSA (*see* **Note 10**).
5. Incubate for 2 h at room temperature and wash 3 times in PBST.
6. Add streptavidin-alkaline phosphatase at a dilution of 1:1000 in PBST-0.1% BSA.
7. Incubate for 2 h at room temperature and wash plates 4 times with PBST, and once with PBS.
8. Add the NBT/BCIP substrate, wait for spots to appear (1/4—1h) and wash with ddH_2O before the background is too strong. Let plates dry and count the spots (cytokine-secreting cells) with a stereoscope.

3.6. Challenge and Protection

Even though immune responses induced in mice and/or the natural host provide a good indication of the efficacy of a particular DNA vaccine, the ultimate goal is to protect the animals from infection, which needs to be tested by challenging them with the appropriate pathogen. This is particularly important in situations where the immune response is not entirely balanced, in which case a protective immune response may not have been generated. In contrast, it is also possible to observe protection in the absence of any measurable immune response. Most of our research on DNA immunization has been carried out with a protective antigen of BHV-1. Although initial efficacy studies may be done in mice and sheep, protection can only be properly assessed in cattle, the natural host of BHV-1 ***(8)***.

1. Challenge: Expose each calf to 4 min of an aerosol of 10^7 pfu/mL of BHV-1 strain 108. We generate the aerosol with a DeVilbiss nebulizer.

2. Following challenge on d 0, attending veterinarians clinically evaluate calves each morning for 10 d. Body weights and rectal temperatures are measured daily. In addition, animals are given a nasal score and a clinical score between 0 (normal) and 4 (severe). The clinical score is based on the levels of depression, conjunctivitis, and rhinitis. Finally, a sick score is determined based on their rectal temperature, depression, conjunctivitis, and rhinitis.
3. Collect nasal secretions daily by cotton swab to determine the amount of virus shed.
4. Obtain blood samples for ELISA and/or cellular assays 10–14 d after challenge.

4. Notes

1. Construction, production, and purification of plasmids: Yields of plasmid may be improved by limiting bacterial growth, such as by growing overnight cultures with reduced aeration. Filling the flask one-half, rather than one-third, full of medium works well in reducing aeration. Other plasmids can be very difficult to grow and may require growth at 30°C rather than 37°C for both the initial screening for positives and large scale growth.
2. In vitro analysis of protein production: The optimal DNA-Lipofectin ratio varies from 1–2 μg of DNA to 2–20 μg of lipofectin and should be experimentally determined for different systems. Other transfection agents like lipofectamine (Gibco-BRL) may be more efficient.
3. Intramuscular (IM) injection: Injections into the muscle do not seem as efficient in large animals as compared to mice, and we find intradermal injections may induce 10-fold stronger immune responses.
4. Intradermal (ID) injection: The injected solution should form blebs in the skin, which differentiates ID from subcutaneous injection. Bleeding should not occur as the DNA solution may leak. There are alternatives to needle injection, such as the Biojector jet injection system (Bioject Inc., Portland, OR), which delivers solution into skin or muscle without a needle. We have no experience with these devices and cannot describe their use other than to acknowledge their availability.
5. Gene gun delivery: Depilatory treatments such as Nair are extreme irritants on sheep and cattle skin and should never be used. To enhance penetration efficiency of the gold particles, pressing scotch tape over the site repeatedly after shaving removes some of the keratin layer and improves penetration of the gold particles.
6. Humoral immunity: Positive and negative sera should be run on each ELISA plate to allow comparison of samples on different plates. ELISA results may be expressed either as absorbance (optical density) values or as titers, which may be expressed as the highest dilution, resulting in a reading of two to three standard deviations above the value of the negative control. Non-specific background staining may show up, which may result from factors like impurities in the antigen preparation, cross-reactivity of the antibodies, or inappropriate dilution of the conjugate. In addition to correcting these problems, it is possible to reduce the background by adding a blocking step after coating of the plates with antigen. FBS, BSA, horse serum, or gelatin at concentrations of 1–10% may be

used as blocking agents. The dilutions for use of commercial conjugates are usually provided, but may have to be experimentally determined, specifically if the assay does not appear to be sensitive enough or if there is non-specific background.

7. Cellular immunity: It is very important to include positive and negative animals in all cellular immunity assays because of the variability inherent in these experiments. This variability is both due to the outbred nature of the animals and to the nature of the sample. In contrast to splenocytes, the PBMCs isolated from peripheral blood are subject to cell trafficking, and therefore, contain a small, possibly variable, fraction of the activated lymphocytes. If the animals are to be euthanized, spleens and/or local lymph nodes may be collected at the end of the experiment and processed to assess antigen-specific proliferation and/or cytokine production.
8. Cells, once isolated, should be processed and plated as quickly as possible, as they begin to clump if left standing too long. Leaving them at room temperature, and keeping them fairly dilute can help reduce clumping. Severe clumping may be reversed somewhat by incubation at 37°C.
9. For cattle and sheep over the age of 3 mo dexamethasone (1–10 n*M*) added to the culture medium for proliferation, and ELISPOT assays can help to control non-specific stimulation.
10. The dilutions for use of commercial conjugates are usually provided, but may have to be experimentally determined.

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9

DNA-Based Immunization of Neonatal Mice

Cynthia L. Brazolot Millan and Heather L. Davis

1. Introduction

Immunization of neonates carries three inherent questions: (i) Is the neonatal immune system mature enough to elicit an immune response, and if not, how early can immunization be carried out? (ii) If an immune response is not elicited, will tolerance be induced? (iii) Can offspring of immune mothers be immunized against an antigen despite high levels of passively transferred maternal antibodies to that antigen?

Despite the earlier fear that the immature neonatal immune system would be tolerized by too early delivery of antigens, and at least one report that DNA-based immunization of newborn mice did in fact lead to tolerance ***(1)***, a number of recent publications have demonstrated that the neonatal murine immune system is indeed immunocompetent and can respond appropriately to vaccination. Various antigens, delivered as purified protein ***(2)***, DNA vaccines ***(3–5)***, live retrovirus ***(6)***, or histocompatibility antigens on transplanted cells ***(7)***, have been shown to induce both humoral and cellular immune responses in mice immunized within the first few days of life.

Similarly, in our own laboratory, we have successfully immunized newborn mice with plasmid DNA vaccines encoding the hepatitis B virus (HBV) surface antigen (HBsAg). With this antigen, we find that the DNA vaccine induces antibodies against HBsAg (anti-HBs) in mice injected as early as 12 h after birth, whereas immunization with recombinant HBsAg protein does not (Brazolot Millan, et al., in press). The strength of the humoral response generated by DNA-based immunization on the first day of life is the same as that with injection of DNA at 3 or 7 d. By comparison, injection of recombinant protein, with or without alum, at d 1, 3, or 7 does not elicit a detectable humoral immune response, nor does it prime a response, even at a dose sufficient

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

to immunize adult mice. Furthermore, we have evidence that DNA- (but not protein-) based immunization of neonates born to anti-HBs positive mothers is capable of priming an immune response, despite the presence of high levels of passively transferred maternal antibodies. Although a primary humoral response was not detected in these neonates (i.e., no measurable anti-HBs after the maternally derived antibodies had disappeared), a pronounced anamnestic response was observed in the mice as young adults upon "challenge" with recombinant HBsAg protein (Brazolot Millan, et al., in press). Thus, in our HBV model, DNA-based immunization does not induce tolerance in neonatal mice and, in fact, it can be used to immunize very young neonates, whether or not they harbor passively transferred anti-HBs antibodies.

2. Materials

1. Vehicle to dissolve DNA such as 0.15 *M* NaCl (Sigma, St. Louis, MO).
2. Alhydrogel "85" (Superfos Biosector, Vedbaek, Denmark; obtained through Cedarlane, Hornby, Ontario, Canada).
3. Heparin, 50 units/mL (Sigma).
4. Insulin syringes ($^3/_{10}$ cc) with an attached 29G$^1/_2$ needle (Becton Dickinson, Franklin Lakes, NJ).
5. Plasmid DNA prepared using Qiagen anion-exchange chromatography columns (Qiagen GmbH, Hilden, Germany). In our HBV vaccine model, we use a plasmid construct encoding the major protein (S) of the HBV envelope under the control of the cytomegalovirus (CMV) immediate early promoter (pCMV-S) ***(8)*** (*see* **Note 1**).
6. Protein antigen in suitable medium for injection (i.e., in saline, alone or adsorbed to alum). For our HBV vaccine model we use recombinant HBsAg, purified from yeast (Genzyme Diagnostics, San Carlos, CA) (*see* **Note 1**).

3. Method

3.1. Formulations

1. Dilute plasmid DNA (stored at –20°C until time of immunization) in physiological saline (0.15 *M* NaCl) to desired concentration and keep on ice until injected into the mice. We use pCMV-S DNA ***(8)*** at 0.5 mg/mL.
2. Dilute protein in physiological saline (0.15 *M* NaCl) to desired concentration. If an adjuvant is desired, add alum (as Alhydrogel; 2.5 µL/µg protein), mix, then maintain on ice for 30 min prior to immunization. We use recombinant HBsAg at 50 µg/mL.

3.2. Immunizations

1. Pups may be immunized very soon after birth. We typically carry this out within the first 12 h of life (*see* **Note 2**). Wear gloves at all times while handling pups and their mothers.
2. First remove the mother from the cage and keep her anesthetized with Halothane gas (Halocarbon Laboratories, River Edge, NJ) while manipulating her offspring.

3. Remove all pups from the nest but do not anesthetize them for injection. Take one pup at a time and drape it over your index finger (of the opposite hand than you will inject with). Use your thumb to restrain the hind legs against your index finger and your middle finger to anchor the head and upper body. Insert the needle through the lateral thigh (in a posterior-to-anterior direction) so that the point ends in the quadriceps muscle mass (anterior thigh). Inject the DNA vaccine in 10 µL per site. We typically carry out bilateral injections and divide the DNA vaccine dose equally between the two injection sites, but still at a volume of 10 µL/site (*see* **Notes 3** and **4**).
4. Place the immunized pup back into the nest in the cage. Once all manipulations are complete, the still-anesthetized mother is placed on top of the litter to recover there.

3.3. Blood Collection

1. Blood can be collected from pups beginning at 1 week following immunization. One-week-old mice are sacrificed by decapitation, and the blood is collected into a microfuge tube containing 10 µL of heparin (50 units heparin/mL) (*see* **Note 5**).
2. Beginning at 2 wk of age, the mice do not have to be killed as it is possible to obtain blood by retro-orbital puncture using a heparinized Pasteur pipet (*see* **Note 5**).

4. Notes

1. We find that a DNA vaccine encoding HBsAg successfully elicits antigen-specific immune responses even in the immature immune system of neonatal mice. Other investigators report similar successes for DNA vaccines encoding several other antigens ***(3–5)***, however, tolerization was reported for a DNA vaccine encoding a malarial antigen ***(1)***. Thus, it is not yet clear how many and which DNA vaccines will work in neonatal mice. Using the reagents and techniques described above, we routinely achieve 80–90% success in immunizing neonates with HBsAg-expressing DNA (pCMV-S). Thus, investigators could use this model to control for their injection technique if they find that their DNA vaccine expressing a different antigen does not appear to be working in their hands.
2. In our experience of handling newborn pups and their mothers, we rarely observe maternal rejection of offspring. Our practice is that we do not handle the pups until the mother has finished giving birth to her entire litter and has reassembled a nest in the cage and retrieved the pups into it. Also, the mother never sees us handle her pups as she is removed from the cage and kept anesthetized while the pups are injected, and we then place the still-anesthetized mother directly onto her litter following all injections. These precautions seem to adequately prevent any awareness of disturbance. To avoid transferring human odors to the pups, they are handled only with gloved hands. However, if problems consistently arise with a mother rejecting her manipulated offspring, a small dab of Vicks VapoRub placed on her nose will prevent her from detecting foreign odors on her pups.
3. Young mouse pups are fairly translucent and the introduction of the needle should be observed carefully. It is easy to insert the needle too far and thus exit the leg from the other side. Nevertheless, the needle must also be inserted far enough.

The entire bevel of the needle must be within the limb, and an extra 1–2 mm depth is advisable.

4. Care must be taken not to stretch the body of the pup too tightly over your finger or to apply too much pressure during immobilization as this will force expulsion of the DNA as soon as you inject it. This can also happen if the DNA is injected too fast or the needle is withdrawn too quickly. Thus, the plunger of the syringe should be depressed relatively slowly, and the needle should be left *in situ* for a few seconds following immunization, prior to being withdrawn.
5. Approximately 50 µL of blood may be routinely obtained from a 1-wk-old mouse; however, it must be killed (by decapitation) in order to recover the blood. Beginning at 2 wk of age, mice do not have to be killed but rather can be bled by retro-orbital puncture. For 2- and 3-wk-old mice, only 100–200 µL of whole blood should be removed from the pup; however, from 4 wk of age onward, 300–500 µL of blood may be routinely removed. Although 2- and 3-wk-old mice are small, their eyes may be bled using Pasteur pipets, without risk of blinding. Capillary tubes or heparinized glass micropipets may be used, but in our experience, it is unnecessary to attempt blood collection with the smaller and more fragile tubes, as Pasteur pipets work quite adequately. It is worth cautioning that young mice often seem to take a long time to succumb to the effects of halothane, but once anaesthetized, they seem to become deeply anesthetized (dangerously so), very quickly. As well, neonatal mice that appear to be over-anesthetized can recover amazingly fast when removed from the gas.

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10

Intramuscular Injection of DNA Vaccines in Fish

Joël Heppell and Heather L. Davis

1. Introduction

The DNA-based immunization technology has only been applied to fish very recently. Though a preliminary study showing reporter gene expression in fish muscles was published in 1991 ***(1)***, the first demonstration of an immune response to plasmid-encoded antigen was not reported until five years later ***(2)***. Thus, relatively little is known about the administration methods, immunological responses, and protective efficacy of DNA vaccines in aquatic animals. In some instances, results obtained with other classes of vertebrates (mammals and birds) can be applied directly to fish, but because of physiological, immunological and structural differences between these animals, this is not necessarily true. For example, it was shown recently that short specific DNA sequences (CpG motifs), in a particular nucleotide context, act as immunostimulants ***(3,4)***. No study has been reported yet assessing these immunostimulatory sequences in fish, but it would be unlikely that the same sequences are effective in all animal species (Weeratna et al., this volume).

Different techniques have been used to introduce DNA into eggs and embryos of fish to create transgenic animals. For vaccination purposes, direct injection with a needle is the simplest and most effective method. Particle bombardment (i.e., biolistic introduction of DNA-coated gold particles using a gene gun which shoots gold particles at high speed into the animal's superficial tissue) has also been tested, but it was shown to be less effective ***(5)***. Though the gene gun technique could be improved for laboratory use, it is unlikely that it will be applied for mass vaccination, due to economical and practical constraints.

Other routes of administration have been used in fish with antigen-based vaccines ***(6)***. Successful transfection of fry or adult fish cells using such methods as immersion (by dip, bath, or spray), or oral administration, with pure

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

DNA solution, or mixed with carrier molecules or microcapsules, has never been reported. Experiments conducted in our laboratory showed that immersion of fish in naked DNA solution (with or without prior hyperosmotic shock), direct application on the gills, and delivery into the digestive tract by intubation do not provide satisfactory results. However, it will be important to develop these alternative methods of vaccination to immunize small fish on a commercial scale.

This chapter summarizes methods for direct transfer of plasmid DNA into fish for the purpose of vaccination. In particular, plasmid constructs, administration methods, and expression of injected genes are discussed in light of results obtained with mammals.

2. Materials

1. Appropriate anesthesia for fish (tricaine or benzocaine).
2. U-100 insulin syringes (0.3 cc) with attached 29G1/2 needle (Becton Dickinson, Franklin Lakes, NJ) (*see* **Note 1**).
3. Polyethylene tubing (PE 20, ID = 0.38).
4. Purified plasmid DNA dissolved in sterile saline (0.15 *M* NaCl) or phosphate-buffered saline (PBS) (*see* **Notes 2** and **3**).

3. Method

1. Dilute DNA solution, if needed, in the same solution used to dissolve it. For each fish, 50 μg of plasmid DNA diluted in a volume of 10 to 25 μL is appropriate for initial testing of the vaccine (*see* **Notes 4** and **5**).
2. Prepare appropriate number of syringes by inserting needles in polyethylene tubing and cutting it to a length such that only 2–3 mm of the needle protrudes. The tubing will prevent the needle from going too deep into the fish tissue (*see* **Note 6**).
3. Aspirate DNA solution into syringes and eliminate air bubbles. Syringes can be refilled a few times if several fish have to be injected with the same vaccine.
4. Anesthetize fish by immersion in 0.0084% tricaine (also called MS-222 or 3-aminobenzoic acid ethyl ester) or 0.01% benzocaine (*see* **Note 7**).
5. The DNA vaccine is to be injected intramuscularly. Remove fish from the anesthetic bath and insert needle in the flanks, approximately midway between the dorsal fin and the lateral line, with the needle pointing toward the tail. Inject slowly, and wait for 1–2 s before withdrawing the needle, to prevent loss of DNA solution (*see* **Notes 8–10**).
6. Immediately transfer fish into well oxygenated water for recovery and test for immune response when appropriate (*see* **Note 11**).

4. Notes

1. Insulin syringes can be used to deliver small volumes accurately. Alternatively, when large numbers of fish have to be injected, it might be more convenient to use a device that can automatically deliver pre-selected volumes repeatedly, without having to refill the device. A repeater pipet fitted with a 28G needle is a good alternative.

Table 1
List of Promoters Tested by Injection into Fish Muscle

Promoter	Fish species	Reference
Glucocorticoid-responsive mouse mammary tumor virus (MMTV)	Rainbow trout (*Oncorhynchus mykiss*)	*7*
Cytomegalovirus (CMV) immediate early (alone or with translational promoter)	Rainbow trout (*Oncorhynchus mykiss*)	*7,8*
	Zebra fish (*Brachydanio rerio*)	*8*
Carp ß-actin	Rainbow trout (*Oncorhynchus mykiss*)	*7*
	Tilapia (*Oreochromis niloticus*)	*9*
SV40 early	Common carp (*Cyprinus carpio*)	*1*
Rabbit ß-cardiac myosin heavy chain (MHC)	Common carp (*Cyprinus carpio*)	*1*
Human MxA	Common carp (*Cyprinus carpio*)	*1*
Artificial (based on human MxA)	Common carp (*Cyprinus carpio*)	*1*
Herpes simplex virus thymidine kinase (tk) with the CMV enhancer	Rainbow trout (*Oncorhynchus mykiss*)	*5*

2. DNA should be dissolved and diluted to the desired concentration with sterile isotonic and non-toxic buffer to avoid any extended or permanent tissue damage.
3. Plasmid constructs currently used to express foreign genes in fry and adult fish tissues are similar to those employed for mammals. Several promoters have been tested in fish (**Table 1**), and although expression of reporter genes under control of these promoters was detected in all cases, efficiency varied greatly. Comparison of the different studies to rank promoters according to the expression level obtained is not possible, because conditions under which they were tested are too different, but, overall, the cytomegalovirus promoter seemed to give very good results ***(5,7,8)***. No comparisons of the other controlling elements (e.g., polyadenylation signal and introns) have been reported, but there is no indication that gene expression from plasmid DNA in piscine muscle cells will be significantly different from that in mammalian myofibers.
4. The total volume of DNA to inject per fish should be kept small. We have shown that dilution of DNA in larger volumes increases consistency between individual fish, but decreases the expression level of the reporter gene (**Table 2**). Anderson et al. ***(7)*** tested larger volumes (100 and 200 µL) and came to the same conclusion. The greater variations observed between individual fish with smaller volumes could come from the inaccuracy of the delivering device and/or the loss of DNA. When very small volumes of DNA are injected into muscles, great care should be taken to prevent DNA from leaking out of the animal when the needle is retracted. Hence, it is important that fish are well anesthetized before injections.

Table 2
Total Luciferase Activity in Rainbow Trout Muscle Injected with 1 μg of Luciferase-Encoding Plasmid, According to the Volume of Saline (0.15 *M* NaCl) Used to Suspend DNA

Volume of DNA injected per fish (μL)	Luciferase activity (RLU/sec)[a]
5	416 554 ± 173 583
10	327 621 ± 89 936
25	174 525 ± 31 206
50	76 867 ± 14 368
100	47 451 ± 13 476

[a]Mean luciferase activity calculated in relative light unit per second in muscle ± standard error of the mean. For each volume tested, n ≥ 5.

Table 3
Optimal Dose of DNA to Inject Intramuscularly for Maximum Expression of Reporter Genes

Reporter gene used	Optimal dose of DNA (μg)	Reference
CAT[a]	50	*1*
luciferase	25	*7*
luciferase	50	*5*
luciferase	1–10	*8*

[a]Chloramphenicol acetyltransferase.

5. The dose-response of foreign gene expression following intramuscular injection has been studied in different fish species (**Table 3**). Maximum activity for a given gene is reached with the same dose of plasmid DNA, independently of the promoter used ***(7)***. Injection of larger amounts of DNA did not result in increased expression levels. DNA is probably taken up only by a limited number of cells, either due to cell membrane damage or other causes. To date, there is no indication that results will vary greatly between fish of different species or size, but age could be an important factor ***(1)***. Nevertheless, the optimal dose of DNA is not directly proportional to the size of animals to be immunized. To test the immune response to a plasmid-encoded antigen, a starting dose of 25–50 μg DNA per fish appears to be sufficient.
6. To minimize tissue damage and injuries to animals, we prefer to limit penetration of the needle to 2–3 mm. This is particularly important when very small fish (<3 g) have to be injected. The reported depth of injection varies from 2 mm ***(5)*** to 10 mm ***(1)***. Larger fish can be injected without depth-limiting devices.
7. Fish have to be completely anesthetized to make handling easier, and to avoid muscle contractions which could expel DNA out of the animal. This is very important when small volumes are injected. Avoid leaving fish for too long in the anesthetic bath as they may die.

8. Various routes of injection were tested, but intramuscular administration seems to work best. Very low or no expression of reporter genes was detected when DNA was injected in the peritoneum or the gills (5; J. Heppell and H. L. Davis, unpublished results).
9. The most common sites for intramuscular injection are the flanks, usually midpoint between the dorsal fin and the lateral line, or immediately rostroventral to the dorsal fin. Injections close to the caudal fin give similar expression level, but are less convenient and less consistent, possibly due to loss of DNA solution caused by manipulation of fish or muscular contraction when animals come out of anesthesia.
10. Before insertion of the needle, the site of injection can be wiped with cotton swabs or other clean absorbent material, to remove excess mucus. On very small fish, mucus can make the needle slip on the fish scales, causing injuries to the animal.
11. Experiments with the luciferase reporter gene showed that expression can last for several weeks ***(5,7,8)***. This was shown to be sufficient for induction of protective immune responses ***(2)***. Specific antibodies can be detected in sera from DNA-vaccinated fish, but this is not an absolute prediction of protection. Delay before antibodies can be detected varies according to the species and temperature. In rainbow trout kept at 12°C, specific antibodies were detected by ELISA starting at 2 wk post-injection (J. Heppell and H. L. Davis, unpublished results).

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11

Development of DNA Vaccines for Salmonid Fish

Eric D. Anderson and Jo-Ann C. Leong

1. Introduction

Vaccination of fish against many different pathogenic organisms has made it possible to rear Atlantic salmon in net pen cages and produce fish commercially around the world. In fact, vaccine use is critical for the continued growth of the aquaculture industry and researchers are continually looking to develop new and improved vaccines for a wide variety of fish pathogens. Fish vaccines have been formulated from killed or attenuated pathogens, recombinant viral proteins or peptides, and most recently, plasmid DNA encoding viral proteins *(1–7)*. The use of DNA vaccines for the control of viral diseases of fish is particularly appealing since this type of vaccine eliminates the need to purify the viral pathogen or immunoprotective antigen. Other advantages are the elimination of any possibility of reversion to virulence since the DNA vaccine encodes only a portion of the viral genome, the relative stability of the DNA preparation; the ease of DNA preparation; and, importantly, the elicitation of a robust immune response in fish.

We have found that injection of fish with a DNA vaccine (pCMV4-G) encoding the glycoprotein gene of infectious hematopoietic necrosis virus (IHNV), a pathogenic fish rhabdovirus, induces a strong protective immune response to virus challenge *(5,6)*. The process of developing this vaccine is outlined in **Fig. 1**. First, the experimental parameters that governed the expression of foreign genes in fish after the injection of naked DNA were determined. These parameters included DNA dose, volume of injection fluid, first appearance of expressed protein, duration of expression, and tissues of expression *(5)*. Then, the efficacy of the DNA vaccine for IHNV was tested in rainbow trout. In this case, antibody production as well as protection against the lethal

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

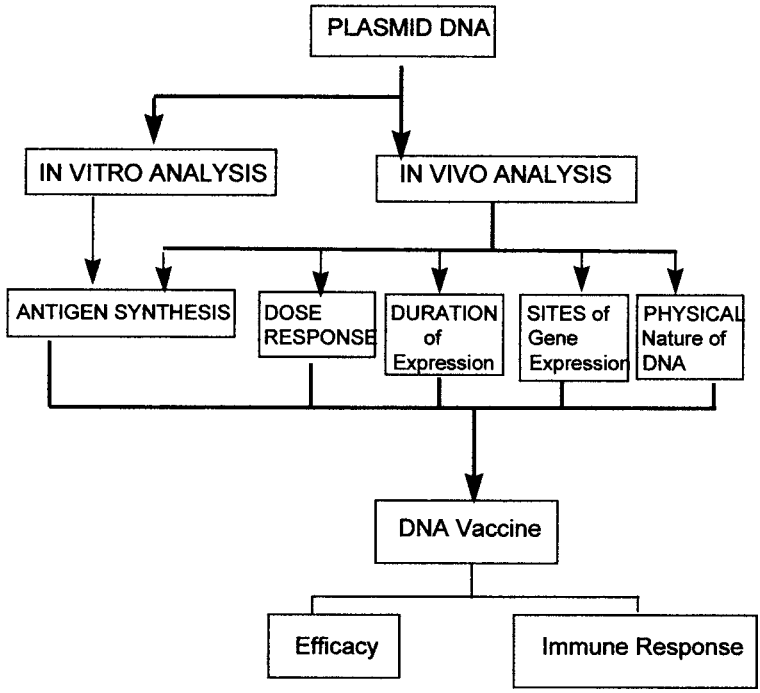

Fig. 1. Flow diagram showing the steps in developing a DNA vaccine.

effects of virus infection were used as measurements of vaccine efficacy *(6)*. In this chapter, we summarize the practical considerations, experimental procedures, and obstacles that may be encountered in developing a DNA vaccine for fish.

1.1. Transgene Expression in Rainbow Trout (Oncorhynchus mykiss)

The selection of the plasmid that will encode the antigen (Ag) or reporter protein is the initial step in DNA vaccine design. While the criteria used in selecting a plasmid may vary, the selection will in most cases be based on the properties of the promoter/enhancer that will drive Ag expression. Any requirements for expression in specific tissues, the inducibility and strength of the promoter, and possible licensing prohibitions on the use of foreign DNA in food animals all have bearing on the selection of the plasmid vector used in the vaccine construction. Our studies indicate that the level of gene expression following the direct injection of DNA into rainbow trout depends on the promoter used to drive the foreign gene expression. The cytomegalovirus immediate early promoter (CMV-IEP) consistently produced very high levels of

luciferase activity in fish muscle tissue. This result with CMV-IEP is consistent with the results obtained in a number of different animal systems ***(8)*** and is the probable reason that plasmids containing the CMV-IEP are widely used for DNA vaccines.

Identifying the minimum effective vaccine dose is important for economic as well as safety reasons. In practical terms, small fish are vaccinated in groups of hundreds to thousands of animals. The use of a single small dose of DNA can make the operation less cumbersome and ensure that the cost of vaccine preparation is not prohibitive. When the reporter plasmid, pCMV-Luc, was used to determine what quantity of DNA was required for maximum luciferase activity in rainbow trout, we found that between 25–50 μg of DNA injected into the dorsal musculature of 50 g rainbow trout produced maximum luciferase activity ***(5)***. There was considerable variability in luciferase activity when fish were injected with 100 μL of solution containing 25 μg of DNA. When the same amount of DNA was injected in a larger volume, i.e., 200 μL, reproducible measurements of luciferase activity were obtained. Thus, larger injection volume provided greater reproducibility. In studies that measured vaccine efficacy through the induction of protective immune response, the smallest dose tested was 10 μg vaccine injected in a 200 μL volume in 1 g fish ***(6)***. The minimum effective dose was not determined in these studies. Heppell and colleagues ***(9)*** have shown recently that DNA vaccine doses at 0.1 μg in 5 μL will also induce protective immunity in rainbow trout at 1 g average weight.

The duration of plasmid driven-antigen production required to induce a strong protective immune response in fish is unknown. Because continuous antigen production may lead to tissue necrosis or immune tolerance, and because the continued presence of plasmid DNA may be perceived as problematic in animals destined for human consumption, the length of time that the plasmid DNA and its encoded antigen persisted in transfected cells in fish was determined. When 1 and 50 g fish were injected with 10 or 25 μg of pCMV-Luc, maximum luciferase activity was observed 7 d later. For the small fish, luciferase activity declined to approximately 10% of the maximum over a 115-d test period. For the larger fish, the activity remained near 50% of the maximum for 28 d and diminished to just 10-fold above background by 63 d postinjection when the experiment was terminated. Thus, in each case, the presence of antigen in transfected muscle tissue was long-lived. No gross histological abnormalities were observed during the experiments and there was no evidence of immune tolerance. Despite these initial findings that DNA vaccines do not have any ill effects on the injected fish, safety concerns may lead to the design of future vaccines that incorporate regulatory components limiting the duration of antigen and DNA persistence in tissues.

The sites of expression and the fate of the injected DNA should be tested when developing the vaccine. We accomplished this by using two plasmids, pCMVßGal with the reporter gene ß-galactosidase fused to the CMV-IEP, and pCMV4-Luc. In 50-g fish injected with 25 µg of pCMVßGal in 200 µL, reporter activity was confined to muscle tissues in the area surrounding the path of injection. When 1-g fish were injected with 10 µg of pCMVßGal in 200 µL, evidence of the enzyme was found along the injection path and in scattered muscle cells up to 5 mm distant from the site of injection. When the small fish were injected with 10 µg of pCMV4-Luc, luciferase activity was observed in numerous tissues at 3–5 d postinjection. The amount of reporter protein activity recovered from the tissues was variable and maximum activity was recovered from muscle cells along the injection path. The role that these transfected tissues play in the overall efficacy of the DNA vaccine is unknown but it may be significant that reporter activity was also recovered from two primary fish lymphoid organs, the kidney and spleen. Thus, when injecting small fish with DNA, special consideration should be placed on determining which cell types are transfected with the DNA vaccine. The physical nature of the DNA in the transfected cells of the internal organs is unknown. However, in transfected muscle cells, we found that the vaccine was maintained as non-replicating, supercoiled plasmid DNA. We found no evidence of plasmid integration into the host genome.

1.2. DNA Vaccination

Methods for testing vaccine efficacy and the immune response elicited by the vaccine are necessarily dependent upon the system under investigation. Several excellent sources of information on general vaccine testing in fish are available (***10***; *see* **Note 1**). We will discuss here only protocols and results relevant to the development of a DNA vaccine for fish.

Several basic points should be considered when choosing a model system for the development of a fish DNA vaccine. There should be available a cloned gene(s) encoding the immunoprotective antigen(s) for the pathogen, i.e., the antigen should be identified and immunological reagents for detecting the antigen should be available. It also is essential that the progression of the disease caused by the pathogen under investigation is well characterized and, importantly, reproducible in the laboratory. In general, the efficacy of fish vaccines is relatively easy to determine because salmonid fish can be reared and vaccinated in large numbers. After vaccination and a suitable period of time for immune response development (3–4 wk in salmonid fish species), the vaccinated fish are challenged with a lethal dose of the pathogen and efficacy is measured by the difference in the percentage of surviving fish in the vacci-

nated vs control fish. The major limitation to our understanding of how fish vaccines work is that there are very few tools available to examine the fish immune response at the cellular level. This limitation is particularly relevant because efficacy and the cellular immune response has been shown to be directly related, for DNA vaccines in mammals ***(8)***.

The first step taken in the development of a DNA vaccine for IHNV was the demonstration that the G and nucleocapsid protein (N) DNA vaccines, pCMV-G and pCMV-N, were functional. Expression of the IHNV G and N proteins by pCMV-G and pCMV-N was confirmed by immunohistochemical staining of epithelioma papulosum cyprini (EPC) cells transiently transfected with the respective plasmids. The transfected cells reacted with the appropriate monoclonal antibodies to N or G. Protein synthesis was similarly confirmed in muscle tissue in vivo in thin sections prepared from vaccinated fish. Both the pCMV-G and pCMV-N transfected cells contained viral protein throughout the cytoplasm. When the pCMV-G transfected cells were briefly reacted with the color substrate, the G protein was seen primarily at the cell surface. These results demonstrated that the plasmids was correctly constructed.

The fish humoral immune response induced by each DNA vaccine was examined in 1 g rainbow trout inoculated with a solution containing 5 μg each of pCMV-G and pCMV-N, or 10 μg of pCMV-G, or 10 μg of pCMV-N. The appropriate control plasmid, pCMV-Luc, was also included in the study. There were high levels of IHNV binding antibody (ELISA) titers in serum collected 4-wk-postinjection in the fish receiving the combination of pCMV-G and pCMV-N. These titers remained high throughout the 14-wk test period. Anti-IHNV antibody in fish receiving pCMV-G was first observed at 6-wk-postinjection with a peak titer at 8 wk. A comparison of anti-IHNV antibody titers between the group receiving only pCMV-G versus the group receiving the combination vaccine at 8-wk-postinjection showed that the serum from the co-injected group contained an average of 26-fold greater anti-IHNV antibody titer than the fish receiving pCMV-G alone. However, the increase in antibody titer in the co-injected group did not correlate with an increase in vaccine efficacy as measured by survival after lethal virus challenge. The relative percentage survival of fish following challenge with IHNV was 75% for pCMV-G and 78% for pCMV-G with pCMV-N. Only the sera from fish that received pCMV-G contained IHNV-neutralizing activity and the activity was very low and near the limits of assay detection. This would suggest that the cellular immune response in vaccinated fish is the important component in the immune response that confers protection. Alternatively, in vitro measurements of virus neutralizing antibodies may not be an accurate predictor of in vivo neutralizing activity.

2. Materials

2.1. Plasmids and DNA Purification

1. Special equipment and materials: Plasmid DNA, spectrophotometer, standard bacteriological supplies, electrophoresis apparatus, and materials/reagents necessary for analyzing electrophoresed DNA.
2. For initial testing, we recommend using either pCDNA3 (Invitrogen) or a similar plasmid. These plasmids should be engineered to encode either the antigen of choice, or one of the reporter proteins, ß-galactosidase or luciferase. These reporter proteins provide a sensitive assay for expression, have low endogenous background in fish, and are relatively simple to measure.
3. LB broth/agar: For 1 L of media, add 10 g bacto-tryptone, 5 g bacto-yeast extract, and 5 g NaCl to double-distilled water (ddH_2O). Adjust the pH to 7.5 with sodium hydroxide. Sterilize by autoclaving. For solid media add 15 g agar to 1 L of LB broth and autoclave. When the autoclaved solution cools to 50–55°C add 50 µg/mL ampicillin when appropriate.
4. 1000 × ampicillin stock: Dissolve 0.5 g ampicillin in 10 mL 70% ethanol. Filter sterilize the solution and dispense into 1 mL volumes and store at –20°C.
5. TE: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4. This reagent should be made with the highest grade water available and this should be at least ddH_2O although triple distilled, reverse osmosis-treated water is better. Autoclave, filter sterilize, and dispense in small volumes. Store at room temperature or –20°C.
6. 3 *M* sodium acetate, pH 5.2. Sterilize the solution by autoclaving and dispense into 10 mL portions and store at room temperature.
7. 95% and 70% ethanol.
8. DNA purification column and reagents: For DNA purification, use the Qiagen-tip 500 (Qiagen). The necessary reagents are supplied with the columns or can be prepared as outlined in the Qiagen-tip 500 manual.

2.2. Cell Transfection

1. Special equipment and materials: A standard cell culture facility and materials must be available.
2. Fish cells: Two different cell lines are recommended, chinook salmon embryo cells CHSE-214 [11; ATCC (American Type Culture Collection) cat no. CRL 1681], and epithelioma papulosum cyprini (EPC) cells derived from the common carp ***(12)***. The EPC cells are not available through ATCC but can be obtained from us upon request.
3. Fish cell line maintenance medium: Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 m*M* L-glutamine.
4. Transfection medium: Opti-MEM 1 reduced serum medium (Gibco-BRL, Gaithersburg, MD).
5. PBS: 0.14 *M* NaCl, 10 m*M* sodium phosphate, pH 7.4. Sterilize the PBS by passage through a 0.2 µm filter and store the solution at room temperature.

6. Lipid: For transfection we commonly use lipofectamine (Gibco-BRL). In our hands, lipid-facilitated transfection of fish cells is much more efficient than calcium chloride methods.

2.3. Analysis of Transfected Tissue Culture Cells

2.3.1. Common Reagents

1. Special equipment : Standard cell culture facility and materials, fume hood.
2. 1% glutaraldehyde cell fixing solution: For 10 mL, add 0.1g glutaraldehyde to a solution containing 0.1 *M* sodium phosphate and 1 m*M* $MgCl_2$, pH 7.0 (1% fixing reagent). The solution is toxic and should be prepared and used under a fume hood. Generally we prepare a fresh solution as needed.
3. Phosphate-buffered saline (PBS).

2.3.2. Histochemical Detection of ß-galactosidase

1. Stock X-Gal solution: For a 20% solution dissolve 2.0g X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) in N, N' dimethylformamide. Dispense the X-Gal solution into 1.5 mL tubes, wrap in aluminum foil to protect the solution from light, and store at –20°C.
2. ß-Galactosidase detection solution: This is made fresh as needed by diluting the stock X-Gal solution to 0.2% in the following solution: 10 m*M* sodium phosphate buffer (pH 7.0), 150 m*M* NaCl, 1 m*M* $MgCl_2$, 3.3 m*M* potassium ferrocyanide, and 3.3 m*M* potassium ferricyanide. The solution is toxic, light sensitive, and should be stored at 4°C.

2.3.3. Luciferase Assays

1. Special equipment: Single photon counter, tissue homogenizer.
2. Lysis buffer: 0.1 *M* potassium phosphate (pH 7.8), 1% Triton X-100, 1 m*M* dithiothreitol (DTT), 2 m*M* EDTA.
3. Assay buffer: 30 m*M* Tris-HCl (pH 7.8), 3 m*M* ATP, 15 m*M* magnesium sulfate, 10 m*M* DTT, and 1 m*M* coenzyme A or 1 m*M* inorganic pyrophosphate.
4. 1 m*M* D-luciferin: Dissolve the D-luciferin in ddH20, adjust the pH to 6.0-6.3. Dispense the solution into 1.5 mL tubes and store in the dark at –20°C.

2.3.4. Antigen Detection

1. Special reagent: Antigen-specific monoclonal antibody.
2. Biotinylated anti-mouse antibody.
3. Avidin-alkaline phosphatase conjugate reagent.
4. Equilibration buffer: 100 m*M* Tris-HCl, pH 8.2. This solution should be sterilized by autoclaving.
5. Alkaline phosphatase color development solution: Numerous reagents are available commercially. We recommend the Vectastain red substrate (Vector Laboratories, Burlingame, CA).

6. Blocking solution: 5% non-fat powdered milk in PBS.
7. Rinsing solution: 0.5% non-fat powdered milk in PBS.

2.4. Injection of Fish

1. Special laboratory needs and materials: Fish-rearing facility and supplies, pathogen-free fish.
2. Injection: For delivery of the DNA use a 27G 1/2 needle and a 1-mL tuberculin syringe or a repeat pipette.
3. Injection solution: The DNA should be suspended in PBS.
4. Anesthetic: Water containing 100 μg/mL of tricaine methane sulfonate (MS222; Argent Chemical Laboratories, Redmond, WA). The anesthetic solution is used once and then discarded.
5. Anesthetic overdose: Water containing 300 μg/mL MS222.

2.5. Analysis of Fish Injected with DNA

1. Special equipment and supplies: fish-rearing facility, histology laboratory, single photon monitor, tissue homogenizer, fish dissection tools.
2. Fish fixative: 10% buffered formalin, which is commercially available or prepared by diluting 37% formaldehyde in PBS and adjusting the solution to pH 7.4.

2.5.1. Preparation of DNA from Fish Tissues

1. Special equipment and reagents: Mortar and pestle, liquid nitrogen.
2. Digestion buffer: 100 m*M* sodium chloride, 10 m*M* Tris-HCl (pH 8.0), 25 m*M* EDTA (pH 8.0), 0.5% sodium dodecyl sulfate, 0.1 μg/mL proteinase K.
3. PBS.
4. 25:24:1 phenol/chloroform/isoamyl alcohol equilibrated in TE.
5. 7.5 *M* ammonium acetate. For sterilization, autoclave the solution.
6. 95% and 70% ethanol (ethanol diluted with sterile distilled water to 70%).
7. 1 μg/mL DNase-free RNase.

2.5.2. Analysis of DNA by Hybridization

The southern blotting method is used to determine the physical nature of the DNA vaccine in transfected fish cells. This method involves standard protocols outlined in Sambrook et al. **(13)**

3.1. Plasmid DNA Preparation

A number of protocols are available for preparing DNA vaccines. We have tested relatively crude preparations of DNA encoding reporter proteins in fish and the resulting protein activity is similar to that achieved with highly purified DNA. All of the protocols have merit. We use Qiagen-tip 500 columns because they are simple, quick, and consistently provide very pure DNA. The following outline is for preparation of approximately 500 μg of pure plasmid DNA.

1. Streak *E. coli* DH5α transformed with the plasmid on LB-agar plates containing 50 μg/mL ampicillin. Incubate at 37°C overnight.
2. Inoculate 10 mL LB-ampicillin (50 μg/mL) medium in a 50-mL flask with 2–3 colonies from the above plate. Incubate 8–12 h at 37°C with vigorous shaking, 225–250 rpm.
3. Inoculate the 10 mL from above into 90–115 mL LB-ampicillin medium in a 500-mL flask. Incubate 8–12 h at 37°C with vigorous shaking, 225–250 rpm.
4. Transfer the bacterial culture to a sterile bottle and pellet the bacteria by low-speed centrifugation for 10 min.
5. Decant the fluid, invert the bottles, and place on paper towels briefly to collect remaining fluid.
6. Purify the DNA from the bacterial pellet with a Qiagen-tip 500 column. Follow the manufacturer's protocol supplied with the column. Resuspend the column-purified DNA in 300 μL TE and transfer to a 1.5-mL tube.
7. To the purified DNA sample, add 30 μL sodium acetate and 750 μL 95% ethanol. Vortex the solution briefly and pellet the DNA in a microcentrifuge (maximum speed) for 15 min.
8. Aspirate the fluid off from the DNA pellet, add 500 μL 70% ethanol, vortex for 15 s, and spin the DNA in a microcentrifuge for 15 min.
9. Repeat step 8. Air dry the DNA in a sterile environment, e.g., under a laminar flow hood or suitable sterile containment environment. Resuspend the DNA in 200–300 μL of TE.
10. Using a spectrophotometer, determine the DNA concentration and purity of the sample by measuring the optical density of the solution at 260 nm and 280 nm.
11. Adjust the DNA sample to 2–5 μg/mL by dilution in TE. Store the DNA sample at 4°C.
12. The final critical step is to demonstrate that the plasmid is intact and predominately circular. This should be determined by gel electrophoresis of 200–300 ng of the plasmid DNA (*see* **Note 2**).

3.2. Cell Transfection Protocol

The recommended cell lines, CHSE-214 and EPC, are commonly used by fish health specialists. The temperature tolerance of the cell lines are broad ***(11,12)***. In general, the CHSE-214 cells should be maintained at 15°C and the EPC cells at 28°C.

1. Split the cells from newly confluent monolayers into 9.4 cm^2 (6-well plates) wells such that the following day the monolayers have reached 90% confluency. The maintenance temperature at this point should be the same as that for the pathogen for which the vaccine is being developed.
2. Thirty minutes prior to transfection, rinse the cells twice in Opti-MEM 1 followed by addition of 2–3 mL of Opti-MEM 1.
3. For lipofectamine transfection prepare the DNA and lipid complex as outlined in the protocol supplied by the manufacturer. The optimum transfection efficiency

of the fish cells in a 9.4-cm^2 well using lipofectamine is achieved using 1 µg DNA and 6 µL or 9 µL lipid for CHSE-214 and EPC cells, respectively.
4. Incubate the cells with the lipid DNA complex for 24 h.
5. Rinse the cells twice with Opti-MEM 1 and then add 3 mL cell line maintenance media.

3.3. Procedures for Analysis of Transfected Tissue Culture Cells

3.3.1. Cell Preparation

1. At the designated times (typically 48–72 h post-transfection), rinse the transfected cells twice with PBS.
2. Under a fumehood aspirate the PBS and add 2 mL of 1% glutaraldehyde to each well. Let the cells sit for 30 min at room temperature.
3. Discard the fixative (this solution is toxic), add 5 mL PBS and place the cells on a rocking platform for 1 min. Aspirate the PBS and repeat this step four more times.

3.3.2. Histochemical Detection of ß-galactosidase

1. To each well add 1 mL of ß-galactosidase detection solution.
2. Incubate the cells at 37°C for 2–4 h.
3. Aspirate the solution, add 5 mL PBS and place the cells on a rocking platform for 1 min. Aspirate the PBS and repeat the rinse two times.
4. Aspirate the PBS and add 1.5 mL glycerol to the wells.
5. Count the number of transfected cells. The transfected cells are stained blue (**Fig. 2**).

3.3.3. Luciferase Assay

1. Add 200 µL lysis buffer to each well. Triturate the solution briefly and place in a 1.5-mL tube. Add an additional 200 µL lysis buffer to the well and transfer the supernatant to the same tube.
2. Using 10 µL of the lysed cells make 10-fold serial dilutions in 90 µL lysis buffer.
3. Measure the luciferase activity in 100 µL of diluted sample by adding 100 µL assay buffer, gently mix, then add 100 µL D-luciferin, mix again, and place in a photon counter.
4. The luciferase activity should be adjusted by calibration with luciferase standards (*see* **Note 3**).

3.3.4. Antigen Detection

This protocol is a slight modification of Drolet et al. ***(14)***.

1. Add 3 mL blocking reagent to each well. Place the plates on a rocker platform and agitate gently for 1 h.
2. Aspirate the blocking solution and add 1 mL of the appropriate concentration of antigen-specific monoclonal antibody. Incubate with gentle agitation on a rocker platform for 1 h.
3. Aspirate the primary monoclonal antibody, add 5 mL rinsing solution, and agitate on a rocker platform for 1 min. Repeat the rinse step an additional four times.

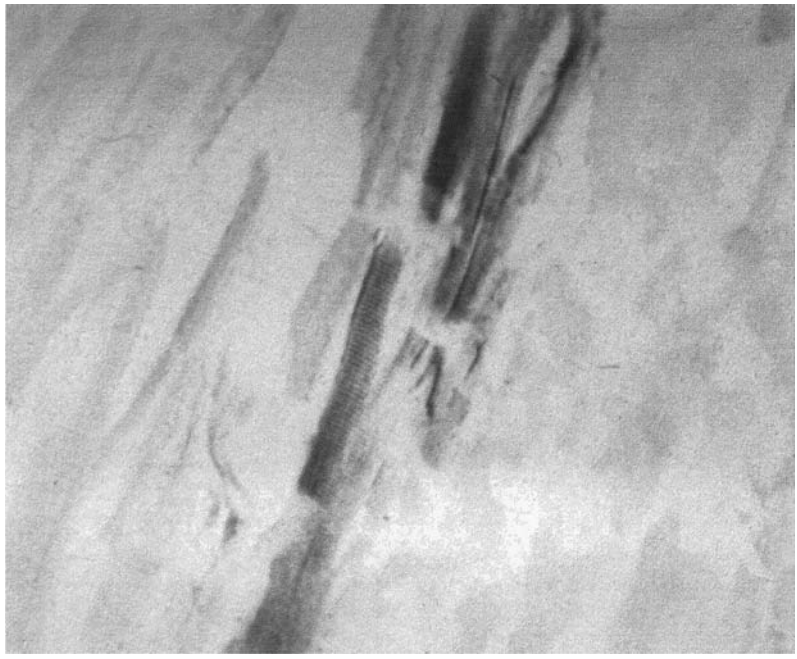

Fig. 2. Tissue section stained for ß-galactosidase activity. The darkly staining muscle tissue expressing ß-galactosidase is shown across the middle of the figure.

4. Aspirate the rinsing solution, and add 1 mL of appropriately diluted biotinylated anti-mouse antibody. Incubate with gentle agitation on a rocker platform for 1 h.
5. Rinse the cells, as in step 3.
6. Add 1 mL of avidin-alkaline phosphatase conjugate and incubate on the rocker platform for 1 h.
7. Rinse the cells, as in step 3.
8. Flood the wells with equilibration buffer and agitate on the rocker for 2 min.
9. Aspirate the wells, and 1 mL of color substrate.
10. Aspirate the wells, add 2 drops of glycerol, and place a coverslip over the cells.
11. Count the number of transfected cells. Using Vector red substrate, the cells containing antigen will stain red.

3.4. Injection of Fish with Plasmid DNA

Special care should be taken to ensure that a minimum amount of time is used when vaccinating fish. Prior to vaccination, a pilot experiment should be performed to determine how rapidly the vaccination process can be done by an individual. A work station should be designed so that the fish can easily and swiftly be moved between the stock tank, anesthetizing tank, and final holding tank.

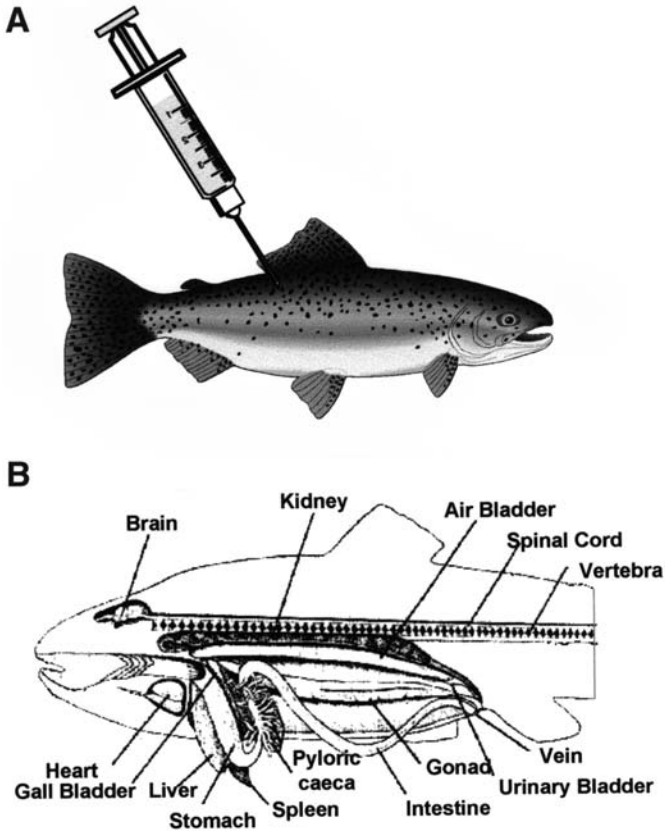

Fig. 3. Injection of DNA into fish. **(A)** Schematic diagram showing injection site at the posterior end of the dorsal fin into the dorsal musculature. **(B)** Cross section of fish showing location of the major organs.

1. Equilibrate the DNA/PBS solution to the water temperature in which the fish are being held.
2. Anesthetize 10–20 fish at a time with MS222. The fish should stop moving in 2–3 min. Remove the fish quickly from the anesthetic.
3. A single fish can be injected while holding it in a gloved hand. Large fish should be injected on a table covered with wet plastic.
4. Inject the fish midway between the anterior end of the dorsal fin and the lateral line (*see* **Figs. 3** and **4**). Gently inject the solution into the muscle tissue of the fish. After the solution has been injected wait 2–3 s. The injection process should take no longer than 10–20 s.
5. As quickly as possible, place the fish back in a holding tank.

Fig. 4. Photograph of rainbow trout fry and tuberculin syringe used to inject DNA into the fish.

3.5. Analysis of Fish Injected with DNA

The fish to be analyzed should be euthanized by an overdose of MS222. Fish to be fixed prior to histochemistry should be placed in 10% buffered formalin after surgically opening their abdomens from their liver to their rectum. Fish to be used for histochemical assays on fresh tissues should be processed immediately as outlined.

3.5.1. Histochemical Detection of ß-Galactosidase in Fixed and Embedded Tissue (*see* **Note 4**)

1. Keep the fish in the formalin fixative for 24 h at 4°C.
2. Remove the fish from the fixative and rinse them in continuously flowing ddH_2O for 20 min.
3. Incubate the rinsed fish in ß-galactosidase detection solution for 4–8 h at room temperature.
4. Rinse the fish as in step 2.
5. Place the fish in fixative for 24 h.
6. Rinse the fish in flowing ddH_2O for several hours.
7. Paraffin embed the fish.
8. For microscopic examination take 6 mm sections from the paraffin block.

3.5.2. Luciferase Assays

1. Surgically remove a small uniform portion of tissue from the fish. The exact size of tissue taken will depend on the outcome of **Subheading 3.5.1.**
2. Place the tissue in a 14-mL polypropylene tube containing lysis buffer (1:5, wt/vol).
3. Numerous methods are available to mince the tissue. We use a polytron homogenizer (Brinkman Instruments, Westbury, NJ) set at 13,000 rpm for 20 s.
4. Clarify the homogenates by centrifugation at 10,000*g* for 10 min.
5. Prepare the samples and measure luciferase activity as described above.

3.5.3. Immunohistochemical Detection of Antigen (*see* **Note 5**)

1. Leave the fish in fixative for at least 24 h.
2. Rinse, paraffin embed, and cut sections as described above.
3. For antigen detection the slides should be placed on a slide tray, in a humid plastic box. Each of the solutions should be added slowly to the slide forming a droplet that completely covers the thin section.

3.5.4. Preparation of DNA from Fish Tissue

1. Surgically remove a small uniform portion of tissue from the fish.
2. As quickly as possible, place the tissue in liquid nitrogen. Once the tissue is frozen, it can be processed as outlined below or stored at –70°C.
3. Grind the frozen tissue to a coarse powder using a mortar and pestle.
4. Place the powdered tissue in a tube containing digestion buffer (100 mg tissue/1 mL digestion buffer).
5. Incubate the samples on a shaking platform at 50°C for 12–16 h. The sample should be gently mixed to avoid shearing of the released DNA.
6. Add an equal volume of phenol/chloroform/isoamyl alcohol to the sample and gently mix.
7. Centrifuge the sample to separate the organic and aqueous phases.
8. Transfer the aqueous (upper) phase to a new tube and repeat steps 6 and 7 and combine the samples.
9. To the aqueous phase add 1/2 vol ammonium acetate and 2 vol 95% ethanol. Gently mix.
10. Immediately centrifuge the sample to pellet the DNA.
11. Aspirate the supernatant and rinse the DNA pellet with 70% ethanol. Repeat this step two times with centrifugation between rinses to re-pellet the DNA.
12. Suspend the DNA in TE to a final concentration of 1–5 µg/mL. Store the DNA at 4°C.

4. Notes

1. The procedures described for the development of a DNA vaccine for fish is based primarily on our experience with salmonid fish species. It is important to remember that there are many different fish species. Some fish prefer habitats of clear,

highly oxygenated water and others can occupy turbid, low oxygen-level water; some can live in marine aquatic environments at temperatures of 2–4°C and others prefer freshwater temperatures at 28–32°C. Genetic studies on fish immunoglobulins and MHC genes indicate that there is considerable diversity in gene arrangements and in the immune response among the different fish species. Even the presence of different immunoglobulin isotypes is unresolved at present for fish. Thus, some of the experimental procedures, such as the period of time required for an immune response to develop before virus challenge, will vary with the fish species.

Although there are some reagents available commercially for detecting rainbow trout immunoglobulin, many investigators purify fish immunoglobulin and make their own antisera for antibody detection. Also, while higher vertebrates immune systems share some common features with fish, one should not assume that the immune response is completely similar and all assays should be tested empirically. Finally, it should be noted that inbred salmonid fish strains are not available commercially although Gary Thorgaard at Washington State University (Pullman, WA) has developed some inbred rainbow trout lines. Without inbred lines, it difficult to develop appropriate assays for the rainbow trout cellular immune response. That is not the case for catfish cells where continuous cell lines are available through Norman Miller and William Clem at the University of Mississippi at Jacksonville, Mississippi. Basic immunological reference and laboratory manuals for the study of the fish immune system are available ***(15–17)*** and many of the protocols can be modified for a particular fish species.

2. The Qiagen-tip 500 column can be used at least twice if it is not allowed to dry out between uses. For multiple use, prepare several crude preparations of DNA containing a maximum of 500 µg DNA. Pass the crude preparation across the column. Immediately equilibrate the column with 2 vol of equilibration buffer and repeat the isolation of DNA.
3. For calibration of luciferase activity, a standard curve should be generated using purified firefly luciferase (Analytical Luminescence Laboratory, Ann Arbor, MI). This step is necessary because luciferase activity can vary depending on uncontrollable environmental conditions. Once a standard curve has been generated, selected control standards should be used each time an assay is performed so that the activity of the luciferase in the test sample can be estimated. Prepare the luciferase in 0.1 *M* phosphate buffer, pH 7.8. To generate the standard curve use 10-fold dilutions, from 0.01–1 pg, of purified luciferase in 100 µL of lysis buffer.
4. In both control and pCMVßGal injected fish, intense b-galactosidase staining is observed throughout the stomach and the intestine. This staining is, more than likely, due to the bacteria inhabiting the intestinal tract. Therefore, if desired, prior to immersion in the X-Gal, the stomach and intestine can be surgically removed. This step helps eliminate bleeding of the staining solution into surrounding tissues. Also, there is no background luciferase activity in the stomach or intestine, so if measurements of protein activity in these organs are critical the fish should be injected with pCMV-Luc.

5. Extensive non-specific staining can occur when either tissue culture cells or fish sections are stained for antigen. This problem can usually be eliminated by keeping the samples moist at all times. For example, when aspirating the rinse solution from tissue culture cells, hold the pipette for aspiration in one hand and a squirt bottle with rinse solution in the other hand, aspirate the solution quickly and immediately squirt the wash solution into the well.

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12

CTL Analysis for Tumor Vaccines

Antonio Rosato, Gabriella Milan, Annalisa Zambon, Paola Zanovello, and Dino Collavo

1. Introduction

Many studies have been conducted with the aim to stimulate a therapeutic immune response against tumors. In most cases, efforts have been directed toward the induction of tumor-specific cytotoxic T lymphocytes (CTL), because this T lymphocyte subpopulation is considered to play a major role in the destruction of tumor cells ***(1)***. In particular, vaccination protocols have been designed to increase the immunogenicity of intact cancer cells by using adjuvants or engineering tumor cells with cytokine or costimulatory molecule genes. A second line of research has employed immunization with tumor-associated antigens (TAA). These antigens are expressed from derepressed or mutated genes in tumor cells, and are recognized by CTL in the form of peptides associated with MHC class I molecules. Genes encoding TAA have been inserted into recombinant viral vectors, which are then used to infect the host's cells and induce expression of the transgene. Moreover, immunization with purified TAA peptides or with antigen-presenting cells, such as dendritic cells, pulsed with TAA peptides have been proposed.

An innovative approach for cancer immunotherapy is offered by DNA vaccination, a procedure that has already been used successfully to induce humoral and cell-mediated immune responses to a considerable number of viral, bacterial, and parasitic agents ***(2)***. Several features make DNA vaccination particularly relevant to the field of cancer therapy: i) the numerous genes encoding TAA that have recently been cloned, and therefore might be used to construct DNA tumor vaccines; ii) the synthesis of TAA within the host's cells, which may favor the presentation of TAA epitopes to CTL in the context of the host's MHC class I molecules; iii) the generation of a strong antigen-specific CTL response.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

In this chapter, we provide some guidelines and suggestions to follow in order to induce high levels of TAA-specific CTL, by injecting DNA-expression vectors containing genes encoding tumor antigens, and we will describe the technical steps to evaluate lytic activity of this T cell subpopulation.

1.1. Choosing the TAA and Experimental Model

The prototypes of human TAA are members of the melanoma-associated antigen (MAGE) family, originally detected on melanoma cells but also shared by other neoplasias. These TAA (and the more recently described BAGE and GAGE antigens) are generated by transcriptional activation of normal genes not expressed in normal tissues, with the exception of testis. A second group of antigens is represented by differentiation antigens, which are expressed not only by cancer cells but also by normal cells of the same histotype. Finally, the third group of antigens arises from mutation of normal genes which may encode new antigens specific for individual tumors *(3)*. Genes encoding each of these different TAA might be cloned into a plasmid vector and used as a potential DNA vaccine to induce an immune response capable of protecting against or eliminating tumors bearing the relevant antigen.

Most of the information obtained thus far in cancer immunotherapy is derived from analysis of the immune reactivity elicited in mice against model tumor antigens, very often represented by xenogenic proteins that are not even related to mouse proteins *(4,5)*. In theory, almost any protein might constitute a potential TAA if it is able to induce an immune response when artificially introduced into a tumor cell.

Technical procedures described here are based on experience acquired with an experimental tumor expressing a TAA encoded by a normal mouse gene, P1A, originally isolated from the mastocytoma cell line P815 *(6)*, and more recently found to be widely expressed by different tumor cell lineages *(7)*. The P1A antigen is recognized by specific CTL clones in the context of the MHC class I molecule L^d. Therefore, this TAA, which shares many characteristics with human antigens of the MAGE, GAGE and BAGE families, represents a useful experimental model to develop and study the efficacy of new vaccination strategies that could find potential applications for therapy of human tumors.

1.2. Plasmid Vectors and Vaccination Procedures

Most of the work done on DNA vaccination has employed plasmid vectors driven by the strong viral CMV or RSV promoters. We have found that the CMV promoter usually drives higher levels of TAA expression compared to other eukaryotic promoters. Thus, cloning the TAA gene of interest into a CMV-driven plasmid vector might be the first choice. Once the gene is cloned,

it is necessary to carefully evaluate its expression from the plasmid in vitro. It is advisable to confirm the sequence of the plasmid insert, especially if the TAA gene was amplified by PCR. Expression of the TAA from the plasmid must always be analyzed in transient transfections in terms of mRNA production and protein expression, using specific antibodies in order to visualize the antigen. If specific antibodies are not available, the TAA protein might be "tagged" by modifying the gene by the addition of a short sequence coding for a tag epitope recognized by a commercially available antibody. Our studies carried out with TAA, modified with a 6-amino acid tag referred as AU-1, showed that this procedure does not modify the physical properties of the TAA or alter the correct processing and presentation of antigenic epitopes. In fact, we observed that cells stably transfected with the AU-1-modified P1A gene expressed the TAA and were lysed by CTL clones specific for the relevant epitope ***(8)***.

The site of plasmid injection into the mouse is a point that also has to be taken into account. Several routes of administration have been tried by others ***(2)*** and ourselves, but we have concluded that intramuscular inoculation (i.m.), e.g., into the quadriceps or tibialis anterior (TA) muscles, remains the best choice to induce CTL sensitization. In this regard, the muscle to be inoculated must be clearly visualized in order to maximize the efficiency of the inoculation; therefore, to gain access to the quadriceps, a small incision of the overlying skin must be performed. Inoculation of the TA does not require surgical procedures and can be carried out transcutaneously (provided that surrounding fur is carefully removed with a trimmer or a depilatory cream) and has now become our standard procedure.

The number of plasmid inoculations necessary to produce an efficient and homogeneous CTL response may vary depending on the mouse strain and the strength of the antigen under study. For example, in DBA/2 mice, 3 i.m. inoculations of 100 μg of plasmid expressing the weakly immunogenic antigen P1A were needed to induce CTL generation, albeit at a very variable extent, in the majority of vaccinated mice ***(8)***. Interestingly, the same protocol of immunization generated a very efficient and homogeneous CTL response in all treated Balb/c mice. Application of the same protocol to immunize mice with plasmid vectors encoding the strong env and gag viral antigens of Moloney-murine leukemia virus (M-MuLV) generated high CTL levels in all vaccinated animals (Rosato et al., unpublished results).

Various pretreatment methods have been proposed to improve the efficiency of the DNA immunization, such as the use of bupivacaine or hypertonic sucrose solutions ***(9)***. In our hands, however, these procedures did not enhance the efficacy of DNA vaccination with the TAA-coding plasmids described above. Instead, a dramatic improvement, in terms of CTL generation, was achieved by

pretreating the TA muscle with cardiotoxin: in this case, a single plasmid injection, carried out 5 d after cardiotoxin treatment, was sufficient to induce impressively high levels of CTL in Balb/c mice and also bypassed the limited responsiveness observed in several DBA/2 animals (Rosato et al., unpublished results). A detailed description of how to perform cardiotoxin treatment and plasmid inoculation currently can be found at The DNA vaccine Web site ***(9)***.

1.3. Analysis of the CTL Response in Vitro and in Vivo

Detailed protocols for performing CTL analysis in allogeneic or viral experimental systems have been reported elsewhere ***(10,11)***. Here we will describe a method for generating and analyzing CTL in bulk cultures following i.m. immunization with plasmid DNA coding for TAA. This analysis may be carried out by setting up a mixed leukocyte tumor cell culture (MLTC), obtained by restimulating splenocytes from DNA-immunized mice with irradiated tumor cells bearing the relevant TAA, or a mixed leukocyte peptide cell culture (MLPC), which employs the antigenic peptide as the stimulator. CTL lytic activity is measured using a ^{51}Cr-release assay. CTL analysis in vitro must be followed by a careful evaluation of the capacity of the immunized mice to reject a syngeneic tumor expressing the relevant TAA, in order to evaluate the overall in vivo efficiency of the vaccination procedure and to study whether a correlation exists between tumor regression and CTL generation.

2. Materials

2.1. Preparation of Spleen Cells and MLTC and MLPC Restimulation

1. Special equipment: ^{137}Cs cell irradiator.
2. Complete culture medium: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2 m*M* L-glutamine, 100 U/mL each penicillin and streptomycin, 10 m*M* HEPES [4-(2-hydroxy-ethyl)-1-piperazine ethanesulfonic acid], 5×10^{-5} *M* ß-mercaptoethanol. Make fresh as required and sterilize by filtration through a 0.22 µm filter.
3. Incomplete medium: DMEM supplemented with 3% (v/v) FCS, 100 U/mL each penicillin and streptomycin, 10 m*M* HEPES. Make fresh as required and sterilize by filtration through a 0.22 µm filter.
4. Petri dishes (3 cm, sterile).
5. Cell strainer with an outside diameter that allows it to rest on the top of a 50 mL conical tube (100 µm Nylon, sterile).
6. Polystyrene round-bottom (10 mL) and polypropylene graduated conical (50 mL) tubes with caps.
7. 25 cm^2 flasks (tissue culture quality).
8. Forceps and scissors (sterile).
9. Syringe plunger (sterile).

10. Neubauer cell counting chamber.
11. Vital dye: prepare a 0.1% (w/v) Eosin Y solution in saline solution (0.9% NaCl).
12. Stimulator tumor cells: grow at 0.8–1 × 10^6/mL if in suspension or to a slightly confluent status if adherent.
13. Peptide (95% purity) stock: prepare a 1 m*M* solution in molecular biology grade DMSO; filter-sterilize, aliquot at 100 µL/vial and store at –80°C.

2.2. ^{51}Cr-Release Assay

1. Special equipment: γ-counter.
2. Incomplete medium as in **Subheading 2.1.**
3. ^{51}Cr as sodium chromate (Na_2CrO_4) in normal saline solution at 1 mCi/mL.
4. Polystyrene round-bottom (10 mL) tubes with caps.
5. Round-bottom 96-well plates with covers.
6. Small plastic tubes (3 cm tall) that fit into 96-well plates.
7. Multichannel pipet.
8. Neubauer cell counting chamber.
9. Eosin Y solution as in **Subheading 2.1.**
10. Target cells: grow at 0.6–0.8 × 10^6/mL if in suspension or to a subconfluent status if adherent.
11. Peptide as in **Subheading 2.1.**
12. Triton X-100: prepare a 5% (v/v) solution in saline solution. Store at room temperature.

3. Methods

3.1. Preparation of Spleen Cells and MLTC and MLPC Restimulation

3.1.1. Collection of Spleens and Preparation of Lymphoid Cells

1. Euthanize mice and disinfect with 70% alcohol. Aseptically remove spleens using sterile forceps and scissors and transfer into a 3-cm Petri dish containing 2 mL of incomplete medium.
2. Position a cell strainer onto a graduated conical tube and rinse the membrane with 1–2 mL of incomplete medium. Transfer the spleen onto the cell strainer and gently press with a plunger to allow dissociation of splenocytes from spleen stroma (*see* **Notes 1** and **2**). Rinse the membrane of the cell strainer with 20 mL of incomplete medium, allowing cells to collect into the conical tube (*see* **Note 3**). Discard the cell strainer with the spleen stroma.
3. Carefully pipet the cell suspension to break clumps. Centrifuge the single-cell suspension for 5–7 min at 250*g*. Resuspend the cell pellet with 20 mL of incomplete medium; place an aliquot of the cells in a Neubauer counting chamber after staining with Eosin Y and count viable cells (*see* **Note 4**). Centrifuge cell suspension as above and resuspend the pellet in complete culture medium, adjusting the cell concentration to 10^7/mL.

3.1.2. Preparation of Stimulator Tumor Cells

1. Collect cells from culture by centrifuging for 5–7 min at 250*g* in a 10-mL polystyrene round-bottom tube. Wash once with 10 mL of incomplete medium, as described in **Subheading 3.1., step 3.**
2. Resuspend pellet in 10 mL of incomplete medium and count viable cells in a Neubauer counting chamber as described above.
3. Centrifuge cell suspension and remove 9 mL of medium, leaving 1 mL over the pellet, then irradiate the pellet at 60–100 Gy (*see* **Note 5**).
4. Wash the cells twice as described above and recount. Resuspend the pellet in complete culture medium, adjusting the cell concentration to 2×10^5/mL.

3.1.3. MLTC Setup

1. In a 25-cm^2 culture flask, add 10 mL of complete culture medium (*see* **Note 6**), 2.5 mL of splenocyte cell suspension (2.5×10^7 total) and 2.5 mL of irradiated stimulator tumor cells (0.5×10^6 total) (*see* **Note 7**).
2. Position the flask vertically and cultivate for 5 d in an incubator at 37°C, with 5% CO_2 and 95% humidity (*see* **Note 8**).

3.1.4. MLPC Setup

1. In a 25-cm^2 culture flask, add 12.5 mL of complete culture medium, 2.5 mL of splenocyte cell suspension (2.5×10^7 total) and 15 µL of sterile peptide stock solution (1 *M* final concentration).
2. Position the flask vertically and cultivate for 5 d in an incubator at 37°C, with 5% CO_2 and 95% humidity.

3.2. ^{51}Cr-Release Assay

3.2.1. Preparation of Effector Cells

1. Without disturbing the cells, remove 5 mL of culture medium from the top of the MLTC or MLPC flasks.
2. Resuspend cells from the bottom of the flask and transfer the entire contents into a 10-mL polystyrene round-bottom tube (*see* **Note 9**), then centrifuge once and resuspend cells in 10 mL of incomplete medium.
3. Count viable cells with Eosin Y and adjust cell concentration to 1.8×10^6/mL in incomplete medium (*see* **Note 10**).

3.2.2. Preparation of Target Cells

3.2.2.1. ^{51}Cr Labeling

1. After counting, collect 1×10^6 target cells from the culture into a 10 mL polystyrene round-bottom tube (*see* **Note 11**), centrifuge and carefully remove all medium away from the pellet.

2. Gently resuspend the pellet with 10 µL of FCS. Add 100 µL of 51_{Cr} and incubate at 37°C for 1 h, with periodic gentle shaking (*see* **Note 12**).
3. After labeling, wash cells three times with incomplete medium and count viable cells. Adjust cell concentration to 2×10^4/mL.

3.2.2.2. Peptide Pulsing of 51_{Cr}-labeled Target Cells

1. After labeling, wash cells two times with incomplete medium, resuspend the pellet in 1 mL of incomplete medium and add 1 µL of peptide from stock solution (1 µ*M* final concentration).
2. Incubate at 37°C for 0.5 h, then wash three times and adjust cell concentration to 2×10^4/mL (*see* **Note 13**).

3.2.3. Coincubation of Effector and Target Cells

1. Distribute 150 µL of effector cell suspension in the first row of a round-bottom 96-well plate (*see* **Note 14**); run each sample in triplicate.
2. Add 100 µL of incomplete medium to the next 3 or 5 rows, depending on the number of dilutions you may want to carry out.
3. With a multichannel pipet, transfer 50 µL of cell suspension from the first row to the next one and mix well to allow complete dilution. Repeat the same operation for the following rows, and discard 50 µL of cell suspension from the last dilution. At this point each well should contain 100 µL of a 3-fold diluted effector cell suspension.
4. Add 100 µL of target cell suspension to wells containing effector cells (*see* **Note 15**).
5. To determine spontaneous release (amount of isotope released by target cells in the absence of effectors) and maximum release (total radioactivity incorporated by target cells), add 100 µL of target cell suspension to 6 additional empty wells. Fill 3 of these wells with 100 µL of incomplete medium (spontaneous release) and the remaining 3 with 100 µL of Triton X-100 solution (maximum release).
6. Centrifuge plates for 3–5 min at 150*g* and incubate for 4 h at 37°C, with 5% CO_2 and 95% humidity.

3.2.4. Collection of Supernatant and Calculation of Lytic Activity

1. After incubation, collect 100 µL of supernatant with a multichannel pipet and transfer into plastic tubes (*see* **Note 16**). Determine radioactivity in a γ-counter.
2. Calculate the specific ^{51}Cr release as follows:

$$\% \text{ specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

3. Plot a graph of % specific release, indicating the effector-to-target ratio on the x-axis (from 90:1 to 3:1 or 0.3:1, depending on whether 4 or 6 dilutions have been carried out) and the corresponding percentage of specific lysis on y-axis.

4. Notes

1. Care should be taken to remove most of the fibrous material from the spleen before pressing with the plunger, as the presence of ligament tissue may reduce cell recovery.
2. If a cell strainer is not available, a 10-cm Petri dish can be equally useful. In this case, the spleen can be pressed on the plastic surface, the stroma removed and cell clumps resuspended before transferring to a tube.
3. Careful rinsing of the cell strainer's nylon membrane is an important step to avoid loss of splenocytes.
4. Viability of splenocytes and correct counting must be carefully checked to allow reproducible results and comparative analysis among different immunized mice.
5. Irradiation is usually the best method to block proliferation of tumor cells and results in good viability for 24–72 h. This aspect is important to induce optimal stimulation of responder spleen cells. Alternatively, mitomycin C can be used to inhibit division of tumor cells, but in this case, cell recovery and viability are inferior and stimulating capacity is reduced.
6. A critical ingredient for successful restimulation of splenocytes is the FCS. Different batches should be tested for optimal results because they may vary greatly in their capacity to support cell proliferation or may even be excessively rich and induce acquisition of non-specific cytotoxic activity.
7. A responder (splenocytes) to stimulator (irradiated cancer cells) ratio of 50, as described, is usually optimal for most experimental tumors. However, it must be kept in mind that a large number of tumors are endowed with immunosuppressive capacity and that excessive quantities of such cells in culture can completely block differentiation of splenocyte into CTL. Therefore, careful titration of tumor cell number to add to the culture will allow the determination of optimal stimulation ratio.
8. If a large number of effector cells are needed, MLTC and MLPC can be set up in 75 cm^2 flasks, tripling the number of splenocytes and irradiated tumor cells to be added. Vertical positioning of flasks allows collection of cells in a narrower surface and better cell-cell interactions.
9. If the entire culture is not needed for the ^{51}Cr release assay, only an aliquot may be used, leaving the remainder for the next day. In fact, one can measure lytic activity as many as 6 d after set-up unless complete exhaustion of culture medium is observed, and cultures may be retested if the first attempts encounter technical difficulties.
10. The presence of a high number of blast cells in cultures is usually a good sign of active proliferation and stimulation and is generally followed by relevant cytotoxic activity. Nonetheless, some cultures may appear devoid of any stimulation and present high levels of cytotoxicity.
11. Cells to be labeled must not be overgrown because this reduces isotope incorporation and increases spontaneous release (which should not exceed 20%). The optimal cell concentration for labeling is that reported in **Subheading 2.2.**, and counts measured in 1 min from 2×10^3 target cells should range between 2000–4000.

12. Labeling of cells growing in suspension does not present particular problems, because their number can be easily evaluated at any moment. Adherent cells, which must be trypsinized in order to be counted before labeling, sometimes form large clumps during the labeling step, which cannot be re-counted later. In this case, the clumps can simply be resuspended and can be assumed not to have undergone any loss in cell numbers. Adherent cells usually incorporate high levels of isotope.
13. Peptide pulsing of target cells can also be carried out before or during the labeling period.
14. When many cultures have to be tested, the distribution of effector cell suspensions into the 96-well plate may require some time, thus allowing time for the cells to settle down at the bottom of the well. Therefore, before starting dilutions, care must be taken to mix well, in order to transfer a volume containing a homogeneous quantity of effector cells.
15. After distributing the target cells, a brief centrifugation allows rapid contact between cells and can be designated as the starting time of the test.
16. Collection of supernatant from wells is a critical step, as removal of pelleted material will completly ruin the test. To correctly collect the supernatant, the plate should be slightly inclined (for example, by placing the plate cover under one side) and the multichannel pipet (whose tips must be aligned properly) should be kept at a 45° angle, with the tips touching the well walls. If doubts exist as to whether the pellet has been disturbed, it is better to discharge the volume into the wells, centrifuge the plate and try again.

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The Use of Bone Marrow-Chimeric Mice in Determining the MHC Restriction of Epitope-Specific Cytotoxic T Lymphocytes

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1. Introduction

Plasmid DNA immunization has emerged as a promising vaccine strategy against infectious agents, as well as a potential intervention for the treatment of cancer, autoimmunity, and allergy ***(1)***. Until recently, however, the cellular events by which injected plasmid DNA elicits potent antibody and cytotoxic T-lymphocyte (CTL) responses were largely unknown. Upon intramuscular (i.m.) injection of naked DNA, predominant expression of transfected DNA occurs in the myofibers ***(2)***, but no direct transfection of antigen presenting cells (APC) has been reported. There are essentially three different mechanisms by which CTLs can be primed by the injected DNA ***(3)***. The first possibility is that the transfected muscle cells directly activate CTLs by presenting the antigenic peptide on their MHC class I molecules. Alternatively, the priming of CTLs may be mediated by professional APC taking up antigen released from muscle cells. Finally, CTL priming may involve direct transfection of APC occurring, albeit at low level, and that the CTLs are activated by the transfected APC.

In an effort to identify the key cellular subset(s) responsible for the induction of CTL responses by plasmid DNA immunization, we created and immunized a set of bone marrow-chimeric mice **(Fig. 1)**. Bone marrow-chimeric mice have proven to be a valuable tool for determining the relevant cell type(s) involved in the activation of CTLs ***(4)***. In a bone marrow chimera, only the bone marrow derived, hemopoietic cells express the MHC haplotype of the donor origin, whereas non-bone marrow derived cells such as muscle cells and skin keratinocytes bear the host MHC molecules. By immunizing defined chi-

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

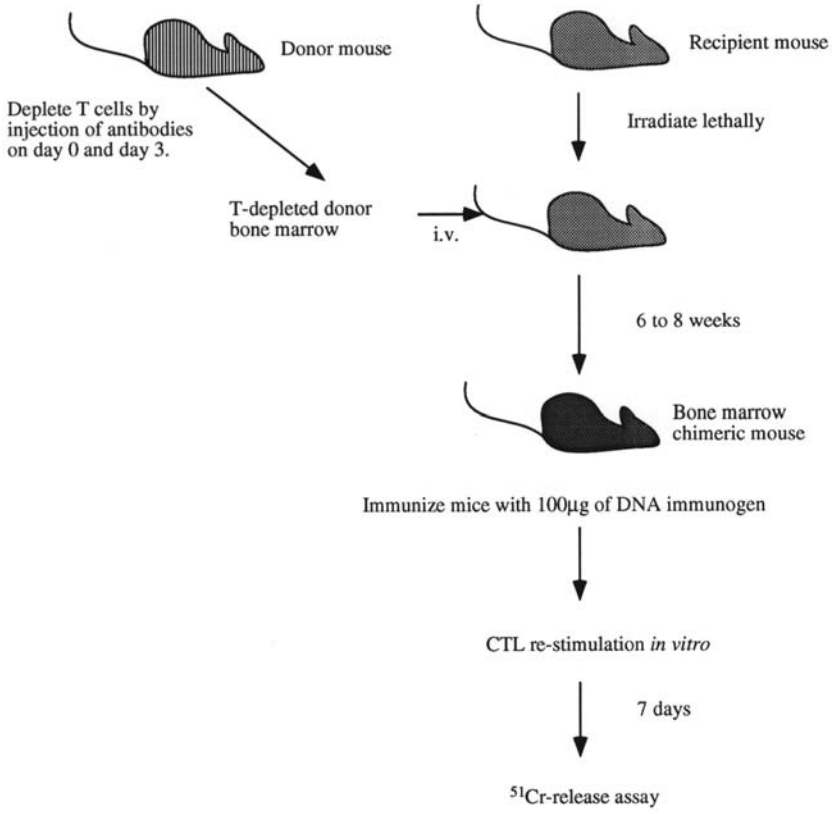

Fig. 1. Schematic diagram for the generation and immunization of bone marrow-chimeric mice.

meric mice and assessing the specificity of the CTLs generated, one is able to determine which of these cell type(s) were involved in stimulating naive CTLs upon DNA immunization. We and others have demonstrated by the use of bone marrow-chimeric mice that the key cells in the presentation of DNA-encoded antigen by both gene gun-mediated epidermal injection ***(5)*** and by needle i.m. injection ***(5,6)*** of plasmid DNA are bone marrow-derived. Thus, this represents another example of how this technique can be used to obtain vital information about the nature of cells responsible for the presentation of antigens to naive T cells.

The main obstacle to the more extensive use of bone marrow-chimeric mice has been the difficulties associated with the successful production of these mice. Problems can arise from death of the infection-prone lethally irradiated mice, and from complications from graft vs host disease (GvHD) by the donor

bone marrow-derived cells. Here we describe a comprehensive procedure for generating bone marrow-chimeric mice, and point out certain measures that may be taken to prevent commonly encountered problems. Further, the procedures for DNA immunization and CTL assays that can be performed with the fully reconstituted mice are also described here.

2. Materials

2.1. Construction of Bone Marrow-Chimeric Mice

2.1.1. Production of Antibodies for the Depletion of T Lymphocytes in Donor Mice

1. In vitro culture equipment: CELLMAX® system (Cellco, Germantown, MD).
2. Culture media: RPMI-1640 supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 m*M*).
3. Lactate assay kit (Sigma, St. Louis, MO).
4. Hybridomas that secrete T-cell depletion antibodies: anti-CD4 (YTS-191) and anti-CD8 (YTS-169) *(7)*. These antibodies are both derived from hybridomas which secrete rat IgG2b antibody isotype.

2.1.2. Depletion of T Lymphocytes with Antibodies Against CD4 and CD8 in Donor Mice

1. Special equipment: flow cytometer.
2. 1 mL Tuberculin syringe fitted with 27G1/2 needle.
3. Concentrated antibodies against CD4 (YTS-191) and CD8 (YTS-169) generated using the CELLMAX system.
4. Donor inbred mice.
5. Detection antibodies for CD4 (FITC-conjugated anti-CD4) and CD8 (Phycoerythrin-conjugated anti-CD8) (Becton Dickinson, San Jose, CA).
6. Protein concentration filter, Centriprep concentrators (Amicon, Beverly, MA).

2.1.3. Isolation and Injection of Bone Marrow Cells from the Donor Mice

1. Special equipment: γ-irradiation source suitable for use with experimental animals.
2. Sterile Petri dishes (60 × 15 mm style).
3. Cold phosphate-buffered saline (PBS) in 50 mL tubes on ice.
4. Pair of surgical scissors and forceps.
5. 70% ethanol in a squirt bottle.
6. 3 mL Tuberculin syringe fitted with 23G1$^1/_2$ needle.
7. Sterile cell strainer.
8. Culture media as in **Subheading 2.1.1.**
9. Infrared heating lamp.
10. Mouse holder.

2.1.4. Treatment of Mice Before and After Lethal Irradiation

1. Antibiotics: Clavulin-125F (SmithKline Beecham, Oakville, ON, Canada) stock solution. Dissolve 1g of Clavulin in 40 mL of 0.9% sterile NaCl under laminar flow hood. This stock solution can be kept at 4°C for up to 10 d.
2. Autoclaved water bottles.
3. Clidox (chlorine dioxide), freshly made. Once the base and the activator are mixed, it must be used within 14 d.

2.1.5. Plasmid DNA Immunization of Reconstituted Mice

1. Endotoxin-free plasmid DNA purified from bacterial sources. Qiagen Megaprep columns are recommended. Store lyophilized DNA at –20°C. Each injection requires 100 μg of DNA.
2. Sterile saline.
3. Insulin syringe.
4. Bone marrow-chimeric mice.
5. 70% Ethanol in a squirt bottle.
6. Gauze.

2.2. Peptide Epitope Specific Cytotoxic T-Lymphocyte Assay

2.2.1. In Vitro Re-stimulation of Splenocytes

1. Special equipment: γ-Irradiation source suitable for use with cells in suspension.
2. Stimulators: syngeneic naive mice.
3. Responders: bone marrow-chimeric mice immunized with DNA vaccine.
4. 70% ethanol in a squirt bottle.
5. Pair of surgical scissors and forceps.
6. Cold PBS in 10 mL tubes on ice.
7. Sterile cell strainer.
8. Culture media as in **Subheading 2.1.1.**
9. Sterile 3 mL tuberculin syringes.
10. 50 mL tissue culture flasks.
11. Chemically synthesized and purified MHC class I-restricted peptide from the antigen encoded by the DNA immunogen, resuspended at 100 μg/mL in sterile PBS.
12. ß-Mercaptoethanol.

2.2.2. ^{51}Cr-Release Assay

1. Special equipment: gamma counter, Skatron cell harvester (Sterling, VA).
2. 96-well v-bottomed plates with lids.
3. Multichannel pipettor.
4. Culture media as in **Subheading 2.1.1.**, and a separate media made with 25% FCS content.
5. [^{51}Cr] Na_2CrO_4 in sodium chloride solution.
6. MHC class I-restricted peptide of the DNA encoded antigen, resuspended at 0.1 μg/mL in sterile PBS.

3. Methods

3.1. Construction of Bone Marrow-Chimeric Mice

3.1.1. Production of Antibodies for the Depletion of T Lymphocytes in Donor Mice

We describe here a practical method of obtaining a sterile, highly concentrated antibody using the CELLMAX system.

1. Grow hybridomas in RPMI-1640 supplemented media to obtain 5×10^7 cells.
2. Pellet down 5×10^7 cells and resuspend in 15 mL of media.
3. In the mean time, equilibrate CELLMAX capillary module with fresh media for at least 48 h.
4. Inoculate CELLMAX capillary module with 5×10^7 cells, making sure that everything that comes in contact with the module is sterile.
5. Monitor the growth of the cells by lactate production, which corresponds to glucose consumption by the cells. Media should be changed when the glucose consumption reaches 50% of the starting glucose concentration. For RPMI-1640 with 2.0g/L of glucose, reservoir media must be changed when the glucose level falls to 1.0g/L. To monitor lactate production, a simple spectrophotometric assay kit is available from Sigma (Lactate Reagents and Lactate Standard Solution).
6. When lactate consumption level reaches 750–2000 mg/day, harvest the extracapillary space (ECS) by flushing out the ECS with sterile syringes and collect the content into a 50 mL tube.
7. Centrifuge the cells down at 210*g* for 5 min and collect the supernatant into a sterile tube.
8. Test various concentrations of the ECS eluate for its ability to deplete T cell subsets in vivo (Subheading 3.1.2.).

3.1.2. Depletion of T Lymphocytes with Antibodies Against CD4 and CD8

The most stringent assessment of T cell depletion can be obtained from the detection of T cells in the lymph node, since the proportion and the concentration of mature T cell population is the highest in the lymph nodes, as opposed to blood, spleen, or bone marrow. In order to obtain a sufficient number of cells to be used for flow cytometric analysis, mesenteric lymph nodes are harvested.

1. Draw up different concentrations of antibodies into 1 mL syringes adapted with $27G^1/_2$ needles. We recommend using 0.5 mL of the most concentrated form, and dilutions of 1/10 and 1/50. Typically, the supernatant from the hybridomas must be concentrated to 150–200 fold the volume of the total culture media used to grow the hybridomas in order for a 0.5 mL volume injected on d 0 and 3 to completely deplete both subsets of T cells.
2. Inject the antibodies intraperitoneally into naive mice at d 0 and d 3.

3. Collect mesenteric lymph nodes and make single cell suspensions by disrupting the lymph node through a cell strainer. Wash the cells three times in cold PBS, and count the number of cells.
4. Perform two-color staining of cells using anti-CD4 and anti-CD8 detection antibodies conjugated with FITC or PE, respectively. Analyze on flow cytometer.
5. Calculate the percent depletion by comparing the percentage of cells seen in either the $CD4^+$ or the $CD8^+$ single positive quadrant from the undepleted mice. The depletion obtained in vivo should be close to 100%, in order to prevent GvHD by the residual donor T cells. If required, concentrate the antibody using a concentrator system such as Centriprep (*see* **Note 1**).

3.1.3. Isolation and Injection of Bone Marrow Cells from the Donor Mice

Isolation of bone marrow cells from the femurs and tibia of donor mice is a laborious procedure that requires meticulous care to remove flesh and tendon from the bones without breaking them under sterile condition. As much as possible, all solutions are to be kept on ice, and all procedures to be done under laminar flow hood.

1. Sacrifice donor mice depleted of mature T cells in vivo.
2. Soak the mice in 70% ethanol, and make small incision in the abdominal skin.
3. Remove the fur all the way to the ends of the hind legs by tearing apart the skin from the incision point.
4. Cut off the feet and remove all remaining fur. Harvest the hind legs and place them into sterile PBS on ice.
5. Trim off the muscle, fat, and tendon carefully, using a pair of surgical scissors and forceps. The femur and tibia should have no remaining flesh by this point.
6. Cut off bone endings (about 4 mm off the ends of the bones) with sharp scissors and place the middle part of the bone in a Petri dish containing fresh medium. Repeat this procedure until all the bones are processed.
7. Flush out the content of the bone marrow using $23G1^1/_2$ needles attached to a 3-mL tuberculin syringe into a new Petri dish containing fresh media. When the content of the bone marrow from all the bones are collected, make single cell suspension using the cell strainer and the end of a sterile syringe plunger.
8. Wash the cells once with media. Count the number of cells obtained. Typically, between 10^7–10^8 bone marrow cells can be obtained from one mouse.
9. Each recipient mouse requires between 5×10^6–2×10^7 bone marrow cells. Resuspend the bone marrow cells in cold sterile PBS at an appropriate concentration that contains the desired number of cells in 0.2 mL per mouse.
10. While the procedure for the donor bone marrow is taking place, irradiate the recipient mice at between 800–1000 rad (lethal irradiation). The lethal dose depends on the mouse strain. For example, BALB/c mice take less irradiation (825 rad) and C57B/6 mice take more (925 rad). Take extra care not to expose the recipient mice to any pathogens.

11. Heat the irradiated recipient mice with a infrared heating lamp for a few minutes. Place a recipient mouse in a mouse holder and clean its tail with an alcohol swab.
12. Inject via the tail vein 0.2 mL of the bone marrow cell suspension (2.5×10^7–1×10^8 cells/mL). Pressure the tail with a gauze to stop bleeding.
13. Place a fresh bottle of water with antibiotics in the cages. Do not feed the recipient mice for 24 h after irradiation.

3.1.4. Treatment of Mice Before and After Lethal Irradiation

Mice irradiated at a lethal dose are very prone to infection. One of the common causes of death after irradiation of mice is *Pseudomonas* infection. In order to avoid death of the mice by bacterial infection, recipient mice should be treated with antibiotic before and after the irradiation until complete reconstitution by the donor bone marrow-derived cells takes place. Again, all procedures are to be done under a laminar flow hood.

1. Autoclave the drinking water and allow it to cool down.
2. Make the antibiotic Clavulin stock by dissolving 1 g of Clavulin-125F into 40 mL of sterile 0.9% NaCl solution. Mix well.
3. Add 5 mL of the stock solution to 100 mL of drinking water in a sterile bottle.
4. Change water with Clavulin three times a week.

3.1.5. Plasmid DNA Immunization of Reconstituted Mice

Once the full reconstitution of the recipient mice is confirmed (Subheading 4.3.), plasmid DNA immunogen can be administered to these mice. The detailed methods and protocols for different ways of immunizing with DNA have been described elsewhere in this volume. We describe here a method that was used to obtain CTLs by i.m. injection of DNA.

1. Prepare DNA from bacterial culture using Qiagen Megaprep columns. Detailed instructions are given in the Megaprep Kit. This kit allows endotoxin-free isolation of plasmid DNA. Store lyophilized DNA at –20°C.
2. Resuspend the lyophilized DNA in an appropriate volume of sterile saline to make concentration of 2 μg/mL (50 mL per mouse).
3. Fill insulin syringes with DNA solution (number of mice injected × 50 mL per mouse).
4. Wipe the leg area with alcohol swab. Inject 50 μL of DNA per quadriceps muscle.

3.2. Peptide/Epitope-Specific Cytotoxic T Lymphocyte Assay

3.2.1. In Vitro Re-stimulation of Splenocytes

In order to generate CTLs specific for a particular class I MHC-restricted peptide, we describe here a method for re-stimulating selectively those memory CTLs that recognize a particular peptide derived from the antigen encoded by the DNA immunogen.

3.2.1.1. Stimulator Cells

1. Sacrifice naive mice that express the MHC haplotype of the chimeric host. The number of mice required for stimulator cells is half of the total number of responder mice of that particular strain.
2. Collect spleens into PBS in 15 mL tubes on ice. Make single cell suspension by mashing it through cell strainers in a Petri dish filled with 10 mL of media using the head of a sterile syringe plunger.
3. Transfer the cells to 15 mL tubes and wash the splenocytes three times with media.
4. Resuspend the cells in 10 mL media, and seal the lid tight. Irradiate the cells at 2000 rads.
5. Wash the cells once with media. Resuspend cells in 2 mL of media.
6. Prepare a sterile peptide solution by resuspending 100 μg of the antigenic peptide in 1 mL of cold PBS, and filtering through a 0.2 micron filter.
7. Add the peptide solution to the stimulator cells.
8. Incubate at 37°C for 1 h.
9. Resuspend the culture in appropriate volume which can be transferred to the flasks containing the responders (1 mL per flask).
10. Transfer 1 mL aliquot of stimulator cells to the flasks containing responders.

3.2.1.2. Responder Cells

1. Sacrifice mice previously immunized with plasmid DNA. Optionally, collect blood before sacrifice for detection of antibody responses.
2. Harvest spleens into cold PBS in 15 mL tubes. Make single cell suspension by mashing it through cell strainers in a Petri dish filled with 10 mL of media, using the head of a sterile syringe plunger.
3. Transfer the cells to 15 mL tubes and wash the splenocytes three times with media.
4. Resuspend the cells in 10 mL of media. Transfer the content into 50 mL tissue culture flasks.
5. Add stimulators.
6. Add enough ß-mercaptoethanol to make the final concentration to 50 μ*M* in 15 mL total volume.
7. Adjust the total volume to 15 mL with media.
8. Culture the cells with flasks standing upright, undisturbed for 6–7 d at 37°C/5% CO_2.

3.2.2. 51_{Cr}-Release Assay

3.2.2.1. Target Cells

1. Count the number of target cells. Use tumor cell lines which express the MHC class I allele to which the antigenic peptide binds.
2. Transfer the number of cells required to cover the number of wells in a 96-well plate. 10^4 target cells per well are incubated in triplicate (three columns) with twofold serial dilutions of effector cells (from 100:1 to 0.78:1, eight rows).
3. Centrifuge the cells at 210*g* for 5 min. Discard supernatant.

4. Resuspend the cells in 70 µL of 25% FCS media. To one of the tubes, add 10 µL of 0.1 µg/µL antigenic peptide in PBS to be used as the peptide pulsed target. Save the other tube as an unpulsed target control.
5. Add 100 µCi of [^{51}Cr] Na_2CrO_4 per tube. Incubate at 37°C for 1 h.
6. Add 10 mL of media and incubate for 30 min more.
7. Wash the cells with media thoroughly (at least three times). Monitor the supernatant for radioactivity by a Geiger counter to make sure that no more radioactivity is detected after the last wash.
8. Resuspend the cells at 10^5 cell/mL. Aliquot 0.1 mL per well on top of effector cells.

3.2.2.2. Effector Cells

1. Resuspend the culture that has been incubated for 7 d.
2. Count the number of cells.
3. Transfer the required number of cells (2×10^6 cells/well × number of wells) to 15 mL tubes.
4. Centrifuge the cells at 210*g* for 5 min. Discard supernatant.
5. Resuspend cells at 10^7 cell/mL in media. Aliquot 0.2 mL per well into the top row only.
6. Fill the rest of the wells with 0.1 mL media. Using a multichannel pipettor, make serial dilutions by transferring 0.1 mL suspension from the top row to the second row to the third row and so on until the last row. Since the top row contains 10^6 cells/well, serial dilution of that would give effector to target ratios of : 100:1, 50:1, 25:1, 12.5:1, 6.25:1, 3.13:1, 1.56:1, and 0.78:1 in the 8 rows of 96 well plates.
7. To obtain maximum possible ^{51}Cr release from each target cell sampl, add to a number of wells 100 µL of 2% Triton-X100. Also, to obtain spontaneous ^{51}Cr release values from the various target cells, add to a number of wells 100 µL of media alone.
8. Add target cells to the appropriate wells, including those for maximum and spontaneous release.
9. Centrifuge the plates at 400 rpm for 3 min to settle the cells down to the bottom of the wells.
10. Incubate the plates with lids on at 37°C for 4 h.
11. Using Skatron cell harvester, collect supernatant from each wells and count the supernatant on a gamma counter.
12. Maximum and spontaneous release can be determined from wells that contained either 2% Triton-X100 or medium alone, respectively. Specific lysis is calculated as (experimental ^{51}Cr release—spontaneous ^{51}Cr release)/(maximum ^{51}Cr release—spontaneous ^{51}Cr release) × 100 %.

4. Notes

1. It is absolutely crucial that the depletion of T cells obtained in vivo to be close to 100% in order to prevent GvHD by the residual donor T cells. The antibody

concentration required to deplete T cells to completion can be obtained by concentrating the supernatant from CELLMAX by Centriprep filter system. Typically, the supernatant from the hybridomas must be concentrated to 150–200-fold the volume of the total culture media used to grow the hybridomas in order for a 0.5 mL volume injected on d 0 and 3 to completely deplete both subsets of T cells.

2. The survival of the irradiated mice depends on the cleanliness of the environment in which they are maintained. Lethally irradiated mice are very prone to infection by common bacteria such as *Pseudomonas*. Due to the irradiation-induced damage to the intestinal epithelia, it is recommended that mice not be fed for the first 24 h after irradiation. Aside from the antibiotic treatment indicated in **Subheading 3.1.4.**, scrupulous care must be taken to handle mice with antiseptic techniques. For example, anything that comes in contact with the mice should either be autoclaved or wiped with Clidox (chlorine dioxide) or other antiseptic agents. Surgical masks should be worn by all those who work with the mice, as well as clean set of gloves for each cage. All procedures should be carried out strictly under laminar flow hood. The first two weeks are the most critical period in determining whether mice will survive without infection. Later on, mice may die of GvHD, in cases where the T cells from the donor mice were not properly depleted.
3. Normally, complete reconstitution of bone marrow injected mice requires between 6–8 wk. Since the nature of the experiment requires that no recipient-derived hemopoietic cells be present in these mice, and that reconstitution by the donor bone marrow be complete, it is recommended that the chimerism of these mice be assessed after 6 wk.

   To determine the extent of reconstitution, peripheral blood cells can be stained for the MHC class I or class II molecules. For example, if the bone marrow-chimeric mice are parent into F1, the peripheral blood cells should only stain positively for the parental donor haplotype but not for the other allele expressed by the F1 cells. **Fig. 2** depicts the flow cytometric profile of peripheral blood cells from reconstituted mice stained with anti-MHC class II antibodies. The results clearly demonstrate complete reconstitution in H-2^{b}→H-2^{bxd} and H-2^{d}→H-2^{bxd} chimeric mice.
4. Although a number of CTL in vitro re-stimulation protocols are available, some of which are illustrated in this volume, we describe a method for selectively restimulating memory CTLs specific for a single class I MHC-restricted peptide. The presence of 50 μ*M* ß-mercaptoethanol in the stimulation culture has been found to be very important for a proper growth environment for CTLs in our hands. Also, the flasks are stood upright during the 7 d incubation to maximize the interaction between the stimulator cells and responder cells. Although other re-stimulation methods involve addition of growth factors such as IL-2 or supernatant from Con A stimulated splenocytes, we have not found this to be necessary. **Fig. 3** depicts representative lysis curves for CTLs obtained, using the protocol described here, from H-2^{b}→H-2^{bxd} and H-2^{d}→H-2^{bxd} bone marrow-chimeric mice immunized with the plasmid DNA encoding the nucleoprotein of influenza.

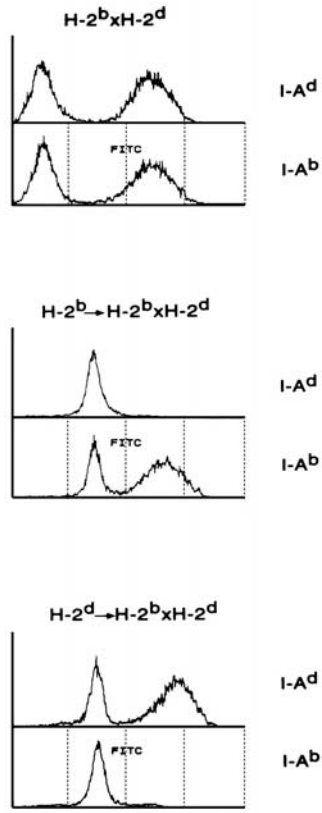

Fig. 2. Anti-class II MHC staining of peripheral blood cells from the bone marrow reconstituted mice. In order to confirm complete reconstitution by donor bone marrow, 7 wk after the bone marrow injection, peripheral blood was collected from F1(H-$2^b \times$ H-2^d) (top), H-$2^b \rightarrow$(H-$2^b \times$ H-2^d) (middle), H-$2^d \rightarrow$(H-$2^b \times$ H-2^d) (bottom), and stained for MHC class II molecules, I-A^b and I-A^d. The FACS profiles depicted here are representative of all the chimeric mice used in this study.

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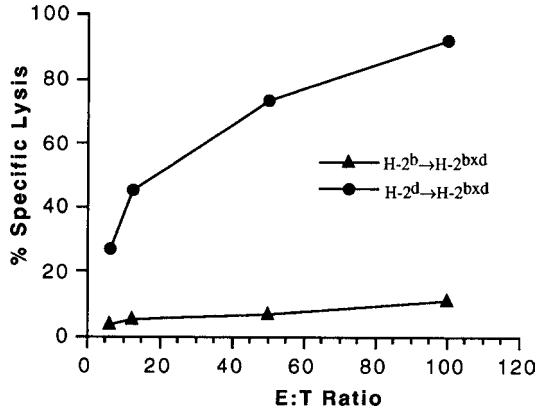

Fig. 3. CTL lysis curves of P815 (H-2^d) cells pulsed with Kd-restricted NP147-155 peptide. Parent into F1 bone marrow-chimeric mice, H-2^b→H-2^{bxd}, and H-2^d→H-2^{bxd}, were immunized with influenza NP gene, and assayed for CTL lysis activity against P815 (H-2^d) cells pulsed with NP147-155. In this example, it is clear that only reconstitution with H-2^d bone marrow has enabled the development of the H-$2K^d$ restricted, peptide specific CTL response.

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14

Immunostimulatory DNA Sequences

An Overview

John H. Van Uden and Eyal Raz

1. Introduction

The biochemical and genetic properties of DNA have been thoroughly investigated, yet only recently has it been appreciated that DNA carries more information than simply a blueprint for the regulation and construction of proteins. Indeed, the immune systems of vertebrates appear to have evolved the ability to distinguish the foreign DNA of bacteria and certain viruses from the self-DNA of the host, a new twist on the self vs non-self detection system already well-known for foreign proteins. Specifically, the frequency of unmethylated CpG motifs (CpG denotes covalently linked CG dinucleotides, not C:G base pairs) is extensively suppressed in vertebrates, including mammals (by at least 20-fold ***[1]***), whereas it is found at the usual frequency ($^1/_{16}$) in most bacterial and viral DNA. There have now been several reports (detailed in **Subheadings 2.** and **4.**) that bacterial DNA or synthetic oligodeoxyribonucleotides (ODNs) containing bacterially derived sequences, stimulate the immune systems of mice and humans to first mount innate, and then antigen-specific (when foreign antigen is present), Th_1-type responses. This adjuvant effect of bacterial immunostimulatory DNA sequences (ISS) appears to be important for the robust Th_1-type immune response usually seen in genetic vaccination ***(2)***. Although the terms CpG motif and ISS are generally used synonymously in this field, CpG motifs are defined structurally, whereas ISS are defined functionally (and therefore include non-CpG sequences that have been found to be stimulatory). A more complete understanding of this phenomena should allow for more efficacious medical applications as well as a better view of the basic mechanisms responsible for immune stimulation.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

2. Early Reports of the Immunostimulatory Properties of Nucleic Acids

As early as 60 years ago, Freund found that dead mycobacteria had antigen-independent immunostimulatory abilities ***(3)***. Although several important immunostimulatory substances have been found since then in crude bacterial preparations, including endotoxin (pyrogenic lipopolysaccharide [LPS]), muramyl dipeptide (MDP), exotoxins (such as diphtheria, tetanus, and cholera toxins), and superantigens (such as staphyloccocal enterotoxins and toxic shock syndrome toxin 1), it is likely that part of the activity of Freund's adjuvant is due to the mycobacterial DNA constituent ***(4)***.

The first report of the direct immunostimulatory properties of nucleic acids came with the demonstration that dsRNA or its analogue, poly-inosinic, poly-cytidilic ribonucleic acid (pIpC), are able to induce the type I interferons (IFN-α/ß) and thus provoke an anti-viral response ***(5,6)***. More recently, the IFN-induced, dsRNA-activated protein kinase (PKR) has been shown to mediate this immune recognition ***(7,8)***. This system is presumed to have evolved to recognize intracellular dsRNA because it is associated with the life cycle of a variety of pathogenic viruses.

The first reports of immunostimulation by a relatively pure fraction of microbial DNA came from Tokunaga and colleagues ***(9–14)***. They found in 1984 that a nucleic acid fraction of Mycobacterium bovis BCG (MY-1) has potent anti-tumor activity in several mouse models, that the DNA is solely responsible for the effect, and that the DNA is not directly cytotoxic, so that the effect is host-mediated ***(9,10)***. It was also shown that the antitumor response is mediated primarily by natural killer (NK) cells activated in vivo by the bacterial DNA ***(11)***.

In vitro experiments have demonstrated that bacterial DNA and poly (dG,dC) ODNs activate macrophages and NK cells, and stimulate the anti-viral response (inhibited by anti-IFN-α/ß antibodies) ***(13,15)***. Histologically, it was shown that injection of bacterial DNA leads to early (day 4) infiltration of macrophages and NK cells, and late (d 14) infiltration of delayed-type hypersensitivity (DTH) T cells ***(14,16)***. Pisetsky and coworkers discovered in 1991 that DNAs from a variety of bacterial species are also potent T-cell independent B-cell mitogens for multiple different mouse strains, whereas DNAs from various mammalian species are not ***(17)***.

3. Attempts to Define the Sequences of DNA that are Immunostimulatory

Tokunaga et al. first demonstrated that particular synthetic ODNs taken from sequences in the mycobacterial genome are able to account for the immune activation seen with bacterial DNA. It was found that 6 of 13 synthetic 45-mer ODNs representing randomly selected sequences of the mycobacterial genome

caused anti-tumor activity, NK activation, and production of IFN-α/ß and IFN-γ. Analysis of shorter overlapping ODNs as well as insertion of active sequences into otherwise inactive ODNs suggested that certain palindromic hexamers, each of which contain CpG dinucleotides, are responsible for the immune activation by the bacterial DNA ***(18–22)***.

Since this original preliminary characterization of the DNA sequences required for immunostimulation, more methodical studies to determine the optimal and required sequences for activation have been carried out. Krieg and co-workers, using mouse B cell stimulation as their primary model to screen over 150 sequences, have found that single-stranded ODNs containing the consensus sequence of a unmethylated hexamer of 5'purine-purine-CG-pyrimidine-pyrimidine3' induce optimal B cell stimulation. Maximal effect was seen when the 5' purines were GA and the 3' pyrimidines were TC or TT, and TGACGTT was found to be the optimal sequence for B cell activation ***(23)***. This group found that the hexamer palindromic structure is not an absolute requirement, although the reported active palindromes were still found to have high activity ***(23)***. In other studies, this consensus model has been shown to hold also for stimulation of production of IL-6, IL-12, and IFN-γ in mouse spleen cells ***(24)*** and for induction of both mouse and human NK cell activity ***(25)***. The most universal requirement is for the inclusion of at least one CpG dinucleotide, but near-terminal CpGs are much less effective ***(21,23)***. However, even this rule has exceptions, as some sequences without the CpG dinucleotide retain sub-optimal ISS properties ***(26)***.

Additionally, several groups have reported that methylation at the C-5 position of the cytosine in the CpG motif (^{me}CpG) almost completely abolishes the immunologic activity of ISS both in vitro and in vivo ***(23,25,27–29)***. This phenomenon is particularly interesting because vertebrate genomic DNA is highly methylated at the cytosines in CpG motifs (approx 70%), whereas non-animal organisms (including pathogens) do not methylate their CpG sequences to any large degree ***(1,30)***, allowing the immune system another way to recognize foreign DNA. In fact, the dominant theory for the mechanism of CpG suppression in vertebrates is that, over evolutionary periods, ^{me}CpG heritably mutates, primarily to TpG and its complement CpA ***(30)***. Methylation of CpG motifs in eukaryotic promoter elements has been shown to nearly completely shut down transcriptional activity of the gene, and is a well-characterized method of long-term transcriptional regulation in development, X-chromosome inactivation, and suppression of both invading and endogenous viral and transposon genomes ***(31)***. Most runs of multiple unmethylated CpG motifs (CpG islands) in the vertebrate genome are strictly associated with the promoters of actively transcribed genes ***(1)***, so it appears that although there are potentially immunologically active CpG sequences in host genomes, evolution has only allowed

the bare minimum to remain. Active ISS may be even further under-represented than non-stimulatory CpG motifs; it has been reported that four of the most stimulatory CpG motifs are less than one-third as common as four of the least stimulatory CpG motifs among human sequences in GenBank, while these sequences occur with similar frequencies in *E. coli* ***(23,32)***.

It is convenient to think of ISS as modular 6-mer units that are necessary and sufficient for immunostimulation, but there is evidence that distal flanking sequences are also important. It has been found, by successive deletions of the 3' end of ISS-containing ODNs (ISS-ODNs) with fixed 6-mer 5' flanking regions, that a minimum length of 18 bases is required, while maximal activity is seen with ODNs of 22 bases or longer ***(33)***. However, it has also been shown that a 15-mer ODN can efficiently stimulate NK activity ***(25)***. Interestingly, cationic liposomal transfection of ODNs consisting solely of the 6-mer ISS motif is stimulatory, implying that this is the minimal active unit inside the target cell ***(22)***. As a further complexity, nuclease-resistant phosphorothioate-modified ODNs (ps-ODNs; see below) have been demonstrated to stimulate without transfection when they are as short as eight bases in length ***(23)***. One of several consistent hypotheses is that, while the 6-mer ISS is the minimal required element, the flanking sequences may allow enough time for the ODNs to enter the cell and trigger the immunostimulatory signaling cascade before exonucleases destroy the active ISS.

The nucleotide composition of flanking sequences has also been shown to affect the relative activity of ISS. Phosphodiester poly-G repeats surrounding the 6-mer ISS have been shown to be able to significantly enhance the activity of ODNs on mouse splenocytes ***(21)***, an effect which appears to be dependent on scavenger receptor binding and internalization of the ODNs ***(34)***. Contrastingly, it has also been reported that ps-ODNs are actually made less active if they are flanked by poly-G ***(25)***, and that ps-poly-G without any ISS (a phosphorothioate homopolymer of G) actually inhibits interferon-γ (IFN-γ) production in response to bacterial DNA, Con A, or PMA/ionophore ***(35)***.

Phosphorothioate-modified ODNs (ps-ODNs) have been shown to be approximately 200-fold more potent than the natural phosphodiester ODNs (po-ODNs) without transfection, while maintaining nearly identical immunomodulatory properties ***(23)***. Ps-ODNs differ from their natural counterparts in that they have a sulfur substituted for one of the two non-bridging oxygens of the phosphate that forms the link between nucleotides. Thus, ps-ODNs are nuclease resistant and have much longer half-lives both in vitro and in vivo ***(36–39)***. These ps-ODNs have been characterized extensively for use in the field of antisense, and are available commercially. For the above reasons, ps-ODNs are gaining in popularity and are currently the ODN of choice in most of the labs working in this field.

Considerably less has been published about the optimization of ISS for humans. Although it is clear that many sequences that stimulate mouse cells also give good responses in human cells, it is equally clear that there is some degree of species-specificity ***(2,12,25,26,40)***. Furthermore, all of the sequence comparisons have used in vitro data, usually using peripheral blood mononuclear cells (PBMCs) instead of splenocytes (where the mouse work is generally done), so it is difficult to extrapolate to in vivo activities. We have found considerably more variability in the responses of human donor cells to ISS than in cells from various mouse strains (E. Raz, unpublished data). One report has compared the efficacy of a variety of different ODNs in stimulating human peripheral blood B cells, and has found that, while the previously described ISS are potent, so are some CpG immunostimulatory motifs that do not fit the 5'pur-pur-CG-pyr-pyr3' model. Also, they show that some sequences that do not contain any CpGs at all yield low levels of stimulation ***(26)***. Interestingly, by far the most potent ODN in this study was derived from an antisense ODN to the *rev* gene of HIV-1. The unexpected and strong ISS effects of this ODN have been independently characterized by the original antisense group ***(28,41,42)***. This and other examples of the unforeseen effects of ISS on experiments designed to address unrelated questions highlight the need, not only for a better understanding of ISS, but also for a broader dissemination of this knowledge.

Recently another paradigm for the immune activity of DNA has been discovered. It has been reported that some sequences containing repeating or alternating Cs and Gs are able to specifically inhibit the effects of ISS both in vitro and in vivo (named CpG-N by the authors) ***(42a)***. Additionally, we have found a different class of motifs that also potently inhibit ISS at all levels, but these sequences have Th2-promoting effects in vivo and in vitro as well (Nguyen, Malek, Van Uden, Tighe, and Raz, submitted for publication). Therefore, there seems to be an important balance between the ratios of different types of immunologically active sequences in large DNA (genomic or plasmid), and a better understanding of this will likely allow for the more rational design of DNA vaccines and gene therapy vectors.

4. Effects of Immunostimulatory DNA Sequences

4.1. Th1-Biased Responses to Antigens

Bacterial DNA (as well as plasmids and ODNs derived from it) stimulates the innate arm of the immune system to activate several non-specific antimicrobial defense systems and sensitizes the specific arm to mount both antibody and cell mediated antigen-restricted responses against any associated foreign antigen, as has been demonstrated in genetic vaccination. Although not complete, there is a clear bias of the immune system toward antigen-specific Th_1-type responses (characterized by $CD8^+$ CTL; $CD4^+$ Th_1 cells; IgG2a>IgG1;

IFN-γ>IL-4, IL-5, IL-10) and away from the opposing Th_2-type responses ***(2,4,43–51)***. One prominent exception to this rule is the generalized Th_0/Th_2-type bias seen with intradermal inoculation using particle-mediated transfer technologies (the gene gun) ***(52,53)***. This exception is likely due to the greatly reduced quantity (approximately 1%) of adjuvant-like DNA delivered by the gene gun relative to injection techniques ***(4,54)***. In fact, the Th_1-inducing immunomodulatory properties of ISS may account for some of the effects seen in many genetic vaccination, antisense, and gene therapy studies (unpublished meta-analysis) ***(2,29,41)***. We have shown that ISS in the plasmid backbone of vectors used for genetic vaccination function as potent Th_1-promoting adjuvants, and are required to allow the minute quantities of antigen that are expressed to be highly immunogenic ***(2)***. Additionally, we and others have shown that ISS-containing plasmids or ODNs also function as Th_1-inducing adjuvants when coadministered with protein antigens, opening up new possibilities of boosting or modifying current protein or subunit vaccines ***(54a–54c)***.

4.2. Splenomegaly

The most striking anatomical change induced by injection of bacterial DNA or immunostimulatory ODNs into mice is the massive transient splenomegaly uniformly observed within 2 wk. Although it has been shown that bacterial DNA is a powerful B cell mitogen (see **Subheading 4.6.**), this probably does not account for the several fold increases in spleen size observed ***(41)***. Indeed, it has been reported that the majority of the increased mass of the spleen can be attributed to extramedullary hematopoesis in the spleen and not to alterations of the quantity or quality of mature immune cells ***(54d)*** (and our unpublished observations). This splenomegaly has not been documented in humans or non-human primates treated with high doses of antisense ODNs ***(55)***.

4.3. NK Cell Activation

As detailed in **Subheading 2.**, early studies found that bacterial DNA possesses potent anti-tumor effects, which are largely attributable to activation of macrophages and NK cells ***(9–11,15,16)***. Purified NK cells were shown to be refractory to stimulation with ISS, implying that they are not directly activated. Indeed, antibody blocking studies have revealed that NK cell activation is decreased by blocking IL-12, TNF-α, or IFN-α/b, and that blocking all three cytokines completely abrogates NK cytolytic activity ***(20,25,56)***. Similarly, it was shown that cytokines from adherent splenocytes stimulated with bacterial DNA are required for secretion of IFN-γ from non-adherent splenocytes (presumed to be NK cells) ***(56)***. Furthermore, B and T cells are not required for activation of NK cells, because the NK inducibility of splenocytes from SCID mice is normal ***(25)***. In addition to becoming cytotoxic, NK cells activated by bacterially-derived DNA secrete large amounts of IFN-γ ***(24,57)***. In the early

response (within days), NK cells have been shown to comprise over 90% of the IFN-γ secreting cells ***(57)***, whereas later on, $CD4^+$ T cells contribute significantly ***(24)***. In addition to mouse cells, NK cells from human peripheral blood mononuclear cells (PBMCs) are also activated by ISS-ODNs ***(25)***.

4.4. Macrophage and Dendritic Cell Activation

Activated cytotoxic macrophages are also seen early in the response to ISS. Although macrophage activation in the response has been shown to be largely abrogated by blocking antibodies against IFN-γ ***(13,15,20)***, other studies have documented direct activation of macrophages and macrophage-like cell lines ***(2,27,56,58)***. It seems likely that macrophages are activated by ISS directly, but that IFN-γ potently augments their response. In addition to becoming cytotoxic, ISS-activated macrophages also produce TNF-α, IL-12, IL-18 (also known as IFN-γ inducing factor, IGIF), IFN-α, and IFN-ß ***(2,4,27,56,58)***. In vitro, IL-12 and IL-18 are potent stimulators of IFN-γ. Following ISS stimulation of splenocytes, IL-12 reaches its maximum expression level at 24 h, whereas IFN-γ peaks at about 48 h, suggesting that primary IL-12 production stimulates later IFN-γ production ***(56)***. The response is not limited to mouse cells; primary human monocytes have also been shown to be activated by ISS ***(2)***. Interestingly, this pattern of cytokine secretion sets up a local inflammatory positive-feedback loop of macrophage and NK cell activation in response to ISS. Macrophages (and possibly other APCs or stromal cells) are activated by bacterial DNA to secrete IFN-α/ß, TNF-α, and IL-12, which in turn activate NK cells to secrete IFN-γ, which stimulates the macrophages further, etc. A host of surface molecules are also induced on macrophages upon their activation by ISS. For example, bone marrow derived macrophages treated with ISS-ODN upregulate their expression of class I MHC, CD40, ICAM-1, and CD16/32, all of which are important in promoting antigen processing and presentation ***(58a)***.

Primary dendritic cells are also activated by ISS to produce IL-12, IL-6, TNF-α and increase their expression of class II MHC and B7-2 ***(58b,58c)***. ISS-activated dendritic cells also become more potent APCs, as seen in their ability to activate T cells in vitro in allogeneic mixed lymphocyte reactions and with superantigen stimulation ***(58b)***. Thus, the professional APCs of the immune system, macrophages and dendritic cells, are directly and potently activated through multiple mechanisms by ISS.

4.5. Indirect T Cell Activation and the Antigen-Specific Response

T cells also contribute to this cytokine positive feedback loop. IFN-α/ß, IL-12, IL-18, TNF-α, and IFN-γ from macrophages and NK cells have been shown to bias naive T helper cells toward the Th_1 phenotype that is associated with cell-

mediated immunity ***(59–64)***. When activated by these cytokines and by appropriate antigen presented on MHC class II, Th_1 cells secrete relatively large amounts of IFN-γ, which activates macrophages in the locality of the Th_1-recognized foreign antigen (delayed type hypersensitivity) ***(65)***. Bacterial DNA and ISS-ODNs do not appear to directly activate T cells to undergo proliferation or cytokine production ***(17,66)***, as they do for B cells (see **Subheading 4.6.**). Also, it has been shown that T cells are not required for the primary antigen-independent activation of NK cells, macrophages, and B cells in response to ISS ***(17,25–27,56,58,66)***.

Nevertheless, both $CD4^+$ and $CD8^+$ T cells have been shown to be instrumental, using depletion and adoptive transfer studies, in the development and maintenance of the characteristic Th_1-type specific immunity induced by genetic vaccination ***(51,67)***. Histological examination of regressing tumors injected with bacterial DNA reveals that activated Th_1 cells are seen in the lesion at late times (d 14), and that they are important for tumor rejection ***(68)***. Due to the fact that the T cell stimulation is indirect and is therefore easier to detect in vivo than in vitro, there are no published reports of T cell activation by ISS in humans yet, but there are reports of Th_1 responses to genetic vaccination in humans ***(68a,68b)***, which may be mediated by ISS in the vector backbone. Additionally, studies have shown Th_1-like responses in non-human primates with genetic vaccination ***(69–71)***.

4.6. B Cell Activation

In contrast to their indirect effects on T cells, immunostimulatory sequences have direct mitogenic and activating effects on B cells. This was first described in 1991 when lymphocytes depleted of T cells were shown to undergo proliferation in response to bacterial DNA ***(17)***. Subsequently, B cell activation has become the most thoroughly investigated effect of immunostimulatory sequences. B cells (both purified and in crude splenocyte cultures) have been demonstrated to undergo polyclonal proliferation in response to bacterial DNA and ISS-ODNs, and this effect is dependent upon unmethylated CpG motifs (ISS) ***(23,26,41,42,66,72)***. In fact, over 95% of B cells in vitro enter the cell cycle under optimal conditions ***(23)***. ISS also cause B cells to secrete high titers of polyclonal IgM antibodies independent of T cell help ***(23,26,41,73,74)***. Additionally, ISS-DNAs are able to rescue the B cell line WEHI-231 from surface IgM crosslinking-induced growth arrest and apoptosis ***(75)***. These results imply that ISS can send an antigen-independent costimulatory signal, allowing rapid low-affinity immune responses, but also creating the possibility of autoimmunity (*see* **Subheading 5.3.**).

Some of the effects of activated B cells are mediated through the cytokines they release, and ISS-stimulated B cells have been shown to produce both IL-6

and IL-12 ***(24,73,74)***. The production of these cytokines coupled with the efficient antigen-presentation abilities of activated B cells suggests that they could be key players in initiating the hallmark responses of the immunostimulatory effects of DNA, including antibody production and Th_1 activation. Blocking IL-6 action by using IL-6 knockout mice (Martin-Orozco and E. Raz, unpublished data), or using anti-IL-6 antibody, does not decrease the proliferative response. However, anti-IL-6 does reduce IgM secretion as much as 90%, indicating that this may be an autocrine stimulation ***(24,74)***. It is not clear whether the macrophage-produced or B cell-produced IL-12 is quantitatively more important in induction of the Th1-type response ***(2,24,56)***, and it may depend in vivo upon the site of contact with bacterial DNA.

Interestingly, low-level B cell receptor crosslinking (simulating specific antigen recognition by surface IgM) combined with ISS administration synergistically potentiates proliferation, IL-6 production, and IgM production by B cells ***(23,74)***. This implies that while bacterial DNA is an antigen-independent B cell activator, it also enhances antigen-specific B cell responses. Exogenous IFN-γ down-regulates IgM in response to LPS, but with ISS-ODNs, IFN-γ actually increases the amount of IL-6 and IgM produced ***(73)***. The surface molecule profile of B cells is also potently stimulated by ISS. Class I MHC, class II MHC, B7-1, B7-2, CD40, CD16/32, ICAM-1, IFN-γR, and IL-2R are upregulated to varying degrees both in vivo and in vitro, and CD23 (FcεR) is downregulated. This remarkable alteration in surface phenotype is largely cytokine independent and would be expected to enhance the ability of B cells to act as activating APCs ***(58a)***. Human B cells also respond to ISS-ODNs, and 95% of optimally stimulated B cells increase the surface expression of the activation markers B7-2 and the IL-2 receptor ***(26)***.

5. Potential Side Effects of Immunostimulatory DNA Sequences

As described above, immunostimulatory DNA sequences are extremely potent antigen-independent activators of a variety of cell types, launching both antibody-mediated and cell-mediated responses. The immune system hangs in a delicate balance, however, and extreme activation or polarization toward either Th1 or Th2 responses could cause unwanted effects. Most of the work in gene vaccination and gene therapy in general is aimed at eventually producing therapeutics for human use, therefore the possible adverse effects of ISS must be examined rigorously.

5.1. Provocation of a Septic Shock-Like Syndrome

Another T-independent B cell mitogen, LPS, induces the immune system to mount early innate responses against gram-negative bacteria, and is thus likely to be useful in the small quantities generated by controlled local infections.

However, large amounts of LPS induce a systemic inflammatory response that can lead to lethal septic shock. The inflammatory cytokines IL-1 and especially TNF-α have been shown to be the primary mediators of LPS-induced septic shock, and although not causative, an elevated level of IL-6 is the most reliable prognostic factor for shock ***(76)***. As outlined earlier, IL-6 and TNF-α have been shown to be produced in large amounts by bacterial DNA. Two published reports have addressed the issue of whether ISS can promote systemic inflammatory responses. In the first study, bacterial DNA or ISS-ODNs were injected i.v., followed by sublethal doses of LPS. They found that TNF-α and IL-6 were massively increased relative to calf thymus (mammalian DNA) pretreated cells, and that the rate of lethal septic shock was increased from 0% to 75%. However, IFN-γ receptor knockout mice were almost completely protected from this potentiation of the LPS response, suggesting that IFN-γ production from NK cells is important in this effect ***(57)***. The second study to address this issue found that mice that were sensitized to TNF-α by pretreatment with D-galactosamine underwent lethal shock when challenged with high doses of bacterial DNA or ISS-ODNs (without additional LPS). This effect was blocked by anti-TNF-α antibody and by using TNF-α receptor knockout mice, but not by SCID beige mice (T, B, and NK cell deficient). This implies that in this model, the TNF-α produced by macrophages is more important than the IFN-γ produced by NK and T cells ***(58)***. Interestingly, these researchers also showed that certain modified ISS-ODNs can retain the ability to stimulate IL-12 release by macrophages while losing their TNF-α stimulating ability. These motifs are able to serve as adjuvants for a Th_1 response but have lost their ability to induce toxic shock ***(76a)***. Although these studies show that ISS can contribute to a systemic inflammatory reaction when the system is otherwise primed, there have not been any reports of any reaction with bacterial DNA or ISS-ODNs alone, regardless of dose in either mouse or human.

5.2. Local Inflammation

In addition to systemic inflammation, excessive local inflammation can also cause serious problems, as seen with bacterially derived adjuvants, such as complete Freund's adjuvant (CFA), and this is conceivable with purified bacterial DNA. There have been literally hundreds of in vivo genetic vaccination and antisense studies with active ISS-containing plasmids and ODNs, without any grossly observable local inflammatory responses reported. Additionally, we have found that intradermal genetic vaccination does not induce local inflammatory infiltrates, despite the fact that it does provoke strong immunity ***(44)***, and that a non-coding immunostimulatory plasmid, pUC-19, does not cause histological inflammation if delivered alone intradermally ***(77)***. In con-

trast, it has been shown that while intramuscular genetic vaccination with a weak immunogen (luciferase) allows expression for greater than 60 d without histological inflammation, a vector encoding a strong immunogen (hepatitis B surface antigen or ß-galactosidase) results in nearly complete immune-dependent destruction of the transfected myofibers between 5–20 d with inflammatory infiltrates ***(78)***. It is important to note that this study shows that ISS in the vector plasmids do not cause destructive inflammation by themselves; the response appears to be an antigen-specific cell-mediated attack on the expressed antigens. However, it has been demonstrated that delivering bacterial DNA or ISS-ODNs without exogenous antigen to the lungs of mice causes moderate increases in numbers of neutrophils and other cells, as well as the inflammatory cytokines TNF-α, IL-6, and MIP-2 in the lavage fluid. Additionally, it was shown that DNA from the lung fluid of cystic fibrosis (CF) patients, who suffer from recurrent bacterial lung infections and chronic inflammation, is able to reproduce the effects of ISS-containing DNA in mouse lungs ***(79)***. This indirectly implies that DNA from lysed bacteria in the lungs of chronically infected CF patients may contribute to the pathogenicity of this disease. It may be that one current therapy used in CF, DNase instillation into the lungs of patients, destroys the immunostimulatory effects of the bacterial DNA present, in addition to decreasing the viscosity of lung fluid to allow better clearance. However, this and previous studies have not directly found bacterially-derived DNA to cause any clinically significant damage to the lung or any symptoms of respiratory distress ***(79,80)***.

5.3. Autoimmune Disease Induction

Another potential adverse effect of the adjuvanticity of ISS is the possibility of precipitating an autoimmune disease in genetically or environmentally prone people. There is some evidence that the immunostimulatory properties of bacterial DNA may be able to play a role in the development of autoimmune diseases such as systemic lupus erthythematosus (SLE). One prominent feature of SLE is the presence of anti-DNA antibodies that are widely cross-reactive (sequence non-specific). Gilkeson and coworkers have shown that when bacterial DNA (but not mammalian calf thymus DNA) is given to mice complexed with methylated BSA in CFA, IgM and IgG anti-DNA antibodies are produced at sustained levels, but are not cross-reactive with mammalian DNA ***(81,82)***. Additionally, this methyl-albumin-bacterial DNA complex in CFA provokes proteinuria and proliferative glomerulonephritis in normal BALB/c mice ***(83)***. However, when this mixture is given to preautoimmune NZB/NZW F1 mice (a model for genetic susceptibility to SLE), anti-DNA antibodies that can cross-react with mammalian DNA are produced ***(84)***, but they do not increase the severity of spontaneous nephritis in these animals. Unexpectedly, treatment

with the complexed bacterial DNA actually gives significant protection from autoimmune nephritis and prolongs survival relative to untreated mice and mice treated with complexed calf thymus DNA ***(85)***. It is important to note, however, that the CFA used as an adjuvant in each group also includes mycobacterial DNA, complicating the interpretation of these results. Another correlation between SLE and ISS activation is that human SLE is associated with high levels of IL-6, and disease initiation and progression in mouse models is dependent upon IL-6 ***(32)***, one of the cytokines produced by ISS activation. Also, because bacterially derived DNA stimulates B cell polyclonal proliferation and activation, as well as resistance to apoptosis of cells that recognize antigen in the absence of proper costimulation, it could theoretically lead to expansion and activation of autoreactive B cells.

ISS are particularly efficient at stimulating cell-mediated immune responses, and precipitation of one of the Th_1-mediated autoimmune diseases is therefore a potential problem. This was addressed recently using a mouse model of multiple sclerosis, experimental allergic encephalomyelitis (EAE). It was found that T cells that have been primed in vivo with myelin basic protein (MBP) in CFA can be activated in vitro to leave quiescence and become capable of transferring EAE to recipient mice by incubation with MBP and bacterial DNA, ISS-ODN, or LPS ***(86)***. This Th_1 cell activation was found to be entirely dependent upon IL-12 ***(86)***. Although a relatively artificial system, this study supports the hypothesis that certain types of infection may contribute to the development of autoimmunity and shows that the potent immunostimulatory properties of ISS-laden DNA are potentially dangerous under conditions of extreme autoimmune sensitivity.

5.4. Reduction in Antigen Expression

An additional concern is that the interferon-mediated antiviral response generated by ISS would be expected to decrease antigen expression levels. Thus, it may be that too much ISS adjuvant as either ODN or in plasmid DNA could inhibit the immune response. Indeed, neutralization of type I IFNs increase expression levels from DNA vaccines in the interferon-sensitive cell line MG-63 (Raz, unpublished data) and it has been reported that coinjection of ISS-ODN with DNA vaccines in vivo can reduce transgene expression and the ensuing immune response in a dose-dependent manner ***(86a)***. Another explanation proposed by these authors for the results is that the ODNs are competing with the plasmid for uptake and therefore transfection efficiency is reduced. This effect could be particular for the phosphorothioate-modified ODNs or for the model antigen (hepatitis B surface antigen) because it has been previously shown that coinjection of the ISS-containing plasmid pUC19 with a plasmid encoding the antigen is effective for increasing the immune response to the

antigen ***(2)***. One potential way to circumvent the very serious issue of antigen expression level while still coupling the antigen to the DNA-based adjuvant is to covalently conjugate the protein antigen to ISS-ODN. We have found that this approach is much more potent and effective than plasmid DNA vaccination or mixtures of protein and ISS-ODN in mice and non-human primates (Tighe and Raz, manuscript in preparation).

6. Molecular Mechanisms of Immunostimulation by DNA

The mechanism of ISS recognition by cells and the way this recognition is translated into cellular activation is still largely unknown. Although it is likely that there are one or more ISS binding proteins that can distinguish ISS from non-ISS, there is still no published experimental evidence of this, or of any other mechanism for cells to sense ISS. Nevertheless, several groups have reported evidence that the DNA must be internalized for activity, as opposed to activating a surface receptor. Phosphodiester (non-modified) ISS-ODNs are much more potent when liposomally transfected into cells ***(87)***, and the minimum active size of these ODNs decreases from approximately 16-mers to 6-mers (containing only the ISS without flanking sequences) when transfected ***(22)***. Also, although no data or methods were shown, it has been reported that ISS-ODNs covalently linked to a solid support lose their stimulatory effects in mouse B cells, and that ODNs with and without ISS bind the surface of B cells equally well ***(23)***. However, another group found that ISS-ODNs bound to sepharose beads did stimulate human B cells ***(26)***. Therefore, it is conceivable that there are different mechanisms of activation in human and mouse B cells. However, further work is required to address this possibility.

The downstream signaling events of ISS activation have also been examined. It has been reported that ISS activation of B cells does not increase tyrosine phosphorylation, inositol triphosphate (IP_3) levels, or calcium ion (Ca^{2+}) levels, therefore these three common pathways of signaling are unlikely to be involved ***(23)***. Inhibitors of activation via the PKC, PKA, and NO pathways do not decrease production of IL-6 in response to ISS, but antioxidants inhibit to baseline levels. Additionally, this study showed that the levels of reactive oxygen species (ROS) were increased within 20 min of treatment (before IL-6 mRNA, which was first detected at 30 min) with ISS-ODN ***(74)***, implying that the generation of ROS is a very early event and that it is required for activation. When activated by ISS-containing plasmid, the mouse macrophage-like cell line RAW-264 expresses mRNA of plasminogen activator inhibitor-2 and, when costimulated with IFN-γ, it expresses inducible nitric oxide synthase. Additionally, these ISS-stimulated cells activate transcription of reporter genes driven by the HIV-1 long terminal repeat (HIV-1 LTR) pro-

moter. NF-κB is one of the transcription factors that binds to and activates the HIV-1 LTR, and has also been shown to be activated by ISS via gel-shift assays *(27)*.

Both NF-κB and ROS are involved in the signaling pathways of a large number of immune and inflammatory systems, including many of those observed as effects of ISS *(88,89)*. Additionally, ROS activate NF-κB, so these studies may be providing insight into the same signaling pathway *(90)*.

More recently the mitogen activated protein kinase (MAPK) and stress kinase pathways have been implicated in ISS signaling. ISS-ODN have been shown to activate c-Jun NH2-terminal kinase (JNK), JNKK1, p38, activating transcription factor-2 (ATF-2), c-Jun, MAPK- activated protein kinase-2, and activator protein-1 (AP-1) *(90a,90b)*. Blockade of the p38 pathway has been shown to block the subsequent signaling cascade and also the generation of cytokines, and is therefore thought to be a crucial component *(90a,90b)*. These signaling pathways are well-characterized regulators of immunological responses, but more difficult to interpret is the finding that blockade of the acidification of endosomes by chloroquine, monensin, or bafilomycin A potently inhibits the entire signaling pathway and the subsequent production of cytokine *(90a,90b)*. It remains to be elucidated exactly how these various relatively nonspecific immune signaling mechanisms combine to yield the characteristic rapid and concerted Th_1-promoting immune response that has been observed.

The previously mentioned study (**Subheading 4.6.**) that showed that ISS can rescue a B cell line from anti-IgM induced apoptosis also found that the expression levels of several genes associated with apoptosis are altered by ISS. Although the mRNA levels and protein levels were not consistently regulated in the same fashion, myn, bcl_2, and bcl-x_L appear to be upregulated by ISS, whereas the IgM-induced downregulation of c-myc is blocked by ISS *(75)*, indicating that these growth and activation regulatory genes may be important in the response to bacterial DNA.

There is also some evidence that the cAMP system may be involved. Forskolin, a cAMP antagonist, blocks IFN induction in response to ISS-ODN *(22)*. It has been hypothesized that, because the cAMP response element (CRE) consensus sequence, TGACGTCA, is so similar to one of the maximally stimulatory ISS, TGACGTT, the ISS may exert its effects by binding to the CRE binding protein complex (CREB/ATF) or a cellular analog. An additional similarity is that cytosine methylation of the CpG motif in the CRE sequence prevents the binding of CREB/ATF to it, just as methylation of the CpG motif in ISS abrogates its activity *(32,91)*.

7. Clinical Applications of the Th1 Bias

There are several situations in which inducing a strong Th_1 response and reducing a Th_2 response in an antigen-specific manner would be therapeutic.

For example, we and others have shown that IgE and other correlates of Th_2-mediated allergy and asthma are downregulated in an antigen-specific manner by ISS-ODN and by genetic vaccination, potentially leading to a new era in immunotherapy ***(2,91a,91b,43,51,67).*** Additionally, Th_1 responses (including CTL) are required to clear many pathogens, and some invaders even bias the immune response toward ineffective Th_2 responses against their dominant antigens ***(92)***. These would clearly be good targets for genetic vaccines with Th_1-inducing ISS as adjuvants because protein vaccines and current adjuvants (which generally bias toward a Th_2 response) are relatively ineffective against them. Many pathogens infect via the mucosal route, and ISS has also been demonstrated to be a potent mucosal adjuvant ***(93–95)***. In addition to prophylactic genetic vaccination, some chronic infectious diseases such as those caused by hepatitis B virus, human immunodeficiency virus, and herpes simplex virus may be treatable with therapeutic genetic vaccination despite the state of functional tolerance that they facilitate in their hosts. ISS-ODN can even be used without antigen as either a prophylactic or a therapeutic treatment for intracellular pathogens such as Leishmania major and Listeria monocytogens ***(96,97)*** due to the activation of the antigen non-specific immune response. Similarly, cancer immunotherapy against tumor-associated antigens may be possible, taking advantage of the strong CTL induced by genetic vaccination with ISS as an adjuvant (***98*** and Cho and Raz, in preparation).

8. Summary and Perspectives

Immunostimulatory DNA sequences provoke a rapid concerted activation of the innate immune system by directly triggering non-specific responses from NK cells, macrophage/monocytes, and B cells (polyclonal). This adjuvant effect produces a local environment that facilitates the anti-viral response (IFN-α/ß, IFN-γ), cytotoxic activity (NK cells and macrophages), polyclonal neutralizing antibodies (B cells), and induction of foreign antigen-specific Th_1-like responses (IFN-α/ß, IFN-γ, IL-12, and IL-18). The ensuing immunity is characterized by long-lasting antigen restricted Th_1 $CD4^+$ cells, cytotoxic $CD8^+$ T cells, and antibody (with relatively more IgG_{2a} than IgG_1 in the mouse) in response to genetic vaccination or coadministration of protein antigen. This profile of responses is particularly valuable because it is difficult to achieve with typical vaccines and seems to be exactly the one required to successfully clear intracellular pathogens ***(64)***.

Therefore, it appears that ISS can be used as discrete DNA-based adjuvants. In general, when more immunostimulation or Th_1 deviation is advantageous, such as in most genetic vaccination applications, more numerous or potent ISS should be used. However, when an immune inflammatory response or anti-viral state would be counter-productive, such as in gene replacement therapy,

or when Th_2 responses are called for, ISS should be engineered out of the vector DNA *(2,4)*. It is not yet clear which ISS are the most effective in humans, although it is clear that human cells are at least similar to mouse cells in their reactivity to ISS.

In addition to being a useful tool and important confound, the recognition of ISS by the immune system appears to be a fundamental mechanism of basic immune surveillance that is likely to be key in the early host response against invading organisms. Further characterization of the phenomenology and mechanism of ISS activation of the immune response is therefore not only required for clinical applications, but also likely to yield significant new insights into as yet unforeseen areas of biology.

Acknowledgments

The authors are supported in part by grant and by Dynavax Tecnologies Corp. AI 40682 from the National Institutes of Health. J.V.U. is a Medical Scientist Training Program Trainee (NIGMS grant GM 07198) and a Lucille P. Markey Charitable Trust Fellow.

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Immunostimulatory CpG Motifs and DNA Vaccines

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1. Introduction

1.1. Role of Immunostimulatory Motifs

Bacterial DNA, but not vertebrate DNA, causes direct stimulation of several components of the vertebrate immune system. This activation is due to the presence of unmethylated CpG dinucleotides ***(1)***, which are present at the expected frequency in bacterial DNA, but are underrepresented ("CpG suppression") and methylated in vertebrate DNA ***(2)***. The immunostimulatory effects include direct induction of B cell proliferation and immunoglobulin (Ig) secretion ***(1)***, as well as activation of monocytes, macrophages, and dendritic cells to upregulate their expression of costimulatory molecules, which drive immune responses, and secreting a variety of cytokines, including high levels of IL-12 ***(3,4)***. These cytokines then, in turn, stimulate natural killer (NK) cells to secrete IFN-γ and to have increased lytic activity ***(5)***. Overall, CpG DNA induces a Th1 like pattern of cytokine production dominated by IL-12 and IFN- γ, with little secretion of Th2 cytokines ***(4,5)***. These effects can also be obtained with synthetic oligonucleotides (ODN) ***(6,7)*** or plasmid DNA vectors ***(8)*** containing CpG immunostimulatory motifs. From a teleological view, it appears likely that the rapid immune activation in response to CpG DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial molecules.

Not all CpG motifs are immunostimulatory, however. Not only is the particular sequence context of the unmethylated CpG dinucleotide important, but there are species-specific motifs ***(9)***. Many motifs which work well on mouse cells do not stimulate human cells, whereas those which do stimulate human cells will also stimulate, at least to some degree, mouse cells. Also, the best motif depends on the backbone used; the best CpG motif with a phosphorothioate

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

backbone ODN will not necessarily be the best with a phosphodiester backbone.

Although the effects of CpG motifs are not antigen-specific, B cell activation by low concentrations of CpG DNA synergizes strongly with signals delivered through the B cell antigen receptor for both B cell proliferation and Ig secretion ***(1)***. This strong synergy between the B cell signaling pathways triggered through the B cell antigen receptor and by CpG DNA promotes antigen-specific immune responses and suggests utility of CpG DNA as a vaccine adjuvant.

1.2. CpG Motifs and DNA Vaccines

DNA vaccines composed of plasmid DNA have numerous CpG motifs (approx 1 per 20 bases), which by virtue of their production in bacterial cells are unmethylated. At least a proportion of these are immunostimulatory and it appears that such CpG sequences act as an autoadjuvant with DNA vaccines, and indeed that they may even be required for the successful induction of immune responses ***(5)***.

It should be possible to augment the immune responses of DNA vaccines by the selective use of CpG motifs. There are two different approaches that could be used to do this: (i) administration of CpG-containing ODN with the DNA vaccine, or (ii) direct cloning of the CpG sequences into the DNA vaccine vector.

2. Methods and Problems

2.1. Mixing CpG ODN with DNA Vaccines

The easiest method to use CpG ODN as adjuvants to DNA vaccines is to use synthetic CpG ODN along with the plasmid DNA vaccine. This approach has worked well with protein vaccines using CpG ODN made with a phosphorothioate backbone (see below), which is preferred over the naturally occurring phosphodiester backbone because it is resistant to nucleases ***(3)***. However, problems have arisen when this approach has been tried with DNA vaccines. We have found that coinjection of ODN with a phosphorothioate backbone and plasmid DNA results in decreased gene expression in a dose-dependent manner (as determined with a luciferase reporter gene construct), and that this in turn results in a dose-dependent decrease in immune responses to a DNA vaccine (as determined with a hepatitis B surface antigen-expressing plasmid). This effect is due to the synthetic backbone and not the CpG motif since it also occurs with a non-CpG control phosphorothioate ODN. There are several possible reasons for the interference effect of the ODN on the plasmid. For example, it could block the entry of the DNA into muscle cells, as has been found in vitro ***(10)***. It might also block transcription of the transfected plasmid DNA or translation of the mRNA. When the ODN contains a CpG motif, the induced secretion of cytokines may also affect expression. For example IFN-γ,

a cytokine that is known to be induced by CpG ODN has been reported to down-regulate the expression by viral promoters ***(11–13)***. However, this cannot explain the results with the non-CpG ODN.

ODN with a phosphodiester backbone does not interfere with expression of foreign genes from plasmid DNA injected into muscle. Unfortunately, they are degraded so quickly ***(10)*** that they do not have a significant adjuvant effect in vivo (Davis et al., unpublished results). We are currently testing alternative modified backbones to see if we can identify one that will not interfere with gene expression yet will cause immune stimulation.

2.2. Cloning CpG Motifs into DNA Vaccine Vectors

There has been confusion and disagreement as to whether it is possible to augment immune responses by cloning CpG motifs into the plasmid DNA vectors. Although the plasmids have a phosphodiester backbone, they are mildly nuclease-resistant by virtue of being in a closed-circular form, and thus in theory, this should be possible. Indeed, a report by Sato et al. ***(8)*** shows a significant improvement in immune responses by the cloning of only two immunostimulatory CpG sequences into a DNA vector. However, this has not been repeatable in other systems (personal communications). It is possible that, for most antigens and doses used, an additional two motifs is simply too few to make a significant difference. When one takes into consideration the fact that most DNA vaccine vectors already contain 200–300 CpG motifs, several of which will be immunostimulatory, it is unlikely that an additional two motifs would have a significant impact on the immune response. The optimal number of CpG motifs to be cloned into a DNA vaccine is yet to be determined.

2.3. Use of CpG ODN as Adjuvant with Protein Vaccines

It is possible to augment both humoral and cell-mediated immune responses to antigen-based vaccines by the addition of CpG ODN. For reasons discussed above, this is best mixed directly with the antigen for coadministration by intramuscular, subcutaneous or intraperitoneal injection. It is best to use synthetic ODN with a phosphorothioate backbone, and for studies in mice, a good pan-activating sequence is TCCATGACGTTCCTGACGTT. Doses as small as 1 μg can be effective, although we routinely use doses of 10–100 μg. The ODN should be ordered as a sodium salt rather than an ammonium salt. If it arrives as a lyophilized powder, it may be redissolved directly into saline, TE, or any other buffer of choice.

3. Notes

CpG ODN appears to work extremely well with protein vaccines, indeed much better than alum ***(6)***, the only adjuvant currently approved for human

use, or even Freund's complete adjuvant (unpublished results). It should be possible to improve the efficacy of DNA vaccines through the rational use of CpG motifs; however, this area clearly needs further study. While few methods are actually given in this chapter, it is hoped that the description of the problems we have encountered will save time and frustration for someone entering into this field.

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In Vitro Assay of Immunostimulatory Activities of Plasmid Vectors

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1. Introduction

1.1. Principles of DNA Vaccination

DNA vaccination represents a powerful new approach for the elicitation of long-lived protective immunity against a broad range of protein antigens ***(1,2)***. In this approach, the vaccine is a plasmid DNA vector that encodes a foreign protein to be targeted for the induction of humoral or cellular responses. Following administration by various routes, the plasmid is taken up by cells to allow intracellular production of the protein for presentation to the immune system. Although the trafficking of the plasmid and its protein product is not well understood, the generation of responses ultimately involves a bone marrow-derived antigen presenting cell ***(3)***.

The effectiveness of DNA vaccines has been impressive and has been subjected to considerable investigation to optimize this strategy and improve vectors. While the intracellular production of antigen may facilitate immune responses, the vectors themselves may be crucial for effectiveness. As shown in recent studies, these vectors have immunostimulatory properties, which can serve as adjuvants, both increasing the magnitude of responses as well as their cellular profile. The immunostimulatory properties of vectors, as with naturally-occurring bacterial DNA, result from DNA sequences whose content differs between mammalian and bacterial DNA.

1.2. Immunological Properties of Bacterial DNA

Contrary to long-held notions on the immunological inertness of DNA, bacterial DNA displays potent immunostimulatory properties that are manifest in both in vivo and in vitro systems. These properties are similar to those of endo-

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

toxin and suggest that, during ordinary encounters with infecting organisms, bacterial DNA can serve as a danger signal and activate the innate immune system ***(4,5)***. As defined originally in the murine system, these properties encompass induction of cytokines, including IFN-α/ß, IL-12, IFN-γ, TNF-α, and IL-6; stimulation of B cell mitogenesis and immunoglobulin production; and downstream effects of cytokines, such as activation of NK cells or promotion of Th1 responses ***(6–11)***. The cytokine effects also occur in human peripheral blood cells, although a direct mitogenic effect on human B cells has not as yet been described. Stimulation by bacterial DNA involves activation of NF-κB by an anti-oxidant sensitive pathway that appears common to other immune activators ***(12,13)***.

1.3. DNA Sequences with Immunological Activity

The immunostimulatory properties of bacterial DNA reflect structural microheterogeneity and the presence of short sequence motifs that are characteristic of prokaryotic DNA. These motifs, also termed immunostimulatory sequences (ISS), or CpG motifs, have the general structure of two 5' purines, an unmethylated CpG dinucleotide, and two 3' pyrimidines. These motifs occur much more commonly in bacterial DNA than mammalian DNA for two reasons. In mammalian DNA, cytosine and guanosine occur in tandem much less frequently than predicted by DNA base composition, a phenomenon called CpG suppression. Furthermore, in mammalian DNA, unlike bacterial DNA, cytosine is commonly methylated ***(9,14,15)***. While the biological advantages of CpG suppression and cytosine methylation are not known, they provide the basis for a recognition system that allows distinction of eukaryotic and prokaryotic DNA for the self–nonself discrimination.

In addition to CpG motifs, other DNA sequences may influence the immunostimulatory properties of bacterial DNA as well as plasmids. Thus, runs of deoxyguanosine can directly stimulate murine B cells as well as promote production of cytokines, as reflected in IFN-γ expression. IFN-γ expression results from the activity of IL-12 and TNF-α, both products of macrophages/monocytes. As shown using synthetic oligonucleotides, runs of dG, while unable to induce cytokine production themselves, can enhance the stimulatory activity of ISS when juxtaposed in the same oligonucleotide ***(16–18)***. This enhancement may result from the increased uptake of dG-rich oligonucleotides by the macrophage scavenger receptor. This receptor has broad ligand specificity and binds a variety of polyanions, including acetylated low density lipoprotein, fucoidan, dextran sulfate and dG-rich oligonucleotides and polynucleotides. By unconventional hydrogen bonding between dG residues, dG-rich compounds can form four stranded arrays called quadruplex DNA ***(19)***. The increase in macrophage production of IL-12 and TNF-α by ISS presum-

ably results from an increased uptake into cells via binding to the macrophage scavenger.

1.4. Vectors as a Source of ISS

As currently formulated, plasmid vectors for vaccination represent a potential source of ISS because: (i) plasmids have unmethylated cytosines due to propagation in bacteria; and (ii) plasmids display bacterial DNA sequences because of the encoded foreign protein as well as genes for replication or antibiotic resistance. The importance of vector ISS to the induction of vaccine responses has been clearly established in in vivo experiments in mice. Thus, methylation of plasmid vectors leads to loss of vaccine activity. Furthermore, the potency of vaccine vectors varies dramatically depending on the content of ISS in antibiotic resistance elements ***(20,21)***.

In addition to potentiating overall vaccine response, ISS in the plasmid may influence the generation of Th1 responses that are characteristic of DNA vaccine responses in mouse models. While IL-12 most likely causes the Th1 predominance, IFN-α/ß may also contribute to this pattern of T-cell responsiveness. In this regard, the presence of ISS could complicate DNA vaccination because of local inflammation, shift in the balance of Th1/Th2 cells, as well as stimulation of anti-DNA antibody production. In these activities, plasmid DNA does not differ from bacterial DNA, which is a normally-encountered foreign antigen and appears well-tolerated ordinarily. While bacterial DNA can induce antibody responses in normal individuals, the antibodies appear non-pathogenic by virtue of their isotype and selectivity for bacterial DNA ***(4)***.

1.5. In Vitro Assessment of Vector Activities

Characterization of in vitro immunostimulatory properties of plasmid vectors is important, especially as vaccination techniques are refined and vectors engineered to promote effectiveness. Although the array of immunostimulatory activities of bacterial DNA is large, they reflect the presence of common motifs. This feature allows selective assay of activities considered most crucial for an adjuvant effect. These activities include expression of IL-12 and IFN-γ as well as B cell mitogenesis. In contrast, assessment of DNA uptake is more problematic because the mechanisms of DNA uptake by cells appear to depend on concentration as well as ambient conditions. Since increased uptake should translate into increased immunostimulatory activity, however, either cytokine responses or B cell mitogenesis can be used as surrogate markers for uptake.

In using in vitro assays to assess ISS, certain caveats should be considered. First, human responses appear more sporadic than murine responses. This variability could result from differences among humans in their intrinsic capacity to respond to DNA. Alternatively, the bacterial DNA sequences causing opti-

mal stimulation may differ in human and murine systems. Most of the work on immunostimulation has utilized oligonucleotides that have been characterized in the murine system, suggesting the need for a search for other active motifs for human study. Furthermore, natural DNA sequences differ significantly in their activities, raising the possibility of active sequences in addition to CpG motifs; of equal importance, bacterial DNA as well as plasmids may contain inhibitory sequences that can affect the response to ISS.

Although cytokine stimulation can be assayed in terms of either mRNA or protein, available assays by ELISA are convenient and allow sampling of multiple specimens. The methods described utilize this approach and have been verified in the murine system with natural DNA, oligonucleotides as well as plasmids.

2. Materials

2.1. Preparation of Mouse Splenocytes

1. Suitable mice are available from The Jackson Laboratory (Bar Harbor, ME) (*see* **Note 1**).
2. 70% (v/v) ethanol.
3. Dissecting tools, scissors and forceps.
4. 60 mm diameter sterile petri dishes (Falcon, Los Angeles, CA).
5. Frosted microscope slides.
6. RPMI 1640 medium with sodium bicarbonate and glutamine (Sigma, St. Louis, MO).
7. Heat inactivated fetal bovine serum (HyClone, Logan, VT).
8. Red blood cell lysis solution consisting of 1 part 0.17 *M* Tris pH 7.6 and 9 parts of 0.16 *M* ammonium chloride filtered through a .22 micron Nalgene filter unit (Nalgene, Rochester, NY).
9. Sterile 15 mL conical polypropylene centrifuge tubes (Costar, Cambridge, MA).
10. Hemacytometer, microscope.
11. Sterile 96 well flat bottom tissue-culture-treated cell culture clusters (Costar).
12. Control mitogens, LPS, ConA (Sigma).
13. Purified sterile DNA, oligonucleotides (*see* **Note 2**).
14. Multi-channel pipettor with capacity of at least 100 µL (Finnpipette) and sterile tips for the pipettor (USA/Scientific Plastics, Ocala, FL).
15. Single-channel pipettors with ranges of 5–40 µL and 40–200 µL (Finnpipette) and sterile tips (USA/Scientific).
16. Complete medium consisting of RPMI 1640, 5% (v/v) heat inactivated fetal bovine serum, and 5×10^{-5} M 2-mercaptoethanol.
17. 5 and 10 mL sterile serological pipets (Costar).
18. Laminar flow hood (Baker, Phillipsburg, NJ).
19. Incubator, 37°C, 5% (v/v) CO_2 (Forma Scientific, Marietta, OH).

2.2. Measurement of Proliferation

1. Thymidine [methyl-^{3}H] 6.7 Ci/mmol aqueous solution (New England Nuclear, Boston, MA).

2. Cells (mouse splenocytes or human peripheral blood cells) plated in 96 well plates stimulated with mitogen.
3. Microharvester (Bellco Glass Inc., Vineland, NJ).
4. Glass fiber strips for microharvester.
5. Distilled water.
6. Scintillation vials, 5.5 mL (USA/Scientific).
7. Scintillation fluid: Safety Solve (Research Products International, Mt. Prospect, IL).
8. Scintillation counter: Packard Tri-Carb (Downers Grove, IL).

2.3. General ELISA Reagents

1. 96 well polystyrene plates. Use Immulon II for cytokines, Microtiter for total immunoglobulin (Dynatech, Chantilly, VA).
2. 96 well polypropylene plates (Sigma).
3. PBS pH 7.2, for washing plates.
4. PBS pH 7.2, 1% (w/v) bovine serum albumin, for blocking plates.
5. Multichannel pipettor, 50–300 µL (Finnpipette), and tips which hold at least 300 µL (USA/Scientific).
6. PBS pH 7.2, containing 0.5% (w/v) bovine serum albumin, 0.4% (v/v) Tween 20 (PBS–BSA–T). Make fresh daily or filter sterilize and store it at 4°C. Use this for diluting samples, standards, and antibodies.
7. 1.2 mL polypropylene tubes in a 8 × 12 array rack with the same spacing as 96 well polystyrene plates for diluting standards and culture supernatants (USA/Scientific).
8. Citrate buffer, 21g citric acid monohydrate/L water. Adjust the pH to 4.2 with 50% (w/v) sodium hydroxide.
9. TMB: dissolve 3,3',5,5'-tetramethylbenzidine dihydrochloride (Sigma) to 0.75% (w/v) in water and filter to 0.22 µ to remove any undissolved material. Store in 1 mL aliquots at –20°C.
10. Hydrogen peroxide, 30% (v/v) (*see* **Note 3**).
11. Plate washer: Skatron Skan Washer (Skatron Instruments, Sterling, VA).
12. Microplate reader with filters suitable for reading at a wavelength of 380 nm (Molecular Devices, Eugene, OR).

2.4. Measurement of Cytokines by ELISA

1. Coating buffer PBS pH 8.5.
2. Capture/biotinylated detection antibody pairs and standards purchased from Pharmingen. Store antibodies at 4°C. Store standards diluted in sterile PBS, 1% (w/v) bovine serum albumin as aliquots frozen at –70°C (**Table 1**).

2.5. Measurement Total Murine Immunoglobulins by ELISA

1. PBS pH 7.2, for coating buffer.
2. Goat anti-mouse polyvalent immunoglobulins for use as capture antibody are available from Sigma.
3. Goat anti-mouse IgG peroxidase conjugate, γ-chain-specific for measuring IgG (Sigma).

Table 1
Reagents for Cytokine Assays[a]

Mouse Cytokines	Capture Ab	Detection Ab(biotin)	Standard
mIL–2	18161D	18172D	19211T
mIL–4	18191D	18042D	19231V
mIL–6	18071D	18082D	19251V
mIL–10	18141D	18152D	19281V
mIL–12 p40	18491D	18482D	19401W, 19371W
mIL–12 p70	20011D	18482D	19361V
mIFN–γ	18181D	18112D	19301T
Human Cytokines			
hIL–6	18871D	18882D	19661V
hIL–12 p40	20711D	20512D	19931V, 19721V
hIL–12 p70	20501D	20512D	19721V
hTNF–α	18631D	18642D	19761T

[a]Numbers refer to Pharmingen Research Products Catalog 1996–1997.

4. Goat anti-mouse IgM peroxidase conjugate, μ-chain-specific for measuring IgM (Sigma).
5. Purified mouse IgG (Sigma).
6. TEPC 183, mouse IgM myeloma protein (Sigma).

2.6. Preparation of Human Peripheral Blood Cells

1. Heparinized whole blood or commercially available fresh buffy coat (Interstate Blood Bank Inc.).
2. RPMI 1640 with l-glutamine and sodium bicarbonate (Sigma).
3. Ficoll-Hypaque lymphocyte isolation medium (Ficoll-Paque, Pharmacia, Piscataway, NJ).
4. Sterile 50 mL polypropylene conical centrifuge tubes (USA/Scientific).
5. Complete medium consisting of RPMI 1640 + 10% (v/v) heat-inactivated fetal bovine serum.
6. Hemacytometer and microscope.
7. Sterile 96-well flat bottom tissue culture treated cell culture clusters (Costar).
8. Multi-channel pipettor with capacity of at least 100 μL (Finnpipette) and sterile tips for the pipettor (USA/Scientific).
9. Single-channel pipettors with ranges of 5–40 μL and 40–200 μL (Finnpipette) and sterile tips (USA/Scientific).
10. Laminar flow hood (Baker).
11. Incubator, 37°C, 5% (v/v) CO_2 (Forma).

2.7. Assessment of Endotoxin in DNA/Oligonucleotide Preparations

1. Quantitative Chromogenic Limulus Amebocyte Lysate Kit (BioWhittaker, Walkersville, MD).

2. 96-well polystyrene microtiter plates.
3. Microplate reader with filters suitable for reading at a wavelength of 405–410 nm (Molecular Devices).
4. Lipopolysaccharide (Sigma).
5. Deoxyribonuclease I (Sigma).

3. Methods

3.1. Preparation of Mouse Splenocytes

1. Sacrifice mice by cervical dislocation.
2. Disinfect the fur by thoroughly saturating it with 70% (v/v) ethanol.
3. With scissors make an incision in the abdominal skin and, grasping either side of this incision with gloved forefingers and thumbs, pull back the skin until the spleen can be observed through the abdominal musculature. Make an incision over the spleen and remove it to a 60 mm Petri dish containing about 5 mL of medium.
4. Flame 2 frosted microscope slides. Pick up the spleen with one of the slides and use the second to express the cells. Start at one end and work towards the middle. Then start at the other end until all the cells are expressed. Frequently dip the slides in the medium in the petri dish to transfer the cells. Discard the remaining connective tissue.
5. Transfer cells to a 15 mL conical tube, make up to 10 mL total volume with medium. Allow large chunks to settle out for 2 min. Carefully remove cell suspension to a new tube and centrifuge 5 min at 400*g* to pellet the cells. Remove supernatant and resuspend cells in RBC lysis buffer. Use 5 mL per spleen. Pellet the cells, 5 min, 400*g*. Resuspend in medium and pellet. Repeat 2 times to thoroughly remove lysis buffer. Resuspend in 10 mL complete medium and count cells with a hemacytometer. Yield should be $0.7–1.5 \times 10^8$ cells/spleen for most mouse strains.
6. Adjust cell concentration for proliferation studies to $2–5 \times 10^6$/mL. and for cytokine assays to $2–5 \times 10^7$/mL. Transfer cells to 96-well plates, 100 µL/well.
7. Prepare DNA and control mitogens/cytokine inducers at 2 times the final concentration in complete medium. Most stimulatory DNAs give a maximum response at a final concentration of approximately 50 µg/mL. LPS will give a maximum response at 1–10 µg/mL and ConA 1–5 µg/mL (*see* **Note 4**). Allow 100 µL/well. Prepare triplicate wells. Pipette onto cells and mix. Place plates in humidified incubator, 37°C, 5% (v/v) CO_2.
8. Incubation times for optimum response vary. Various cytokines are at their maximum from 4–48 h. Proliferation is generally greatest at 48 h but time courses should be established.

3.2. Preparation of Human Peripheral Blood Cells

1. Dilute anti-coagulant treated blood with an equal volume of RPMI 1640.
2. Carefully pipet 15 mL Ficoll-Paque into the bottom of a 50 mL conical centrifuge tube.
3. Carefully layer the diluted blood on top of the Ficoll-Paque.

4. Spin at 400*g* for 30 min at 20°C.
5. Inspect tubes. There should be a pink upper layer, an interface of cells, a clear or slightly cloudy layer of Ficoll-Paque and a pellet of red blood cells. If separation is not adequate, spin for an additional 20 min.
6. Carefully remove the interface of cells with a pipet trying not to remove any Ficoll-Paque and dilute with 4 volumes of RPMI.
7. Spin at 400*g* for 5 min. Discard supernatant and suspend cells in fresh RPMI. Centrifuge. Repeat twice to ensure cells are thoroughly washed.
8. Remove a sample and count with a hemacytometer.
9. Centrifuge cells and resuspend in RPMI-1640/10% (v/v) FBS to appropriate concentration and transfer to 96 well plates, 100 μL/well.

3.3. Tritiated Thymidine Incorporation

1. Prepare 2 mL of diluted label for each 96 well plate by adding 40 μL of [^{3}H]-thymidine stock to 1.96 mL serum free RPMI 1640. Add 20 μL to each well (0.5 μCi), mix gently, label plate to denote that it contains radioactive material and return it to the incubator for 6 h.
2. At the end of the incubation period, harvest cells on glass fiber strips. Wash with distilled water for at least 10 pulses of the harvester rocker valve.
3. Remove filters and allow to dry overnight.
4. With gloved hands and a pair of forceps remove individual filter disks from the glass fiber strip and place them in scintillation vials. (Vials placed in a rack in an 8×12 array simplifies this procedure.)
5. Add 3 mL of scintillation fluid to each vial and cap vial.
6. Count in scintillation counter using the standard program for tritium customized to give the means of three successive vials.

3.4. Analysis of Secreted Cytokines

1. Carefully remove 150 μL of fluid from each well using a multi-channel pipettor, being careful not to disturb the cell layer. Transfer to polypropylene 96 well plates, cover and freeze at –20°C until assayed.
2. Dilute capture antibody in PBS pH 8.5 to a concentration of 0.5–5.0 μg/mL. The optimum concentration should be established for each lot but satisfactory results can usually be obtained at 1 μg/mL. Place 100 μL of diluted antibody in each well, wrap plates in aluminum foil or cover with plate covers and place in the refrigerator (4°C) overnight.
3. The next morning prepare standards in PBS–BSA–T. The standards will occupy two rows of eleven wells on each plate. The twelfth wells are left as blanks. Include these standards on every plate. Dilutions are made in polypropylene tubes racked in the same spacing as the wells of the plates.
4. Make 1:2 dilutions. Start with 0.5 mL of standard diluted in the first tube and 0.25 mL PBS–BSA–T in each successive tube. Suggested standard ranges are indicated in **Table 2**.
5. Wash the plates 3 times with PBS with the plate washer.

Table 2
Detection Range of Cytokine Assays

mIL–2	2000–2 pg/mL	hIL–6	5000–5 pg/mL
mIL–4	5000–5 pg/mL	hIL–12p40	10,000–10 pg/mL
mIL–6	2000–2 pg/mL	hIL–12p70	5000–5 pg/mL
mIL–10	100–0.1 ng/mL	hTNF–α	5000–5 pg/mL
mIL–12p40	5000–5 pg/mL	hIFN–γ	5000–5 pg/mL
mIL–12p70	5000–5 pg/mL		
mIFN–γ	1000–1 U/mL		

6. Place 75 µL PBS–BSA–T in each sample well and transfer 25 µL of each sample from the storage plate to its corresponding position on the assay plate. Mix well by pipetting. Transfer the standards to designated positions on plate, 0.1 mL/well. Incubate 2 h at room temperature.
7. Wash plates 3 times and add 100 µL biotinylated detection antibody diluted in PBS–BSA–T to each well. The optimum concentration of biotinylated detection antibody must be determined for each lot, but in general will be in the range of 1–40 µg/mL. Incubate the plates for 2 h at room temperature, then wash them 3 times with PBS with the plate washer.
8. Add 0.1 mL avidin/peroxidase diluted 1:5000 in PBS–BSA–T and incubate for 30 min at room temperature.
9. Wash the plate 3 times with PBS, reverse the plate and wash it 3 times more with the plate washer.
10. Add 0.1 mL TMB solution to each well (1 mL TMB stock, 17 µL H_2O_2/50 mL citrate buffer pH 4.0). This should be made fresh just before use.
11. Incubate the plate at room temperature 5–30 min. Timing must be determined for each assay. Read the OD_{380} with the plate reader.
12. Plot the log of the concentration of standards vs. OD_{380} and use the curve to determine the sample concentrations.

3.5. Determination of Total Murine Ig

1. Coat 96 well Microtiter plates overnight with goat anti-mouse polyvalent immunoglobulins, diluted to 5 µg/mL in PBS pH 7.4. Use 100 µL of diluted antibody/well. Set up a plate to measure IgG and another to measure IgM.
2. Next morning, wash the plates 3 times with PBS in the plate washer and add 200 µL PBS–BSA to each well. Incubate the plate for 1 h at room temperature.
3. Make standards dilutions. Start at 1.0 µg/mL and make 10 twofold dilutions in PBS–BSA–T. Dilute samples 1:5 in PBS–BSA–T and make four 1:5 dilutions. Make adequate volumes to run in triplicate on two plates (at least 600 µL of each)
4. Wash the plates and add the diluted standards and supernatants, 100 µL/well. Incubate at room temperature for 1 h.
5. Wash the plates and add diluted anti-IgG peroxidase to one plate of the set and anti-IgM peroxidase to the second. Each antibody should be titrated but gener-

ally 1:1000 in PBS–BSA–T is adequate. Use 0.1 mL per well. Incubate for 1 h at room temperature.
6. Wash the plates, reverse and wash them again.
7. Add 0.2 mL TMB/H_2O_2/citrate solution to each well and incubate for 30 min at room temperature (*see* **Subheading 3.4.10.**)
8. Read the OD_{380} on the plate reader.
9. Plot the log concentration of the standards vs OD_{380}. Pick a dilution of sample which gives an OD that falls in the straight portion of the curve and calculate the initial sample concentration.

3.6. Control for Possible Endotoxin Contamination

1. Establish the endotoxin concentrations in oligonucleotides and DNA samples using the Limulus amebocyte lysate assay (*see* **Note 5**).
2. Set up cytokine/proliferation assay cultures and stimulate them with serial dilutions of endotoxin, bracketing the concentration detected (if any) in the DNA sample in its range of stimulatory concentrations (*see* **Note 6**).

3.6.1. DNase Control for Endotoxin Contamination

1. Dilute DNA to 100 μg/mL and DNase I to 200 Kunitz units/mL in complete medium. Also set up controls which include medium and DNase but no DNA and medium and LPS plus DNase. Incubate them for 2 h at 37°C (*see* **Note 7**).
2. Set up cytokine/proliferation assay cultures and stimulate them with DNA and with DNA that has been treated with DNase.
3. A response that is still obtained after DNase treatment suggests endotoxin contamination.

4. Notes

1. Although any strain of mouse may be used, the C3H/HeJ strain is recommended as these mice have reduced responses to endotoxin. While the use of these mice may eliminate confusion with contaminating endotoxin, it does not prevent possible immunostimulatory effects of other bacterial products. It is useful to confirm results with other mouse strains using Polymixin B as an inhibitor of endotoxin.
2. Commercial DNA preparations often have residual RNA and protein and should be further purified by conventional methods. DNA may be sterilized by ethanol precipitation. The precipitated DNA is then redissolved in sterile buffer. Oligonucleotide solutions are conveniently sterilized by filtering through a 0.22 micron Millex-GV low binding filter unit (Millipore).
3. Hydrogen peroxide should be stored at 4°C, but has a limited shelf life, and should be replaced every 6 mo.
4. It is recommended that dilution curves are prepared for all mitogens and inducers of cytokine as well as for controls.
5. The Limulus amebocyte assay is a convenient and well-accepted method of measuring endotoxin contamination. Some oligo- or polynucleotides, however, may

be scored as positive in this assay. If a false positive is suspected, an immunoassay for endotoxin can be used as an alternative.

6. If the presence of endotoxin is detected by the Limulus assay, it is important to determine whether the level measured can account for the biological effects observed on cells. Dilution curves should therefore be established to include the concentration of endotoxin detected. Curves should also be constructed using endotoxin plus a DNA to rule out synergistic effects.
7. Conventional agarose gel electrophoresis may be used to confirm that digestion was complete.

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17

Adjuvants for Plasmid DNA Vaccines

Jon Norman, Jukka Hartikka, Pamela Strauch, and Marston Manthorpe

1. Introduction

In the late 1980s, Jon Wolff of the University of Wisconsin and Phil Felgner here at Vical were screening cationic lipids for their ability to encapsulate and deliver purified plasmid DNA into mouse tissues. They discovered that direct injection of lipid-DNA complexes into muscle resulted in measurable protein expression. A belated control experiment without lipid led to the serendipitous discovery that "naked" plasmid DNA was taken up and expressed in muscle to a greater extent than DNA-lipid complexes ***(1)***. This key observation led to the demonstration that i.m. injection in mice of a standard 50 µg of plasmid DNA encoding a reporter gene becomes readily expressed exclusively in myofiber cells at 180 pg of gene product per muscle ***(2)***. More recently, plasmid DNA expression vectors were improved such that an average of 300 ng of gene product could be produced from single intramuscular (i.m.) injections of plasmid DNA, and up to 40 µg of gene product could be produced after multiple injections (***3*** and J. Hartikka, unpublished observations).

One of the first applications of plasmid DNA injection technology was the induction of an immune response. Since transduction of cells with plasmid DNA injection would lead to intracellular synthesis of antigen, it was expected that antigen could be processed for Class I and Class II MHC presentation, which would lead to a subsequent stimulation of cell-mediated immunity. The first validation of this concept was published in January 1991 by Felgner and Rhodes ***(4)*** who reported preliminary experiments indicating that mice could be immunized against HIV gp120 by i.m. vaccination with gp120 plasmid DNA. Extending this concept to an animal model of infectious disease, Ulmer and colleagues ***(5)*** showed that mice could be protected from a lethal

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

challenge of influenza virus after DNA immunization with influenza nucleoprotein (NP) antigen. Protection obtained after immunization with the highly conserved NP antigen extended across 2 different viral strains, and protection required $CD8^+$ CTL *(6)*. Several human clinical trials are in progress to evaluate DNA vaccination.

In the past several years, agents have been described that facilitate plasmid DNA expression or act as adjuvants to DNA vaccination (*see* **Note 1**). One of these agents, bupivacaine (BP), is a local anesthetic which is also a myotoxin *(7–11)*. Injected BP destroys myofiber cells leading to macrophagic clearance of cell debris and proliferation of muscle precursor cells (satellite cells or myoblasts) which then fuse to one another and to the remaining viable myofiber syncytium, all as the normal part of skeletal muscle regeneration. When injected into muscle prior to injection of plasmid DNA, BP was shown to increase plasmid DNA expression in muscle and presumably stimulate the resulting immune response. Another agent which has been reported to stimulate luciferase expression is polyvinyl pyrollidone (PVP) which is formulated and co-injected with plasmid DNA *(12)*. Additionally, neutral, anionic and cationic lipids are reported to enhance plasmid DNA expression in non-muscle tissues *(13–15)*. Only recently have lipids been shown to enhance plasmid DNA expression and immunity after injection into muscle *(16)*.

Although the above studies using "facilitating agents" with plasmid DNAs are encouraging, there remains a need to systematically establish the comparative potency of these agents in enhancing gene expression and immunization. In this study, a quantitative comparison of the effects of the co-injection of BP, PVP or DMRIE:DOPE cationic liposomes on plasmid DNA-mediated luciferase gene expression and anti-NP antibody responses is performed.

1.1. Effects of Bupivacaine on Luciferase Expression in Mouse Skeletal Muscle

Two plasmids, RSV-Lux and VR1223, each encoding the luciferase enzyme were chosen for study. Previous work had shown that VR1223 expressed over 70-fold higher than RSV-Lux in standard in vivo muscle expression assays *(3)*. Here, the plasmids were individually injected as 50 µg DNA/50 µL saline into mouse rectus femoris muscle, either (i) alone, (ii) alone, but 5 d after injection of 0.75% BP, or (iii) mixed with 0.75% BP. Muscles were collected 7 d later and assayed for luciferase enzyme activity. The chosen BP dose corresponded to that used by others *(17,18)*. The results are shown in **Fig. 1A,B**. The DNAs injected alone (white bars in **Fig. 1**) yielded expression levels of 0.74 (RSV-Lux) and 60 (VR1223) ng of luciferase/muscle, representing an 80-fold higher expression from VR1223. When injected 5 d after BP treatment (gray bars in **Fig. 1**), the RSV-Lux and VR1223 DNAs yielded 5.1 and 99 ng luciferase/

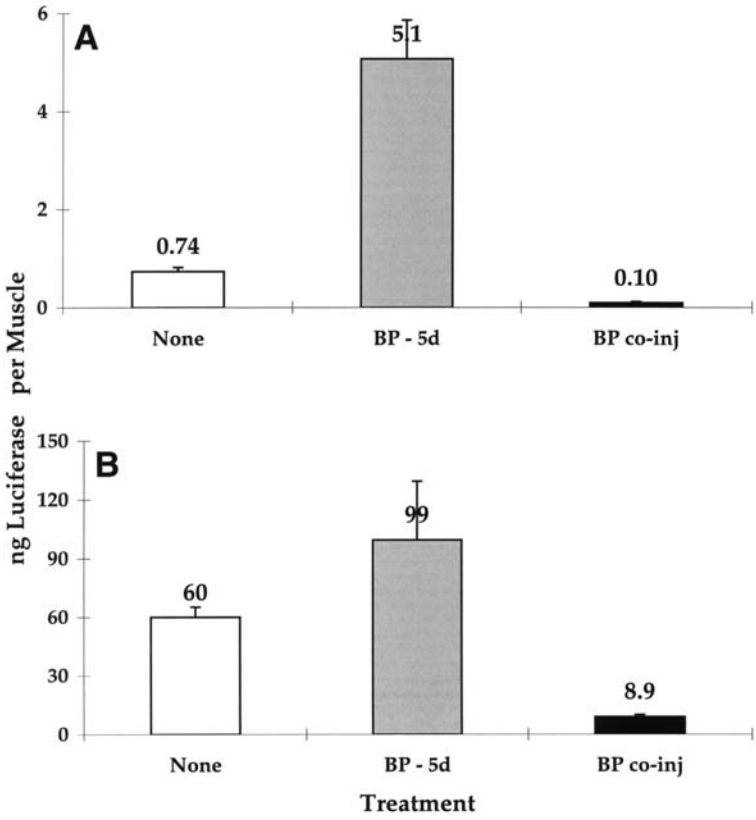

Fig. 1. Adult BALB/c mice were each injected i.m. with 50 mg of the indicated luciferase plasmid DNA either alone (None, white bars) or formulated with 0.75% Bupivacaine (BP co-inj, black bars) or alone but 5 d after injection of 0.75% BP without DNA (BP–5d, shaded bars) (**Subheadings 2.** and **3.**). The plasmid DNAs were previously reported to yield low (RSV-Lux) or high (VR1223) expression levels after injection into mouse muscle ***(3)***. At 7 d post-DNA injection, muscles were extracted and luciferase enzyme activity was measured. Values shown above each bar represent the averages of 20 muscles (2 experiments, 10 muscles each) and error bars are Standard Errors of the Mean.

muscle, respectively. Thus, the lower expressing RSV plasmid expressed about seven-fold more luciferase when injected into BP-treated muscle. This "facilitated" RSV-Lux expression was still an order of magnitude less than the injection of VR1223 in BP untreated muscle (5.1 vs 60). Pre-injection with 0.75% BP enhanced VR1223 expression by less than twofold from 60–99 ng/muscle, but with the normal variation in expression from different muscle, the BP-treated muscle was not statistically different ($P = 0.53$; $n = 20$). Co-injection of

plasmid and 0.75% BP decreased expression with both vectors by 7.4-fold (RSV-Lux: from 0.74–0.1) and 6.7-fold (VR1223: 60–8.9 ng luciferase/muscle), respectively. In summary, 0.75% BP pre-injection enhanced the expression of a poor expression vector, but had no effect on a good expression vector and BP co-injection with either vector decreased expression by nearly an order of magnitude.

Thus, the data confirm previous reports *(7–11,17,18)* that the pre-injection of mouse skeletal muscle with bupivacaine myotoxin followed 5 d later by plasmid DNA injection results in an increase in the expression of the DNA. However, this enhancement only occurs with a plasmid construct that expresses in muscle at relatively low levels. The high efficiency muscle expression vector used in these experiments was not significantly enhanced by BP pre-injection. Furthermore, the injection of a high-efficiency plasmid like the CMV-driven VR1223 can express 10-fold more gene product than the BP-enhanced low-efficiency-expressing RSV-driven plasmid. Thus, the preferred method for intramuscular expression of an i.m. transgene is the injection of a high-expressing naked DNA without the use of bupivacaine.

1.2. Effects of PVP and Cationic Lipids on Luciferase Expression in Mouse Skeletal Muscle

Luciferase expression after intramuscular injection of VR1223 formulated with 5% PVP as reported *(12)* or DMRIE:DOPE cationic lipid was evaluated. The results are shown in **Fig. 2A,B**. VR1223 expression 7 d after injection was decreased by 5.0- and 8.8-fold (from 38–7.6 or 37–4.2) respectively, by PVP or DMRIE:DOPE. Thus, PVP or lipid co-injection, like BP co-injection, greatly decreased plasmid DNA expression in muscle.

These results with PVP contradict those of Mumper and colleagues *(12)*, who showed that co-injection of PVP with plasmid DNA in rat tibialis muscle can enhance transgene expression up to fivefold. A variety of conditions using different DNAs and dosages were attempted but with the same result (*see* **Note 2**). This discrepancy could be due to differences in animal species (mouse vs rat), muscle type (rectus vs tibialis), reporter gene (luciferase vs CAT), plasmid vector ("CMV-CAT" vs VR1223) or other less defined experimental conditions.

1.3. Comparative Adjuvant Effects of Bupivacaine, PVP and Cationic Lipid on DNA Vaccination

A direct comparison of BP, PVP and DMRIE:DOPE was made by co-injecting these agents i.m. with DNA to facilitate the generation of antibodies to the encoded gene product, influenza nucleoprotein (NP). To minimize animal toxicity to BP (*see* **Note 3**) the co-injected BP dose was decreased from 0.75% to 0.25% according to the literature *(19)*. The results are shown in **Fig. 3**. Anti-

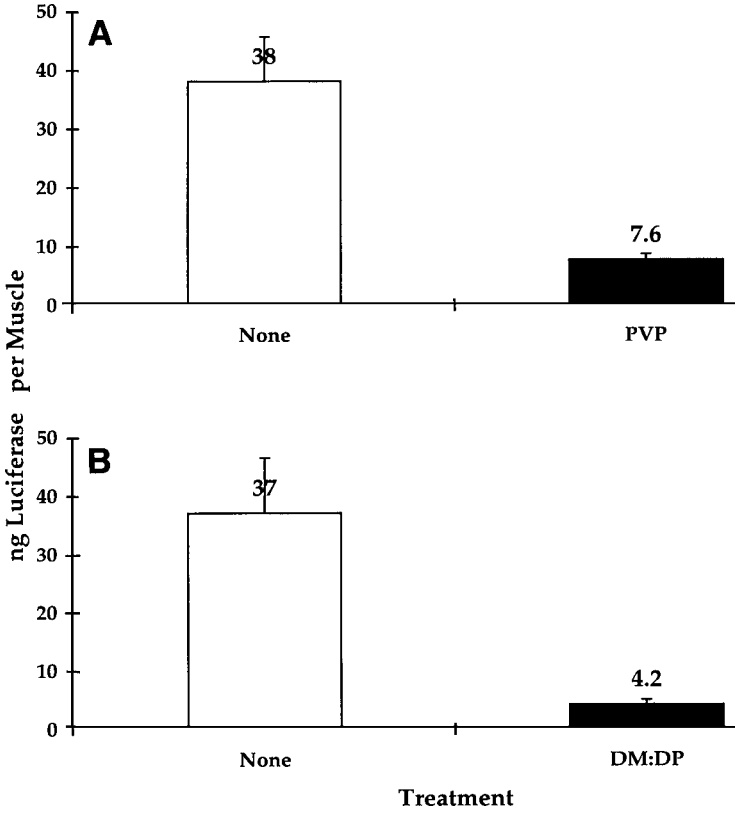

Fig. 2. Adult BALB/c mice were each injected i.m. with 10 μg of VR1223 luciferase plasmid DNA alone (None, white bars) or formulated with 5% PVP (PVP, A. black bar) or 50% DMRIE : 50%DOPE at a 2:1 molar/1:1 mass ratio of DNA:DMRIE (DM:DP, B. black bar) (**Subheadings 2.** and **3.**). At 7 d post-DNA injection, muscles were extracted and luciferase enzyme activity was measured. Values shown above each bar represent the averages of 20 muscles (2 experiments, 10 muscles each) and error bars are standard errors of the mean.

NP antibody titers at one month post-i.m. injection of VR4700 (**Fig. 3A**) were decreased by BP and PVP by 12-fold (from 1312–107) and 17-fold (from 1664–96), respectively. In contrast, DMRIE:DOPE co-injection with VR4700 resulted in a 3.3-fold increase (from 1664–5632) in average anti-NP titer. Although BP, PVP, and DMRIE:DOPE all decreased reporter gene expression levels, only DMRIE:DOPE increased the humoral immune response resulting from gene expression. These results were the same using the less potent vector, CMV-NP (**Fig. 3B**). BP and PVP decreased anti-NP titers by 4.6-fold (from 1392–304) and 3.2-fold (from 240–64), respectively, whereas DMRIE:DOPE increased

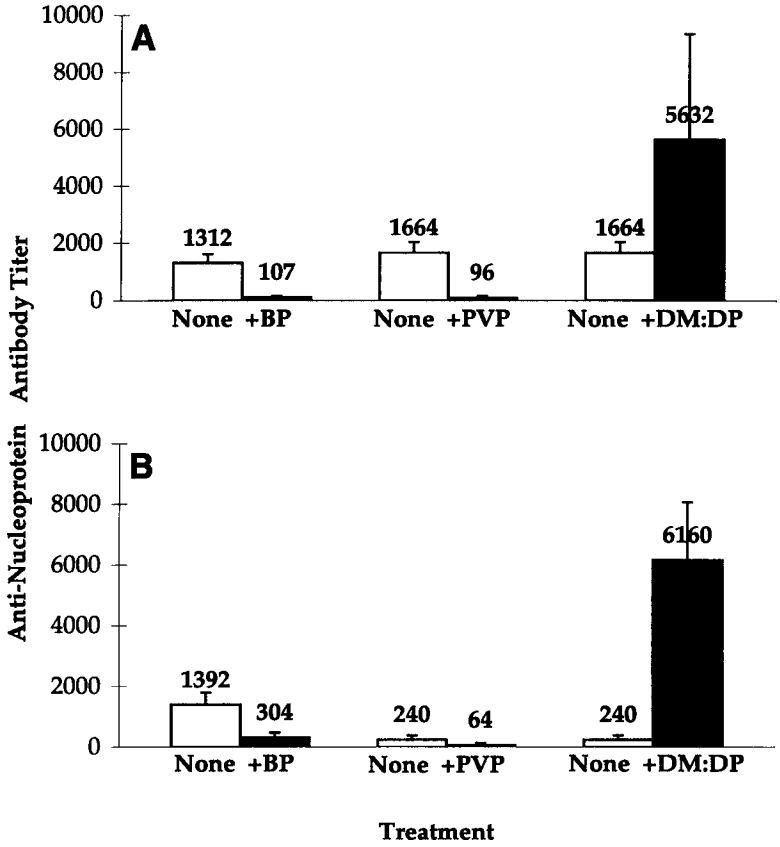

Fig. 3. Adult BALB/c mice were each injected i.m. once with 10 μg of VR4700 or CMV-NP DNAs encoding influenza nucleoprotein (NP). DNAs were injected either alone (None, white bars) or formulated with 5% Polyvinyl pyrrolidone or DMRIE:DOPE (PVP or DM:DP, black bars) (**Subheadings 2.** and **3.**). The corresponding luciferase DNA to VR4700 NP (VR1223) is about 27 times more potent than the corresponding luciferase DNA to CMV-NP (VR1205) via the muscle expression assay *(3)*. At 28 d post-DNA injection, sera were collected and diluted serially for anti-NP IgG antibody assay using ELISA. Values represent average reciprocal anti-NP titers defined as the dilution giving an O.D. value twice the average background (n = 5 mouse sera; error bars are standard errors of the mean). Note that for both vectors, BP and PVP decrease, but DM:DP increase the antibody response.

anti-NP titers 26-fold (from 240–6160). Similar results were achieved using various other conditions (*see* **Note 2**).

This unique activity of DMRIE:DOPE may be due to its ability to slowly release plasmid DNA for uptake by muscle. This possibility could be tested by

conducting an expression kinetic study. DMRIE:DOPE may also act as an adjuvant that induces inflammation, resulting in the death of many transduced muscle fibers, but at the same time stimulating the immune response to the antigen. This could be examined by characterizing the immune response in more detail with respect to the nature of inflammatory intramuscular infiltrating cells, Th_1 or Th_2 responses, CTL activities, etc.

Another potential explanation of the ability of DMRIE:DOPE to decrease transgene expression in muscle, and, at the same time, enhance immunity to the transgene product, may relate to the nature of the transduced cells. It is well documented that i.m. injection of naked plasmid DNA results in the exclusive transduction of myofiber cells ***(2,7,17,20)***. This restricted transduction of myofiber cells occurs even when BP is pre-injected before DNA injection ***(8,21)***. The DMRIE:DOPE/DNA complex, which is quite efficient in transducing non-muscle cells, may be more capable than naked DNA in transducing other cells within the muscle ***(22,23)***. Alternatively, the DNA-lipid complex may be better able to exit the muscle and transduce distal tissues ***(13,16,24)***.

Since intramuscular myofiber cells do not express Class I or Class II MHC antigens or B7 costimulatory molecules, it is unlikely that myofiber cells transduced with a foreign gene would be able to present antigen and stimulate humoral immunity, or engage or activate T cells and induce cellular immunity ***(25)***. Studies with haplotypic chimeric mice with reconstituted bone marrow have provided evidence that naked DNA-transduced myofiber cells release antigen that is picked up by resident dendritic cells, which then process the antigen for presentation to the immune system ***(26–29)***. Since plasmid DNA is rapidly degraded once injected into muscle ***(30)***, bone marrow reconstitution was carried out 3 wk after i.m. injection of DNA to disallow local transduction of intramuscular dendritic cells by residual DNA ***(27)***. In this case, immunity to the transgene epitopes were still characteristic of the transplanted dendritic cell haplotype and not the host muscle haplotype. These results support the idea that transduced muscle provides antigen to dendritic cells, which then processes it for presentation to the immune response. This mechanism, whereby myofiber cells synthesize and provide antigen to dendritic cells, might be greatly disturbed if DNA is provided as a liposome complex. Traditionally, liposomes are viewed as very effective systems for delivery of proteins or drugs to the reticuloendothelial system, in particular to dendritic and macrophagic cells ***(31–34)***. Thus DMRIE:DOPE may directly and efficiently deliver i.m. plasmid DNA to professional antigen-presenting cells. This may be tested experimentally by following the fate of DNA transduction after i.m. injection of lipid-DNA complexes.

In summary, we report that the i.m. injection of DMRIE:DOPE/plasmid DNA complexes induce better humoral immunity than injection with naked

DNA. Future experiments are aimed at a better characterization of such lipid induced adjuvancy, and the testing of a battery of other cationic lipids for their innate ability to increase immune responses when complexed with plasmid DNA.

2. Materials

1. Luciferase substrate and cell culture lysis reagent are available from Promega (Madison, WI).
2. Firefly luciferase enzyme reagent is available from Analytical Luminescence Labs (San Diego, CA).
3. 0.9% saline is available from Radix Labs (Eau Claire, WI).
4. Euthanasia-5 solution is available from H. Schein Inc. (Port Washington, NY).
5. Female 8–12-wk-old BALB/c mice are available from Harlan Sprague Dawley (Indianapolis, IN).
6. Bupivacaine hydrochloride (Marcaine) is available from Abbott Labs (N. Chicago, IL).
7. PVP (Plasdone™–C30; MW 50 kDa) is available from ISP Technologies, Wayne, NJ.
8. DMRIE ((±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide) is synthesized and mixed with co-lipid as described ***(20)***.
9. The luciferase VR1223 plasmid DNA is constructed as outlined in detail previously ***(3)***. The CMV-NP and VR4700 are constructed by replacing the luciferase genes in, respectively, VR1205 and VR1223, with the influenza/A NP gene ***(3)***.
10. Disposable, sterile, plastic insulin syringes and 28G $^1/_2$ needle (Becton-Dickenson, Rutherford, NJ, Cat. No. BD9430) fitted with a plastic collar cut from a micropipet tip.
11. Microplate luminometer (Dynatech, Chantilly, VA, model ML2250).
12. Firefly luciferase enzyme reagent is obtained from Analytical Luminescence Labs (Cat. No. 2400; e.g. 1.69×10^{13} Relative Light Units/mg protein).
13. Alkaline phosphatase-conjugated, Fc-specific, goat anti-mouse IgG is obtained from Jackson Immuno Res Labs (Bar Harbor, ME).
14. ELISA plate reader is available from Molecular Devices (Menlo Park, CA).

3. Methods

3.1. Plasmid DNA Purification

1. Transform plasmid DNA into *Escherichia coli* DH5a or DH10B competent cells and grow in Terrific Broth ***(35)*** complemented with 100 µg/mL ampicillin or 50 µg/mL kanamycin in a 5-L fermentor (Applikon, Foster City, CA). Control temperature and pH at 30° ± 0.5°C and 7.0 ± 0.5, respectively. Set the stirring speed at 600 RPM ± 50 and the air flow at 5L/L/min.
2. Harvest cells by centrifugation at the end of the exponential growth phase. Isolate covalently closed circular plasmid DNA by a modified lysis procedure ***(36)*** followed by standard double CsCl-ethidium bromide gradient ultracentrifugation.
3. Determine that the endotoxin content by the *Limulus* Amebocyte Lysate (LAL, Associates of Cape Cod, Falmouth, MA) assay is less than 0.6 Endotoxin Units/µg of plasmid DNA.

4. Determine protein content using the bicinchoninic acid assay (Pierce Chem. Co., Rockford, IL). The spectrophotometric A_{260}/A_{280} ratio should be between 1.75–2.0.
5. Ensure all plasmid preparations are free of detectable chromosomal DNA, RNA and protein impurities based on gel analysis and the bicinchoninic assay, respectively.
6. Ethanol precipitate plasmids and re-solubilize in USP saline at 4°C. Store the DNA at –20°C.

3.2. DNA Injections

1. Equilibrate injection fluids and syringes to room temperature and inject single 50 or 100 µL volumes in 1–2 s.
2. Inject the quadriceps muscle of restrained, awake mice with 50 µL of DNA in saline, using the syringe and needle fitted with a collar, into the central part of the rectus femoris muscle. Extensive histological analyses of muscle injected with LacZ DNA shows that the rectus femoris is the primary target of DNA transfection ***(20)*** (*see* **Note 4**).

3.3. Extraction and Assay of Luciferase

1. Add 100 µL of luciferase substrate to 20 µL of muscle extract ***(3)***.
2. Record sample Light Units several times within 5 s after addition.
3. Calculate the luciferase content of the samples from Relative Light Units using a standard curve of purified firefly luciferase, which is diluted in pooled extract from uninjected muscles to control for quenching ***(38)***. Luciferase values are expressed as ng luciferase per mL of muscle extract (*see* **Note 5**).

3.4. ELISA for Anti-NP Antibodies

1. Levels of anti-NP IgG Ab in mouse sera are determined by standard ELISA assay. Coat ELISA plates with recombinant NP protein (expressed in baculovirus) at 0.125 µg/well overnight at 4°C.
2. Wash, block with 5% nonfat milk, and incubate with sera for 2 h at room temperature (r.t.).
3. Wash, and then incubate plates with alkaline phosphatase-conjugated goat anti-mouse IgG for 2 h at r.t.
4. Wash again, and incubate with a substrate (p-nitrophenylphosphate) for 2 h at r.t. Take O.D. readings at 405 nm using an ELISA plate reader. Titers are determined to be that serum dilution yielding an O.D. twice that for background non-immune serum.

4. Notes

1. Over the past 7 y, we have performed i.m. luciferase plasmid DNA injections into over 20,000 mouse muscles. Analyses of the expression data reveal that the levels of expression of a given luciferase plasmid DNA in the mouse rectus femoris muscle are not statistically different based on the animal's sex, age (4 wk–1 yr), strain (BALB/c vs C57/Bl), size (20–50 gm), or the level of aerobic activity, or

affected by the season, time of day, temperature of injected solution or even co-injection of the plasmid with a wide variety of pharmacological agents (***36*** and J. Hartikka, unpublished observations).

2. Similar experiments were repeated using medium doses of BP (0.5%), lower or higher DNA doses (10 μg and up to 150 μg) with BP and PVP, and with different cationic lipids. In all cases, BP and PVP and the lipids decreased luciferase expression, the BP and PVP decreased anti-NP titers and the lipids increased anti-NP titers (data not shown). In addition anti-NP titers were measured at 2 wk and 2 mo with the same result (data not shown).
3. Preliminary experiments with mice showed that a standard i.m. injection of 0.75% BP as reported in the literature was lethal in mice. The above results, therefore, could only be obtained using a slow (over several minutes) injection of the BP that ultimately resulted in the loss of only about 40% of the animals, with the rest of the surviving mice undergoing various degrees of apparent cardiac stress. Thus, BP is severely toxic, even at doses used consistently in the literature to enhance the expression of i.m. injected plasmid DNA. However, unlike injections with BP, neither PVP nor DMRIE:DOPE caused visibly-untoward effects on the mice.
4. Animal care throughout the study is in compliance with the "Guide for the Use and Care of Laboratory Animals," U.S. Department of Health and Human Services, National Institutes of Health (NIH Publication No. 86-23, revised 1985) as well as with local regulations (e.g., Vical's Institutional Animal Care and Use Committee).
5. The luciferase assay yields <5% variation in light units within triplicate samples. In control experiments, representative muscle extracts containing high, medium and low levels of luciferase activity were stored at –78°C in replicate aliquots and periodically assayed over a 3-yr-interval, yielding <10% variation in light units.

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18

Cytokine and Costimulatory Factor-Encoding Plasmids as Adjuvants for DNA Vaccination

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1. Introduction

The induction of a potent and long-lasting immunity is one of the most important elements to consider in developing an effective vaccine. DNA vaccines induce markedly stronger $CD8^+$ cytotoxic T lymphocyte (CTL) activity than do traditional peptide vaccines through their particular mechanism of antigen presentation mediated by MHC class I molecules. Induction of CTL specific to pathogenic viruses is thought to provide a reliable means of protecting a host from infection and halting disease progression, as these cells can directly recognize and lyse infected cells. However, in most of the early studies showing induction of pathogen-specific CTL, antigen-encoding immunogenic DNA alone was used and DNA vectors encoding immunomodulating molecules were not considered. It now appears that various types of immunomodulatory molecules such as cytokines (IL-1 ***[1]***, IL-2 ***[2]***, IL-12 ***[3]***, IFN-γ ***[4]***, IL-7 ***[5–7]***, and GM-CSF ***[8,9]***), chemokines (TCA-3 ***[10]***, RANTES ***[11]***, MIP-1 ***[11]***), and costimulatory molecules (CD40L ***[12]***, B7-1 ***[13]*** and B7-2 ***[14]***) could enhance or modify the specific immune responses elicited by DNA immunization (*see* **Table 1**).

In this chapter, we describe an approach for achieving more effective DNA vaccination through the use of plasmids expressing such immunostimulatory molecules. These plasmids have unique immunomodulatory properties, and coinoculation of both immunogenic and adjuvant plasmids can result in augmentation of an antigen-specific humoral and/or cell-mediated immune response. Several groups have recently reported that coadministration of plasmids expressing cytokines can have significantly enhanced vaccine-specific immune responses. Although the precise mechanism responsible for their

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

Table 1
Summary of Effects of Cytokines after Conventional Vaccination and of Expression Plasmids following DNA Immunization

Immunomodulatory Molecules	Effect	Ref.
A. Cytokine proteins		
IL-1	Antibody (Ab) ↑	***(23,24)***
IL-2	Ab ↑	***(2,25,26)***
IL-12	TH1(DTH) ↑	***(3)***
IFN-γ	Ab, DTH ↑	***(4,25,27)***
GM-CSF	Ab ↑	***(28,29)***
B. Expression plasmids		
IL-12	CTL ↑ (i.m. and i.n.)	***(15,21,22,30)***
	DTH ↑ (i.m. and i.n.)	***(15,21)***
	Ab → (i.m. and i.n.)	***(15,21,22)***
GM-CSF	Ab ↑ (i.m.)	***(9,18,22)***
	CTL ↑ (i.m.)	***(18)***
	^{3}H-TdR uptake ↑ (i.m.)	***(9)***
TCA3	CTL ↑ (i.m.)	***(31)***
	DTH ↑ (i.m.)	***(31)***
	Ab → (i.m.)	***(31)***
B7-1	CTL → (i.m.)	***(19)***
	DTH → (i.m.)	***(19)***
	Ab → (i.m.)	***(19)***
B7-2	CTL ↑ (i.m.)	***(19)***
	DTH ↑ (i.m.)	***(19)***
	Ab → (i.m.)	***(19)***
CD40(L)		
	Ab ↑ (i.m.)	(A. Ihata et al., unpublished data)
	CTL ↑ (i.m.)	(A. Ihata et al., unpublished data)

i.m., intramuscular administration; i.n., intranasal administration; Ab, antibody production; DTH, delayed type hypersensitivity; ^{3}H-TdR, incorporation of ^{3}H-Thymidine; ↑, activated immune response.

remarkable enhancement of immunity is not yet clear, it is possible that these expression plasmids can induce continuous cytokine production for more than several weeks ***(15)***. Generally speaking, the biological half-life of recombinant cytokines is short following injection, compared with that of cytokines continuously synthesized after injection of expression plasmids. This simple and effective approach for enhancing DNA vaccination may allow new ways of

controlling infectious diseases. Several reports have also shown that bacterial plasmids containing the CpG motif enhance the Th1-type immune response ***(16,17)***.

We have focused here on the use of cytokine-expression plasmids as well as certain costimulatory molecules as a means of enhancing the immunogenicity of protein antigens derived from plasmid DNA. For the systematic development of vaccination strategies using cytokines or their expression plasmids as adjuvants, it is essential to understand the precise involvement of Th1 and Th2 cells in protective immunity against specific microbial infections. Using Th1 (IL-2, IFN-γ, and IL-12) and Th2 (IL-4, and IL-10) type cytokines, we can also define the respective roles of Th1 and Th2 cells at the various stages of each infection. Recent reports have indicated that certain costimulatory molecules also enhance the immunity conferred with plasmid DNA immunogens ***(18,19)***. In **Table 1**, we have summarized the immunomodulatory effects observed with co-inoculation of various plasmids.

2. Materials

2.1. Expression Plasmids

1. HIV-1IIIB gp160 (pCMV160IIIB) and Rev (pcREV) ***(20)***: each injection mixture contains 20 μg pCMV160IIIB and 5–10 μg of pcREV.
2. Interleukin-12 (pCAGGSIL-12) ***(15)***: donated by Dr. J. Miyazaki (Osaka University, Japan) (**Fig. 1**). We use 10–20 μg per mouse of this plasmid. The pCAGGS vector gives higher expression levels than the pCMV vector ***(15)***.
3. Interleukin-2 (pBCMGNeo-mIL2): donated by Dr. H. Karasuyama (Tokyo Metropolitan Institute of Medical Science, Japan). This plasmid enhances the Th1 type immune response. We use 10–20 μg per mouse as cytokine adjuvant.
4. Interleukin-4 (pCAGGS/IL-4): donated by Dr. J. Miyazaki (Osaka University, Japan) (**Fig. 1**). This plasmid enhances Th2 type immune responses at a dose of about 10–20 μg per mouse.
5. Interferon-γ (nkCMVintMuIFNγ): donated by Dr. H. Kohsaka (Tokyo Medical and Dental University, Tokyo, Japan). This cytokine enhances the Th1 type response.
6. TCA3 (pPGKXbaI/Neo/TCA3): donated by Dr. M. E. Dorf (Harvard Medical School, Boston, MA) ***(10)***. TCA3 enhances Th1 type immune responses at a dose of 1–20 μg per mouse.
7. B7-1 and B7-2: donated by Dr. G. J. Freeman (Dana-Farber Cancer Institute, Boston, MA) ***(13,14)***. B7-2 enhances Th1-type immune responses ***(18)*** when used at around 10–100 μg per mouse.
8. GM-CSF (VR-1701): donated by Dr. R. H. Zaugg (VICAL Inc., San Diego, CA). It strongly enhances Th2-type immune responses, and substantial activation of the Th2-type response occurs with around 5–20 μg per mouse. There are other reports in which 50–100 μg of GM-CSF plasmid were used ***(9)***.

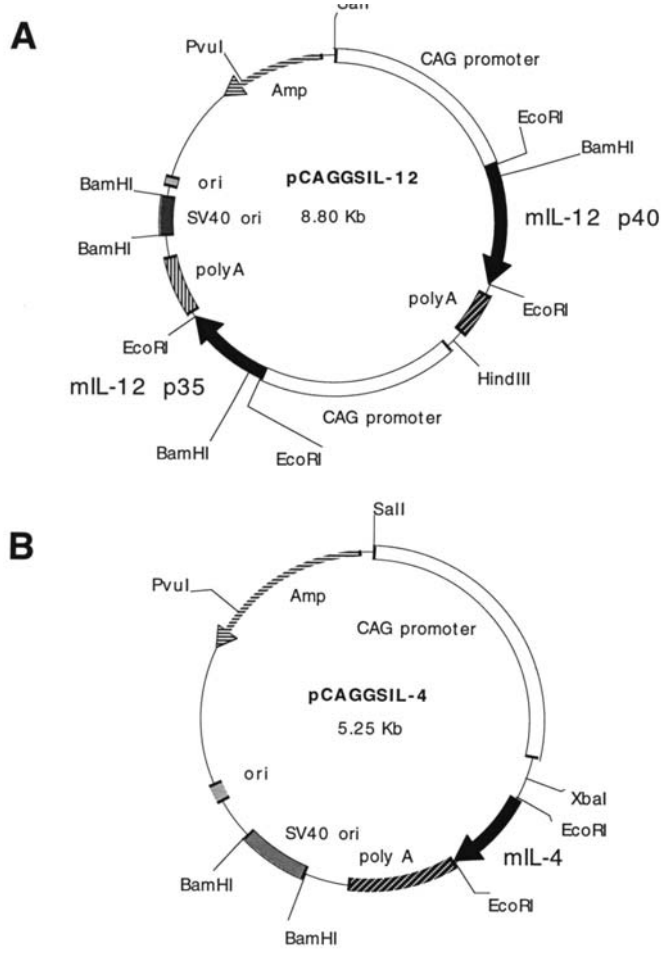

Fig. 1. Constructs of cytokine expression plasmids. **(A)** Expression plasmid of IL-12; **(B)**, Expression plasmid of IL-4.

9. CD40L: donated by Dr. J. Inoue (Institute of Medical Science, University of Tokyo, Japan). This plasmid enhances both Th1 and Th2 type immune responses when 10–50 μg per mouse is administered.

2.2. Other Materials

1. 6–10-wk-old BALB/c mice.
2. 27 gauge needle syringe.
3. Hematocrit capillary tube.
4. 100 μL pipettor.
5. 70% (v/v) ethanol in a spray bottle.

3. Methods

We generally use two routes for vaccine delivery. Intramuscular immunization enhances the Th1-type immune response. The intranasal route of immunization activates the production of intestinal and vaginal IgA ***(21)***, an important feature for sexually-transmitted diseases.

3.1. Immunization and Sample Collection

The biceps femoris muscle of BALB/c mice is inoculated with naked DNA constructs. In our early experiments, to enhance muscle cell uptake of plasmid DNA, muscle was injected with 100 µL of 25% (w/v) sucrose in PBS 15–30 min before DNA inoculation. However, our recent data show that the injection of 25% sucrose does not significantly modify immune responses. A total of 100 µL of the DNA mixture, containing 5–30 µg of pCMV160IIIB and pcREV and expression plasmids of cytokine (1–50 µg) or costimulatory substance (1–50 µg), is injected into the muscle using a 27-gauge needle syringe. Blood is collected by retro-orbital puncture using a capillary tube, and sera are obtained by centrifugation. Sera are stored at –80°C until ELISA assays are performed.

3.2. Intramuscular Immunization (see Note 1*)*

1. Pour about 20–30 mL of diethyl ether into a jar with a volume of about 500 mL. Gauze should be spread on the bottom.
2. After the diethyl ether has fully evaporated, introduce the mice into the jar.
3. When the mouse is no longer active (after about 20 s), allow several more seconds to pass. When the animal begins breathing deeply, remove it from the anesthetic jar. When breathing becomes deep, the mouse should not be kept in the anesthetic jar any longer than 10 s since death may ensue at this stage of anesthesia (*see* **Notes 2** and **3**).
4. Place the mouse in the prone position and slowly inject the vaccine preparation into the biceps femoris muscle (*see* **Notes 4** and **5**).
5. Withdraw the needle from the muscle approx 5 s after completing the injection. This is thought to prevent the injected solution in the muscle from leaking through the needle pore.

3.3. Intranasal Administration (see Notes 1–5*)*

1. Anesthetize mice by inhalation of diethyl ether and spray the injection site with 70% (v/v) ethanol for disinfection.
2. Grasp the mouse gently with one hand and turn it face upward (*see* **Note 6**).
3. Drop the mixture of DNA vaccine and cytokine plasmids into the nasal cavity with a micropipetter. Allow the mouse to inhale the DNA as it breaths (*see* **Note 7**).
4. Allow the mouse to inhale one drop (5–7 µL) before another drop is introduced (*see* **Note 8**).
5. Repeat **steps 5** and **6**.

4. Notes

1. We used 1–50 μg of DNA per mouse whereas other groups ***(9,22)*** use 20–100 μg per mouse. We have not obtained a significant body of data regarding the use of over 50 μg of DNA.
2. Too deep or too long a period of anesthesia will often kill mice.
3. It is important to ventilate the room or to perform the anesthesia step in a ventilated hood since inhalation of ether gas represents a health risk.
4. Injecting hypertonic sucrose solution into muscle results in muscular edema that is thought to help to retain the DNA solution. However, results of previous work on the effect of this treatment on reporter gene expression are ambiguous; one showed that it was highly useful whereas another found that it had no effect. Moreover, there has been no published study showing conclusively that this treatment enhances antigen-specific immune responses. Our current thinking is that sucrose-pretreatment of muscle does not greatly enhance DNA-based immunity.
5. For ease and convenience, large muscle is preferred when choosing the site for the DNA injection. Previous work has demonstrated that quadriceps femoris muscle is superior to gastrocnemius muscle for reporter gene expression. However, injection into quadriceps requires that the mouse be placed in a supine position, which is difficult to maintain once the animal awakens from anesthesia. Since it is easier to keep a mouse in the prone position, we usually use the biceps femoris muscle.
6. The neck should not be tightened or pressed for more than a few seconds as this will easily kill the mouse.
7. It is important not to wet the face of the mouse with alcohol or ether since moisture on the hair around the nasal cavity will often cause the vaccine solution to leak away by widening the drops of the DNA vaccine.
8. It is easy to drop the DNA solution into only one nasal cavity because the mouse can inhale freely through the other nostril without distress. A 30-μL volume of the DNA mixture is the maximum administered in one i.n. immunization. If the mouse starts to discharge the DNA solution from its nose, it is safe to stop the inhalation process. If additional inhalation steps are desired using the same animal, it is better to wait 30 min before repeating the procedure. If the total volume employed is over 30 μL, i.n. administration should be carried out in several treatments spaced 4–5 h apart. If the volume inhaled is too great, the mouse will die by asphyxiation.

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Optimization of DNA Vaccines Through the Use of Molecular Adjuvants

Jong J. Kim and David B. Weiner

1. Introduction

Although the injection of DNA into tissues was originally reported in the 1950s, the technology has gained more attention in recent years as a safe means of mimicking in vivo protein production normally associated with natural infection ***(1–3)***. Nucleic acid or DNA inoculation is an important vaccination technique that delivers DNA constructs encoding specific immunogens directly into the host ***(4–11)***. These expression cassettes transfect the host cells, which become the in vivo protein source for the production of antigen. This antigen then is the focus of the resulting immune response. This vaccination technique is being explored as an immunization strategy against a variety of infectious diseases as well as cancer.

Nucleic acid immunization may afford several potential advantages over traditional vaccination strategies such as whole killed or live attenuated virus and recombinant protein-based vaccines. Since DNA vaccines are nonreplicating and the vaccine components are produced within the host cells, they can be constructed to function safely with the specificity of a subunit vaccine. However, DNA vaccine cassettes should produce immunological responses that are more similar to live vaccine preparations. By directly introducing DNA into the host cell, the host cell is essentially directed to produce the antigenic protein, mimicking viral replication or tumor cell marker presentation in the host. This process has been reported to generate both antibody and cell-mediated, particularly cytotoxic T-cell-mediated, immunity. Unlike a live attenuated vaccine, conceptually there is little risk from reversion to a disease-causing pathogen from the injected DNA, and there is no risk for secondary infection as the material injected is not living and non-infectious. In

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

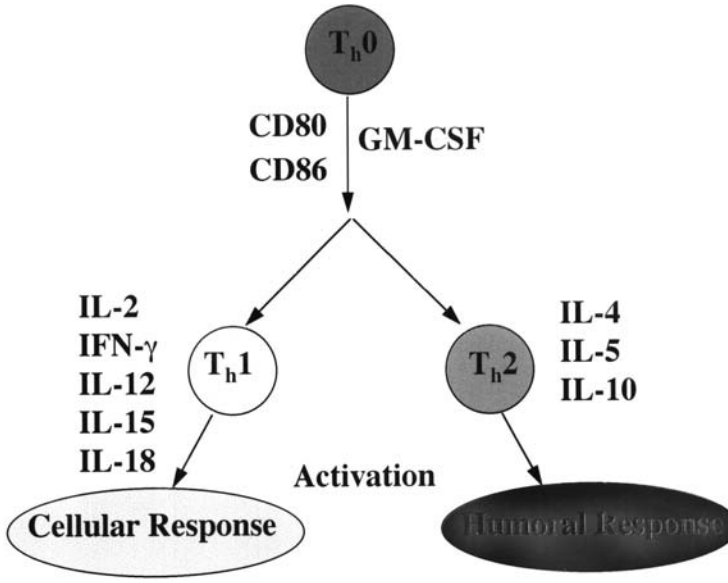

Fig. 1. Cytokines as immune modulators. Cytokines play critical roles in the immune and inflammatory responses. Based upon their specific function in the immune system, these cytokines can be further grouped as Th_1 and Th_2 cytokines. Along with costimulatory molecules, these cytokines also play important roles in the activation and proliferation of T and B cells.

addition, genes that lead to undesired immunologic inhibition or cross-reactivity (autoimmunity) may be either altered or deleted altogether. Finally, DNA vaccines can be manipulated to present a particular genome of the pathogen or display specific tumor antigens in non-replicating vectors.

The goal of the first generation DNA immunization was to demonstrate the DNA vaccines' ability to elicit humoral and cellular responses in vivo in a safe and well-tolerated manner in model systems. As we explore the next generation of DNA vaccines, our goal is to refine the current strategy to elicit more clinically efficacious immune responses. The next generation immunogens, for example, may require finer control of the magnitude and direction (humoral or cellular) of the immune responses induced. Such refinement could be accomplished by co-delivering genes for immunologically important molecules, such as costimulatory molecules and cytokines that play critical regulatory and signaling roles in immunity ***(12)*** (**Fig. 1**).

There have been several reports of immune modulation by protein delivered cytokines. However, the results in general appeared marginal. More recently, we and others have focused on analyzing immune responses induced to such gene delivery. Raz et al. observed that intramuscular injection of plasmids

encoding IL-2, IL-4, or TGF-ß1 modestly modulated immune responses to transferrin protein delivered at a separate site ***(13)***. IL-2 immunization resulted in an enhancement of antibody and T helper proliferative responses while TGF-ß1 immunization reduced anti-transferrin responses. Xiang and Ertl reported that intramuscular co-inoculation of plasmid expressing the glycoprotein of rabies virus and plasmid-encoding mouse GM-CSF enhanced the B and T helper cell activity to rabies virus, while co-inoculation with plasmid-expressing IFN-γ resulted in a decrease of these responses ***(14)***. Similarly, Kim et al. reported that co-immunization of GM-CSF genes with DNA vaccine constructs increased antigen-specific antibody and T helper cell proliferation responses while co-immunization with IL-12 genes resulted in weaker antibody responses and enhanced T helper cell proliferation ***(15)***. In addition, IL-12 co-immunization resulted in a significant enhancement of CTL responses. Kim et al. also reported on the effects of costimulatory molecules, another set of immunologically relevant molecules, as DNA vaccine adjuvants ***(16)***. Co-immunization of DNA vaccines with CD86 results in increased T helper cell proliferation and CTL ***(8)***. Iwasaki et al. more recently reported that GM-CSF and IL-12 co-delivery, with DNA immunogen encoding for influenza NP, resulted in enhanced cellular immune responses. Furthermore, two other reports in addition to Kim's independently confirmed that co-delivery of CD86, and not CD80, with a DNA immunogen-enhanced T cell mediated immune responses ***(8,17,18)***. In addition to these reports, Chow et al. reported that either injection of plasmid co-expressing hepatitis B surface antigen (HBsAg) and IL-2 or co-injection of IL-2 genes with plasmid expressing HBsAg resulted in the enhancement of both antibody and T helper cell responses ***(19)***. More recently, we investigated the induction and regulation of immune responses from the co-delivery of proinflammatory cytokines (IL-1α, TNF-α, and TNF-ß), Th_1 cytokines (IL-2, IL-15, and IL-18), and Th_2 cytokines (IL-4, IL-5, and IL-10) ***(20)***. We observed enhancement of antigen-specific humoral response with the co-delivery of Th_2 cytokines IL-4, IL-5, and IL-10, as well as that of IL-2 and IL-18. A dramatic increase in antigen-specific T helper cell proliferation was seen with IL-2 and TNF-α co-injections. In addition, we observed a significant enhancement of the cytotoxic response with the co-administration of TNF-α and IL-15 genes with HIV-1 DNA immunogens. These increases in CTL response were both MHC class I-restricted and CD8+ T cell-dependent.

These results collectively demonstrate that antigen-specific immune responses can be modulated by the co-injection of costimulatory molecule and cytokine genes with DNA immunogen cassettes. More generally, they demonstrate the potential of this strategy of co-delivering immunologically important genes as a vehicle for the development of important immunogens and the investigation of immunologically important molecules in vivo.

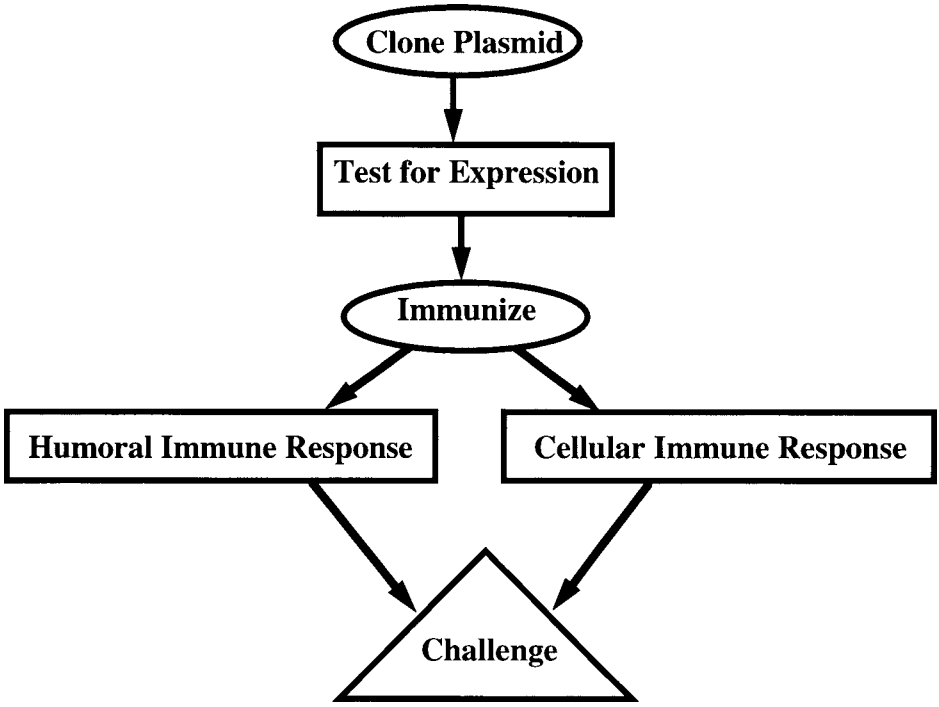

Fig. 2. Flow diagram for using molecular adjuvants.

1.1. Preparation of Molecular Adjuvants

The first step is to clone targeted genes into a mammalian expression vector (**Fig. 2**) *(21)*. Such genes can be cloned using PCR with a set of primers and a cDNA template. For instance, CD80 and CD86 genes were cloned from human B cell cDNA library (Clontech, Inc., Palo Alto, CA) and placed into an expression vector under the control of a CMV promoter *(8)*.

In cloning a molecular-adjuvant gene into an expression vector, two different approaches can be utilized. One strategy is cloning directly several different inserts into a single vector under the control of one or more independent promoter *(18,19)*. The second strategy is cloning into a separate construct for each insert and then mixing together the desired combinations at the time of injection *(8,15)*. The advantage of cloning different inserts into a vector with a single backbone is a greater likelihood of co-expressing the various proteins in the same cell. In addition, the preparation of a single plasmid may save time and materials. The major disadvantage of this method is that each antigen/adjuvant plasmid must be independently cloned for each combination. Such a specific utilization of an antigen/adjuvant combination is inflexible for investi-

gating various antigen and adjuvant combinations. Although cloning separate constructs affords variability in constructs, the ability to express all of these in the same cells is still a reasonable concern ***(8,15)***. We have shown, however, both in vivo and in vitro that the mixture of plasmids prior to transfection leads to co-expression of different plasmids within the same cell ***(22)***.

Once a gene insert is cloned into an expression vector, it can then be sequenced to test the fidelity of the insert. If the sequencing result is satisfactory, the plasmid expression cassette can be tested for the correct protein expression by a variety of methods. The test of expression in vitro requires transfection of a cell line with the desired plasmid construct. Transfection of mammalian cells can be done with the liposomal or electroporation methods. The liposomal method involves encapsulation of the plasmid DNA into a liposomal artificial membrane vesicle. The liposomes fuse with the target cell membrane and introduce DNA to the cytoplasm of the cell. Some transfected DNA will localize in the nucleus through an uncharacterized process and this nuclear-localized DNA generates the RNA transcripts that are transported to the endoplasmic reticulum for translation into antigenic proteins. In the electroporation method, a brief, high voltage electric pulse creates nanometer-sized pores in the cell membrane. DNA is taken directly into the cell cytoplasm either through these pores, or as a consequence of the redistribution of membrane components that accompanies closure of the pores. The transfected cells can then be tested for expression of the protein encoded through the construct by any of the following methods:

1.1.1. Immunoprecipitation

The expression of protein produced by the transfected cells can be assayed by immunoprecipitation. This involves radiolabeling the transfected cells. Following labeling, the cells are lysed, and specific antibodies are added to precipitate the desired protein. The antigen-antibody complex is then analyzed by electrophoresis and visualized by radiography. Alternatively, following electrophoresis, unlabelled protein can be transferred to nitrocellulose. The specific antigen detection then can be accomplished by the immunoblotting technique (Western blotting).

1.1.2. Flow Cytometer

The expression of proteins can be tested on the transfected cells with a fluorescence-activated cell sorter (FACS) assay. FACS uses the ability of flow cytometer to detect and count individual cells passing in a stream in front of it. These instruments are used to study the properties of cell subsets identified with monoclonal antibodies to cell-surface proteins. Individual cells within a mixed population are first tagged by treatment with specific monoclonal anti-

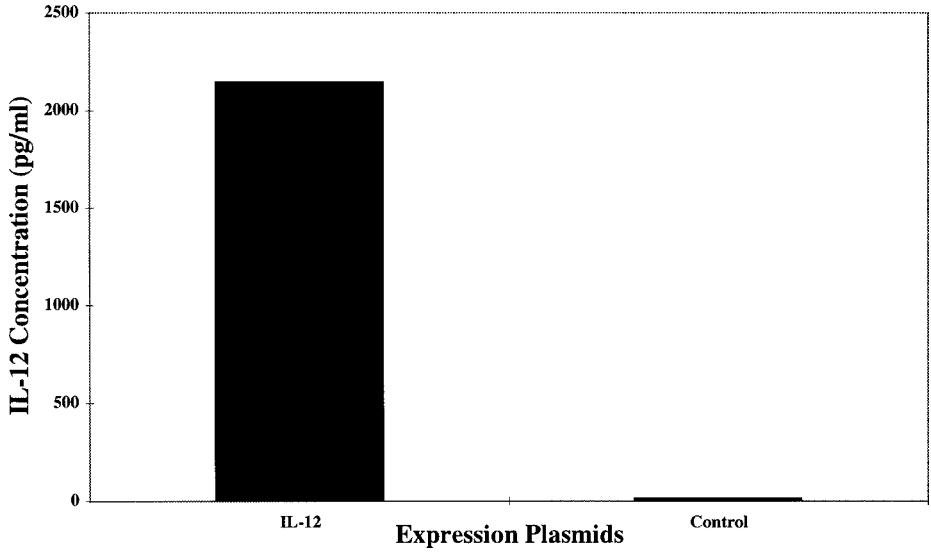

Fig. 3. Detection of murine IL-12 by ELISA. IL-12 genes (encoding both the p35 and the p40 chains) were cloned into expression plasmids under the control of a CMV promoter. The resulting plasmid was transfected *in vitro* into RD cells. Expression of IL-12 in the culture supernatant was identified using ELISA.

bodies labeled with fluorescent dyes. The mixture of labeled cells is then forced with a much larger volume of saline through a nozzle, vibrated at high frequency in order to break the stream into droplets, some of which will contain cells. As each droplet with a cell passes in front of a laser beam, it scatters the laser light, and dye molecules bound to the cell will be excited and, if appropriately tagged, will fluoresce. Sensitive photomultiplier tubes detect both the scattered light, which gives information on the size and granularity of the cell, and the fluorescent emissions, which give information on the specific binding of the labeled antibodies and hence on the expression of cell-surface proteins by transfected cells.

1.1.3. ELISA (Enzyme-Linked Immunosorbent Assay)

Detection of soluble protein such as cytokines can be conducted using ELISA (**Fig. 3**). Numerous vendors offer cytokine ELISA kits. These assays involve capturing the protein of interest by a monoclonal antibody coated onto a polystyrene microtiter plate. A second antibody conjugated to an enzyme reacts with the captured molecule, immobilizing the enzyme in the microtiter well. Substrate is added to the well, and the enzyme generates a color proportional to the amount of molecule. One advantage of this assay is that the amount of protein expression can be quantified.

1.1.4. Immunohistochemistry/Immunofluorescence

Immunohistochemistry or immunofluorescence can be used to analyze the transfected cells. Immunohistochemical analysis involves the binding of specific antibodies to a protein expressed on cells and detecting them by microscopy. Immunofluorescence is a technique in which molecules are detected using antibodies labeled with fluorescent dyes. The bound fluorescent antibodies can be detected by microscopy. In vivo detection of proteins by immunohistochemistry or immunofluorescence techniques are similar to those of in vitro methods. Animals are immunized with cDNA constructs and the tissues at the site of expression are removed. For intramuscular injection, the expression of plasmid-encoded protein can be detected as early as 72 h post-injection *(8)*. The fresh tissue is then frozen, and the slides prepared by sectioning the tissues (**Fig. 4**).

1.2. Analyzing Immune Responses Following Immunization.

Once the antigenic and adjuvant DNA vaccine constructs are prepared, they can be administered to animals to study the induction of immune responses. The animals can be immunized via different routes (intramuscular, intradermal, or mucosal), or different delivery mechanisms (particle gun, or syringe and needle). The co-administration of various gene expression cassettes involves the mixing of chosen plasmids prior to injection. To better study the modulation effect of the molecular adjuvant constructs, a sub-optimal dose of antigenic gene construct should be utilized.

The host immune system responds to infection or immunization via induction of specific and regulated humoral and cellular immune responses. The immune responses induced by immunization using DNA vaccine constructs can be analyzed.

1.2.1. Humoral Response

B cells mediate the humoral immune response. Mature B cells carry surface immunoglobulins that act as their antigen receptor. They then move through the circulation to secondary lymphoid tissues, where they respond to antigenic stimuli by dividing and differentiating into plasma cells under control of cytokines produced by T cells. Plasma cells are terminally differentiated B cells that are entirely devoted to the production of antigen-specific antibodies. Antibodies are serum proteins secreted by plasma cells that are induced following contact with antigen, and they bind specifically to the inducing antigen. The analysis of the antibody responses can be done by ELISA with a specific antigen to determine the presence of antigen-specific antibodies in the serum of the immunized animals (**Fig. 5**).

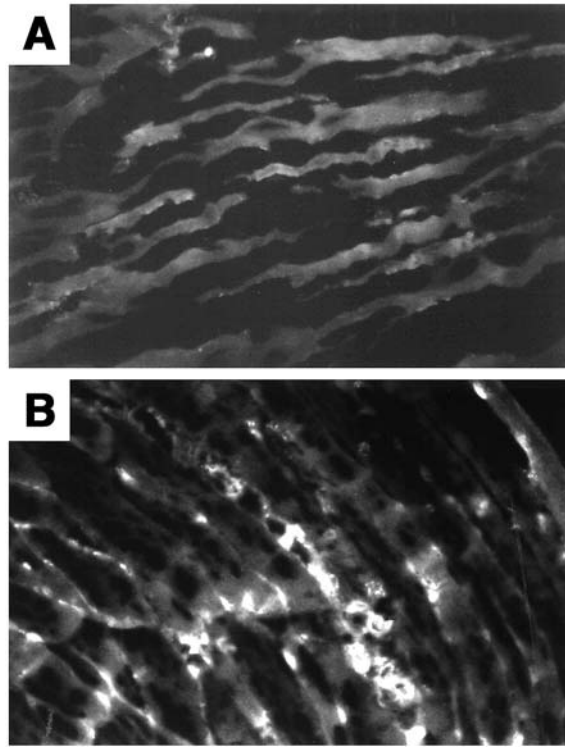

Fig. 4. Co-expression of HIV-1 envelope gp120 protein with CD80 or CD86 on muscle cells. Frozen muscle sections were prepared from DNA-injected animals and stained with FITC-labeled (green) α-CD86 antibodies and Texas Red-labeled (red) α-gp120 antibodies. A) A slide from a leg immunized with pcDNA3 (control vector) was stained with α-CD86 and α-gp120 antibodies. B) A slide from a leg immunized with pCEnv+pCD86 was stained with α-CD86 and α-gp120 antibodies.

1.2.2. T Helper Cell Proliferative Response

Activation and proliferation of T helper (Th) lymphocytes play critical roles in inducing and expanding both humoral and cellular immune responses by activating both B and cytotoxic T cells, respectively. T cells from immunized animals proliferate when exposed to specific antigens, but not to unrelated protein. Proliferation can be determined by measuring the level of incorporation of ^{3}H-thymidine into the DNA of actively dividing cells. We can analyze the vaccine's ability to elicit $CD4^{+}$ Th cell immunity by measuring the antigen-specific proliferation in this manner (**Fig. 6**).

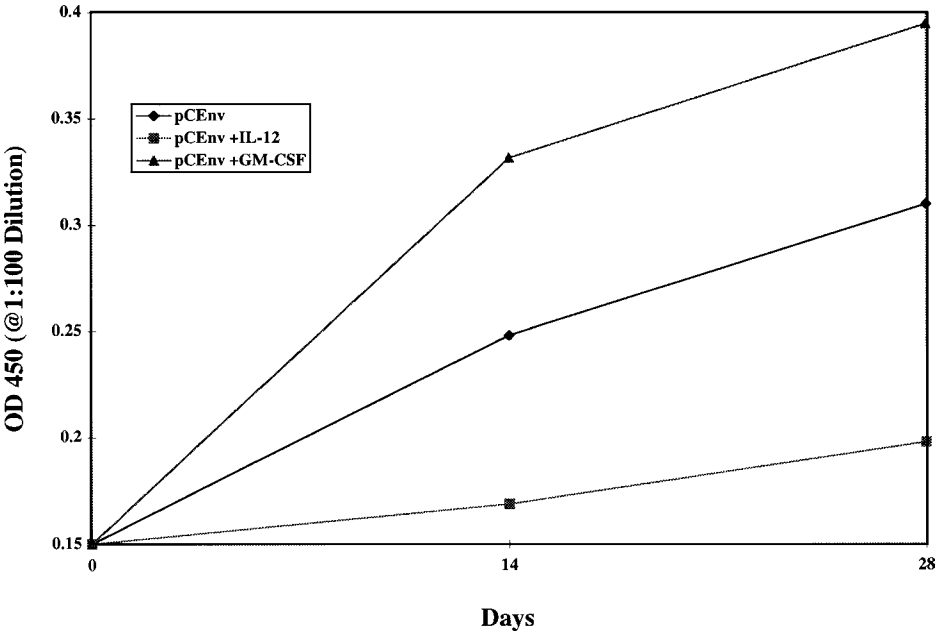

Fig. 5. HIV-1 envelope-specific antibody response following co-immunization with IL-12 or GM-CSF genes. Fifty μg of IL-12 or GM-CSF genes were co-administered with pCEnv cDNA expression cassettes intramuscularly at day 0. Prior to injection and at days 14 and 28, the mice were bled and the sera collected. The mouse sera were tested for envelope-specific antibody responses, with the ELISA as described using HIV-1 gp120 protein.

1.2.3. Cytotoxic T Lymphocyte (CTL) Response

$CD8^+$ cytotoxic cells or CTLs kill target cells that display antigenic fragments of cytosolic pathogens, most notably viruses, bound to MHC class I molecules at the cell surface. Specific recognition of peptide:MHC complexes on a target cell by a cytotoxic CD8 T cell can lead to the killing of the target cell through two different mechanisms. First, the killer T cells release granzymes, which are toxic substances that puncture holes in the membranes of target cells. Second, the cytotoxic T cells signal the target cells to apoptosis via the fas-fas ligand interaction. Cytotoxic T cells can recycle to kill multiple targets. CD8 T-cell function can be determined by a T-cell bioassay measuring the killing of a target cell by a cytotoxic T cell. This is usually detected in a ^{51}Cr-release assay. Live cells will take up, but do not spontaneously release, radioactively-labeled sodium chromate, $Na_2{}^{51}CrO_4$. Target cells are labeled with radioactive $Na_2{}^{51}CrO_4$ and exposed to cytotoxic T cells. When these labeled cells are killed by either mechanism of CTL induced killing, the radio-

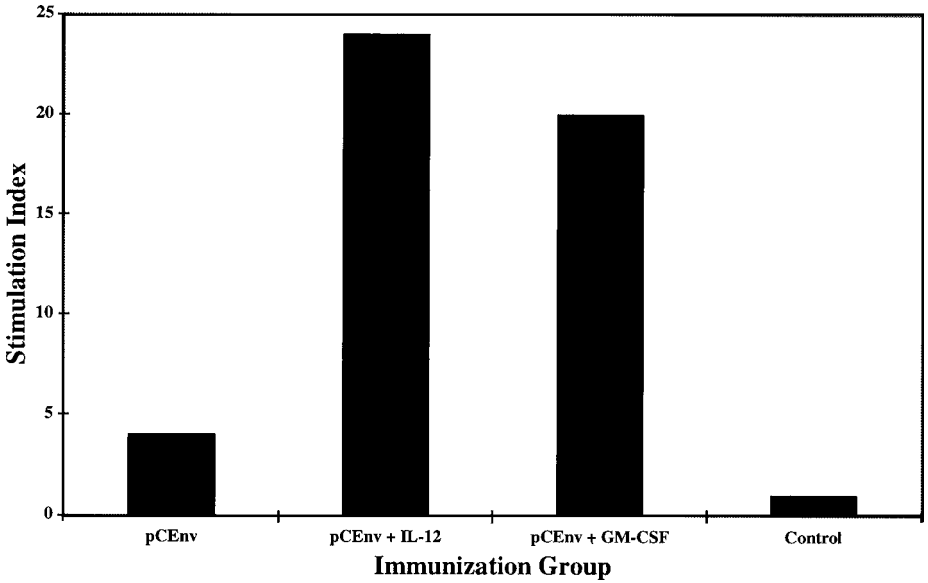

Fig. 6. T-helper cell proliferation responses following co-immunization with IL-12 or GM-CSF genes. Two weeks after the first DNA immunization with pCEnv, the mice were boosted with same amount of plasmid. After one additional week, spleens were collected from immunized mice and the lymphocytes were isolated. Recombinant gp120 protein (5 μg/mL final concentration) was added to each well to stimulate the proliferation of T helper cells.

active chromate is released and its presence in the supernatant of mixtures of target cells and cytotoxic T cells can be measured. Cell destruction is measured by the release of radioactive chromium into the medium, detectable within four hours of mixing target cells with T cells (**Fig. 7**).

1.2.4. Challenge Models

The overall goal of an immunization strategy is to induce specific immune responses that confer lifetime protection from the pathogen of interest. Thus, an important test of vaccine's immunogenicity is the ability to protect the host from challenge. For infectious diseases, it would be optimal to challenge the immunized animals with the actual pathogen of interest in the host of interest. However, for safety and practical reasons, this is usually not done. Accordingly animal model system of infection can provide us with important evidence of vaccine utility. Several DNA vaccine constructs have been reported to protect small animals from viral or bacterial pathogens such as influenza, malaria, and TB pathogens ***(7,10,23)***. On the other hand, not all models are

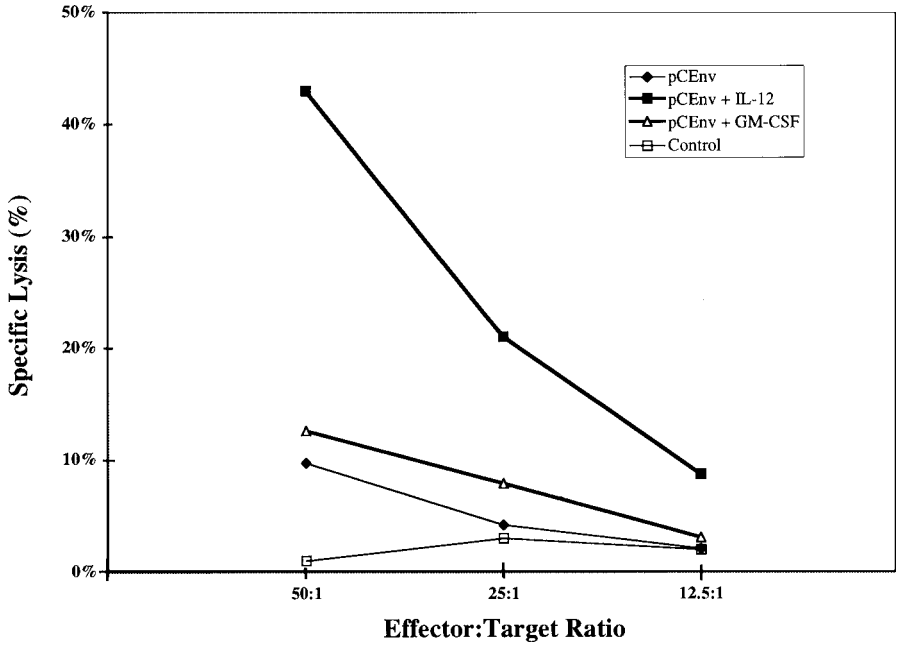

Fig. 7. HIV-1 envelope-specific CTL responses in mice immunized with pcDNA3, pCEnv, pCEnv+IL-12, or pCEnv+GM-CSF. CTLs were analyzed against vaccinia-infected targets within vitro stimulation of effector cells.

relevant for different infectious agents. Because HIV-1 does not infect mice and other small rodents, primates represent the most relevant challenge system for HIV vaccine evaluation. Specifically, there are currently three different primate models for HIV vaccine studies. They are the SIV and chimeric (SIV)/HIV-1 (SHIV-1) challenge models in macaques and HIV challenge model in chimpanzees. Our laboratory has observed protection of macaques from a SHIV-1 challenge as well as protection of chimpanzees from an HIV-1 challenge *(24,25)*. Challenging of immunized animals with tumor cells expressing relevant pathogenic or tumor antigens also represents a relevant protection model *(26,27)*.

2. Materials

2.1. Immunoprecipitation

1. RIPA buffer: 50 m*M* Tris-HCl pH 7.6; 150 m*M* NaCl; 0.2% (v/v) Triton X-100; 0.2% (w/v) Deoxycholic acid; 0.1% (w/v) SDS and 1 m*M* PMSF.
2. ^{35}S protein labeling mix is available from NEN/DuPont (Boston, MA).
3. SDS-PAGE materials.

2.2. Flow Cytometer

1. FACS buffer: PBS containing 1% (w/v) BSA and 0.1% (w/v) sodium azide.
2. Antibodies.

2.3. Immunohistochemistry/Immunofluorescence

1. Leica 1800 Cryostat (Leica Inc., Deerfield, IL).
2. ProbeOn Plus Slides (Fisher Biotech, Pittsburgh, PA).
3. 1.5% (v/v) goat serum (Vector Labs, Burlingame, CA).
4. Streptavidin-Biotin HRP (Research Genetics, Huntsville, AL).
5. DAB (Ventana Biotech, Tucson, AZ).
6. Microscope.

2.4. Detecting Humoral Response Using ELISA

1. 0.1M carbonate-bicarbonate buffer (pH 9.5).
2. PBS-0.05% (v/v) Tween-20.
3. 3% (w/v) BSA in PBS with 0.05% (v/v) Tween-20.
4. HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO).
5. 3'3'5'5' TMB (Sigma).
6. Plate reader.

2.5. T Helper Cell Proliferation Assay

1. Harvested splenocytes.
2. Culture media: RPMI (Gibco-BRL, Grand Island, NY), 10% (v/v) fetal calf serum (Gibco-BRL).
3. 96-well microtiter flat bottom plate.
4. Tritiated (^{3}H) thymidine.
5. Plate reader.

2.6. CTL Assay

1. Harvested splenocytes.
2. CTL culture media: RPMI (Gibco-BRL), 10% (v/v) fetal calf serum (Gibco-BRL), and 10% (v/v) RAT-T-STIM without Con A (Becton Dickinson Labware, Bedford, MA).
3. $Na_2{}^{51}CrO_4$.
4. Gammacounter.
5. 5% (v/v) Triton X-100.

3. Methods

3.1. Immunoprecipitation

1. Transfect cells with the plasmid construct of interest.
2. Wash the cells twice with PBS, starve them for one hour in DMEM lacking serum, methionine and cysteine, and then label them with 200 μCi/mL (1200 Ci/mmole) of ^{35}S protein labeling mix.

3. Lyse the labeled cells in 0.5 mL of RIPA buffer on ice and then clarify the lysate by centrifugation at top speed in a microcentrifuge for 10 min.
4. Incubate the clarified lysates with specific antibody for 90 min on ice.
5. Add Protein A-Sepharose to the resultant antigen-antibody complexes and mix by shaking at 4°C for 90 min.
6. Resuspend the protein pellet in 50 µL of sample buffer, extensively washing it in buffers containing high salt and BSA and heat the final suspension at 100°C for 3–5 min after.
7. Analyze the protein sample by 12% (w/v) SDS-PAGE.

3.2. Flow Cytometer

1. Wash cells (1×10^5) three times with FACS buffer and incubate with FITC- or PE-conjugated mAbs at a saturating conditions for 30 min on ice.
2. Wash cells three times with FACS buffer and analyze them using a FACScan.

3.3. Immunohistochemistry/Immunofluorescence

1. Inoculate mouse quadriceps muscles with the plasmid construct of interest ***(8)***.
2. Remove the quadriceps muscles 72 h later.
3. Cut 6 µm cryostat sections using a Leica 1800 cryostat and place on ProbeOn Plus slides (Fisher Biotech).
4. Fix the slides in acetone, wash, and air dry.
5. Block the slides with 1.5% (v/v) goat serum and incubate in primary antibody at room temperature.
6. Incubate the slides with a biotinylated secondary antibody followed by incubation with streptavidin-biotin HRP.
7. Treat the slides with DAB and counter-stain them.
8. View the slides with a microscope.

3.4. Detecting Humoral Response Using ELISA

1. Dilute the protein antigen to 2 µg/mL diluted in 0.1*M* carbonate-bicarbonate buffer (pH 9.5).
2. Adsorb 50 µL of the diluted protein onto microtiter wells overnight at 4°C.
3. Wash the plate with PBS-0.05% (v/v) Tween-20 and block with 3% (w/v) BSA in PBS with 0.05% (v/v) Tween-20 for 1 h at 37°C.
4. Add mouse antisera diluted with 0.05% (v/v) Tween-20 and incubate the plate for 1 h at 37°C.
5. Add HRP-conjugated goat anti-mouse IgG and incubate the plate for 1 h at 37°C.
6. Wash the plate and develop it with 3'3'5'5' TMB buffer solution.
7. Read the plate on a plate reader with the optical density at 450 nm.

3.5. T Helper Cell Proliferation Assay

1. Harvest lymphocytes from the spleen and remove the erythrocytes.
2. Resuspend the isolated cell suspensions to a concentration of 5×10^6 cells/mL.

3. Add a 100 µL aliquot containing 5×10^5 cells to each well of a 96-well microtiter flat-bottom plate containing a specific protein at various concentrations.
4. Incubate the plate at 37°C in 5% (v/v) CO_2 for 3 d.
5. Add 1 µCi of tritiated thymidine to each well and incubate the plate for additional 12–18 h at 37°C.
6. Harvest the plate and measure the amount of incorporated tritiated thymidine on a Beta Plate reader.
7. The stimulation index was determined from the formula: Stimulation Index (SI) = (experimental count/spontaneous count)

3.6. Cytotoxic T Lymphocyte Assay

1. Harvest lymphocytes from spleens and remove the erythrocytes.
2. Stimulate the effector cells with stimulator cells for 4–5 d in CTL culture media at 5×10^6 cells per mL.
3. CTL culture media consists of RPMI, 10% (v/v) fetal calf serum, and 10% (v/v) RAT-T-STIM without Con A.
4. Perform a standard chromium-release assay in which the target cells are labeled with 100 µCi/mL $Na_2{}^{51}CrO_4$ for 60–120 min and used to incubate with the stimulated effector splenocytes for 4–6 h at 37°C.
5. Determine CTL lysis at effector:target (E:T) ratios ranging from 50:1 to 12.5:1.
6. Following incubation, harvest supernatants and count on a LKB CliniGamma gamma-counter.
7. Percent specific lysis was determined from the formula:

$$100 \times \left(\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \right)$$

8. Maximum release is determined by lysis of target cells in 5% (v/v) Triton X-100 containing medium. An assay should not be considered valid if the values for the 'spontaneous release' counts are in excess of 20% of the 'maximum release.'

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Cytokine Fusion Constructs as DNA Vaccines Against Tumors

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1. Introduction

1.1. Tumor Antigens

1.1.1. Overview of Types of Tumor Antigens

Various studies have used DNA vaccination as a method of immunizing against tumors ***(1–12)***. As with any tumor vaccine, one challenge is to find a truly tumor-specific antigen ***(13,14)***. The majority of immunologically targeted tumor antigens are also expressed on a subset of normal host cells. Examples of such antigens include prostate-specific antigen, and CD20, a B cell marker. Some tumor antigens are specific for activated cells of certain types, such as carcinoembryonic antigen (CEA) or the IL-2 receptor. These are often found on embryonic or fetal cells as well as tumor cells. The carbohydrate antigens of melanomas and the immunoglobulin (Ig) idiotype of B cell lymphomas represent tumor-specific antigens (TSA). Unfortunately, TSA have not been identified in more common malignancies. Furthermore, the antigenic determinants of known TSA may differ between patients; for example, the tumor idiotype (Id) of B cell lymphoma is highly patient-specific and must be determined for each case.

In addition to protein and carbohydrate antigens, peptide determinants have also been exploited as tumor-specific vaccines. These include the MAGE antigens of certain melanomas as well as peptides derived from activated oncogenes such as ras or myc. Using this approach, presentation of peptide in the context of host MHC leads to activation of antigen-specific T cells. Peptide sequences have been used successfully as DNA vaccines ***(3)***. However, the polymorphic nature of MHC complicates the design of peptide vaccines, and

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

the absence of the native protein from which the peptide was derived most likely precludes the induction of humoral immunity.

In an effort to further explore DNA immunization against TSA, work in our laboratory has focused on DNA vaccination against Id using the murine B cell lymphoma, 38C13. Immunization with plasmids encoding the heavy and light chain variable regions of 38C13, which contain the tumor Id, can protect mice against subsequent tumor challenge ***(11,12)***. Induction of anti-Id antibodies by DNA vaccination, but not tumor protection, has been demonstrated in another mouse B lymphoma model, BCL-1 ***(9,10)***. In addition to the 38C13 model, we have used ovalbumin. as an antigen for DNA vaccination. Although it is not a true tumor antigen, ovalbumin. is advantageous in that proliferative and cytotoxic T cell responses against it can be readily measured.

1.1.2. Problems of Immune Responses to Tumor Antigens

Ig idiotypes, as well as many of the other "tumor antigens" described above, are only weakly immunogenic. The antigenic differences that confer specificity for tumor are subtle when compared to similar proteins normally found in host tissue. In addition, there may be a need to overcome host tolerance to these antigens. For example, a B cell lymphoma-bearing host will have already been exposed to large quantities of the tumor idiotype. Thus, potentially responsive host cells may have been tolerized by deletion or anergy ***(15,16)***. The host may, therefore, not respond to these antigens unless they are presented in a novel, immunostimulatory context. This may consist of a foreign carrier protein such as keyhole limpet hemocyanin, or a foreign immunoglobulin (Ig) constant region. Alternately, cytokine sequences, whose products can provide stimulatory signals to the proper immune cells, can be used to make fusion constructs. In the case of the 38C13 system, foreign constant region sequences are required for tumor protection when vaccinating with DNA encoding whole Ig ***(12)***. A single-chain Fv (scFv) construct, which contains the V regions of heavy and light chains connected by a flexible linker, is poorly immunogenic on its own. However, addition of a sequence derived from IL-1ß to the scFv DNA construct renders it immunogenic and results in good tumor protection ***(11)***.

1.2. Advantages of DNA Vaccination

1.2.1. No Need to Purify Protein

Unlike protein immunization, DNA immunization eliminates the need to produce a recombinant protein in substantial quantities. Ig production is often a limiting step when making customized vaccines for patients with B cell lymphoma. The simplicity of cloning the necessary sequences and the ease of producing large quantities of plasmid make production of a DNA vaccine much faster in such cases.

1.2.2. Ability to Induce Cellular Immunity

DNA vaccination also has the advantage of inducing cellular immune responses. Since DNA immunization results in endogenous production of antigen by host cells, the target antigen can be presented on both class I and class II MHC. In fact, DNA immunization has been shown to induce strong cytotoxic T cell (CTL) responses in a number of systems *(17–32)*. By contrast, immunization with soluble protein can sometimes induce CTL responses, but generally only in the presence of strong adjuvants *(33,34)*. This is presumably due to the difficulty of getting soluble proteins introduced into the intracellular pathway for presentation by class I MHC, a requirement for CD8-restricted CTL responses.

1.3. Construction of Plasmids

1.3.1. Immunostimulatory Sequences

The immunostimulatory qualities of certain CpG-containing sequences derived from bacterial DNA have been widely reported *(35–37)*. It is therefore advantageous to choose an expression plasmid for DNA vaccination that contains optimal immunostimulatory sequences (ISS). We have chosen to work with the plasmid pId *(12,38)* (**Fig. 1**), which contains an ampicillin-resistance gene that has been shown in other systems to encode a powerful ISS *(36)*.

1.3.2. Foreign Constant Regions for Immunoglobulins

When constructing an idiotype-specific DNA vaccine, it is possible to combine the heavy and light chain V regions of the tumor Ig in an scFv construct. This eliminates the need for a plasmid expressing two separate proteins for heavy and light chains. However, because of the immunostimulatory effect of foreign Ig constant regions mentioned above, it may be more desirable to construct vectors that encode whole Ig molecules. For this purpose, a bicistronic plasmid that independently codes for Ig heavy and light chains was designed *(38)*. This plasmid contains appropriate restriction sites into which Ig heavy and light chain V regions can be cloned (**Fig. 1**).

1.3.3. Dual Promoter vs IRES for Multiple Chain Constructs

In lieu of vectors encoding two promoters, one for heavy and one for light chain, it is also possible to link the heavy and light chain coding regions with an internal ribosomal entry site (IRES). The IRES allows for translation of two coding regions (heavy and light chain in this case) from the same RNA message. In theory, this should coordinate the expression levels of the two chains most precisely, since ribosomes will be able to initiate translation of both chains with equal efficiency. In practice, we have found no advantage in tumor protection in vivo, when comparing a dual promoter plasmid to the same plasmid modified to contain a single promoter with an IRES (unpublished data).

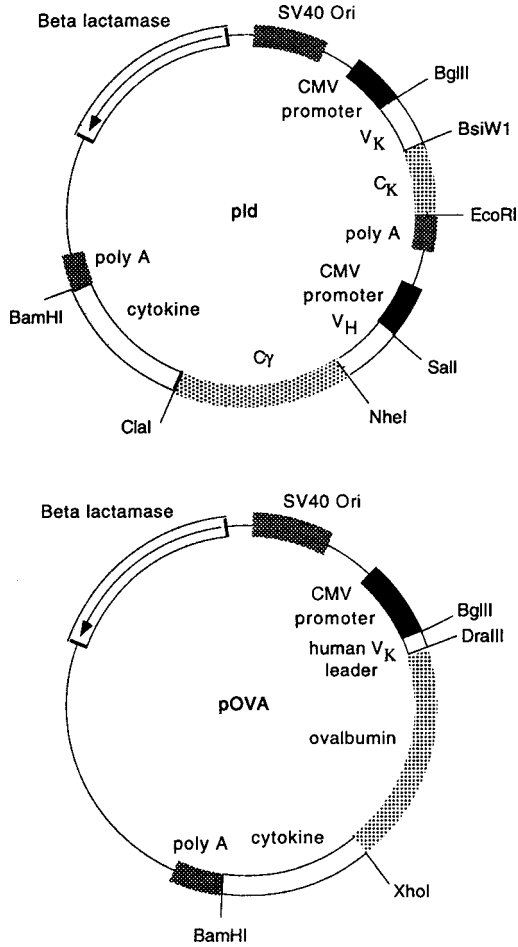

Fig. 1. Schematic of the pId plasmid used for DNA immunization of 38C13 Ig-cytokine constructs, and pOVA plasmid used for DNA immunization with ovalbumin.

1.3.4. Leader Sequences for Secretion of Protein

Leader sequences, found at the 5' end of the coding region of membrane and secreted proteins, ensure that the synthesized protein is targeted to the endoplasmic reticulum and thus enters the secretory pathway. Ig molecules have endogenous leaders, which can be cloned from tumor cDNA together with the variable region sequences, as we have done for 38C13. We have also used exogenous leaders, such as the human kappa-chain leader sequence, with non-Ig coding regions, such as ovalbumin. ***(32)***.

Is a secreted product necessary for efficient DNA vaccination? If DNA is transfected into resident host tissue, such as muscle or skin cells, these cells

can secrete the protein for uptake by host APC. However, this may not be necessary for DNA vaccination to work, since resection of the target tissue even minutes after DNA injection did not alter antibody or CTL responses in two different systems ***(39)***. In fact, direct transfection of APC in the tissue and/or draining lymph nodes has been implicated as critical to the efficacy of DNA vaccination ***(40)***. However, we have compared DNA vaccination against 38C13 idiotype using two similar plasmids, one of which led to efficient secretion of the encoded protein, while the other did not. In this system, antibody responses and tumor protection were not detected in the animals that received the plasmid encoding the nonsecreted version of 38C13 (our unpublished data). Thus, it may be helpful in at least some systems to ensure that cells taking up the plasmid DNA secrete the target antigen.

1.3.5. Choice of Cytokines

The use of a cytokine gene linked to the coding region for the target antigen has been shown to be an effective way of inducing anti-tumor immune responses when using soluble protein vaccination ***(41,42)***. In the first case, the GM-CSF coding region was linked to the 3' end of the heavy chain constant region. This resulted in production of a protein that consisted of the entire Ig molecule with two GM-CSF molecules, one attached to each of the heavy chain constant regions. The tumor protection observed with a 38C13-GM-CSF fusion protein was dependent upon the bioactivity of the cytokine, as human GM-CSF, which is inactive in mice, could not induce protection ***(41)***. GM-CSF was chosen for its ability to enhance antigen presentation by dendritic cells. However, other cytokines, including IL-2 and IL-4, have also been shown to be effective in the same system ***(42)***.

The cytokines that we have tested in fusion constructs with target antigens can be divided into three groups: (i) those that can potentially enhance antigen presentation, such as GM-CSF and IL-1b; (ii) those that can enhance Th_1 immunity, such as IL-2, IFNγ, and IL-12; and (iii) those that can enhance antibody production or Th_2 immunity, such as IL-4. The most extensive comparison of these cytokines has been done in the ovalbumin system ***(32)***. In this system, antibody production was relatively unchanged regardless of the choice of cytokine, while significant CTL activity could be found with all constructs. However, IL-12 and a nonapeptide sequence derived from IL-1ß appeared to induce the strongest and most rapid CTL responses ***(32)***. In the 38C13 system, the IL-1ß peptide construct was able to induce tumor protection in a scFv system, whereas scFv alone was not protective ***(11)***.

In protein vaccination with cytokine fusion constructs, vaccine efficacy was dependent upon covalent linkage of the antigen and cytokine ***(41,43)***. This was

true when measuring tumor protection in the 38C13 system ***(41)*** as well as when measuring the conversion of a Th_2-dominated immune response to a Th_1-dominated immune response in the ovalbumin. system ***(43)***. However, this was not the case for DNA vaccination. No difference could be seen in antibody responses when using a 38C13-GMCSF fusion construct versus coinjection of 38C13 and GM-CSF on separate plasmids (our unpublished data). In either case, the addition of GM-CSF resulted in a higher proportion of immunized animals ***(12)***. Similarly, others have shown that coinjection of a DNA vaccine with a cytokine protein ***(5)*** or with a cytokine-encoding plasmid ***(9,44)*** could enhance antibody responses to tumor or viral antigens. The difference between protein and DNA vaccines regarding the need for cytokine fusion may be explained because coinjection of two DNA plasmids results in cotransfection of the same host antigen-presenting cells. This would result in the cytokine acting on the same cells as are processing the antigen. Alternatively, it may be sufficient to express the antigen and cytokine in the same microenvironment even if not by the same cells.

1.3.6. Linkers

If antigen and cytokine are to be fused in a recombinant construct, native antigenic conformation as well as cytokine bioactivity must be preserved. We have used a linker consisting of two glycine residues to connect the antigen and cytokine. While this almost always works, bioactivity of the cytokine is diminished in some cases, possibly due to conformational problems. A longer linker, $[(Gly)_4Ser]_3$, was used to fuse the heavy and light chain V regions in scFv constructs.

1.3.7. Tags: His, Myc

A variety of "tag" sequences can be added to recombinant constructs to facilitate purification or identification of the protein product. Two tags studied in our laboratory are the hexahistidine tag (his tag) and a sequence of six amino acids from the myc oncoprotein (myc tag). The his tag can bind to nickel chelate resins, while the myc tag encodes an epitope recognized by the 9E10 anti-myc antibody. These tags have been added to the 3' end of a construct, although alternative placements may also be feasible. Although DNA vaccination does not require a tag, since there is no need to purify protein, it may be convenient to introduce one so that the construct can be easily tested for secretion in vitro. Following transient transfection into COS-7 cells, for example, the cell supernatant can be assayed by ELISA or Western blot for production of the proper protein. The myc tag is useful when the protein product is to be detected by Western blotting, as the 9E10 antibody is a superior reagent for Westerns. In

certain cases, we have found these tags to fail in that the recombinant proteins are not detected in such assays, although the constructs are detected by antibodies to the antigen itself. This may occur through masking of the tag due to the conformation of the protein. Thus, the efficacy of such tags must be tested on a case-by-case basis.

The presence of foreign tag sequences may also introduce new antigenic epitopes into the recombinant protein or DNA vaccine. Although we have never demonstrated this definitively, the possibility cannot be discounted. We therefore recommend that appropriate controls be included when comparing vaccines that contain tags.

In the majority of our experiments, mice were immunized intramuscularly with 100 μg of naked DNA. When compared to intradermal immunization, we found that equivalent levels of specific antibody could be raised by either method, but that intramuscular injection yielded a higher ratio of IgG2a to IgG1 antibodies ***(12)***. This is consistent with a Th_1-dominated immune response for intramuscular injection, and a Th_2-dominated response for intradermal injection. This has been found for both 38C13 and ovalbumin. antigens (our unpublished data). However, in other systems, investigators have reported that the isotype ratio and cytokine profile of immunized animals was unaffected by the route or dose of immunization but instead corresponded to the method of vaccination (gene gun versus injection) ***(45)***. Other investigators have found that the injected muscle cells can be resected after DNA vaccination without detrimental effect, suggesting that the target tissue is not important in inducing the immune response ***(39)***.

2. Materials

2.1. Plasmid Construction

2.1.1. Expression Plasmids

The mammalian expression plasmids pId ***(12,38)*** and pOVA ***(32)*** are depicted in **Fig. 1**. The pId plasmid is designed for cloning of heavy and light chain V regions (human or mouse) into cassettes which contain human Ig constant regions. Appropriate restriction sites have been included in order to clone Ig V regions as well as cytokines into this plasmid. A single promoter version of this plasmid containing an IRES (*see* **Subheading 1.3.3.**) has also been made. The pOVA plasmid is a modified version of pId designed for expression of ovalbumin or other non-immunoglobulin genes ***(32)***.

2.2.2. Ig V Regions

The primers shown in **Table 1** were used to clone the V regions of 38C13 from cDNA of a hybridoma expressing 38C13 Ig. The heavy chain primers contain *Sal*I and *Nhe*I sites at their 5' and 3' ends, respectively, to allow in-

Table 1
Primers for Cloning 38C13 V Regions, Ovalbumin, and Cytokine genes[a]

Gene	Restriction sites	Forward primer (restriction sites) Reverse primer (restriction sites)
38C13 V_K	BglII	5' GTAGATCTCTCACCATGGGACCGTCT 3'
	BsiW1	5' TCTACGTACGTTTTATTTCCAACTTGGTCCC 3'
38C13 V_H	SalI	5' ACAGTCGACATGGAGTTGTGG 3'
	NheI	5' AGGTGCTAGCTGAGGAGACGGTGA 3'
ovalbumin	DraIII	5' GGCCCACGATGTGGCTCCATCGGCGCAG 3'
	XhoI[b]	5' CCTCTCGAGGGGGGAAACACATCT 3'
	XhoI[c]	5' CCTCTCGAGGGGGAAACACATCT 3'
mGM-CSF	ClaI	5' GTCATCGATGGCGGAGCACCCACCCGC 3'
	BamHI	5' CCGGATCCTCATTTTTGGACTGGTTT 3'
hIL-1ßpep	ClaI	5' GTCATCGATAGTTCAGGGTGAAGAAAGTAA CGATAAATAAG 3'
	BamHI	5' GGGGATCCTTTATCGTTACTTTCTTCACCCTG AACTCCGC 3'
mIL-2	ClaI	5' CTTATCGATGGCGGAGCACCCACTTCAAG 3'
	BamHI	5' CCGGATCCTTATTGAGGGCTTGTTG 3'
mIFNγ	ClaI	5' GTCATCGATGGCGGATGTTACTGCCACGGC 3'
	BamHI	5' CCCGGGGATCCTCAGCAGCGACTCCTTTTC 3'
mIL-12p40	ClaI	5' GTCATCGATGGCGGAATGTGGGAGCTGGAG 3'
	BamHI	5' GGGGATCCCTAGGATCGGACCCTGCAG 3'
mIL-4	ClaI	5' GTCATCGATGGCGGACATATCCACGGATG 3'
	BamHI	5' CCCGGGGATCCCTACGAGTAATCCATTTG 3'

[a]For cytokine primers used for the pOVA plasmid, see ***(32)***.
[b]With stop codon.
[c]Without stop codon, for linking to cytokine gene.

frame cloning of the pId plasmid with the human IgG1 constant region. The light chain primers contain *Bgl*II and *Bsi*W1 sites at their 5' and 3' ends, respectively, to allow in-frame cloning with the human kappa constant region.

2.2.3. Cytokine Genes

The primers shown in Table 1 were used to clone the genes for murine GM-CSF, IL-2, IFNg, and IL-4 by PCR from Concanavalin A-stimulated spleen cells. IL-12 p35 and p40 genes were amplified in a similar way from previously cloned plasmids (a generous gift of Steven Wolf, Genetics Institute, Cambridge, MA), using the primers shown in Table 1. For the IL-1b nonapeptide sequence (46), the primers shown in Table 1 were annealed together and extended using Pfu polymerase without the need for an exogenous template. All cytokine primers contain ClaI and BamHI restriction sites at their 5' and 3' ends, respectively, to allow cloning into the pId vector downstream of and in

frame with the coding sequence of the heavy chain constant region. In the ovalbumin system, cytokine genes were cloned into XhoI and BamHI sites so as to be downstream of and in frame with the ovalbumin. coding sequence. All Ig and OVA sequences were recloned without stop codons, which were then incorporated into the cytokine reverse primers just upstream of the BamHI site.

1. RNAzol B reagent is available as from TelTest "B" Inc. (Friendswood, TX).
2. Promega RT kit is available from Promega Corp. (Madison, WI).
3. Cloned Pfu polymerase Pfu buffer is available from New England Biolabs (Beverly, MA).
4. Restriction enzymes are available from New England Biolabs.
5. Concanavalin A is available from Sigma Chemical Co. (St. Louis, MO).
6. MegaPrep column is available from Promega.

2.2. Vaccination

2.2.1. Tumor Model

The murine B lymphoma cell line 38C13, derived in C3H/eB mice, has been previously described ***(47)***.

1. Culture the cells in RPMI-1640 medium with 10% (v/v) fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 m*M* glutamine, and 50 µ*M* 2-mercaptoethanol (cRPMI).
2. Thaw the cells from a common stock 3 d prior to tumor challenge and grow them in log phase.

2.2.2. Chemotherapeutics

1. Dissolve cyclophosphamide (Sigma) in sterile saline and inject it intraperitoneally (i.p.) at 100 mg/kg.

2.3. Readouts

2.3.1. Direct Readouts of Tumor

Plot survival using Kaplan-Meier plots, with significance determined by a log-rank or Gehan score ***(48)***.

2.3.2. Readouts of Immune Response

2.3.2.1. ELISA of Serum Antibodies

1. 38C13 IgM: purify this from ascites of a hybridoma-rescue fusion secreting 38C13 Ig ***(49)***.
2. Microtainer serum separator tubes, Cat. No. 36-5960, are available from Becton Dickinson (Rutherford, NJ).
3. Coat microtiter plates, Maxisorb, are available from Nunc, Inc. (Naperville, IL).
4. Anti-mouse IgG-peroxidase, or subclass specific antibodies are available from Southern Biotechnology Associates (Birmingham, AL) or Caltag Laboratories (South San Francisco, CA).

5. Anti-38C13 monoclonal antibodies: mix S4C8 (IgG1), S1C5 (IgG2a), and S5A8 (IgG2b) ***(49)*** at a 2:1:1 ratio and use as a standard.
6. Microplate reader is available from Molecular Devices (Sunnyvale, CA).

2.3.2.2. T Cell Proliferation Assays

1. [^{3}H]-Thymidine is available from Amersham Corp. (Arlington Heights, IL).
2. Lympholyte M is available from Accurate Chemical Co. (Westbury, NY).
3. 96-well cell harvester and scintillation counter for 96-well format are available from Wallac (Turku, Finland).

2.3.2.3. CTL Assays

1. Ovalbumin-transfected cell line EG.7-OVA (a gift of M. Bevan, University of Washington, Seattle, WA): grow the cells in DMEM medium with 10% (v/v) fetal calf serum and 200 µg/mL G418 (Gibco-BRL, Grand Island, NY).
2. Parental cell line EL-4 for use as a negative control target is available from the American Type Culture Collection: grow it in DMEM medium with 10% (v/v) fetal calf serum.
3. Recombinant human IL-2 is available from Cetus Corporation (Emeryville, CA).
4. [^{51}Cr]-sodium chromate is availale from Amersham.
5. Gamma counter is available from Packard Instruments (Meriden, CT).

3. Methods

3.1. Plasmid Construction

3.1.1. Cloning of Ig V Regions

1. Extract RNA by centrifuging up to 10^7 hybridoma cells expressing the desired Ig and resuspending them in 1 mL of RNAzol B reagent. Follow the manufacturer's directions.
2. Use 1 µg of total RNA in a total volume of 20 µL to produce cDNA using the Promega RT kit. Incubate the reaction at 42°C for 60 min, then keep it at 4°C or frozen.
3. Use 1 µL of cDNA, or approximately 1 µg of cDNA, in a PCR reaction containing 84 µL water, 10 µL of cloned Pfu buffer, 4 µL of 10 m*M* dNTP mix, 2 µL of a pair of 50 µ*M* primers as shown in **Table 1**, and 1 µL of Pfu polymerase. Overlay the PCR reaction with mineral oil and cycle as follows:

   1 cycle: 5 min at 94°C, 5 min at 50°C, 5 min at 72°C.
   35 cycles: 30 s at 94°C, 30 s at 50°C, 1 min at 72°C.
   1 cycle: 7 min at 72°C.
4. Extract the PCR product with chloroform, precipitate with 0.1 volume of 3 *M* sodium acetate and 2 volumes of ethanol, spin, wash in 70% (v/v) ethanol, and resuspended in 10 µL water. Digest with restriction enzymes as shown in **Table 1**, using the manufacturer's recommended conditions.
5. Purify the digested PCR product on a gel and ligate it into the pId vector; cut the vector with matching enzymes to allow directional ligation of the V region.
6. Transform the ligated DNA into bacteria by conventional methods. Screen colonies by restriction digests of miniprep DNA to identify those that contain a correct insert. Verify the identity by DNA sequencing before proceeding with a given clone.

7. Repeat the restriction digestion, ligation, and transformation to allow cloning of the remaining (heavy or light chain) V region. When cloning new V regions into the plasmid, note whether the heavy or light chain V regions contain either of the restriction sites used to clone the opposite chain. This will determine the order in which the V regions must be cloned into the plasmid.

3.1.2. Cloning of Cytokine Genes

1. For cloning most cytokine genes, harvest C3H.HeN mouse spleen cells as described below. Resuspend the lymphocytes at 5×10^6 cells per mL in cRPMI medium and add 5 μg/mL Concanavalin A. Incubate the cells for 24 h at 37°C.
2. Extract RNA by centrifuging the cells and then resuspending them in 1 mL of RNAzol B reagent. Carry out RNA purification, cDNA synthesis, and PCR as described above for cloning of V regions.
3. Once a clone containing the proper insert is obtained, grow the plasmid in large-scale culture, and purify by either cesium chloride banding or by use of a MegaPrep column (*see* **Notes 1** and **2**).

3.1.3. Testing for Cytokine Bioactivity

Before using a new construct for DNA vaccination, it is prudent to test it for expression and cytokine bioactivity.

1. Transfect COS-7 cells.
2. Two to three days after transfection, harvest the supernatant.
3. Test for protein by immunoprecipitation, Western blotting, and ELISA.
4. Check the bioactivity of the cytokine by using an indicator cell assay (*see* **ref. *32*** for cell lines and procedure) (*see* **Note 3**).

3.2. Vaccination

3.2.1. Tumor Challenge Experiments

1. Vaccinate mice intramuscularly, with 100 μg of DNA in 100 μL of 0.9% (w/v) saline given bilaterally, 50 μL into each quadriceps muscle, 2–3 times at 1–3 wk intervals, then challenged with tumor cells 2–3 wk after the last vaccination.
2. Draw blood approximately 18 d after each vaccination, and assay the serum for specific antibodies by ELISA (*see* **Subheading 3.3.2.1.**).
3. About two wk after the last vaccination, wash 38C13 cells in log phase growth three times and resuspend them in RPMI-1640 medium without serum.
4. Serially dilute the cells to 400 cells per mL and inject 0.5 mL (200 cells) i.p. or s.c. per mouse (*see* **Notes 4–6**).

3.2.2. Use of Chemotherapy and Vaccination

A more rigorous test of vaccine efficacy is the ability to induce an anti-tumor immune response in animals with already established tumors. However, this is difficult because of the fast growth rate of experimental tumors such as 38C13. To more closely mimic the human clinical situation, as well as contain tumor growth, we have performed chemotherapy and vaccination experiments.

1. Inject 200 38C13 cells s.c.
2. Vaccinate with DNA as described, beginning several hours to days post tumor challenge.
3. When palpable tumors are evident on all mice (usually about 10–12 d), inject the animals i.p. with a non-curative dose of 100 mg/kg cyclophosphamide (Sigma).
4. Record mouse survival as above.

3.3. Readouts

3.3.1. Direct Readouts of Tumor

The endpoint of all tumor challenge experiments is survival.

1. Record the date of death of each animal.
2. Plot survival on a Kaplan-Meier plot (**Fig. 2**).
3. Calculate the statistical significance of differences between groups by the Gehan or log rank algorithm ***(48)*** which takes into account both prolongation of survival and the appearance of long-term survivors (*see* **Notes 7** and **8**).

3.3.2. Readouts of Immune Response

3.3.2.1. Antibody Response: Serum ELISA (*see* **Notes 9** and **10**)

1. Bleed mice from the tail vein into Microtainer serum separator tubes. Spin the tubes for 30 s in a microfuge to separate serum from blood cells, and freeze the sera for use in later assays.
2. Coat microtiter plates with 38C13 or a similarly purified control IgM at 2.5 µg/mL in PBS for 24 h at 4°C. Store coated plates wrapped in parafilm at 4°C for up to several months.
3. Wash the coated plates three times by flicking out the contents of the wells over a sink, and immerse the plates in a bucket of ELISA wash buffer (0.9% (w/v) saline with 0.5% (v/v) Triton-X100).
4. Saturate the washed plates with 100 µL per well of 5% (w/v) nonfat milk in PBS and incubate at room temperature for 30 min, and flick out nonfat milk/PBS without washing.
5. Prepare serial dilutions of the sera from vaccinated mice in 2% (w/v) bovine serum albumin. (BSA) in PBS, starting at 1:20–1:200 dilution, with serial 1:2 dilutions in a final volume of 50 µL, over 8–16 wells. Include a standard anti-38C13 antibody cocktail containing IgG1, IgG2a, and IgG2b isotypes in a 2:1:1 ratio over 8 wells on each assay plate, starting at 1 µg/mL. Incubate the plates an additional 1 h at room temperature.
6. Wash the plates as in step 3, then add 50 µL per well of anti-mouse IgG (gamma chain specific) at a 1:3000 dilution in 2% (w/v) BSA in PBS. Incubate the plates for 1 h at room temperature. Note that IgM antibodies are not detected by this assay, since an IgM-binding detector would cross-react with the 38C13 IgM used to coat the ELISA plate.
7. Wash the plates as in step 3, then add 100 µL per well of a freshly made substrate solution (100 µL of 15 mg/mL ABTS (Sigma), 3.3 µL of 30% (v/v) hydrogen

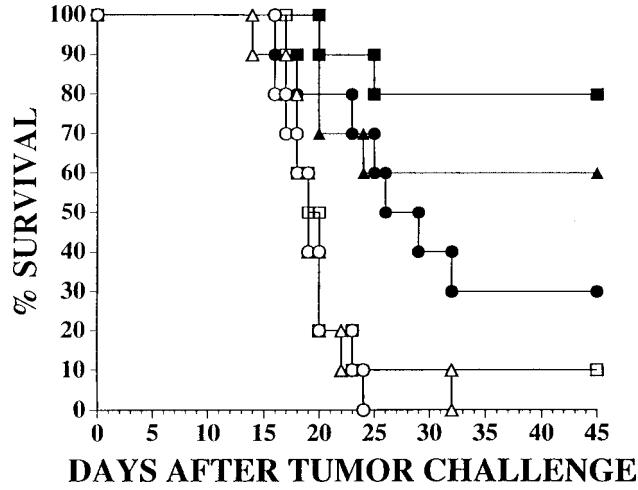

Fig. 2. Representative Kaplan-Meier survival plot comparing protein and DNA vaccination with idiotype or idiotype-GM-CSF fusion constructs. Reprinted from ***(12)***.

peroxide, and 10 mL of 0.1 *M* citrate buffer, pH 4.0). Observe the plates for color development, and read them on a microplate reader at a test wavelength of 405 nm and a reference wavelength of 490 nm. Maximum color usually develops after 15–30 min incubation at room temperature. Use SOFTmax software (Molecular Devices) to calculate antibody responses in each animal based upon the standard curve.

3.3.2.2. T Cell Proliferation Assays

1. Harvest the spleens of representative vaccinated mice from each group 2 wk after the last immunization for cellular assays of immune response. Remove spleens using sterile technique and place them in a tube containing 5–6 mL of cRPMI medium.
2. Prepare a single cell suspension by gently squeezing the spleen in a frosted glass tissue homogenizer.
3. Allow debris to settle for 1–2 min, remove the supernatant containing the spleen cells and pipet it into a fresh polypropylene 15 mL conical tube.
4. Underlay 3–5 mL of Lympholyte *M* into each tube with a 5 mL pipet, thereby creating a step gradient. Spin the tubes at 400*g* for 20 min, and collect the lymphocytes from the interface with a sterile 5 mL pipet.
5. Wash the collected lymphocytes two times with RPMI-1640 medium without serum, then resuspend at 5×10^6 cells per mL in RPMI-1640 containing 1% (v/v) fresh normal mouse serum.
6. Add 100 µL of cells in each well of a microtiter plate, along with 100 µL per well of medium containing titered amounts of 38C13 Ig or a control Ig.

7. Use Ig doses of 100 μg/mL, 10 μg/mL, and 1 μg/mL (final concentrations) to establish a dose-response curve. Use a class-matched control Ig and purify it in a manner similar to that used for the test 38C13 Ig (*see* **Note 11**).
8. Incubate the plate at 37°C for 3 d and add 1 μCi/well of ^{3}H-thymidine in ~25 μL of medium to each well on d 3.
9. Approximately 16–20 h later, harvest the wells onto filters using a cell harvester and determine the proliferation by quantifying [^{3}H]-thymidine incorporation into DNA. Dry the filters and measure the bound radioactivity in a scintillation counter. Calculate specific proliferation as cpm of 38C13-stimulated wells with reference to cpm of irrelevant Ig-stimulated wells.

3.3.2.3. CTL Assays

1. Harvest spleen cells from immunized mice as for T-cell proliferation assays (steps 1–4 above).
2. Wash the cells twice after recovering them from Lympholyte M gradients, then resuspend them at 5×10^6 cells per mL in cRPMI containing 10–15 U/mL recombinant human IL-2. Add up to 8 mL per well of a 6-well plate.
3. Harvest EG.7-OVA cells and irradiate them (6000 rad) in a cesium irradiator. Resuspend the cells at 5×10^6 cells per mL, and add one-tenth volume to each spleen cell culture (10:1 responder:stimulator cell ratio). Incubate the cultures at 37°C for 5–6 d, adding fresh medium to the wells as needed (usually every 2 d).
4. On the day of assay, recover 10^6 cells of both EG.7-OVA and EL-4 cells by centrifugation. Resuspend them in approximately 100 μL residual volume of medium, and add 200 μCi [^{51}Cr]-sodium chromate. Incubate the cells at 37°C for 1–2 h, shaking them occasionally.
5. While the target cells are incubating with ^{51}Cr, transfer the stimulated spleen cells to 15 mL tubes by gentle pipetting, leaving adherent cells behind. Count the harvested cells and resuspend them at 5×10^6 cells per mL in cRPMI medium. Prepare two serial 1:5 dilutions to allow effector:target ratios of 50:1, 10:1, and 2:1. Add 100 μL of cells from each dilution in triplicate in each of two 96-well round-bottom microtiter plates (one for EL-4 and one for EG.7-OVA). Prepare triplicate wells for each plate containing 100 μL medium (for spontaneous lysis) and 100 μL 0.1% (v/v) Triton-X (for maximum lysis).
6. Wash the labeled target cells three times with cRPMI medium and resuspend them at 10^5 per mL. Add 100 μL of cells to each well of the microtiter plates containing the spleen cells. Spin the plates briefly and incubate them for 4 h at 37°C.
7. Following the 4 h incubation, spin the plates briefly to pellet cells, then remove 100 μL of supernatant from each well and place it in a tube for counting on a gamma counter. Use 2 mL tubes that are racked in a 96-well format for convenient transfer from plates to tubes. After counting each tube for 1–2 min, calculate the mean cpm of triplicate tubes. Calculate specific lysis for each sample as: [cpm (sample) – cpm (spontaneous lysis)]/[cpm(maximum lysis) – cpm (spontaneous lysis)].

4. Notes

1. DNA made from bacterial cultures contains endotoxin, which can act as an adjuvant for DNA immunization. Different methods of DNA preparation, as well as different batches made by the same method, can vary in their endotoxin content. Although endotoxin effects have not been detectable in our system, it would be prudent to test batches of DNA that are to be used for vaccination to ensure that their endotoxin content is low. We have found that cesium chloride banding generally yields low levels of endotoxin (<100 U/mg DNA). Alternately, low-endotoxin DNA purification columns (Qiagen Corp., Chatsworth, CA) may be used. It is recommended that a large batch of each DNA be prepared and frozen for repetitive use.
2. Test for endotoxin using a Limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). Follow the manufacturer's instructions. This assay relies upon the gelation activity of a lysate of amoebocytes from the Limulus crab. By making several dilutions of each DNA sample being tested, the highest dilution that still causes gelling can be found. Record the endotoxin concentration as a range between the calculated amount of DNA in this dilution and the amount in the next higher dilution. Run all samples in duplicate.
3. It is possible to quantify the amount of fusion protein in the supernatant by comparison with a standard in an ELISA assay. When the supernatant is used in an indicator cell assay alongside a standard of free recombinant cytokine, one can then calculate the bioactivity of the cytokine fusion protein on a molar basis.
4. Like other cell lines, 38C13 cells can vary from batch to batch with regard to their tumorigenicity in vivo. Prepare and titer frozen stocks prior to use in tumor challenge experiments. Avoid prolonged in vitro culture. We recommend freezing multiple aliquots of cells, then thawing a vial, growing the cells for 3 d, and testing for tumorigenicity in a titration experiment.
5. Tumorigenicity is also dependent upon the health and growth phase of the cell culture. Harvest cells during logarithmic growth (e.g., 3 d after thawing), then wash three times in cold RPMI-1640 medium without serum to avoid any carrier effect of fetal calf serum. Count and dilute the cells and keep them on ice prior to injection to avoid loss of viability.
6. To allow for statistically significant comparisons between groups, at least 10 mice per treatment are used for tumor challenge experiments. Mice vaccinated with saline or an irrelevant DNA construct should die between d 15 and 25 post-challenge.
7. The two statistical analyses commonly used for survival data are log-rank and Gehan scores ***(48)***. Both methods will yield *p* values for pairwise comparisons of survival curves. However, they may yield slightly different *p* values and thus different levels of significance for the same comparisons, due to differences in the algorithms. In general, the Gehan algorithm places greater significance on differences at the top of the survival curve, that is, when animals first start to die. Thus, two survival curves that are similar in the initial part of the curve may not be significantly different by Gehan score, even though the base of the two curves

may appear quite different. By contrast, the log-rank algorithm places equal weight on all parts of the survival curve, so that such groups may yield a more significant *p* value by log-rank than by Gehan score.

8. It is important to titer the tumor-cell doses so that control animals die within a reasonable period of time (15–25 d has proven optimal for 38C13). On the other hand, injecting too many tumor cells will make it difficult to show vaccine-specific effects, as the tumor may overwhelm the immune response. Experiments as described can be terminated 60 d post-tumor challenge, with no change in long-term survival.
9. With 38C13, as with other systems studied in our laboratory, the immune response as measured by serum antibodies can vary between individual mice, despite using an inbred strain such as C3H/HeN. It is therefore important to use large groups of mice (10 or more mice per group) to obtain meaningful results for comparison.
10. It is possible to compare the antibody titer and survival data from individual mice by ear-punching or toe-clipping the mice in each group. When this has been done, we have found that variability in antibody titers exists within mice that were all equally protected from tumor challenge. Mice that do not make detectable antibody are almost never protected; however, it is difficult to determine a precise threshold of antibody level required for tumor protection. The isotypes of the specific antibodies have been measured ***(42)***, but a clear correlation of isotype profile with protection cannot be established.
11. Contaminants that the two preparations may have in common can lead to non-specific proliferation, or high backgrounds, which may obscure a weak idiotype-specific proliferative response. Use normal mouse serum instead of fetal calf serum to avoid the high background reactivity observed with fetal calf serum.

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21

The Use of Conventional Immunologic Adjuvants in DNA Vaccine Preparations

Shin Sasaki and Kenji Okuda

1. Introduction

The term "adjuvant" originates from the Latin word *adjuvare*, which means to help or aid. An immunologic adjuvant is defined as any substance that acts to accelerate, prolong, or enhance antigen-specific immune responses when used in combination with specific vaccine antigens ***(1)***. In a broad sense, immunologic adjuvants include certain cytokines and other immunomodulatory molecules (i.e. chemokines and costimulatory factors) or their expression vectors. Since these adjuvants are described elsewhere in this book, here, we are concerned only with adjuvants that are derived from microorganisms and plants or are synthesized chemically.

Our group has attempted to boost the immune response to a DNA vaccine for HIV-1 using several immunologic adjuvants and results have been promising. The search for adjuvants for enhancing DNA-derived immunity has just started and knowledge on this topic is beginning to accumulate. In this chapter, we briefly characterize the immunologic adjuvants evaluated in our DNA vaccine study and summarize the findings of others. We also give an account of the preparation and usage of these adjuvant-DNA vaccines. This section does not aim at providing an all-inclusive review of the current status of adjuvants; for this readers can refer to various excellent review articles and books ***(1–4)***. There are a number of candidate adjuvants for DNA vaccines other than those referred to here.

1.1. A Compendium–Classification of Immunologic Adjuvants

The immunologic adjuvants include various chemical compounds with unique immunological or physicochemical properties. They exert their adjuvanticity through a plurality of mechanisms. In addition, many have over-

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

lapping mechanistic features together with unique physicochemical properties, and are therefore not easily classified. As an expedient measure, we have chosen here to follow the classification system of Vogel *(1)*. The efficacy of adjuvants in DNA vaccination does not always correlate with their usefulness in conventional polypeptide vaccination as **Table 1** shows. This is clearly because DNA vaccination involves a unique mode of antigen presentation distinct from that of peptide-based vaccination. We present the method for preparing our adjuvant-DNA combination using the adjuvants indicated in bold style in the Table.

1.2. Mechanism of Adjuvant Action in DNA Vaccination

The mechanisms responsible for eliciting specific responses to a DNA vaccine remain conjectural. As attempts to use immunologic adjuvants with DNA vaccines have just begun, it is impossible to present here the precise adjuvant mechanism operative in DNA vaccination. However, a few findings afford insight into this issue and we now know of three mechanisms by which adjuvants enhance DNA-derived immunity. These are: (i) recruitment of macrophages or other lymphoid cells, (ii) induction of cytokines which can amplify responses generated by immunogenic DNA, and (iii) facilitation of DNA entry into host cells.

Antigen-presenting cells (APC), such as macrophages or dendritic cells, have been shown to prime the cytolytic response to DNA-derived antigens *(5,6)*. Our adjuvant studies on DNA vaccines demonstrated that mononuclear cell recruitment in muscles injected with DNA and adjuvants was associated with substantial enhancement of DNA-derived immunity *(7–9)*. Interferon gamma and IL-2 synthesis by antigen-restimulated splenocytes was observed with a QS-21 adjuvant-DNA combination *(9)*, and enhanced IL-4 and IL-5 production was reported using cholera toxin adjuvant-DNA preparation *(10)*. Concurrent administration of cytokine expression plasmids and immunogenic DNA is reported to be effective for enhancing the response to an encoded antigen *(11–14)*. These findings suggest that APC recruitment at the DNA-injection site as well as cytokine induction, help to enhance antigen-specific immune responses. Carriers such as cationic liposomes are reported to be useful as adjuvants for DNA vaccination *(15,16)*. Their mechanistic utility is thought to arise from the incorporation of DNA into the liposomal vesicle, and the subsequent facilitation of DNA entry into host cells. Further studies are necessary to clarify the mechanism for adjuvant-mediated enhancement of the immunity induced by DNA vaccination.

2. Materials

2.1. Gel Type Adjuvants

1. Special equipment: spectrophotometer to measure DNA adsorption onto aluminum or calcium gels.

Table 1
Immunologic Adjuvant—Classification and Efficacy in DNA Vaccination

Category	Adjuvant name	Effectiveness in DNA vaccination	Reference
Gel-type adjuvant	**Aluminum hydroxide (alum)**	Injection prior to DNA is effective	Saski and Okuda, unpublished observation
	Calcium phosphate (CPA)	Injection prior to DNA is effective	Saski and Okuda, unpublished observation
Bacterial adjuvant	Monophosphoryl lipid A (MPL)	Effective in intramuscular and intranasal immunization	*8*
	Cholera toxin (CT)	Effective	*10*
	Muramyl peptides (e.g., MDP, MTP-PE)	Not evaluated	
Particulate adjuvant	Immunostimulatory complexes (ISCOMS)	Not evaluated	
	Cationic liposomes	Effective in intramuscular and intranasal immunization	*15,16,23*
	Mannan-coated liposomes	Effective in intramuscular and intranasal immunication	*18,19*
	Biodegradable polymers	Effective in oral and nasal immunization	*23*
Emulsifier-based adjuvant	Incomplete Freund's adjuvant (IFA)	Not evaluated	
	QS-21	Effective in intramuscular immunization and particle bombardment	*9,21*
Synthetic adjuvants	Nonionic block copolymers	Not evaluated	
	Ubenimex (UBX)	Effective in intramuscular immunization	*22*

The adjuvants indicated in bold are referred to in the text for preparing a combined adjuvant-DNA vaccine.

2. Aluminum hydroxide (alum) is available from Sigma (St. Louis, MO).
3. Calcium phosphate adjuvant (CPA) is available from Superfos Biosector a/s (Vedbaek, Denmark).
4. Sterile phosphate buffered saline (PBS), pH 7.4.

2.2. Liposome-Associated Adjuvants

2.2.1. DC-Chol:DOPE Liposome Adjuvant

1. DC-Chol:DOPE liposome, prepared as described previously ***(17)***.
2. Sterile PBS, pH 7.4.
3. Sterile 10 m*M* HEPES buffer, pH 7.8.

2.2.2. diC14-Amidine Adjuvant (Vectamidine™

1. N-t-butyl-N'-tetradecyl-3-tetradecylaminopropionamidine (diC14-amidine) is available from ULB-Interface (Brussels, Belgium).
2. Sterile PBS, pH 7.4.
3. Sterile 10 m*M* HEPES buffer, pH 7.8.

2.3. Mannan-Coated Cationic Lipids

1. {N-[2-(Cholesterylcarboxyamino)-ethyl]carbamoylmethyl}mannan (Chol-AECM-Mannan) is available from Wako Chemicals (Osaka, Japan).
2. DC-Chol:DOPE liposome adjuvant.
3. diC14-amidine (Vectamidine).

2.4. Ready-to-use Adjuvant Products

1. MPL-SE (Monophosphoryl lipid A in a stable emulsion) is available from Ribi ImmunoChem Research Inc. (Hamilton, MT).
2. MPL-AF (Monophosphoryl lipid A in an aqueous formulation) is available from Ribi ImmunoChem Research Inc. (Hamilton, MT).
3. QS-21 is available from Aquila Biopharmaceuticals (Worcester, MA).
4. Ubenimex is available from Nippon Kayaku Co. Ltd. (Tokyo, Japan).

3. Methods

Our standard inoculation procedures for intramuscular and intranasal delivery are described in detail in Chapter 18. We describe here only the methods for adjuvant-DNA vaccine preparations.

3.1. Gel Type Adjuvants (Aluminum and Calcium Gels)

This class of adjuvants are known to act in polypeptide-based vaccination by adsorbing antigen onto their surfaces. Although the extent of their activity in DNA vaccination has not been established, they consistently adsorb plasmid DNA. The method for preparing adjuvant-adsorbed plasmid DNA is presented here.

3.1.1. Adsorption of Plasmid DNA onto Mineral Gels

Experience has shown that approximately 80–90% of 10 to 100 μg of plasmid DNA can be adsorbed by over 30–300 μL of alum or CPA irrespective of the DNA-encoded antigen. We describe here the procedure for adsorbing 10 μg of plasmid DNA onto adjuvant gels.

1. Transfer 30 μL of alum or CPA to a 1.5 mL-microcentrifuge tube.
2. Add 10 μg of TE-dissolved DNA into the tube, then fill the tube with an appropriate volume of PBS to obtain the desired volume for inoculation.
3. Briefly touch the tube to a vortex mixer to ensure a homogenous gel suspension.
4. Incubate the mixture at 4°C for 30 min. Time-dependent adsorption kinetic analysis has shown that the optimum incubation time is 30 min or 24 h.

3.1.2. Measurement of DNA Adsorbed to the Gels

1. After incubation, centrifuge the gel at 10,000 rpm (1000*g*) for 3 min to obtain a gel-free supernatant (*see* **Note 1**).
2. Measure the absorbance of the supernatant by spectrophotometry at 260 nm.
3. The percent adsorption efficiency is calculated as follows:
   [1 – (10–DNA μg in the supernatant)/10] × 100.
4. Before inoculation, resuspend the precipitated gels by briefly touching to the vortex mixer.

3.2. Cationic Liposome Species

The liposome/DNA ratio employed in synthesizing liposome-DNA complex should be such that the effect of the adjuvant is optimized. Our experience has shown that the appropriate liposome/DNA ratio for enhancing a DNA-derived immune response is 4:1 to 6:1.

3.2.1. Synthesis of the DC-Chol:DOPE Liposome-DNA Complex

1. Transfer the required amount of TE-dissolved DNA for immunization into a 1.5 mL-microcentrifuge tube.
2. Add an appropriate amount of DC-Chol:DOPE liposomes in 10 m*M* HEPES buffer (pH 7.8) into the tube. The recommended liposome dose is 4–6 times the DNA dose. Mix the solution gently by pipetting several times (*see* **Note 2**), then fill the tube with an appropriate volume of PBS to obtain the desired liposome-DNA complex concentration.
3. Stand the tube at room temperature for at least 1 h for complex formation, overnight incubation may be necessary. This preparation is now ready for inoculation.

3.2.2. Synthesis of the diC14-Amidine-DNA Complex

1. The diC14-amidine is in crystalline form and should be hydrated with 10 m*M* of the HEPES buffer in the kit according to the manufacturer's instruction.
2. Transfer the amount of TE-dissolved DNA that is required for immunization to a 1.5 mL-microcentrifuge tube.

3. Add an appropriate amount of diC14-amidine in 10 m*M* HEPES buffer (pH 7.3) to the tube. The recommended liposome dose is 4–6 times the DNA dose. Mix the solution gently by pipetting several times (*see* **Note 2**), then fill the tube with an appropriate volume of PBS to yield the desired liposome-DNA complex concentration.
4. Stand the tube on ice for 15 to 30 min to allow for complex formation. This preparation is now ready for inoculation.

3.3. Mannan-Coated Cationic Lipids

Although we have used Chol-AECM-Mannan to modify the adjuvant effect of DC-Chol:DOPE and diC14-amidine liposomes ***(18,19)***, other neoglycolipids are thought to have substantial immunomodulatory effects as described elsewhere ***(20)***. The procedure for coating mannan onto liposomes is the same, irrespective of type of liposome used. The term liposome in this section is applied to both DC-Chol:DOPE and diC14-amidine liposomes.

3.3.1. Coating Mannan onto Liposomes

1. Dissolve Chol-AECM-Mannan with PBS to a concentration of 1 mg/mL, then transfer 25 µL into a 1.5 mL-microcentrifuge tube.
2. Add 75 µL of liposomes in HEPES buffer (1 mg/mL), and mix the solution gently by pipetting (*see* **Note 2**).
3. Add 400 µL of PBS to the tube and mix it by inversion, then stand the tube at 4°C overnight to allow the mannan to attach to the liposomes. The use of a rolling mixer for this incubation is recommended.
4. Centrifuge the tube at 3,000 rpm for 5 min and discard 250 µL of the supernatant.
5. Resuspend the pellet by pipetting. This preparation can be used as the stock solution of mannan-coated liposomes.

3.3.2. Synthesis of the Mannan-Coated Liposome-DNA Complex

1. Transfer the amount of TE-dissolved DNA required for immunization to a 1.5 mL-microcentrifuge tube.
2. Add an appropriate amount of the mannan-coated liposome preparation to the tube. The recommended liposome dose is 4–6 times the DNA dose. Mix the solution gently by pipetting several times, then fill the tube with an appropriate volume of PBS to yield the desired mannan-coated liposome-DNA complex concentration.
3. The incubation time and temperature required for complex formation are the same as those described in **Subheadings 3.2.1.** and **3.2.2.**

3.4. Monophosphoryl Lipid A (MPL)

We have used two preparations of MPL: MPL-SE and MPL-AF. Both preparations have exhibited similar adjuvant activity in our murine DNA vaccination system ***(8)***. MPL adjuvant-DNA vaccine can be prepared simply by mixing

the adjuvant solution with immunogenic DNA dissolved in TE. No incubation and emulsification are necessary. The optimum MPL dose for augmenting DNA-derived immunity is 50 mg per mouse in our experience.

3.5. QS-21

QS-21 adjuvant-DNA vaccine can also be prepared simply by mixing the adjuvant solution with immunogenic DNA without incubation and emulsification. However, we have found that there are different optimum QS-21 doses for antigen-specific cytolytic activity and antibody production; a 5 μg and a 25 μg QS-21 dose are recommended for the cytolytic and the antibody response, respectively. To enhance both the antigen-specific cytolysis and humoral immunity, 10 μg QS-21 per mouse is recommended ***(9)***. Use of QS-21 as an adjuvant for DNA vaccination by particle bombardment is also reported ***(21)***. Although some peptide-based vaccination studies have shown that alum-adsorbed antigen can be formulated with QS-21, our experience suggests that concurrent administration of alum-adsorbed DNA mixed with QS-21 does not augment an antigen-specific immune response.

3.6. Ubenimex (UBX)

UBX as supplied by the manufacturer is a powder that is difficult to dissolve in an aqueous solution. Using ultrasonication and heating, a maximum 4.5 mg of UBX can be dissolved with 1 mL of water or PBS. The UBX solution can be filter-sterilized through a 0.2 micron filter and can be frozen and thawed without denaturation. In our study, 100–500 μg of UBX per mouse had an adjuvant effect on antigen-specific humoral and cell-mediated immune responses ***(22)***. Incubation and emulsification are not necessary, and UBX adjuvant-DNA vaccine can be prepared simply by mixing the immunogen and UBX solution.

4. Notes

1. Aluminum or calcium gels that have precipitated naturally by gravity can be easily resuspended with a vortex mixer. However, a tight gel pellet formed by centrifugation is harder to resuspend. We therefore used only part of the solution to measure A260.
2. Gentle mixing of the DNA-liposome(-mannan) mixture is important for consistent synthesis of the complex. Rough mixing with the vortex mixer seems to reduce the effect of the adjuvant in our experience.

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22

Genetic Adjuvants

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1. Introduction

In 1992, the era of DNA vaccines began with the report of antibody production upon intradermal injection of mice with a plasmid vector expressing a foreign antigen *(1)*. A rapid succession of subsequent manuscripts showed stimulation of immune responses, including cytolytic T cells, upon inoculation of expression-vectors specific for antigens derived from viruses, bacteria, protozoa and tumor-associated antigens *(2–7)*. Plasmid DNA can be applied through various routes of injection including: intradermal, intramuscular, subcutaneous, intravenous, or directly on mucosal membranes *(1,2,8,9)*. The most commonly used methods of inoculation involve the use of DNA-coated gold beads propelled into the skin by a gene gun or intramuscular inoculation of the vector in saline solution.

Both T and B cell-mediated immunity can be elicited to different forms of antigen, including secreted and cell surface antigens, as well as antigens with nuclear targeting domains *(2,9)*. Most manuscripts report successful immunization with DNA vaccines showing potent, long-lasting immunity and, when the appropriate animal models are available, protection to subsequent challenge *(2,4,6–8)*. Nevertheless, in some models including our own, which uses DNA vaccines expressing rabies virus antigens, immunization with vector DNA resulted in an immune response that was by no means as potent as the one seen upon immunization with traditional inactivated or recombinant viral vaccines *(10)*. We therefore investigated avenues to enhance the immune response by using genetic adjuvants, i.e., vectors expressing cytokines *(11,12)* or chemokines.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

1.1. A Rabies Virus Model

Rabies virus infects and causes a potentially fatal disease in all warm-blooded mammals including mice, the host of our experiments. The rabies virus strain used in our challenge studies, i.e., CVS-24, is mouse-adapted by serial passages through mouse brain; it is therefore highly virulent for mice, i.e., a small number of infectious particles can induce an encephalitic fatal disease. Antibodies mediate vaccine-induced protection to rabies virus and cytolytic T cells do not play a role; $CD4^+$ T cells are only needed to promote B cell responses ***(13)***. Only low levels of antibodies are needed to neutralize the small dose of challenge virus, thus making this system highly amenable for the demonstration of protection even when using comparatively weak vaccines including anti-idiotypic antibodies ***(14)*** or peptide vaccines ***(15)***.

2. Materials

2.1. Mice

1. Obtain inbred mice from Jackson Laboratories (Bar Harbor, ME). Immunize them at 6–12 wk of age.
2. Purchase Lewis rats from Harlan Sprague Dawley Inc. (Indianapolis, IN). Use at 6–8 wk of age.

2.2. Bacteria

1. Grow plasmid vectors in the *E. coli* strain DH5α.
2. Purchase competent bacteria from Gibco-BRL (Gaithersburg, MD).
3. Propagate transformed bacteria in LB medium supplemented with antibiotics (*see* **Note 1**).

2.3. Vectors

1. Purchase the pcDNA3 vector from Invitrogen (San Diego, CA).
2. Purchase the pSG5 vector from Stratagene (LaJolla, CA).
3. Obtain the vectors containing the IL-2 and IL-4 genes from the ATCC (American Tissue Culture Collection, Rockville, MD).

2.4. Cells

1. Grow baby hamster kidney (BHK)-21 cells, WEHI cells, L929 mouse fibroblasts and murine hybridoma cell lines 11-B-11 (secreting anti-mouse IL-4) and S4B6 (secreting anti-mouse IL-2) in Dulbecco's modified Eagle's medium (DMEM), high glucose version, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS).
2. Generate antibody-containing ascitic fluid in pristine-primed SCID mice by inoculation of 5×10^6 hybridoma cells.
3. Grow the IL-2- and IL-4-sensitive HT-2 cell line in DMEM supplemented with 10% (v/v) FBS and 10% (v/v) rat Concanavalin A supernatant (RCAS).

4. Maintain the IL-4-dependent CT4S cell line (originally obtained from Dr. W. Paul, NIH, Bethesda, MD) in DMEM supplemented with 10% (v/v) FBS and 10 units of recombinant mouse IL-4 (Becton Dickinson Labware Collaborative, Bedford, MA).
5. Grow the IL-3 and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) dependent 32DC13G/GM cell line ***(16)*** in DMEM supplemented with 10% (v/v) FBS and 10% (v/v) WEHI supernatant (*see* **Note 2**).

2.5. Cytokines

1. Generate WEHI supernatant, a source of mouse IL-3 and GM-CSF, by culturing WEHI cells at 5×10^5 cell/mL for 48 h in DMEM supplemented with 10% (v/v) FBS. Collect cell-free supernatant, titrate on the 32DC13G/GM cell line, and then aliquot and store at –20°C (*see* **Note 3**).
2. Generate RCAS, a source of IL-2, by culturing rat splenocytes at 1.25×10^6 cells/mL for 48 h in 2.5 µg/mL of Concanavalin A (Pharmacia Fine Chemicals, Piscataway, NJ) (17). Harvest cell-free supernatants, adsorb twice to Sephadex G-25 beads (Pharmacia Fine Chemicals) at 0.2 g/100 mL of supernatant to remove ConA, titrate using HT-2 cells, and then aliquot and store at –20°C. Sterile-filter the batch before each use and supplement with a-methyl mannoside to inactivate any residual ConA.
3. Purchase recombinant human GM-CSF and IL-4 from Genzyme Diagnostics (Cambridge, MA).
4. Purchase recombinant mouse GM-CSF and IL-4 from Sigma Chemical Co. (St. Louis, MO) (*see* **Note 4**).

3. Methods

3.1. Construction of Expression Vectors

1. Clone cytokine genes into the multicloning site of an expression vector such as pSG5 or pcDNA3.
2. Excise the cDNA of the cytokines from the original plasmid by use of appropriate restriction enzymes. Select the restriction enzymes according to the following criteria: enzymes that do not cut within the coding region, enzymes that cut upstream of the ATG start codon and downstream of the stop codon, and, if available, enzymes that allow unidirectional cloning into the alternate vector backbone.
3. Purify the excised DNA fragments by gel electrophoresis using a 1% (w/v) agarose gel supplemented with ethidium bromide. Visualize the DNA band of the appropriate size by longwave UV light and cut it from the gel. Isolate the DNA fragment using the Gene Clean Kit (Bio 101, Vista, CA) according to the manufacturer's protocol.
4. Cut the expression plasmid within the multicloning site downstream of the promoter using, when possible, the same enzyme(s) used for excision of the insert.
5. After dephosphorylation and purification by the Gene Clean Kit, ligate the backbone and insert, using direct ligation or blunt end ligation. For blunt end ligation,

fill in the overhangs of both insert and fragment using the large fragment of Klenow enzyme prior to dephosphorylation of the backbone.
6. Carry out the ligations overnight using T4 ligase enzyme at 14°C ***(18)***.
7. Transfect the ligation mixture into competent DH5α cells and plate onto agarose plates supplemented with the appropriate antibiotics. Isolate colonies the next day and amplify them in 2 mL liquid cultures (LB medium + antibiotic) (*see* **Note 5**).

3.1.1. Construction of a Vector Expressing a Chemokine Whose Sequence Was Recently Published ***(19)***

1. Isolate human peripheral blood mononuclear cells by purification of heparinized whole human blood over a Ficoll-Isopaque gradient.
2. Wash the cells and then suspend them in RPMI 1640 medium supplemented with 10% (v/v) FBS in Petri dishes.
3. Remove non-adherent cells 1 h later. Incubate the adherent cells for 3–7 d with RPMI 1640 medium supplemented with recombinant human GM-CSF (50 ng/mL) and recombinant human IL-4 (1000 units/mL).
4. Harvest the cells and isolate total RNA using Ultraspec™ RNA (Biotecx Laboratories, Houston, TX). Reverse transcribe and amplify the resulting cDNA using oligonucleotide primers delineated from the 5' and 3' non-coding regions of the published sequence in a 40-cycle polymerase chain reaction.
5. Sequence the PCR product, which should appear as a single band of the expected size upon gel electrophoreses, in an automated DNA sequencer to ensure faithful amplification. Clone the insert into the pcR2.1 vector (Stratagene Cloning Systems) (*see* **Note 6**).

3.2. In Vitro Biological Characterization of Vectors

3.2.1. Transient Transfections

1. Transiently transfect BHK-21 cells to ensure that vectors express functionally active cytokines. Generate stable transfectants to further quantitate expression.
2. Plate 5×10^5 trypsinized BHK-21 cells in 3 mL of DMEM supplemented with 10% (v/v) FBS in tissue-culture grade culture dishes.
3. The next day, precipitate 1 μg of plasmid DNA (quantitated by agarose gel electrophoresis and optical density reading against a known standard) with Lipofectin (Gibco-BRL) with some modifications of the manufacturer's protocol.
4. Dilute 10 μL of Lipofectin in 100 μL of serum-free DMEM and mix with vector DNA also diluted to 100 μL in DMEM. Use as a control a vector expressing an unrelated sequence.
5. Precipitate DNA for 30–60 min at room temperature. During this time, wash the adherent BHK-21 cells 4–5 times with 5 mL of serum-free DMEM.
6. Add an additional 0.8 mL of serum-free medium to the precipitated DNA and then add this mixture to the BHK-21 cells for 12–16 h at 37°C.
7. Remove the supernatant and resuspend the cells in DMEM supplemented with 10% (v/v) FBS. Harvest the supernatants 48 h later and test for cytokines.

3.2.2. Stable Transfections

1. Incubate 1×10^5 BHK-21 cells with a mixture of 1 μg of cytokine expressing vector and 0.2 μg of pSV2neo ***(20)***. Co-precipitate the two vectors with Lipofectin as described above.
2. Add G418 (1 mg/mL) to the cultures two days after the transfection. Replace medium containing G418 in 4–5 d intervals until colonies become visible.
3. Remove the medium once the colonies reach a size of >1 mm^2. Wash the plates once in serum-free DMEM and add 2 mL of trypsin (0.25 % (w/v) trypsin) in Versene (BioWhittaker, Walkersville, MD). Harvest individual colonies and transfer them to 24-well plates (Costar, Thomas Scientific, Swedesboro, NJ).
4. Expand the cells in 1.6 mL of DMEM. Harvest supernatants once the cells have become confluent (*see* **Note 7**).

3.3. Cytokine Assays

1. Assay cytokines on appropriate indicator cell lines, i.e., GM-CSF on 32DC13G/GM cells, and IL-2 and IL-4 on HT-2 cells. For other cytokine vectors, use either indicator cells (IL-12), other biological assays (reduction of virus-induced plaque formation on L929 cell monolayers for IFN-γ) or sandwich ELISAs using specific MAbs (IL-5, IL-10).
2. To test for GM-CSF, dilute 2×10^3 32DC13G/GM cells in 75 μL of DMEM supplemented with 10% (v/v) FBS. Incubate with 75 μL of serial dilutions of cell-free supernatants from transfected cells in 96-well round bottom microtiter plate wells (Limbro, Thomas Scientific). Use serial dilutions of WEHI supernatant or recombinant purified mouse GM-CSF as a positive control; use supernatants from sham-transfectants as a negative control.
3. Pulse the cells 48 h later for 6 h with 0.5 μCi of [^{3}H]thymidine, harvest onto filtermats, and analyze in a ß-counter.
4. Consider as positive those supernatants that induce proliferation (i.e., [^{3}H] thymidine incorporation) three times above background (*see* **Note 8**).

3.4. Immunization of Mice

1. Use a GM-CSF expressing vector to assess the ability of cytokines to serve as genetic adjuvants in the initial experiments. Inoculate mice intramuscularly with a mixture of 50 μg of an antigen-expressing vector, such as the pSG5rab.gp vector, expressing the rabies virus glycoprotein, with various amounts of the GM-CSF expressing vector (10–250 μg) in a total volume of 100 μL of saline buffer. Alternatively, inject mice with the cytokine-expressing vector, then inject the pSG5rab.gp plasmid 24 or 48 h later into the same muscle. In some experiments, you can boost the mice either once or twice at 14 d intervals with an additional 50 μg of pSG5rab.gp.
2. Analyze 2–3 different mouse strains (e.g., C3H/He, C57Bl/6 and Balb/c) to determine the effect of a cytokine.
3. Test the cytokine vectors at three different doses (e.g., 10, 50 and 250 μg).
4. For some cytokines, test different vector constructs expressing the cytokine gene under the control of the SV40 promoter (IL-4, IL-2), the Rous sarcoma LTR (GM-CSF) or the CMV promoter (IL-2, IL-4 and GM-CSF) (*see* **Note 9**).

3.5. Analysis of the Immune Response

3.5.1. Antibody Titers to the Antigen Measured by an ELISA (21)

1. Bleed the mice at varied intervals and analyze serum antibody titers to the antigen such as rabies virus. To determine potential shifts in the kinetic of the antibody response, bleed the mice by retro-orbital puncture early in the time course, e.g., 2 wk after immunization, and then in monthly or bimonthly intervals for at least 4–6 mo.
2. Coat plates with antigen, such as ERA-BPL virus (i.e., purified inactivated rabies virus) and incubate with duplicate or triplicate samples of serial dilutions of pooled sera using sera of naive mice for comparison.
3. Use an alkaline phosphatase-conjugated goat anti-mouse Ig as a second antibody.
4. Test the subisotype profile of antibodies to the antigen (rabies virus) using the Calbiochem Hybridoma Subisotyping Kit (La Jolla, CA) with some modifications of the manufacturer's protocol as follows. Coat microtiter plate wells with antigen (ERA-BPL) virus overnight and then block with a 2% (w/v) BSA-PBS solution. After washing, add a 1:200 dilution of serum diluted in the buffer provided with the kit. Incubate the plates at room temperature for 1 h. Next, add 100 µL of each of the typing antisera to the different wells. Incubate plates for 1 h at room temperature. Add the peroxidase conjugate and incubate for 1 h at room temperature. Then add TMB substrate and stop color development after 5–10 min by the addition of 50 µL of 1 *M* HCL. Read plates at 450 nm (*see* **Note 10**).

3.5.2. T-Cell Cytokine Secretion in Response to Restimulation in Vitro

1. Assay single cell suspensions of lymphocytes, isolated from draining lymph nodes or spleens, either for cytokine release or cytolytic activity. The best results are obtained in mice tested 2–4 wk after the last immunization.
2. To test for cytokine release *(23)*, culture cells at 6×10^6 splenocytes or 2×10^6 lymph node lymphocytes in 1.6 mL DMEM, supplemented with 10^{-6} *M* 2-mercaptoethanol and 2% (v/v) FBS. Use 24-well Costar plates without antigen or with 5 µg/well of antigen, such as purified, inactivated rabies virus.
3. Harvest supernatants 24 h later and test for IL-2 and IL-4 on HT-2 cells.
4. To distinguish between the two cytokines, retest supernatants that score positive in the initial assay by addition of a 1:200 and 1:1000 dilution of ascitic fluid containing the 11-B-11 monoclonal antibody to IL-4 or the S4B6 antibody to IL-2. Alternatively, test supernatants on the IL-4-sensitive CT4S cell line using a proliferation assay. Test GM-CSF and IL-3 on the 32DC13G/GM cell line in a proliferation assay. Test IFN-γ for plaque reduction on equine encephalitis virus infected L929 cells (*see* **Note 11**).

3.5.3. Testing for Cytolytic T Cell Activity

1. Restimulate the same sets of splenocytes used for cytokine release with a live, non-cytopathic virus expressing the target antigen, such as with 0.1 pfu of an adenoviral recombinant expressing the rabies virus glycoprotein *(25)*.

2. Alternatively, harvest cells early after immunization and restimulate with 1 μg/mL of inactivated virus such as ERA-BPL virus. The inactivated preparation is not appropriately presented for activation of memory $CD8^+$ cells but it stimulates cytokine secretion for expansion of activated cytolytic effector T cells.
3. Harvest cells after 5 d and test at various effector:target cell ratios (100:1–12:1) on ^{51}Cr-labeled H-2 compatible cells expressing the same antigen (i.e., rabies virus). Prepare these cells by stable transfection with a plasmid vector or infect with a viral recombinant in 100 μL of DMEM supplemented with 10% (v/v) FBS in V-bottom microtiter plate wells.
4. Incubate control target cells in medium to determine spontaneous lysis, or add 1% (w/v) SDS to measure maximal release.
5. Harvest supernatants 6 hrs later and assay for ^{51}Cr ***(4)***.

3.6. Discussion of the Results

We have, to date, analyzed nine different cytokines for their ability to serve as genetic adjuvants for DNA vaccines. GM-CSF consistently gives the best results ***(11)***. This cytokine enhances the antibody and T-cell response to both the pSG5rab.gp and the pVR1012rab.np vector. The pVR1012 rabies vector expressed the rabies virus nucleoprotein under the control of the CMV promoter; Vical Inc. (San Diego, CA) kindly provided the vector backbone.The type of the immune response, as measured by antibody isotype mapping and cytokine secretion pattern, is not affected by GM-CSF.

We only obtain an increase in the immune response if the GM-CSF-expressing vector and the antigen-expressing vector are injected simultaneously as a mixture. In some experiments where the GM-CSF vector was injected prior to the antigen expressing vector we saw a dramatic reduction of the response. This might reflect a depletion of the muscle of professional antigen-presenting cells, migrating to lymphatic tissue upon activation by GM-CSF (and the CpG sequences present in the vector), however, this is speculation.

The most impressive results were obtained using either low doses of the antigen-expressing vector or a vector expressing a poorly immunogenic protein. We tested two different vectors expressing GM-CSF under the control of either the Rous Sarcoma virus LTR (pRJB-GM) or the CMV promoter (pcDNA3GM-CSF). Both vectors enhanced the early B- and T-cell immune response. The effect of GM-CSF as a genetic adjuvant was transient, while the immune response was only enhanced when tested early after immunization. Mice inoculated with the antigen-expressing vector alone had slightly better antibody titers after several months. The effect of GM-CSF was dose-dependent and the best results were obtained with 250 μg of DNA, but 50 μg also gave a clear increase.

IL-4 causes a shift in the kinetics of the immune response, at least in C3H/He mice. The response is reduced when tested early after immunization but,

after several months, a significant increase for antibodies, T-helper and cytolytic T cells is observed. IL-4 expressing vectors do not affect the phenotype of the T-helper response.

IFN-γ normally regulates the expression of class I and II MHC molecules as well as that of the co-stimulatory molecules, proteasomes, and transporters involved in antigen processing. It consistently reduced the B- and T-cell response to the DNA vaccine ***(12)***.

In summary, cytokine-secreting vectors can serve as genetic adjuvants for DNA vaccines. Due to the localized effects of vector-encoded cytokines at the site of antigen presentation and due to the relative ease of constructing such vectors, they provide an attractive alternative to systemic use of the often toxic, and always expensive recombinant cytokines. Nevertheless, extensive safety studies are needed to assess their potential usefulness as vaccine adjuvants in humans.

4. Notes

1. Stock bacteria are stored frozen at –80°C in LB supplemented with 30% (v/v) glycerol.
2. Most of these cell lines are maintained as frozen stocks in liquid nitrogen. The HT-2 cell line is difficult to recover from frozen aliquots; we have not yet been able to successfully recover the CT4S cell line upon freezing; this cell line has therefore been kept in continuous culture.
3. Batches in use can be kept for up to 4 mo at 4°C.
4. Lyophilized cytokines are supplemented according to the manufacturer's specifications, aliquoted, and stored at –20°C.
5. For small-scale amplification of bacteria (1–5 mL), plasmid DNA is purified using the Promega Wizard™ Minipreps DNA Purification System (Promega Corporation, Madison, WI) according to the manufacturer's specifications. The miniprep plasmid DNA is cut with the appropriate restriction enzymes to ensure the presence and appropriate orientation of the insert. Bacteria carrying the vector with the correct orientation of the cytokine cDNA are expanded into a 1:10 dilution and isolated using the Promega Wizard Maxipreps DNA Purification System.
6. From this point on, the further procedures to construct an expression vector are identical to those described above.
7. In general, 10–20 colonies are analyzed for each stable transfection. Cells that are shown to secrete cytokine are then plated at a defined cell number; after 48 h the supernatants are retested for better quantification of cytokine secretion.
8. Units are determined by comparison to a defined standard. IL-2 and IL-4 are tested on HT-2 cells using the same procedure.
9. In general, groups of five mice are used for antibody and T cell assays. In most experiments, control mice are immunized with "empty" vector to account for an effect of additional plasmid. Initially we used a lacZ-expressing construct that

gave consistently high background, possibly due to bacterial CpG sequences present in the insert.

10. We initially tested this kit by using an immunoglobulin control kit provided by Calbiochem Co. to show that it has comparable sensitivity for the different immunoglobulin isotypes ***(22)***. In addition, sera from pSG5rab.gp immune mice are tested by a neutralization assay ***(21)*** that generally gives results comparable to those of the ELISA.
11. We have on occasion used ELISPOT assays ***(24)*** to determine cytokine secretion patterns. The assay is too labor-intensive for routine use. Alternatively, sandwich ELISAs are available for cytokines; these tests are significantly more expensive than the relatively simple indicator cell assays outlined above. Nevertheless, for laboratories with limited tissue culture experience or a reluctance to subculture CT4S and HT-2 cells every 3–4 d, indicator cell assays might not be suitable.

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DNA Immunization in Combination with the Immunostimulant Monophosphoryl Lipid A

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1. Introduction

The use of the MPL® immunostimulant, a monophosphoryl lipid A preparation derived from the lipopolysaccharide (LPS) of *Salmonella minnesota* R595, began with the studies of Johnson et al. ***(1)***. It was shown that LPS was a potent adjuvant for protein antigens, even if administered at a different site and a different time than the antigen ***(2,3)***. Nonetheless, the toxicity of the LPS precluded its usefulness as a practical adjuvant. Studies by Ribi and co-workers ***(4–6)*** and others ***(7)*** resulted in the attenuation of the toxicity through exposure to mild acid hydrolytic conditions. The resulting acid hydrolysate was shown to be the 4'-monophosphoryl derivative of the lipid A moiety ***(8)***. Numerous biological studies confirmed that this 4'-monophosphoryl lipid A derivative was a potent immunostimulant which lacked many toxic properties of the parent LPS. Subsequent studies determined that mild alkaline treatment resulted in removal of one fatty acid from the MPL, resulting in additional attenuation of toxicity without changing the immunostimulating activity ***(9)***. These observations led to the development of the product MPL which is presently undergoing trials as an adjuvant for several human vaccines. The manufacture, chemical composition and structure of MPL has been detailed by Ulrich and Meyers ***(10)***. We will describe our techniques for using MPL as an immunostimulant in mice with the aim of enhancing the magnitude and duration of the protective neutralizing antibody response elicited by a DNA vaccine encoding the glycoprotein of the CVS rabies virus.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

2. Materials

2.1. Plasmid Construction

1. TA cloning vector PCRII is available from Invitrogen (San Diego, CA).
2. Geneclean II is available from Bio 101 (Vista, CA).
3. Plasmid pRABpreG is a construct from Dr. Joseph Esposito (Centers for Disease Control and Prevention, Atlanta, GA); plasmid pCMV4 is a construct from Dr. David Russell (University of Texas Southwestern Medical Center, Dallas, TX,); plasmid pcDNA3 is available from Invitrogen (*see* **Note 1**).

2.2. DNA Purification

1. Luria broth (LB): make 10 g Bacto tryptone (Difco, Detroit, Ml), 5 g Bacto yeast extract (Difco), 10 g NaCI and 1 g glucose, up to 1 L with distilled water, adjust pH to 7.2 and autoclave.
2. Terrific broth (TB): make 12 g Bacto tryptone and 24 g Bacto yeast extract up to 896 mL with distilled water, sterilize by autoclaving, add 100 mL of 0.17 *M* KH_2PO_4, 0.72 *M* K_2HPO_4 sterile solution and 4 mL sterile glycerol.
3. Make stock solution of ampicillin (e.g., 25 mg/mL), filter sterilize and store at –20°C. Dilute in media to appropriate concentration (e.g., 100 ug/mL).
4. Grow plasmids in high-efficiency competent cells. These should be recA (–) end (–) such as Max Efficiency DH5α cells available from Life Technologies (Grand, Island, NY).
5. Make Tris-EDTA (TE) buffer: 0.16g Tris-HCL (10 m*M*), 0.37 g EDTA (1 m*M*) in 100 mL distilled water, adjust pH to 8.0 with NaOH and autoclave.
6. DNA purification buffers and columns are available from Qiagen (Santa Clarita, CA) (*see* **Note 2**).

2.3. MPL

These products may be obtained from Dr. Terry Ulrich (Ribi Immunochemicals, Hamilton, MT).

2.4. Accell

1. The Accell-gene delivery system (genegun) is available from powderject Vaccines, Inc. (Madison, Wl).
2. Spermidine (free base) and polyvinal-pyrrolidone (PVP-360) are available from Sigma (St. Louis, MO).
3. Ethanol (dehydrated, punctilious) is available from Pharmco, Inc. (Brookfield, CT).
4. Gold beads, (0.95 μ or 2.6 μ) are available from Powderject Vaccines.
5. Masterflex tubing L/S (size 16) is available from Cole Palmer (Vernon Hills, IL).
6. Salve emulsions are available from Ribi Immunochemicals.
7. Intramedic Tygon tubing (PE-60, ID 0.76 mm, OD 1.22 mm) is available from Cole Palmer (Vernon Hills, IL).
8. Cyanoacrylate (super glue) is available from Chemence, Inc. (Alpharetta, GA).
9. Metofane (methoxyflurane) is available from Mallinckrodt Veterinary (Mundelein, IL).

10. 1 cc tuberculin syringes and hypodermic needles [$26^3/_8$ (9.5 mm) with intradermal bevel] are available from Becton Dickinson (Bedford, MA).
11. Small animal clippers, Model A-2, are available from Oster (Milwaukee, WI).

3. Methods

3.1. DNA Mega-Prep Plasmid Preparation

1. Pick one colony from a plate of freshly transformed high-efficiency competent cells (*see* **Note 3**) and inoculate a starter culture of 3 mL of LB or TB medium (*see* **Note 4**) containing an appropriate concentration of selective antibiotic (*see* **Note 5**). Incubate for 8 h at 37°C in a shaking incubator set at 200–300 rpm.
2. Dilute starter culture into 500 mL of selective LB or TB medium in a 1 L sterile culture flask and incubate culture at 37°C in a shaking incubator at 200-300 rpm overnight.
3. Pellet the cells at 6000 × g for 10 min and resuspend the pellet completely by pipetting up and down in 50 mL of resuspension buffer containing RNase A (P1-Qiagen).
4. Add 50 mL of lysis buffer (P2-Qiagen), mix by inverting 2–3 times and incubate at room temperature for 5 min (*see* **Note 6**).
5. Add 50 mL of pre-chilled (4°C) precipitation buffer (P3-Qiagen), mix by inverting 4–5 times and incubate on ice for 30 min (*see* **Note 7**).
6. Split the solution containing the precipitate into 50 mL conical Falcon tubes, and centrifuge at approximately 20,000*g* for 30 min at 4°C. Equilibrate a DNA binding column with 35 mL equilibration buffer (tip 2500 column, Qiagen, QBT buffer, Qiagen).
7. Filter supernatant fluid from centrifugation through sterile gauze to remove floating precipitate and apply to column by gravity flow.
8. Wash the column with several column vol of wash buffer (200 mL QC buffer-Qiagen) and elute DNA from the column with elution buffer (35 mL QF buffer-Qiagen). Precipitate DNA by adding 0.7 vol of isopropanol, mix by inversion and split the volume into 15 mL Corex tubes (no more than 12 mL/tube) and centrifuge at maximum rpm in a swinging bucket rotor for 30 min at 4°C (*see* **Note 8**).
9. Resuspend the pellets in 200–500 µL of TE buffer (depending upon the pellets), make appropriate dilutions in distilled water and quantitate by UV spectrophotometry at 260 nm.
10. Analyze purity of DNA by A_{260}/A_{280} ratio (*see* **Note 9**), enzyme digestion and agarose gel electrophoresis.
11. Store DNA at 4°C in TE buffer (*see* **Note 10**) and dilute as needed for vaccinations in sterile double-distilled water or sterile PBS (e.g., 1 mg/mL).

3.2. Intramuscular Vaccination

1. Fit 26 gauge $^3/_8$ in. (intradermal bevel) needles with Tygon tubing sleeves.
2. Shave the rear legs of Metofane anesthetized mice with animal clippers.

3. Use a tuberculin syringe and needle fitted with a tygon tubing sleeve (*see* **Note 11**) to inoculate each tibialis anterior muscle with 50 µg of MPL in 50 µL (stock MPL solution supplied by Ribi Immunochem) or with 50 µL of PBS without MPL (*see* **Note 12**).
4. Forty-eight hours later, inject 1–100 µg of DNA in 50 µL of PBS into the previously treated muscle. Booster vaccinations, if given, are administered using the identical procedure.

3.3. Intradermal Vaccination

1. Shave the area over the dorsal surface of each hindquarter of metofane anesthetized mice.
2. Combine 50 µg of MPL and varying concentrations (1–100 µg) of plasmid DNA per 50 µL of PBS and inoculate the shaved area ID (*see* **Note 13**). If booster vaccinations are given use an identical protocol.
3. Inoculate control mice similarly, but without the MPL.

3.4. Genegun Vaccination

1. Prepare 2.6 µm gold beads coated with 2 µg of DNA according to the specific instructions provided by Powderject Vaccines.
2. Shave the abdomen of Metofane anesthetized mice with animal clippers.
3. With a gloved finger, massage 40 µL of a salve emulsion containing 48 µg of MPL within micellar nano-particles into a 1.5 cm^2 area of the shaved abdomen. Use salve without the MPL to similarly treat control mice.
4. Immediately shoot the DNA-coated beads through the salve-treated skin into the epidermis using the Accell gene delivery system at a helium pressure setting of 400 psi. A helium pressure setting of 300 psi is used for mice 24 h to 10 d of age.

4. Notes

1. Plasmid Constructions: cDNA encoding the glycoprotein (G) gene of the CVS strain of rabies virus was amplified by polymerase chain reaction (PCR) with *Taq* DNA polymerase. The cDNA had previously been cloned into pBR322 resulting in the recombinant plasmid pRABpreG. The 28 base 5' primer used for amplification was designed to include a *Bam*HI site for cloning and, for improved expression, a Kozak sequence more closely matching the Kozak consensus sequence. The 22 base 3' primer included a pre-existing *Bam*HI site located 3' of the termination codon. Tenfold dilutions of a solution containing 10 ng of pRABpreG were amplified. One microliter of PCR amplified DNA encoding G was used to clone into the TA cloning vector PCRII. Resulting clones were sequenced by DNA double-strand dideoxy sequencing. One mutation-free clone was used for subsequent subcloning. The 1.6 kb fragment containing the entire coding sequence for G was excised from PCRII with *Bam*HI, purified with Geneclean II methodology, and subcloned into the *Bgl*11 site of pCMV4, or the *Bam*HI site of pcDNA3. Recombinant clones were sequenced and determined to be mutation-free. Both plasmids contained the G insert downstream from the

CMV major immediate-early promoter. All recombinant DNA was transformed into competent DH5α cells. Recombinant plasmid DNA was isolated from cultures grown in either LB or TB containing 100 μg/mL of ampicillin. Wizard minipreps and restriction enzyme analyses were done to screen potential clones. Recombinant DNA used for sequencing and vaccination was prepared using Qiagen Maxi and Mega kits.

2. Qiagen Plasmid Mega Kits, which contain all the necessary buffers, yield approximately 1–2 mg of highly pure DNA. Low copy number plasmids give lower yields. Endo-free kits are available from Qiagen to prepare endotoxin-free DNA.
3. Continuous passaging of transformed cells may result in lower yields with certain plasmids.
4. Some TB cultures produce too much protein, which can interfere with plasmid purification. In such cases use LB.
5. Antibiotic choice depends on selective resistance of the plasmid.
6. Solution should become clear and viscous, do not exceed 5 min.
7. A white flocculant precipitate is formed that contains genomic DNA, protein and SDS.
8. A clear to white pellet should be concentrated at the bottom of the Corex tube.
9. Pure plasmid DNA should have an A_{260}/A_{280} ratio of approximately 2.0.
10. Plasmid DNA is more stable in TE buffer since it contains EDTA, which chelates Mg^{2+} ions away from nucleases, inhibiting their activity.
11. The tubing is pulled away from the hub of the needle (2 mm), cut with scissors and then slipped back to the needle hub where it is firmly attached with super glue. The tubing below the unsheathed tip of the needle controls the depth of the IM inoculation to 2 mm. Tubing with a smaller ID will fit tightly to the needle, eliminating the need for super glue.
12. Animals must be anesthetized to a state in which there is total relaxation of the muscles. If the muscle flexes at the time of the injection, there is the possibility that some of the inoculum will be squeezed from the site.
13. Use 26 gauge $^3/_8$ in. needles with ID bevels that are not sheathed with Tygon tubing. It is very important that needles with ID bevels are used for this inoculation. Without the ID bevel it is very difficult to see the tip of the needle below the skin The needles are inserted through the skin and then upward toward the skin surface until the bevel is observed just below the dermis. Expulsion of the 50 μL at this position leaves a typical ID "bleb" in the skin. ID injections are more difficult and take more time than IM injections. Thus, each mouse must be anesthetized well enough to remain absolutely quiet for 30–45 s.

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Controlled Plasmid Delivery and Gene Expression

Applications for Nucleic Acid-Based Vaccines

Russell J. Mumper, Harry C. Ledebur, Jr., Alain P. Rolland, and Eric Tomlinson

1. Introduction

After the concept of genetic immunization was first demonstrated by Johnston's group in 1992 ***(1)***, numerous studies have reported the potential prophylactic and therapeutic use of nucleic acid-based vaccines for combating various infectious diseases ***(2–4)***. Vaccines of this composition appear to be both efficacious in the short term, and able to elicit a prolonged anamnestic response capable of preventing or resolving infection when challenged at up to one year after vaccination ***(5)***. Nucleic acid-based vaccines elicit a broader immune response than do subunit vaccines, inducing both cellular and humoral responses that are reminiscent of attenuated and whole-killed viral vaccines. Further, nucleic acid-based vaccines can be prepared with relative ease of synthesis and production. Expression plasmids can be generated quickly once the antigen's coding sequence is known and small- and large-scale purification methods are well established. Nucleic acid-based vaccines also avoid some of the safety concerns of conventional vaccines in that there is no chance of disease due to co-purification of contaminating virus or reversion of the attenuated strain in the patient. This is not to claim that the safety issues surrounding nucleic acid-based vaccines are minimal. The major theoretical concerns surrounding the safety of this technology include plasmid integration into the host genome, transformation of somatic or stem cells, and tolerability. However, there is no published evidence that administration of unformulated or 'naked' plasmid produces a severe short or long term deleterious effect ***(6)***.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

Even though there is a tremendous amount of expectation surrounding the use of nucleic acid-based vaccines for the prevention and treatment of infectious diseases, several aspects of the technology need to be significantly improved before such approaches will have widespread use. One area centers on the delivery of plasmid to control precisely its deposition in the body. The second area focuses on control of gene expression to limit the cell types capable of producing the gene product. With the heightened focus on the safety of nucleic acid-based vaccines by the FDA *(7)*, control of plasmid delivery and gene expression is paramount not only to enhance the overall efficacy of these vaccines but also to eliminate various safety concerns.

In **Fig. 1**, we have summarized the six key product requirements for a nucleic acid-based vaccine. Essentially five critical factors need to be considered to achieve these product requirements. This paper discusses how the critical factors of formulated nucleic acid-based vaccines can be controlled by using well-defined plasmid delivery and gene expression systems.

2. Mechanism of Action

Several different routes have been investigated for the administration of nucleic acid-based vaccines. These routes include subcutaneous, intravenous, intradermal, nasal, oral and intramuscular administration. Of these routes, the most widely used route for the administration of a nucleic acid-based vaccine in animal studies and human clinical trials is direct intramuscular administration of plasmid. Initially, it was hypothesized that transfected muscle cells expressing the antigen after intramuscular administration served as an antigen depot or factory, capable of initiating both the cellular and humoral responses to a particular antigen. Antigen released from transfected myocytes (by secretion or cell death) into the circulation or lymph nodes was supposedly taken up by macrophages or B cells and used to initiate a T-helper dependent antibody response. CTL priming was believed to occur via endogenous proteasome-dependent processing of the antigen and presentation of the antigen on the surface in association with MHC class I molecules. In accordance with this model, transplantation of transfected myoblasts into syngeneic mice led to the generation of both an antigen-specific antibody and a CTL response *(8)*. However, subsequent adoptive transfer studies in mice with mix-matched and matched haplotypes demonstrated that the cells actually presenting class I restricted antigens were derived solely from the bone marrow *(9–10)*. In similar studies, Doe et al. reported that bone marrow reconstitution could occur several days to weeks after vaccination, thus demonstrating that bone marrow derived antigen presenting cells (APCs) could present antigens synthesized at other sites in the body and not from antigen synthesized endogenously *(11)*. From these studies, it has been postulated that transfected myocytes serve as an

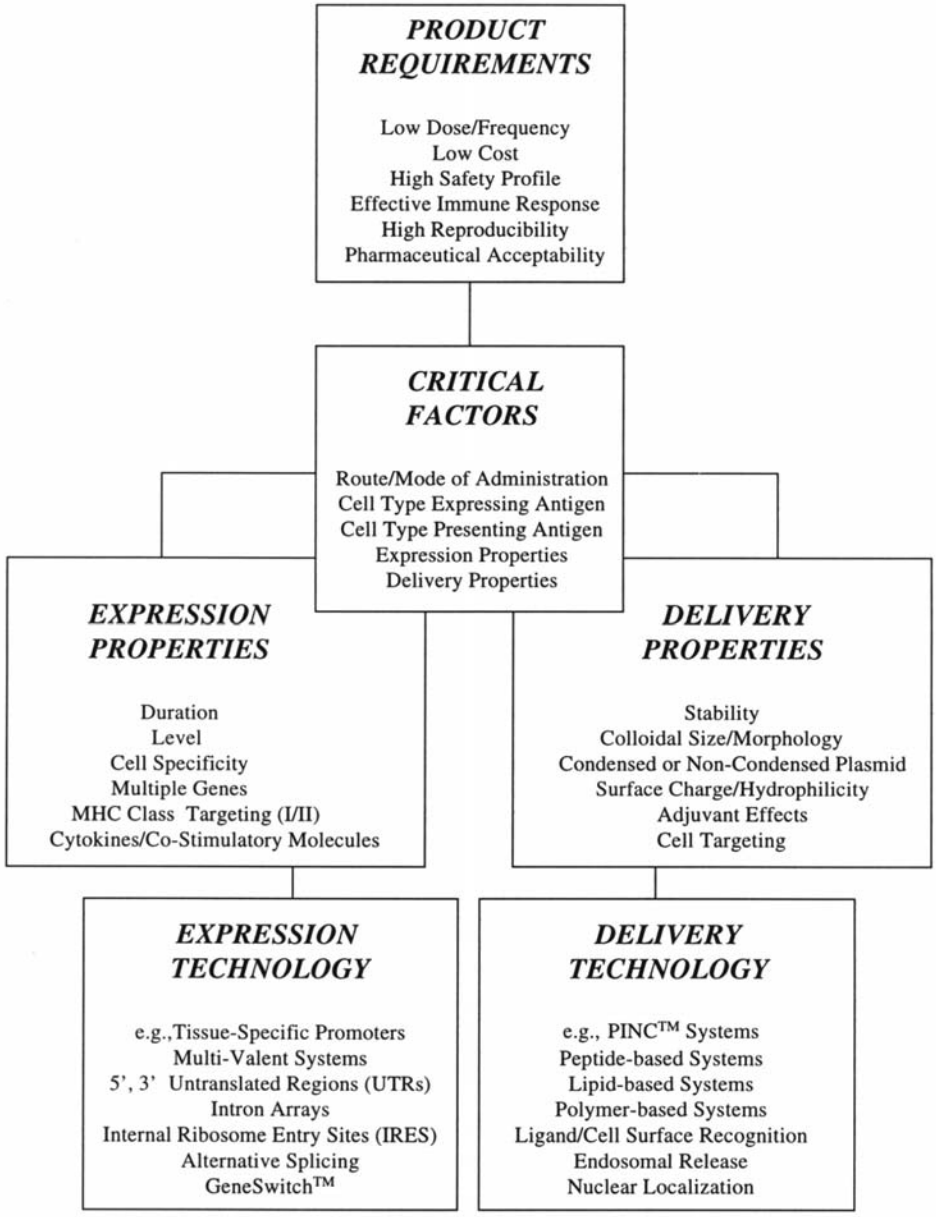

Fig. 1. Elements of a formulated nucleic acid-based vaccine.

endogenous factory producing antigen that is picked up and processed by the bone marrow derived APCs. Interestingly, Torres et al. have recently demon-

strated that antigen expression in the muscle is not required for the elicitation of an immune response to an intramuscularly administered nucleic acid-based vaccine ***(12)***. This experiment involved removing the injected muscle within minutes after administration and demonstrating that this had no effect on the immune response to the encoded antigen. If these studies accurately reflect what is happening in vivo, it is quite possible that there may be redundant pathways for the elicitation of an immune response after intramuscular administration of a nucleic acid-based vaccine. For example, gene expression in the muscle appears to be sufficient for the elicitation of the immune response, but is not required. Promiscuous deposition of plasmid ensures that other cells or tissues in the body are capable of producing the antigen and stimulating the immune response, even when the primary site of expression is removed.

One possible explanation for the results observed by Torres et al. is direct transfection of APCs after intramuscular administration of plasmid. Theoretically, APCs could express the antigen endogenously for presentation on their surface rather than relying on antigen produced by transfected cells in the periphery (i.e., muscle cells). This hypothesis is supported by studies using a gene gun to administer plasmid. After intradermal administration of plasmid with a gene gun, plasmid directly transfects the resident APCs of the skin (Langerhans cells), resulting in the subsequent migration of these cells to the draining lymph node, where antigen is expressed ***(13)***.

All current evidence suggests that direct transfection of APCs using the gene gun is responsible for the initiation of the antigen-specific immune response to the nucleic acid-based vaccine.

3. Approaches to the Control of Plasmid Delivery

A key issue for the development of effective nucleic acid-based vaccines is the identification of those tissues or cells where plasmid needs to be delivered in order to elicit the desired response. If plasmid could actively and specifically be targeted to critical sites rather than relying on the random pharmacokinetics of 'raked' plasmid or plasmid-laden gold particles, one could theoretically reduce the dose of plasmid required for a prophylactic or therapeutic immune response. These dose reductions could translate into a more cost-effective product. In addition to reducing the effective dose for nucleic acid-based vaccines, targeted delivery of plasmid would eliminate certain safety concerns about the distribution of plasmid after administration. Data suggest that plasmid can be detected in numerous tissues after various routes of administration. For example, plasmid DNA can enter the bloodstream or lymphatic system after intramuscular administration and traffic to the spleen, liver, kidney, draining lymph nodes, and bone marrow ***(14)***. The deposition of plasmid in the body can be controlled by use of targeted delivery systems. For

successful targeting of plasmid within the body, one must be able to effectively target plasmid to the appropriate cell type by controlling plasmid stability, colloidal size, and surface charge/morphology (*see* **Fig. 1**). This strategy may potentially alleviate safety concerns dealing with random distribution of plasmid in vivo.

Presently, it is unclear whether production of antigen in muscle has unique properties with respect to the elicitation of a prolonged immune response or whether expression in any tissue in the periphery is sufficient for the induction of an antigen-specific immune response. It is conceivable that one may circumvent the need for peripheral expression of antigen and directly target plasmid and subsequent gene expression to APCs as a means of inducing the desired immune response. Although the exact identity of which APC should be targeted has yet to be determined, in vitro and in vivo evidence suggests that the ideal cells to target are dendritic cells (DCs). DCs have been demonstrated to be potent initiators of immune responses and possess the co-stimulatory and adhesion molecules required for T cell activation ***(15)***. In addition, DCs possess a unique ability to process and present extracellular antigen in the context of both class I and class II molecules ***(16)***. If plasmid were to transfect these cells directly, it is likely that these cells would elicit both the cellular and humoral response observed (i.e., generation of a CTL response, antigen production for activation of B cells and the T cell help necessary for a humoral response). Systems that can efficiently target and deliver plasmid to these cells may provide the necessary technology leap to provide effective and low cost vaccines (*see* **Fig. 1**). In the next sections, the use of protective interactive non-condensing (PINC) systems, peptide-based systems, and lipid-based systems will be discussed as potential delivery systems to control the in vivo deposition of plasmid and potentially target plasmid to DCs (*see* **Table 1**).

3.1. PINC™ Systems

It is estimated that less than 0.01% of plasmid injected into muscle is taken up by muscle cells and expressed ***(14,17–20)***. The majority of injected "naked" plasmid appears to be rapidly degraded by extracellular nucleases and/or removed from the muscle via the lymphatic system. To increase the bioavailability of intact plasmid to muscle cells and protect it from nuclease digestion, protective, interactive, non-condensing (PINC; Gene Medicine Inc., The Woodlands, TX) polymers have been developed to interact with plasmids without condensation into compact particles ***(21–23)*** (*see* **Table 1**). Recently, a PINC polymer/plasmid complex formulation was approved by the FDA for a Phase I human clinical trial for direct intramuscular administration of a human insulin-like growth factor (hIGF-I) expression plasmid ***(24)***. Unlike condensed plasmid particles, these PINC complexes are able to diffuse throughout the

Table 1
Potential Approaches to the Control of Plasmid Delivery for Nucleic Acid Vaccines

PINCTM Systems
Non-condensing amphipathic polymers
Approved by the FDA in Phase I human clinical trials for non-viral gene therapy
Interact with plasmid to form polymer/plasmid complexes
Protect plasmid from rapid extracellular nuclease degradation
Designed for intramuscular, subcutaneous, and intradermal routes of vaccination
Increase both the levels and extent of gene expression in rodent muscle
Facilitate the uptake of plasmid into cells
Cell-specific targeting (using a ligand-PINC conjugate) may be possible using PINC
Peptide-Based Systems
Short cationic peptides synthesized by solid phase chemistr'
Highly pure and homogeneous; easy to characterize
Controlled colloidal and surface properties of peptide/plasmid complexes
Designed for multiple routes of vaccination
Lipophilic moieties have been attached to provide added colloidal stability
Improved intracellular delivery of plasmids has been achieved using endosomolytic peptides
Structural motifs that potentiate immune responses can be included
Cell-specific targeting has been achieved
Lipid-Based Systems
Amphiphilic cationic lipids/biodegradable cationic lipids
Approved by the FDA in Phase I human clinical trials for non-viral gene therapy
Controlled colloidal and surface properties of lipid/plasmid complexes
Designed for multiple routes of vaccination
Improved intracellular delivery of plasmids using fusogenic co-lipids and/or endosomolytic peptides
Demonstrated potentiation of immune responses
Cell-specific targeting achievable

tight lattice of muscle to increase the extent of plasmid deposition in the muscle. In addition, the interaction of PINC polymers with plasmid results in increased resistance to nuclease degradation. Complexation of the polymers and plasmids may occur by any one or several mechanisms including hydrogen bonding (accepting or donating), Van der Waals, ionic interaction, dipole-dipole (or inducible dipoles), ionic-dipole, or hydrophobic interactions. PINC polymers have a common feature in that they are amphipathic in nature, having a functional group that can bind to plasmid and a backbone that provides a hydro-

phobic surface on the polymer/plasmid colloid. We hypothesize that the hydrophobic surface of the polymer/plasmid complex facilitates the cellular uptake of plasmid through increased hydrophobic interaction of the polymer/plasmid complex with cell membranes. There is a similar phenomenon reported by Kabanov et al. whereby uptake of plasmid complexes by cells has been shown to be increased by the use of cationic polyvinylbased polymers that condense plasmid ***(25)***. These condensing cationic polymers of Kabanov et al. result in a polymer/plasmid complex with a hydrophobic surface due to the presence of the polyvinyl backbone.

Prototype PINC polymers, such as polyvinyl pyrrolidone (PVP; 50 kDa) and polyvinyl alcohol (PVA; 18 kDa), bind to plasmid via hydrogen bond accepting and donating mechanisms, respectively. Optimized stoichiometry for complexation of these polymers with plasmids has been shown (by microtitration calorimetry, zeta potential modulation, and ethidium bromide fluorescence) to be 1:17 w/w (plasmid/PVP) and 1:10 w/w (plasmid/PVA). Injection of these complexes into rat muscle results in up to a 10-fold increase in the both the level and extent of gene expression in rat muscle. Using a series of co-polymers consisting of polyvinyl-pyrrolidone and co-vinyl acetate, we have shown that polymer structure, independent of all other variables, affects the level of reporter gene expression in rat muscle. This structure-activity relationship is being used as the basis for designing advanced PINC polymers that bind to plasmid with increased affinity. It is thought that the increased binding between the polymer and plasmid will result in increased resistance of the plasmid to nuclease degradation. In addition, the improved stability may result in further increases in the levels and extent of gene expression in muscle as a result of the enhanced access of intact plasmid to a greater number of muscle cells. Increased binding affinity to micromolar range or lower may also afford the possibility of attaching cellspecific ligands to novel PINC polymers for targeting cells with enhanced immune function. such as DCs.

PINC systems have been tested as delivery systems for nucleic acid-based vaccines by administration of PINC polymer/plasmid complexes via intramuscular, subcutaneous, and intradermal routes. These studies have included the use of needle-free injection devices to reproducibly deposit nuclease-protected plasmid at these sites. We have shown that plasmid complexed to PINC polymers gains access to the draining lymph nodes after intramuscular and subcutaneous administration (unpublished results). Further, we have shown that a complex between PVP and an expression plasmid for human growth hormone (hGH) formulated in 150 m*M* NaC1 and injected using a needle-free injection device (Medi-Ject Corporation; Minneapolis, MN) into dog muscle results in up to a five-fold increase in anti-hGH antibody titers at wk 8 as compared to the same dose of plasmid formulated as "naked" plasmid in 150 m*M* NaC1

(26). Further, anti-hGH antibody titers using the needle-free injection device were 20-fold greater than with needle injection. The mechanism for this increase in anti-hGH antibody titers using a PVP/plasmid complex is currently under investigation in our laboratory.

PINC polymer/plasmid complexes have promise in the field of nucleic acid-based vaccines since they add to the usefulness of 'naked' plasmid technology that has been shown to work in multiple animal models as a prophylactic vaccine *(2–4)*. Potentially, second generation, more interactive PINC polymers will enable the dose of plasmid to be greatly reduced as a result of increased bioavailability of plasmid administered by these routes. It is also feasible that PINC polymers will enable the targeting of DCs via conjugation of the PINC polymers with DC-specific ligands.

3.2. Peptide-Based Systems

Proteins, such as higher molecular weight poly-L-lysine (PLL), have been investigated to condense plasmid into a compact structure. For many applications, various cell-specific targeting ligands have been covalently attached to PLL, including transferrin, folate, CD3 antibody, asialoorosomucoid, lactose, galactose, and mannose *(27–34)*. These approaches have shown that cell-specific targeting can be achieved in vitro and, in some cases, in vivo. PLL has also been used to enhance the uptake of peptide antigens into antigen presenting cells in vitro, which suggests that polycationic carriers may also be utilized to target plasmid to important cells of the immune system *(35)*. Major limitations of PLL-based systems include the heterogeneity of the PLL used, cytotoxicity, and the irreproducibility in both plasmid complexation and biological effect. As a result, several attempts have been made to utilize shorter and more well-defined peptides for plasmid delivery.

We have developed proprietary peptide-based systems derived from the original work reported by Smith and colleagues *(36–38)* (*see* **Table 1**). For example, the peptide backbone, YKAKnWK, has been extensively modified and conjugated to obtain multiple families of "lipophilic" condensing peptides. Other lipophilic PLL or peptide derivatives for plasmid delivery have been described in the literature *(39–41)*. These short lipophilic condensing peptides are synthesized by solid phase chemistry, highly pure, and unlike PLL, easy to characterize. These peptides have been shown to have significantly less cytotoxicity compared to PLL. These lipophilic condensing peptides have also been covalently modified to obtain condensing peptides with cell-specific targeting ligands such as mono- or multi-antennary galactose or mannose. For example, we have developed a plasmid complexed with a tri-antennary galactose modified-lipophilic condensing peptide, combined with a lipophilic lytic peptide, which can selectively target mouse hepatocytes after tail vein injection

(unpublished results). This plasmid complex, with an average particle size below 100 nm, has shown to be stable to both salt and salt/serum challenge, a characteristic that is critical for successful in vivo receptor-mediated endocytosis. Lipophilic lytic peptides, which promote pH specific lysis of endosomal vesicles, are also being developed as a means to enhance cytoplasmic entry of plasmid.

The colloidal and surface properties of condensed systems, and the stability in biological milieu, can be controlled to ultimately affect the biodistribution of these systems after in vivo administration via various routes. Control of the colloidal properties is made possible by the inclusion of stabilizing moieties including: (i) peptide sequences that are more resistant to protease degradation, (ii) spacer linkages between the condensing group of the peptide and the cell-specific targeting moiety that provide for steric stabilization, and (iii) lipophilic moieties that provide stabilization via hydrophobic contribution. For use in delivery of nucleic acid-based vaccines, we envision the possibility of including in the lipophilic condensing peptides motifs that potentiate immune responses, attract DCs, and/or that selectively bind DCs, as described by Barry et al. ***(42)***.

3.3. Lipid-Based Systems

Of the fifteen clinical protocols approved by the FDA for non-viral gene therapy as of September 1996, twelve involved the use of cationic lipid formulations of plasmid ***(43)***. Initial human clinical trials ***(44–46)*** using several different cationic lipids have demonstrated that no significant toxicity results from lipid-based formulations, even for protocols involving repeated administration. Thus, it is feasible that cationic lipid-based systems have immediate applicability as delivery systems for nucleic acid-based vaccines if these systems are shown to meet other product requirements (*see* **Fig. 1**) .

Cationic lipids may serve many functions including: (i) protection of the plasmid from nuclease degradation, (ii) modification of the size and surface charge of the plasmid, (iii) enhancement of the cell uptake of plasmid, (iv) enhancement of the release of plasmid from the endosomes, and v) enhancement of the entry of intact plasmid into the nucleus (either directly or indirectly) ***(37)***. Several alternative lipid-based systems, including cochleates ***(47–48)*** and emulsion-based systems ***(49–51)*** for gene therapy, and nucleic acid-based vaccines, have also been described. These novel systems may also possess similar functions as mentioned above.

Targeting motifs may also be included in lipid-based systems to enhance cell-specific uptake of biomolecules. For example, antibodies ***(52–53)***, asialofetuin ***(54–56)***, transferrin ***(57)***, lactosylceramide ***(58)***, N-acetyl glucosamine ***(59)***, fucose ***(59)***, and mannose ***(59–64)*** have been attached to lipids and shown to increase the efficiency of cell uptake.

Specific targeting of dendritic cells residing in the lymph nodes may be a viable strategy for nucleic acid-based vaccines. Lipid-based systems (cationic and non-cationic) have shown preferential (non-specific) targeting to the draining lymph nodes after subcutaneous administration. These liposomes were found to be taken up largely by macrophages ***(65)*** and possibly other antigen-presenting cells ***(66)***. Several parameters can govern the efficiency of lymph drainage after intramuscular and subcutaneous administration of colloidal formulations, including the particles' size, surface charge, and surface hydrophobicity/hydrophilicity balance ***(67–70)***. Maximum lymphatic uptake by a colloidal system after intramuscular and subcutaneous administration has been reported to be potentially up to 40% of the administered dose, depending on the colloidal properties, as mentioned above ***(70)***.

Liposomes, especially those containing cationic lipids, have also been shown to have strong immunopotentiating properties that may prove suitable for their use as adjuvants ***(71–72)***. For example, the use of cationic lipids to deliver a plasmid encoding for the S (small) region of hepatitis B surface antigen (HBsAg) resulted in 100-fold greater IgG_1 antibody titers and 10-fold greater IgG_{2a} and IgG_{2b} antibody titers when delivered via a cationic lipid-based system as compared to 'naked' plasmid ***(66)***. In addition to elevated immunoglobulin titers, plasmid formulated with a cationic lipid-based delivery system significantly increased the levels of certain cytokines compared to 'naked' plasmid, most notably INFγ and IL-4.

Lipid-based systems, as delivery systems for nucleic acid-based vaccines, may serve many diverse functions including controlling the surface and colloidal properties of plasmid, plasmid deposition, cell-specific targeting, and immunopotentiation. It is expected that a considerable amount of effort will continue to be devoted to the development of these delivery systems for nucleic acid-based vaccines.

4. Approaches to the Control of Gene Expression

Presently it is unknown whether the sites of plasmid deposition harbor plasmid fragments or intact plasmid capable of producing the viral/bacterial antigen. Potentially, uncontrolled antigen expression could have toxic effects depending on the nature of antigen (e.g., bacterial toxins or viral oncogenes) or lead to the destruction of non-professional APCs presenting the gene product in the context of class I by antigen-specific CTLs. The second area for improvement in the development of nucleic acid-based vaccines centers on the control of gene expression. Two different aspects of gene expression will be discussed. The first deals with tissue-specific expression to limit antigen production to a particular cell type. The second focuses on the expression of multiple antigens from a single plasmid.

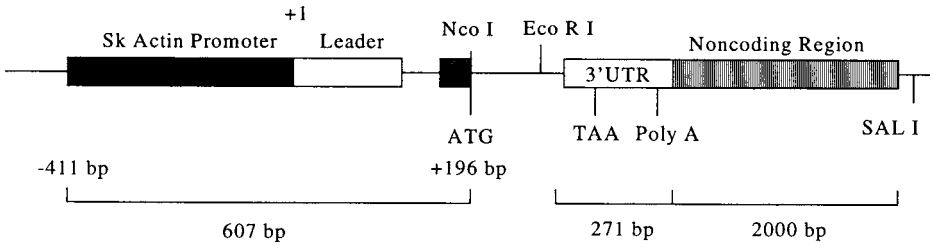

Fig. 2: Muscle-specific expression system. The expression cassette was derived from the chicken skeletal α-actin gene and contains the following components: the chicken skeletal otactin promoter (–411 to +1), the first exon [5' untranslated region (UTR)] of the skeletal (α-actin gene (+1 to +196), the first intron of the skeletal α-actin gene, restriction enzyme cleavage sites for insertion of gene coding sequence and 2 kilobases of the skeletal α-actin gene 3' (UTR) ***(79)***.

4.1. Tissue-Specific Expression

All current nucleic acid-based vaccines described in the literature rely on viral promoters for the expression of a gene product. These genes not only include the antigen of choice, but genetic adjuvants being tested as ways to potentiate the immune response like CD80, CD86, IL2, GM-CSF, IL-4 and IL-12 ***(73–78)***. Since plasmid can potentially access several tissues throughout the body, uncontrolled expression of antigen from promiscuous viral promoters may result in serious side-effects. These side-effects could range from an autoimmune-like reaction (i.e., destruction of the expressing tissue) to expression of an oncogenic viral protein. In theory, restricting the site of expression of genes through the use of tissue-specific regulatory elements would minimize or eliminate risks related to aberrant expression of a gene product.

Cell-specific expression systems have yet to be exploited for use in nucleic acid-based vaccines, but are presently being developed for the expression of therapeutic gene products. These systems are centered on tissue-specific regulatory elements that lay outside the coding region of a gene and, as such, can be used to restrict the expression of various genes, regardless of their intended purpose. We have developed one such system for the expression of hIGF-I in skeletal muscle after intramuscular administration ***(79–80)***. This novel muscle-specific expression system contains the promoter and portions of the 5' and 3' untranslated region (UTR) of the chicken skeletal α-actin gene (*see* **Fig. 2**). This system may have applicability to nucleic acid-based vaccines, as antigen expressed solely in muscle is capable of eliciting a prolonged prophylactic and therapeutic immune response. In addition, other systems that restrict expression to sites in the periphery (i.e., sites of administration) would have benefit as other routes become suitable for the delivery of a nucleic acid-based vac-

cine. For example, restriction of expression to airway epithelium may be of importance for intranasal delivery, as portions of the dose may end up in the digestive tract.

Regardless of the route of administration, the critical cell types to target with a nucleic acid-based vaccine may be DCs. If specific targeting of plasmid to DCs proves to be difficult, one may want to limit expression of antigen to these cells as an added safety measure. Dendritic cell-specific expression systems have not yet been developed, but information is being generated as to what proteins are restricted to these cell types. To date, only a few proteins have been identified whose expression is limited to DCs. These include DEC-205, S-100, p55, and CD83 ***(81–82)***. Regulatory elements of these genes are now being identified and characterized to determine their role in limiting expression to these cell types. As a better understanding of the tissue-specific expression of proteins in DCs is obtained, novel expression cassettes will be generated to restrict the expression of antigen to these cells.

4.2. Expression of Multiple Antigens: Multivalent Systems

It is conceivable that for some pathogens expression of a single gene will not suffice for an effective nucleic acid-based vaccine. Additional pathogen antigens may be needed to provide broader immunological coverage or cytokine/co-stimulatory molecules to function as genetic adjuvants. Single plasmids encoding for the transcription of each gene can be created, but this could significantly increase production costs associated with the vaccine. In addition, it would be difficult to reproducibly formulate several plasmids as a single product for administration or transfect a single cell with multiple plasmids in vivo. One alternative approach to multiple expression plasmids is a single plasmid producing all the desired gene products. Several strategies can be employed to accomplish this feat (*see* **Fig. 3**). The first strategy uses multiple independent transcriptional units on a single plasmid, each defined by its own promoter and polyadenylation signal (Poly A). Each transcriptional unit functions independently of the other to produce high levels of both gene products. The promoters driving the transcription of each unit do not have to be identical to one another and can be used to direct expression in a tissue-specific or non-specific manner.

The second strategy makes use of the internal ribosome entry site (IRKS) of picornaviruses. Placement of an IRKS sequence between two coding regions on a single transcript facilitates the entry of ribosomes at this site and translation of the downstream coding sequence. IRKS sequence elements eliminate the need for a 5' cap structure or a free 5' end on the message for translation ***(83,84)***. This strategy has been employed for the production of IL-12 from a

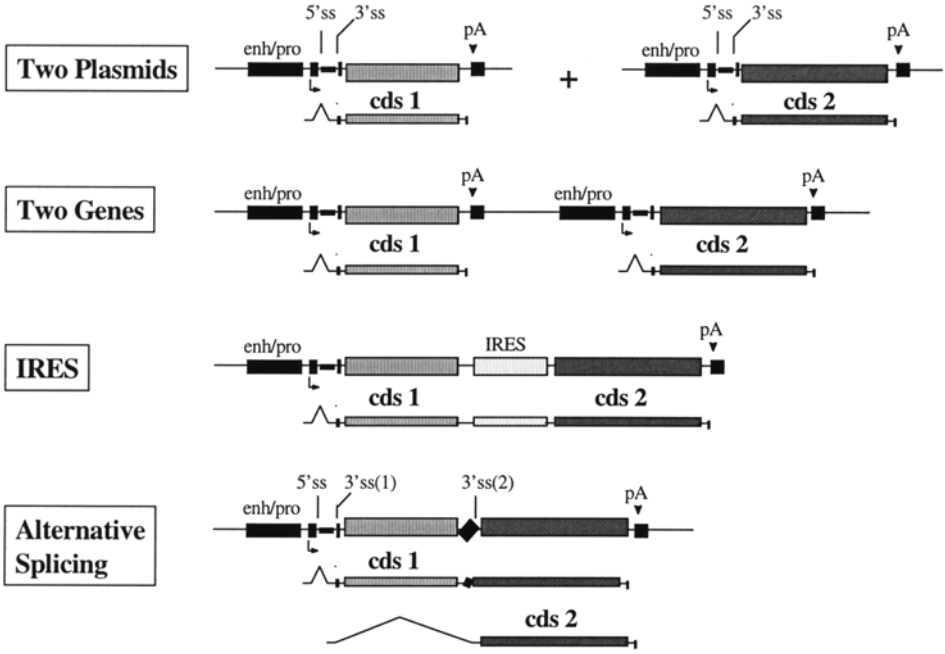

Fig. 3. Multivalent expression systems. Two plasmids: two coding sequences are encoded as single transcriptional units on individual plasmids. Two genes: two coding sequences are encoded as independent transcriptional units on a single plasmid. IRKS: two coding sequences are encoded as a single transcriptional unit with an IRKS sequence locating between the two coding sequences. Alternative splicing: two coding sequences are encoded as a single transcriptional unit with an intron (5' ss/3' ss) placed upstream of the first coding sequence and an alternative 3' splice site [3' ss (2)] located upstream of the second coding sequence.

retroviral vector ***(85)*** and an adenoviral vector ***(86)*** and has been tested in murine tumor models ***(87)***.

The third strategy utilizes alternative splicing of a single transcript to produce the individual gene products. In this system, an intron containing a consensus 5' and 3' splice site is placed upstream of the first coding sequence. A second 3' splice site is placed after the stop codon of the first coding sequence, upstream of the second coding sequence. A single promoter directs the transcription of this unit and produces a single pre-mRNA species containing both coding sequences. Depending on the strength of the individual 3' splice sites, the endogenous splicing machinery will either excise the smaller upstream intron, producing a transcript with two coding sequences, or excise a larger intron that deletes the upstream coding sequence. Translation of transcripts

that result from the first splicing scenario will only generate gene product from the first coding sequence, as only that coding sequence which is proximal to the 5' end of the mRNA is efficiently recognized by the translation machinery. Removal of the first coding sequence in the second splicing scenario places the second coding sequence proximal to the 5' end of the mRNA. Subsequent translation of this transcript produces only gene product from the second coding sequence. In addition to the production of multiple gene products, the alternative splicing construct can be used to generate different ratios of the gene products. By varying the strength of the 3' splice sites, one can regulate the levels of gene expression with respect to one another by controlling the splicing preference of the transcript. This would be advantageous for the synthesis of multi-subunit gene products where there is an uneven stoichiometry of the subunits.

Unpublished in vitro and in vivo data from our group has shown that a single plasmid harboring multiple independent transcriptional units is capable of producing the desired gene products at levels equal to or greater than those observed when individual plasmids with single transcriptional units are utilized. In addition, we have demonstrated that the IRKS and alternative splicing systems are effective means of producing gene products at different ratios. Presently, all of these systems could be readily used in a nucleic acid-based vaccines to generate multiple gene products from a single plasmid. The choice of which system to use will ultimately depend on the nature of the selected genes, the desired ratio of gene products and how they perform and interact with the expression elements that comprise each system.

5. Concluding Remarks

Advances in the field of nucleic acid-based vaccines will stem from the delivery and expression technologies currently being developed by those in the non-viral gene therapy field for the production of therapeutic gene products in vivo. First and foremost, the use of advanced plasmid delivery technologies allows one to control the site of plasmid deposition in the body and accurately target gene expression to the most appropriate cells. For nucleic acid based-vaccines this may be professional antigen presenting cells like DCs. Second, the use of tissue-specific expression cassettes allows one to restrict expression of a gene product to a specific cell population. This may become of paramount importance if plasmid deposition remains uncontrollable and promiscuous gene expression is a concern. In addition, the ability to encode multiple gene products on a single plasmid may prove to be of a tremendous benefit as several gene products may need to be expressed for the elicitation of an effective prophylactic or therapeutic immune response. It is likely that the combination of these technologies will generate safer and more potent vaccines in accordance with the criteria put forth in **Fig. 1**.

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Mucosal Immunization with DNA Vaccines

Michael J. McCluskie and Heather L. Davis

1. Introduction

1.1. Advantages of Mucosal Immunization

The mucosal surface area of the gastrointestinal, genitourinary and respiratory tracts is more than 200 times greater than that of the skin and is the primary site of transmission of numerous diseases. The entry of pathogenic organisms at mucosal surfaces can be prevented by mucosal, but not systemic immunity. Vaccines which are delivered by intramuscular (IM) or subcutaneous (SC) injection induce strong systemic responses but generally no mucosal immunity. In contrast, vaccines delivered at mucosal surfaces trigger both mucosal (at local and distant sites) and systemic responses ***(1,2)***. Other advantages of mucosal immunization include a broader age range of recipients, the vaccines are easy and non-invasive to administer and there is no risk of needle stick injury and cross contamination ***(3)***.

There have been only a limited number of reported cases of DNA-based immunization at mucosal surfaces. Intranasal (IN) administration of pure plasmid DNA (often referred to as "naked" DNA) expressing influenza virus hemagglutinin glycoproteins protected both mice and chickens against lethal influenza challenge although mucosal-specific responses were not measured ***(4)***. In another study, both systemic and mucosal immunity were generated following IN immunization with "naked" plasmid DNA expressing herpes simplex virus type 1 glycoprotein B, but not enough to protect against vaginal challenge with HSV ***(5)***.

1.2. Direct Gene Transfer in the Lung

Numerous studies have indicated that gene transfer may be carried out in the lung using plasmid DNA either alone or formulated with cationic liposomes,

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

however reports often conflict as to their relative efficiencies. Some studies report that liposome-formulated but not naked DNA can transfect lung tissue ***(6–8)***. Others state that transfection is possible with naked DNA but this is less efficient than with lipids ***(9–11)***. Yet other studies report that naked DNA and lipid associated DNA are equally efficient ***(12,13)***. We have shown that the efficiency of transfection of lipid-formulated DNA depends very much on the nature of the lipid (i.e., neutral and cationic lipid components, length of carbon chains ***[14]***), and this likely explains the discrepant findings reported heretofore.

Numerous approaches for delivering DNA (formulated or not) to the lung have been reported. The most common methods of administration are IN (inhaled or instilled), which are indirect but non-invasive, or intratracheal (IT) (injected or via a cannula) which is more direct but invasive. Each of these techniques is described below.

In order to determine the effectiveness of a mucosally delivered DNA vaccine, mucosal washes can be collected (as described in **Subheading 2.2.**) and antibodies specific for the expressed protein measured by ELISA, the hallmark of mucosal immunity being the local production of secretory immunoglobulin A (S-IgA) antibodies.

2. Materials

2.1. Direct Gene Transfer to the Murine Lung

2.1.1. Intranasal Instillation

1. Appropriate anesthesia for mice (e.g., Halothane, Halocarbon Laboratories, River Edge, NJ) (*see* **Note 1**).
2. Micropipettor with suitable tips (*see* **Note 2**).
3. Plasmid DNA vaccine dissolved in endotoxin-free saline at appropriate concentration (*see* **Note 3**).
4. Liposome formulation, if applicable, at appropriate concentration (*see* **Note 4**).

2.1.2. Intranasal Inhalation

1. Appropriate anesthesia for mice (e.g., Halothane, Halocarbon Laboratories) (*see* **Note 1**).
2. Micropipettor with suitable tips (*see* **Note 2**).
3. Plasmid DNA vaccine dissolved in endotoxin-free saline at appropriate concentration (*see* **Note 3**).
4. Liposome formulation, if applicable, at appropriate concentration (*see* **Note 4**).

2.1.3. Intratracheal Injection

1. Appropriate anesthesia for mice (e.g., Somnotol, 75 mg/kg IP; MTC Pharmaceuticals, Cambridge, ON) (*see* **Note 1**).
2. Fine-tipped surgical scissors (Fine Science Tools Inc., North Vancouver, BC).

3. Insulin syringe, 0.33 cc with a 29G1/2 needle attached (Becton Dickinson, Franklin Lakes, NJ).
4. Plasmid DNA vaccine dissolved in endotoxin-free saline at appropriate concentration (*see* **Note 3**).
5. Liposome formulation, if applicable, at appropriate concentration (*see* **Note 4**).
6. Fine degradable surgical sutures.

2.1.4. Intratracheal Cannulation

1. Appropriate anesthesia for mice (e.g., Somnotol, 75 mg/kg IP) (*see* **Note 1**).
2. Fine-tipped surgical scissors.
3. A 20-gauge olive tip steel feeding tube (Fine Science Tools Inc.) attached to a 1 cc tuberculin syringe (Becton Dickinson).
4. Plasmid DNA vaccine dissolved in endotoxin-free saline at appropriate concentration (*see* **Note 3**).
5. Liposome formulation, if applicable, at appropriate concentration (*see* **Note 4**).
6. Fine degradable surgical sutures.

2.2. Collection of Mucosal Secretions

2.2.1. Vaginal Wash

1. Micropipettor with standard 200 µL tip attached.
2. Appropriate anesthesia for mice (e.g., Halothane).
3. Phosphate-buffered saline (PBS) or Tris-buffered saline (TBS) (*see* **Note 6**).
4. Microcentrifuge tubes for sample collection.
5. Centrifuge (15,690*g*).

2.2.2. Fecal Pellets

1. Individual cages for mice (*see* **Note 7**).
2. Micropipettor with standard 200 µL tip attached.
3. PBS or TBS (*see* **Note 6**).
4. 50 mL Centrifuge tubes for sample collection (one for each mouse).
5. Sodium azide (10 µL per 0.1 mg fecal material).
6. Centrifuge (3340*g*).

2.2.3. Lung Wash

1. Appropriate anesthesia for mice (*see* **Note 8**).
2. Fine-tipped surgical scissors.
3. Two surgical microclamps (Fine Science Tools Inc.) (*see* **Note 9**).
4. Insulin syringe, 1 cc with a 29G1/2 needle attached.
5. Polyethylene tubing (PE 20, ID = 0.38 mm).
6. PBS or TBS (*see* **Note 6**).
7. Microcentrifuge tubes for sample collection.
8. Centrifuge (15,690*g*).

2.2.4. Nasal Wash

1. Appropriate anesthesia for mice (*see* **Note 8**).
2. Fine-tipped surgical scissors.
3. Two surgical microclamps.
4. Insulin syringe, 1 cc with a 29G1/2 needle attached.
5. Polyethylene tubing (PE20, ID = 0.38 mm) (Becton Dickinson).
6. PBS or TBS (*see* **Note 6**).
7. Microcentrifuge tubes for sample collection.
8. Centrifuge (15,690*g*).

3. Methods

3.1. Direct Gene Transfer to the Murine Lung

3.1.1. Intranasal Instillation

1. Prepare DNA and liposome solution (*see* **Notes 3, 4, 10,** and **11**).
2. Draw 25 µL DNA solution into pipet tip and ensure there are no air bubbles (*see* **Note 2**).
3. Anesthetize mice (*see* **Note 1**).
4. Hold mouse on its back and carefully insert the tip of the gel loading tip a few mm into the nasal cavity, so as not to damage nasal epithelium, and slowly instill DNA solution. Immediately repeat instillation into second nostril (*see* **Note 12**).
5. Apply pressure to the lower mandible to reduce swallowing and hold the mouse in this position for 10 s to ensure that the fluid is not expelled (*see* **Notes 13** and **14**).

3.1.2. Intranasal Inhalation

1. Prepare DNA and liposome solution (*see* **Notes 3, 4, 10,** and **11**).
2. Draw DNA solution into pipet tip and ensure there are no air bubbles (*see* **Note 2**). A volume of 25 to 200 µL can be administered at one time (*see* **Note 15**).
3. Anesthetize mice (*see* **Note 1**).
4. Deposit the DNA solution as droplets applied bilaterally directly over the external nares of mice during a period of approximately 5 s. Apply pressure to the lower mandible to reduce swallowing during administration (*see* **Note 14**). Although the DNA solution is delivered by a pipet tip, this is not placed inside the nasal cavity.
5. Hold mouse for a further 10 s to ensure that fluid is not expelled.

3.1.3. Intratracheal Injection

1. Prepare DNA and liposome solution (*see* **Notes 3, 4, 10,** and **11**).
2. Anesthetize mice (*see* **Note 5**).
3. Draw desired volume of DNA solution (100–200 µL) into the syringe and ensure there are no air bubbles. Set syringe aside.
4. Using fine-tipped surgical scissors expose the mouse trachea through an anterior midline incision.

5. Inject the DNA solution through the anterior wall of the trachea taking care not to puncture posterior wall (*see* **Note 14**).
6. Suture the skin incision and place the mouse in an incubator until fully recovered from the anesthetic (approx 45 min).

3.1.4. Intratracheal Cannulation

1. Prepare DNA and liposome solution (*see* **Notes 3, 4, 10,** and **11**).
2. Anesthetize mice (*see* **Note 5**).
3. Attach feeding tube to syringe.
4. Draw desired volume of DNA solution (100–200 µL) into the syringe and ensure there are no air bubbles. Set syringe aside.
5. Using fine-tipped surgical scissors expose the mouse trachea through an anterior midline incision. This incision is used to visualize the insertion of the cannula into the trachea.
6. Gently pass the feeding tube through the oral cavity and into the trachea.
7. Slowly inject the DNA solution directly into the lungs (*see* **Note 14**).
8. Suture the skin incision and place the mouse in an incubator until fully recovered from the anesthetic (approx 45 min).

3.2. Collection of Mucosal Secretions

3.2.1. Vaginal Wash

1. Draw 30 µL PBS or TBS (*see* **Note 6**) into pipet tip and ensure there are no air bubbles.
2. Anesthetize mice.
3. Hold mouse by the back of neck, stomach upward. Carefully insert pipet tip a few mm into the vaginal cavity.
4. Instill and withdraw solution into the vaginal cavity 10 times.
5. Centrifuge samples at 15,690*g* for 7 min to remove vaginal lavage sediments.
6. Remove supernatant and store at –20°C.
7. Repeat procedure over 5 consecutive d (*see* **Note 16**).
8. Pool supernatants and store at –20°C until assayed by ELISA (*see* **Note 17**).

3.2.2. Fecal Pellets

1. Isolate mice in individual cages (*see* **Note 7**) for a 24 h period.
2. Collect and weigh fecal pellets.
3. Add 1 mL TBS or PBS (*see* **Note 6**) and 10 µL sodium azide per 0.1 mg of fecal material.
4. Rehydrate samples for 30 min at RT.
5. Centrifuged at 3340*g* for 15 min to remove fecal debris.
6. Collect supernatant and store at –20°C until assayed for S-IgA.

3.2.3. Lung Wash

1. Draw 1 mL TBS or PBS (*see* **Note 6**) into the syringe and ensure there are no air bubbles. Attach PE tubing to needle. Use length of tubing 1 cm longer than needle. Set syringe aside.

2. Kill mouse by cervical dislocation or anesthesia overdose (*see* **Note 8**).
3. Using fine-tipped surgical scissors expose the mouse trachea through an anterior midline incision.
4. Make a small incision in trachea and place a clamp above it (*see* **Note 9**).
5. Pass PE tubing a few mm through incision in direction of the lungs and place a second clamp below incision to hold PE tubing in trachea.
6. Slowly instill and withdraw solution into lungs three times (80% recovery expected).
7. Centrifuge samples at 15,690*g* for 7 min, collect supernatant and store at –20°C until assayed for S-IgA.

3.2.4. Nasal Wash

1. Draw 0.5 mL TBS or PBS (*see* **Note 6**) into the syringe and ensure there are no air bubbles. Attach PE tubing to needle. Use length of tubing 1 cm longer than needle. Set syringe aside.
2. Kill mouse by cervical dislocation or anesthesia overdose (*see* **Note 8**).
3. Using fine-tipped surgical scissors expose the mouse trachea through an anterior midline incision.
4. Make a small incision in trachea and place a clamp below it (*see* **Note 9**).
5. Pass PE tubing a few mm through incision in direction of nares and place a second clamp above incision to hold PE tubing in upper trachea.
6. Invert mouse and slowly inject solution into the trachea. Allow solution to flow through nares and collect in a microtube.
7. Centrifuge samples at 15,690*g* for 7 min, collect supernatant and store at –20°C until assayed for S-IgA.

4. Notes

1. Mice must be anesthetized as they will swallow the instilled material if they are awake. Halothane is a suitable anesthetic as recovery time is short and thus the risk of choking is reduced.
2. We have found that a fine-tipped gel loading tip is best as it has a smaller tip to be inserted into the nasal cavity for IN instillation and it allows very small droplets for the IN inhalation method.
3. Plasmid DNA may be purified by cesium chloride (CsCl) or anion-exchange chromatography. The quality of the DNA by these two methods is equivalent for DNA immunization, however, anion-exchange methods are considerably quicker, easier to perform and scale-up than CsCl methods and avoid the use of hazardous chemicals ***(15)***. In our laboratory, DNA is purified on Qiagen anion-exchange chromatography columns (Qiagen GmbH, Hilden, Germany) and resuspended in sterile saline (0.15 *M* NaCl, Sigma). An endotoxin removal kit is also available from Qiagen which yields DNA with extremely low levels of endotoxin. The concentration of DNA is calculated based on absorbance of ultraviolet light (OD 260) with final concentrations usually being 5–10 mg/mL, purity determined at OD260/280, and DNA verified by restriction enzyme digest and agarose gel elec-

trophoresis. For mucosal delivery, it is important to use concentrated DNA solutions to minimize administration volume. DNA solutions can be stored at –20°C until required for in vivo delivery.

4. Various cationic liposome formulations are commercially available. We routinely use Cellfectin (Life Technologies, Inc., Gaithersburg, MD) at a DNA:lipid w/w ratio of 1:1 which we have found to be efficient for gene transfer to lungs. However, other liposome formulations, such as Lipofectin or Lipofectamine (Life Technologies, Inc.), resulted in lower levels of expression than plasmid DNA alone ***(14)***. It is important to allow time for DNA/lipid complex formation before administration (usually 30–60 min) and thereafter to use as quickly as possible.
5. For intratracheal administrations where surgery is involved, it is necessary to use a long lasting general anesthetic. We have found Somnotol (75 mg/kg IP) to be suitable for this procedure. During recovery time (approx 45 min) mice can be placed in a 37°C incubator to maintain body temperature.
6. Phosphate can act as an inhibitor in certain commercial kits that are used to detect low levels of antibody in washes (e.g., ELISA Amplification System, Life Technologies, Inc., Gaithersburg, MD). Phosphate containing buffers should be avoided when these kits are used.
7. Individual cages are required to prevent mixing of fecal material from different mice. We have found that if we replace the regular bedding material (sawdust) with thick paper towels fecal pellet collection is greatly facilitated.
8. Mice can be killed by cervical dislocation while under Halothane anesthesia; however to avoid damage to the trachea, an overdose with a suitable anesthesia (e.g., Somnotol) may be preferable.
9. We have found that surgical microclamp is an efficient and easy way to close the trachea. Alternatively, a surgical suture may be used to tie the trachea off at the desired place.
10. When DNA is formulated with cationic liposomes, it is desirable to determine the lowest DNA:lipid ratio at which all the DNA is complexed, as we have found this optimal for gene transfer in the lung. Free plasmid DNA can be visualized on a 0.5% agarose gel stained with 0.04 μg/mL ethidium bromide as a migrated band at the expected distance (based on molecular size) whereas DNA-lipid complexes fail to enter the gel.
11. India ink, trypan blue or methylene blue may be used initially to visualize the distribution of fluid in the airways. In this case, mice are killed and lungs harvested 1 or 2 min after administration. The success of direct gene transfer in the lung can be evaluated using any of the routine reporter genes, such as beta-galactosidase (*lacZ*), luciferase (*luc*), chloramphenicol acetyl-transferase (CAT) or green fluorescent protein (GFP).
12. The gel-loading tip must be carefully inserted to ensure the nasal epithelial layer is not damaged otherwise a systemic immunization, rather than mucosal, may result.
13. The instillation procedure can be repeated as necessary but it is best to leave 15 min intervals between administrations to allow time for the animals to recover.

14. We have shown essentially no differences between IN inhalation, IN instillation and IT cannulation for reporter gene expression, except for higher variability with the latter. IT injection provides somewhat lower transfection efficiency than the other three methods, possibly due to DNA leakage ***(14)***. Thus our method of choice is IN inhalation since it is the easiest to perform and least invasive. Other investigators have described adaptations on these techniques; for example, the tongue can be pulled out with IN administration to limit swallowing ***(16)***, a small volume of air can be injected after IT administration to disperse the fluid throughout lung ***(12)***, or an aerosol or nebulizer system can be used for delivery of a mist rather than a liquid ***(17)***.
15. For multiple administrations, at least 15 min should be allowed between administrations particularly when larger volumes (e.g., 200 µL) are used.
16. This is necessary in order to account for variations in S-IgA levels during the murine estrus cycle ***(18)***.
17. Mucosal antibody levels can be measured by ELISA using a primary antibody specific for the expressed protein and an anti-IgA secondary antibody. Since IgA levels are typically low, a more sensitive antibody assay is the ELISA amplification system (EIA) (Gibco-BRL, Gaithersburg MD, USA). Alternatively, the presence of antibody-secreting cells (ASC) may be determined in single cell suspensions obtained from lung tissue or lymph nodes using the ELISPOT technique ***(5)***, and antigen-specific proliferative T-cell responses or the induction of cytotoxic T lymphocytes may also be measured.

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Preparations for Particle-Mediated Gene Transfer Using the Accell® Gene Gun

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1. Introduction

Particle-mediated delivery involves coating materials onto the surface of dense sub-cellular sized (0.5–5 mm) particles and accelerating the particles to sufficient velocity to penetrate target cells. The technique was invented by Sanford and Wolf at Cornell University ***(1)*** to transfer DNA into intact plant cells ***(2)***, and was further developed into an effective process for producing genetically engineered crop plants by several groups (reviewed in ***3***). Subsequent work has shown that this method is generally applicable for transferring materials including DNA, RNA, proteins, peptides and pharmacological compounds into a wide variety of tissue and cell types in vivo, ex vivo, or in vitro (reviewed in ***4***).

The topic of particle-mediated gene transfer to epidermis as a means of nucleic acid immunization is specifically addressed in an accompanying chapter. This chapter describes procedures for preparation of the necessary materials (*see* **Note 1**). The procedures are divided into two sections: bead preparation and tube preparation. The first section describes procedures for making a slurry of DNA-coated gold particles, and the second section procedures for loading the DNA-coated particles into "cartridges."

Particle-mediated gene transfer has primarily employed gold particles as carriers. Particles in the sub-cellular size range must be dense to achieve the momentum necessary for adequate penetration. Among the materials with sufficient density (e.g., gold, iridium, platinum, tungsten) gold is preferred because of its low chemical reactivity and low toxicity. Moreover, high purity gold powders in the desired size range are commercially available.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

Powder injection is a conceptually distinct technology that is, in several aspects, complementary to particle-mediated delivery. Rather than using dense sub-cellular sized carrier particles, as in particle-mediated delivery, powder injection employs larger low-density particles that can be formulated without a dense carrier. Sarphie et al. ***(5)*** demonstrated the systemic distribution of inulin administered dermally in the form of low-density (1.5 g/cm^3) particles. Particles in the 30–50 μm size range were required, thus delivery was likely extra-cellular; however, significant systemic bio-availability was achieved. These results suggest that powder injection is suitable for delivery of conventional pharmaceuticals, conventional vaccines, and other substances where extra-cellular delivery is effective and cellular uptake of the delivered material is efficient. Particle-mediated delivery, on the other hand, is an effective means of direct intra-cellular delivery. The use of powder injection for nucleic acid immunization is under investigation.

A number of parameters that influence particle-mediated gene delivery can be readily adjusted to optimize performance in a particular system. These parameters include the accelerating force, the size of the particles, the number of particles per target site, the distribution of particles within the target, and the amount of DNA loaded onto the particles. Manipulation of these parameters provides versatility, allowing adaptation of the method to target tissues with widely differing physical characteristics. A number of conveniently assayed reporter genes (e.g., human growth hormone, β-galactosidase, green fluorescent protein) are available that can be used to optimize parameters for a specific system. Reporter genes that can be assayed histologically are especially valuable for identification of transfected cells within a tissue as well as measurements of transfection efficiency.

Several devices have been developed to accelerate the coated particles; these include the original Biolistic Device of Sanford and Wolf ***(2)***, the PDS-1000/He device and Helios Gene Gun manufactured by Bio-Rad Laboratories Inc. (Hercules, CA), a hand-held helium-driven device designed by John Sanford and Stephen Johnston ***(6)***, and the Accell electric-discharge ***(7)*** and helium-powered ***(8)*** devices designed by Dennis McCabe and co-workers at Agracetus, Inc. A disposable device (PowderJect), described by Sarphie et al. ***(5)*** for powder injection, offers several attractive features in terms of practical clinical application of the technology. A device that combines these features of the PowderJect device with the delivery capabilities of the Accell helium device is currently under development.

This chapter describes procedures for using the helium-driven Accell device ***(8)***. A similar device, the Helios Gene Gun, and associated equipment and supplies are commercially available from Bio-Rad. The Helios device was developed in collaboration with PowderJect Vaccines Inc. (Madison, WI) to provide

devices for the life sciences research community. Particle-mediated gene transfer is a proprietary technology covered by patents held by PowderJect Vaccines, Inc. (previous names or affiliates include Geniva Inc., Auragen Inc., and Agracetus Inc.) and E. I. DuPont de Nemours & Co. (Wilmington, DE). Devices from Bio-Rad that implement this technology are licensed for research purposes only, and are not intended or approved for clinical use.

2. Materials

2.1. Bead Preparation

1. Analytical balance capable of accurately weighing milligram quantities of gold.
2. 0.5–4 μm gold particles.
3. 1.5 mL Eppendorf tubes.
4. Micro-pipettors.
5. 0.05 *M* spermidine (Sigma).
6. Vortex mixer.
7. Ultrasonic cleaner (Model FS3, Fisher Scientific, Pittsburgh, PA).
8. Plasmid DNA solution in TE (DNA concentration should be >1 μg/μL).
9. Microcentrifuge.
10. Dehydrated alcohol, 200 proof (ET107, Spectrum, New Brunswick, NJ).
11. 1 mg/mL ethanolic polyvinylpyrrolidone K-90 (PVP) (P1416, Spectrum).
12. 5, 10 and 25 mL pipettes and pipette-aid.
13. 22 mL glass scintillation vials and Teflon caps.

2.2. Tube Preparation

1. Tefzel tubing: ID 0.09375 ± 0.002 in., OD 0.127 ± 0.001 in.
2. 5 mL syringe with Masterflex size 14 silicon tubing adapter.
3. Stopwatch.
4. Tubing Prep Unit (Bio-Rad).
5. Compressed helium gas grade 4.5 (99.995%) or higher.
6. Compressed nitrogen gas grade 4.8 (99.998%) or higher.
7. Helium pressure regulator (delivery pressure range 10–800 psi).
8. Nitrogen pressure regulator (delivery pressure range 0–1000 psi).
9. Gas manifold.
10. Peristaltic pump capable of pumping 1–10 mL/min.
11. Cutting board with ruler.
12. Scalpels.
13. Humi-cap desiccant pellets (United Desiccants, Belen, NM).

3. Methods

3.1. Bead Preparation

1. Weigh the appropriate amount of gold into a 1.5-mL Eppendorf tube. For a bead loading rate (BLR) (*see* **Note 2**) of 0.5 mg of gold/cartridge, weigh ~20 mg gold for 35–40 cartridges.

2. Pipette 100 µL of 0.05 *M* spermidine into the Eppendorf tube containing the gold.
3. Vortex the spermidine/gold mixture for 2–3 s to suspend the gold in the spermidine solution.
4. Sonicate the spermidine/gold mixture for 10 s to disperse any aggregated gold particles.
5. Add the volume of DNA required to achieve the desired DNA loading rate (DLR) (*see* **Note 3**) to the spermidine gold mixture. The volume of DNA should not exceed the volume of spermidine (100 µL). If a greater volume of DNA is required, increase the volume of spermidine to equal that of the DNA solution (*see* **Note 4**).
6. Invert the DNA/spermidine/gold mixture several times to thoroughly mix.
7. Quickly add 100 µL of 1 *M* $CaCl_2$, dropwise, to the DNA/spermidine/gold mixture while gently vortexing. Close cap and vortex vigorously for 2–3 s after the completion of addition of the $CaCl_2$. The DNA/gold precipitate should rapidly sediment to the bottom of the tube when vortexing is stopped. The volume of the 1 *M* $CaCl_2$ should be equal to the volume of spermidine added; i.e., if the volume of spermidine was adjusted in step 5, the volume of $CaCl_2$ should be adjusted accordingly.
8. Allow the mixture to continue to precipitate at room temperature for 5–10 min.
9. Centrifuge the bead prep for 10 s to pellet the gold and carefully remove the supernatant.
10. Disrupt the DNA/gold pellet by raking the bottom of the tube across the holes of an Eppendorf rack.
11. Wash the DNA/gold pellet by vortexing in 700 µL of dehydrated alcohol (*see* **Note 5**). Pellet the gold by centrifugation for 10 s and remove the supernatant with a pipette. Repeat the wash two times.
12. Prepare 10 µL of fresh 1 mg/mL ethanolic PVP stock solution (*see* **Note 6**). Weigh 10 mg of PVP in a glass scintillation vial. Add 10 mL of dehydrated alcohol. Sonicate the suspension until the PVP is dissolved (*see* **Note 5**).
13. Mix the appropriate amount of dehydrated alcohol and ethanolic PVP stock to give the desired final PVP concentration (*see* **Note 6**). Pipet the total volume of the final PVP solution needed for the desired BLR (*see* **Note 2**) into a 22-mL glass scintillation vial with a Teflon cap. Add 500 µL of this solution to the DNA/gold pellet and resuspend the particles thoroughly. Transfer this suspension to the bottom of the scintillation vial. Using another 500 µL of the solution from the vial repeat the suspension, carefully rinsing the sides of the Eppendorf tube, until all of the DNA/gold has been transferred from the Eppendorf tube into the scintillation vial.
14. Tightly screw-on the Teflon cap and seal with parafilm. The bead prep is ready for tube preparation, or it can be stored in the dark at –20°C.

3.2. Tube Preparation

1. Purge the Tubing Prep Unit overnight by flushing with dry nitrogen gas at a flow rate of 0.1 L/min (LPM). If tubes are made frequently the tube turner should be

left purging with nitrogen at a flow rate of 0.1 LPM. In addition, the tefzel tubing should be purged overnight with nitrogen at 0.01 LPM.
2. Cut the tefzel tubing to a length that is approximately 2.5 cm longer than the Tubing Prep Unit.
3. Vortex the DNA/gold suspension for 3 s and briefly sonicate to disrupt any aggregates.
4. Turn off the nitrogen flow to the Tubing Prep Unit.
5. Attach the cut tefzel tube to the 5-mL syringe via the Masterflex adapter.
6. Vortex the DNA/gold suspension until the DNA/gold is completely, homogeneously suspended.
7. Quickly remove the cap from the vial and draw the DNA/gold slurry into the length of the tube.
8. Quickly slide the tube into Tubing Prep Unit then start the stop watch.
9. Settle for 15 s then rotate the tube approximately 90° by twisting the end (use the syringe markings to indicate the degree of rotation).
10. Settle for 30 s then rotate the tube an additional 90° in the same direction.
11. Settle for another 45 s then rotate the tube another 90° in the same direction. Allow the tube to settle for 2 min after this rotation.
12. Rotate the tube 45°, remove the syringe, attach the end of the tube to a peristaltic pump and draw off the liquid at approximately 3.5 mL/min.
13. Detach the tube from the peristaltic pump and turn on the Tubing Prep Unit at a rate of 20 rpm (0.05*g*).
14. After 30 s start N_2 flow at 0.4 LPM. After 3 min, remove the tube from the Tubing Prep Unit and attach it to a gas manifold that is attached to a nitrogen tank. Flush with dry N_2 for at least 1 h.
15. Cut the tube at each end, 4 cm in from the edge of the gold bead coating, and discard the end pieces.
16. Cut into 1.27 cm (0.5 in) lengths. Test segments from each end of tube (*see* **Note 7**).
17. Pool tubes and store in a sealed container with a desiccant pellet. A tightly capped glass scintillation vial wrapped with parafilm makes a good storage container.

4. Notes

1. DNA for particle-mediated gene transfer has most frequently been in the form of plasmid molecules in the 5–15 kilobase (kb) size range, however recombinant lambda phage clones and cosmid clones (≅ 50 kb), as well as defective viral genomes and uncloned genomic DNA fragments in the range of 150–200 kb have also been successfully transferred by this method. The use of very large DNA molecules requires that great care be taken in the manipulations to avoid shearing. Mixtures of plasmids can also be coated onto the gold particles ***(9)***.
2. The bead-loading rate (BLR) is the amount of gold in each cartridge. In most DNA vaccination experiments the BLRs are 0.25 mg to 0.5 mg of gold per cartridge. However, the BLRs may range between 0.0625 to 2 mg per cartridge in treatment parameter studies. The BLR is determined by calculating the volume of the inside of the tefzel tubing used to prepare the cartridges.

BLR	DNA/gold suspension
0.25 mg/shot	4.42 mg of gold/mL of EtOH/PVP solution
0.5 mg/shot	8.84 mg of gold/mL of EtOH/PVP solution
1.0 mg/shot	17.68 mg of gold/mL of EtOH/PVP solution

3. The DNA loading rate (DLR) is the amount of DNA, usually in micrograms, per milligram of gold. For DNA vaccination experiments the DLRs usually range between 1–2.5 μg of plasmid DNA per milligram of gold and can range between 5 ng to 5 μg of plasmid DNA per milligram of gold using the standard protocol. At the lower DLRs the addition of carrier DNA is helpful to efficiently precipitate the plasmid onto the gold beads. Aggregation of the gold particles can become a problem at DLRs greater than 3 μg of DNA per milligram of gold. Increasing the overall volume of the preparation may reduce aggregation somewhat. Higher DLRs can also be achieved using alternative formulation procedures.
4. The volume of DNA solution needed for a bead prep can be calculated as follows:

$$\text{Volume of DNA Stock} = [(\text{milligrams of gold in prep}) \times (\text{DLR})] / (\text{plasmid concentration})$$

5. After the first ethanol wash special care should be used to ensure that the ethanol and bead prep is not exposed to moisture. Bead and tube preps are extremely sensitive to humidity. Generally, bead and tube preps are not performed if the relative humidity is greater than 60%.
6. PVP is used as a binding agent to prevent premature release of the DNA/gold particles during the rise-time of the helium jet. The PVP concentration for most applications is 0.05 mg/mL, however, adjustment of the PVP concentration in the range 0.01–0.1 mg/mL may be helpful if the tubes are not releasing properly. The effect of PVP on performance is greatest in situations that require maximum penetration.
7. The cartridges can be tested for proper release by shooting the DNA/gold into parafilm at the appropriate pressure. A 0.5 mg shot should produce a circular pattern of particles approximately 1 cm in diameter. The pattern from a 0.25 mg shot is slightly smaller. In addition, the spent cartridge can be examined for complete release of DNA/gold. Penetration can be tested by shooting the particles into water-agar and visualizing the particles microscopically, or by shooting the particles through a barrier that approximates the intended target tissue. The quality of the DNA precipitation and tube preparation can be assayed by eluting the DNA bound to the gold in the cartridge and analyzing the eluted DNA by agarose gel electrophoresis. The biological activity of the prep can be determined by analyzing transient gene expression after transfection of tissue culture cells or an appropriate in vivo model.

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Entrapment of Plasmid DNA Vaccines into Liposomes by Dehydration/Rehydration

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1. Introduction

Intramuscular injection of naked plasmid DNA is known ***(1–3)*** to elicit humoral and cell-mediated immune responses against the encoded antigen. It is thought ***(2,3)*** that immunity follows DNA uptake by muscle cells, leading to the expression and extracellular release of the antigen which is then taken up by antigen presenting cells (APC). In addition, it is feasible that some of the injected DNA is taken up directly by APC. Disadvantages ***(1–3)*** of naked DNA vaccination include: uptake of DNA by only a minor fraction of muscle cells, exposure of DNA to deoxyribonuclease in the interstitial fluid thus necessitating the use of relatively large quantities of DNA, and, in some cases, injection into regenerating muscle in order to enhance immunity. We have recently proposed ***(1,4)*** that DNA immunization via liposomes (phospholipid vesicles) could circumvent the need of muscle involvement and instead facilitate ***(5)*** uptake of DNA by APC infiltrating the site of injection or in the lymphatics, at the same time protecting DNA from nuclease attack ***(6)***. Moreover, transfection of APC with liposomal DNA could be promoted by the judicial choice of vesicle surface charge, size and lipid composition, or by the co-entrapment, together with DNA, of plasmids expressing appropriate cytokines (e.g., interleukin 2), or immunostimulatory sequences.

To that end, a method has been developed ***(6)*** which allows for the quantitative entrapment of plasmid DNAs into neutral, anionic and cationic liposomes that are capable of transfecting cells in vitro with varying efficiency ***(6)***. Moreover, it was shown ***(1,4)*** that immunization of Balb/c and outbred (T.O.) mice

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

by a variety of routes with (cationic) liposomal DNA leads to much greater humoral immune responses (as indicated by splenocyte IL-4 production and circulating IgG subclasses) and cell-mediated immune responses (splenocyte IFN-γ production) than those obtained with naked DNA or DNA complexed to similar pre-formed liposomes. We have observed ***(1,4,7)*** that a cationic lipid is essential for the promotion of strong responses and that PE and DOPE are equally effective in inducing immune responses in genetic vaccination. Here we describe methodology for the incorporation of plasmid DNA into liposomes of varying lipid composition, vesicle size and surface charge. The dehydration-rehydration procedure is characterized by its mildness and is thus compatible with most labile materials. It can also be applied for the entrapment of DNA into non-phospholipid liposomes in which PC (or DSPC) is replaced by non-ionic surfactants ***(8)***. Furthermore, as already shown ***(9,10)*** for other entrapped solutes, DNA-containing liposomal suspensions as prepared here can be freeze-dried (for storage) in the presence of a cryoprotectant without significant loss of material from within the vesicles on reconstitution with 0.9% NaCl.

2. Materials

1. Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidyl glycerol (PG), and phosphatidylserine (PS) (more than 99% pure) are available from Lipid Products (Nutfield, Surrey, UK), or Lipoid GmBH (Ludwigshaten, Germany).
2. Dioleoyl phosphatidylcholine (DOPE), distearoyl phosphatidylcholine (DSPC) and stearylamine (SA) are available from Sigma Chemical Co. (Poole, Dorset, UK).
3. 1,2-dioleyloxy-3-(trimethylamonium propane) (DOTAP) and 1,2-dioleyl-3-dimethyl- ammonium propane (DODAP) are available from Avanti Polar Lipids Inc. (Alabaster, AL).
4. 1,2-Bis (hexadecylcycloxy)-3-trimethylamino propane (BisHOP) was a gift from Syntex Corp. (Palo Alto, CA).
5. N[1-(2,3-dioleyloxy) propyl]-N,N,N-triethylammonium (DOTMA) was a gift from GeneMedicine (Houston, TX).
6. 3β(N,N,-dimethylaminoethane)-carbamyl cholesterol (DC-CHOL) was a gift from Dr. C. Kirby.
7. Sepharose (CL) 4B and polyethyleneglycol 6000 are available from Pharmacia (Hertfordshire, UK) Ltd.
8. Lipid solutions: dissolve 16 μmoles of PC and 8 μmoles of DOPE (or PE) in ~2–5 mL chloroform. For negatively charged (anionic) liposomes, add 4 μmoles of PA, PG or PS. For positively charged (cationic) liposomes add 4 μmoles of SA, BisHOP, DOTMA, DOTAP, DODAB or DC-CHOL. Greater amounts of charged lipids can be added, depending on the amount of vesicle surface charge required.

9. DNA solution: dissolve up to 500 μg of plasmid DNA in 2 mL distilled water or 10 m*M* sodium phosphate buffer pH 7.2 (PB) if needed. The nature of buffer in respect to composition, pH, and molarity can be varied as long as this does not interfere with liposome formation or entrapment yield. Amounts of added DNA can be increased in proportion to the total amount of lipid used.

3. Methods

The procedure for the entrapment of plasmid DNA into liposomes entails the preparation of a lipid film from which multilamellar vesicles (MLV) and, eventually, small unilamellar vesicles (SUV) are produced. SUV are then mixed with the plasmid DNA destined for entrapment and dehydrated. The dry powder is subsequently rehydrated to generate multilamellar "dehydration-rehydration" vesicles (DRV) ***(11)*** containing the plasmid DNA. On centrifugation, liposome-entrapped DNA is separated from non-entrapped DNA. When required, DNA-containing DRV are reduced in size by microfluidization in the presence or absence of non-entrapped DNA.

3.1. Preparation of the Lipid Film

1. Place the chloroform solution of lipids in a 50-mL round-bottomed spherical Quick-fit flask and remove the solvent in a rotary evaporator at about 37°C to leave a thin lipid film on the walls of the flask.
2. Flush the film for about 60 s with oxygen-free nitrogen (N_2) to ensure complete solvent removal and to replace the air.

3.2. Preparation of MLV

1. Pre-warm some distilled water and the flask containing the lipid film plus a few glass beads to a temperature above the liquid-crystalline transition temperature (Tc) of the phospholipid (>Tc).
2. Add 2 mL of the warm distilled water into the flask, replace the stopper and shake vigorously by hand or mechanically while maintaining the temperature >Tc until the lipid film has been transformed into a milky suspension.
3. Allow the suspension to stand at >Tc for about 1–2 h whereupon multilamellar liposomes of diverse sizes are formed.

3.3. Preparation of SUV

1. Remove the glass beads and sonicate the milky suspension at >Tc (with frequent intervals), using a titanium probe slightly immersed into the suspension. Keep the flask flushed with N_2 (achieved by the continuous delivery of a gentle stream of N_2 through thin plastic tubing). This step (*see* **Note 1**) should produce a slightly opaque to clear suspension of SUV of up to 80 nm in diameter.
2. Centrifuge the sonicated suspension of SUV for 2 min at 3000 rpm to remove titanium fragments, and allow the supernatant to stand at >Tc for about 1–2 h.

3.4. Dehydration of SUV Mixed with Plasmid DNA

1. Mix the SUV with the DNA solution and rapidly freeze the flask in liquid nitrogen while rotating it.
2. Freeze-dry overnight under vacuum (<0.1 torr).

3.5. Rehydration of the Powder

1. Add H_2O, pre-warmed at >Tc, (0.1 mL per 16 μmoles of PC) and swirl the mixture vigorously at >Tc (*see* **Note 2**). Keep the sample at >Tc for about 30 min.
2. Repeat the process with a further 0.1 mL H_2O.
3. After 30 min at >Tc, repeat with 0.8 mL PB (pre-warmed at >Tc) and allow the sample to stand for 30 min at >Tc. It now contains multilamellar DRV (size range about 0.5–5.0 μm in diameter) with entrapped and non-entrapped plasmid DNA

3.6. Separation of DRV Liposome-Entrapped from Non-Entrapped DNA

1. Centrifuge the suspension at 40,000*g* for 60 min at 4°C and resuspend the pellet (DNA- containing DRV) in H_2O (or PB).
2. Repeat the process at least once to remove the remainder of the non-entrapped material and resuspend the final pellet in an appropriate volume (e.g., 2 mL) of H_2O or PB (*see* **Note 3**).

3.7. Measurement of DNA Entrapment

The extent of DNA entrapment in DRV liposomes is monitored by measuring the DNA in the suspended pellet and combined supernatants. The easiest way to monitor entrapment is by using radiolabelled (^{32}P or ^{35}S) DNA (e.g., *see* **Table 1**). If a radiolabel is not available or cannot be used, DNA can be liberated from liposomes by adding Triton X-100 (up to 5% final concentration) or, preferably, isopropanol (1:1 volume ratio). If Triton X-100 or the solubilized liposomal lipids interfere with the assay of the DNA, it must be extracted using appropriate techniques ***(6)***. Entrapment values range between about 40–100%, depending on the amounts of lipid and DNA used and the presence or absence of a cationic charge. Highest values are achieved when DNA is entrapped in cationic DRV (**Table 1**) (*see* **Note 4**).

3.8. Microfluidization of DNA-Containing DRV

This step and those following are needed when DNA-containing DRV liposomes are to be converted to smaller vesicles (down to about 100 nm average mean diameter) while still retaining a substantial proportion of the DNA.

1. Take the rehydrated liposome preparation from 3.5 or above (prior to separation of trapped from non-trapped DNA) and dilute it to 10 mL with H_2O.

Table 1
Incorporation of Plasmid DNA into Liposomes by the Dehydration-rehydration Method

Liposomes	Incorporated plasmid DNA (% of used)					
	pGL2	pRc/CMV HBS	pRSVGH	pCMV4.65	pCMV4. EGFP	VR1020
PC, DOPE*a*	44.2	55.4	45.6	28.6		
PC, DOPE*b*	12.1		11.3			
PC, DOPE, PS*a*	57.3					
PC, DOPE, PS*b*	12.6					
PC, DOPE, PG*a*			53.5			
PC, DOPE, PG*b*			10.2			
PC, DOPE, SA*a*	74.8					
PC, DOPE, SA*b*	48.3					
PC, DOPE, BisHOP*a*	69.3					
PC, DOPE, DOTMA*a*	86.8					
PC, DOPE, DC-Chol*a*		87.1	76.9			
PC, DOPE, DC-Chol*b*			77.2			
PC, DOPE, DOTAP*a*		80.1	79.8	52.7	71.9	89.6
PC, DOPE, DOTAP*b*		88.6	80.6	67.7		81.6
PC, DOPE, DODAP*a*			57.4			
PC, DOPE, DODAP*b*			64.8			

^{35}S-labeled plasmid DNA (10-500 μg) was incorporated into, (*a*) or mixed (*b*) with neutral (PC, DOPE), anionic (PC, DOPE, PS or PG), or cationic (PC, DOPE, SA, BisHOP, DOTMA, DC-Chol, DOTAP or DODAP) dehydration-rehydration vesicles (DRV). Incorporation values for the different amounts of DNA used for each of the liposomal formulations did not differ significantly and were therefore pooled (values shown are means of values obtained from 3–5 experiments). PC (16μmoles) was used in molar ratios of 1:0.5 (neutral), and 1:0.5:0.25 (anionic and cationic liposomes).

Plasmid DNAs used encoded luciferase (pGL2), hepatitis B surface antigen (HBsAg, subtype ayw) (pRc/CMV HBS, available from Dr. R. Whalen), human growth hormone (pRSVGH), *Mycobacterium* leprosy protein (pCMV 4.65, available from Dr R. Tascon), "fluorescent green protein" (pCMV 4.EGFP) and Schistosome protein (VR1020) (from ***[1]*** with permission).

2. Cycle the suspension several times through a Microfluidizer 110S (Microfluidics, Newton, MA). The pressure gauge is set at 60 psi throughout the procedure to give a flow rate of 35 mL per min (*see* **Note 5**).

3.9. Separation of DNA Entrapped in Microfluidized DRV from Non-entrapped DNA

1. Reduce the volume of the microfluidized sample by placing it in dialysis tubing which is then covered with polyethyleneglycol 6000 in a flat container (*see* **Note 6**).
2. When the required volume has been reached, separate entrapped from free DNA by molecular sieve chromatography using a Sepharose CL 4B column, or by centrifugation as in **Subheading 3.6.**
3. Estimate the DNA within liposomes as % of DNA relative to the original amount as described above (*see* **Note 7**). Liposome size measurements can be carried out by photon correlation spectroscopy as described elsewhere ***(6,12)***. The DNA-containing cationic liposomes can also be subjected to microelectrophoresis in a Zetasizer to determine their net surface charge.

4. Notes

1. The time required to produce SUV varies, depending on the amount of lipid used and the diameter of the probe. For the amounts of lipid mentioned above, a clear or slightly opaque suspension is usually obtained within up to four sonication cycles, each lasting 30 s, with 30 s rest intervals in between, using a probe of 0.75 inch diameter. The process of sonication is considered successful when adjustment of the settings in the sonicator is such that the suspension is agitated vigorously.
2. The volume of H_2O added must be kept at a minimum, with just enough H_2O to ensure complete hydration of the powder under vigorous swirling.
3. When the liposomal suspension is destined for in vivo use (eg. intramuscular injection), NaCl is added to a final concentration of 0.9%.
4. As part of the liposome-associated DNA may have interacted with the liposomal surface (e.g., cationic groups) during the entrapment procedure, it may be necessary to determine actual entrapment of the DNA (as opposed to surface-bound DNA). This can be achieved by the use of deoxyribonuclease which will degrade most of the external material ***(6)***. Our experience ***(6)*** shows that most of the DNA entrapped within liposomes remains intact on exposure to ribonuclease, presumably protected by the surrounding bilayers.
5. The number of cycles used depends on the vesicle size required ***(6)*** or on the sensitivity of the plasmid DNA. In the case of pGL2 (*see* **Table 1**), microfluidization for more than three cycles resulted in progressive smearing of the DNA and failure to transfect cells in vitro ***(6)***. It is likely that other plasmid DNAs will behave similarly on extensive microfluidization. Microfluidization of the sample can also be carried out after the removal of non-entrapped DNA as in **Subheading 3.6.** ("washed liposomes"), although DNA retention in this case may be reduced: the presence of non-entrapped DNA during microfluidization (a pro-

cess that destabilizes liposomes, which then reform as smaller vesicles) is expected ***(6)*** to diminish DNA leakage, perhaps by reducing the osmotic rupture of vesicles ***(11)***.

6. Removal of excess H_2O from the tubing is relatively rapid and it is therefore essential that the sample is inspected regularly.
7. When the sample is microfluidized following **Subheading 3.5.**, i.e., before the estimation of entrapment, it is necessary that a small portion of the sample which is to be microfluidized, be held aside for the estimation of entrapment, according to **Subheading 3.7.**

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DNA-Based Vaccination with Polycistronic Expression Plasmids

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1. Introduction

DNA-based vaccination is a potent technique to prime cellular (T-cell mediated) immune responses (reviewed in ***1***). Many details of the priming of T-cell precursors by antigen translated from injected expression plasmid DNA are unknown. The relevant cell that is transfected in situ after DNA vaccination and that can process and present the protein in an immunogenic form has not yet been identified. Alternatively, the transfected cell may initiate 'cross-priming' in vivo by transferring processed antigen to a professional antigen-presenting cell (APC).

Normally only a single protein is translated from a eukaryotic mRNA. However, efficient internal ribosomal entry sites (IRES) that support cap-independent initiation of translational have been identified in certain viruses (e.g., picornaviruses) (*see* **Notes 1**,**2**). This observation has been used to construct polycistronic expression plasmids in which different proteins are translated from a single mRNA species. The translational efficiency of individual cistrons in multicistronic mRNAs is influenced by the IRES element used and by the RNA sequences located between the IRES elements (or the cap) and the AUG initiation codon (*see* **Note 3**).

Expression of different cistrons in polycistronic expression plasmids is strictly coupled. Although the cotransfer of different genes into a cell can be achieved by mixing different plasmids, gene expression from such mixtures of plasmids is never balanced. Protein expression of the different genes, and there-

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

fore antigen presentation, will vary significantly (even if the same promoter/enhancers are used to control expression). To circumvent these problems, we used polycistronic plasmids to deliver different antigens by injection of a single DNA species (*see* **Notes 4** and **5**). As model antigens, we used the surface (HBsAg) and core (HBcAg) antigen of the hepatitis B virus (HBV). We have characterized the murine cellular (CTL), and humoral (serum antibody), response that can be elicited by DNA-based vaccination in H-2^d and H-2^b mice ***(2–5)***.

Polycistronic expression plasmids contain a promoter/enhancer element, an IRES element, selected IRES-flanking sequences, different sets of multiple cloning sites, intron-encoding sequences, and/or a polyA signal (*see* **Note 3**). To facilitate construction of polycistronic plasmids, we designed different monocistronic expression plasmids from which elements can be taken to construct di- and tricistronic plasmids (see **Note 6**).

2. Materials

2.1. Construction of Monocistronic pCI-1 and pCI-2 Expression Plasmids

1. pCI-1: The high copy number plasmid pCI (Promega, catalogue no. E1731) was digested with *Eco*RI and *Not*I. A 675 bp *Not*I–*Eco*RI insert derived from pVBC3, containing the IRES and approximately 80 bp of flanking sequence, was cloned into pCI, resulting in the pCI-1 plasmid (**Fig. 1**).
2. pCI-2: The pCI plasmid was digested with *Sal*I and *Xho*I and religated, resulting in the pCI$^-$ plasmid. A 687 *Eag*I fragment of the pVBC2 vector containing the IRES sequence and about 50 bp of flanking regions was cloned into the *Not*I site of pCI$^-$, resulting in plasmid pCI-2 (**Fig. 1**).

2.2. Construction of Dicistronic Expression Plasmids Containing Two Different Antigens

1. The pCI-1 plasmid contains a unique *Not*I-site following the IRES sequence. Into this site, the *Eag*I fragment from pCI-2 (containing the gene of interest followed by another IRES element) was inserted. This eliminates the upstream *Not*I site, but leaves the downstream *Not*I site intact. This strategy can therefore be used to construct tri- or polycistronic plasmids. **Figs. 2A** and **B** demonstrate representative examples of the cloning strategy.
2. The *Sma*I/*Hind*III fragment from plasmid pCMV-2/C ***(6)*** encodes the complete core antigen (HBcAg) sequence.
3. This fragment was inserted into the *Sma*I site of pCI-1 to generate the vector pCI-1/C.
4. The *Sal*I/*Xho*I fragment from plasmid pCMV-S is derived from pSBC-1 ***(6)*** into which the *Stu*I fragment encoding the HBsAg of HBV was cloned. The SV40 promoter of this plasmid was replaced by the HCMV promoter of plasmid BCMG*neo* (a generous gift of Dr. H. Karasuyama, Basel, Switzerland).

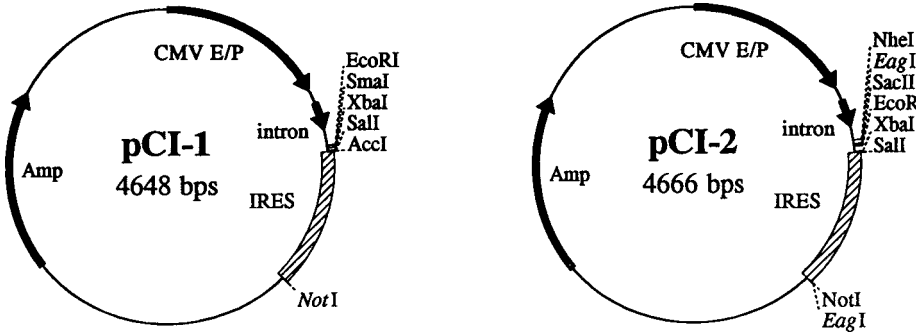

Fig. 1. Monocistronic expression plasmids pCI-1 and pCI-2.

5. This fragment was cloned into the *Sal*I-site of pCI-2 to generate the pCI-2/S vector.
6. The *Eag*I fragment of pCI-2/S (containing the HBsAg-encoding region and the IRES) was inserted into the *Not*I site of pCI-1/C to generate the dicistronic expression plasmid pCI/C-S.

2.3. Special Materials

1. Target cells: monkey COS-7 kidney cells (ATCC; CRL-1651), murine L929 fibroblasts (ATCC, CCL-1), chicken LMH hepatoma cells.
2. Antisera: polyclonal rabbit anti-HBsAg antiserum (a generous gift of the Behring AG, Marburg, Germany), polyclonal rabbit anti-HBcAg antiserum Hc-1 (a generous gift of Dr. H. J. Schlicht).
3. Protein-A Sepharose (cat. no. 17-0780-01, Pharmacia, Freiburg, Germany).
4. Ultrapure 100 anion exchange chromatography columns (Qiagen, Hilden, Germany).
5. Cardiotoxin solution (Latoxan, catalogue no. L8102, Rosans, France).
6. Anti-HBsAg IMxAUSAB test kit (catalogue no. 2262-20, Abbott, Wiesbaden, Germany)
7. Anti-HBcAg CORE anti-HBc test kit (catalogue no. 2259-20, Abbott).
8. A reference sample from the Paul-Ehrlich-Institute (Langen, Germany).

3. Methods

3.1. Coexpression of HBsAg and HBcAg from Dicistronic Expression Plasmids

Coexpression of HBsAg and HBcAg from the pCI/S-C and pCI/C-S can be analyzed in transient transfection assays, using COS-7, L929, or LMH cells. Antigens cloned into the first cassette of the bicistronic vectors are expressed at levels comparable to those in monocistronic constructs. In contrast, antigens cloned into the second cassette are expressed at 3–20-fold lower levels than observed in monocistronic vectors.

1. Grow cells to a density of 10^4-10^5 cells/mL in 100 mm tissue culture dishes in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) FCS.

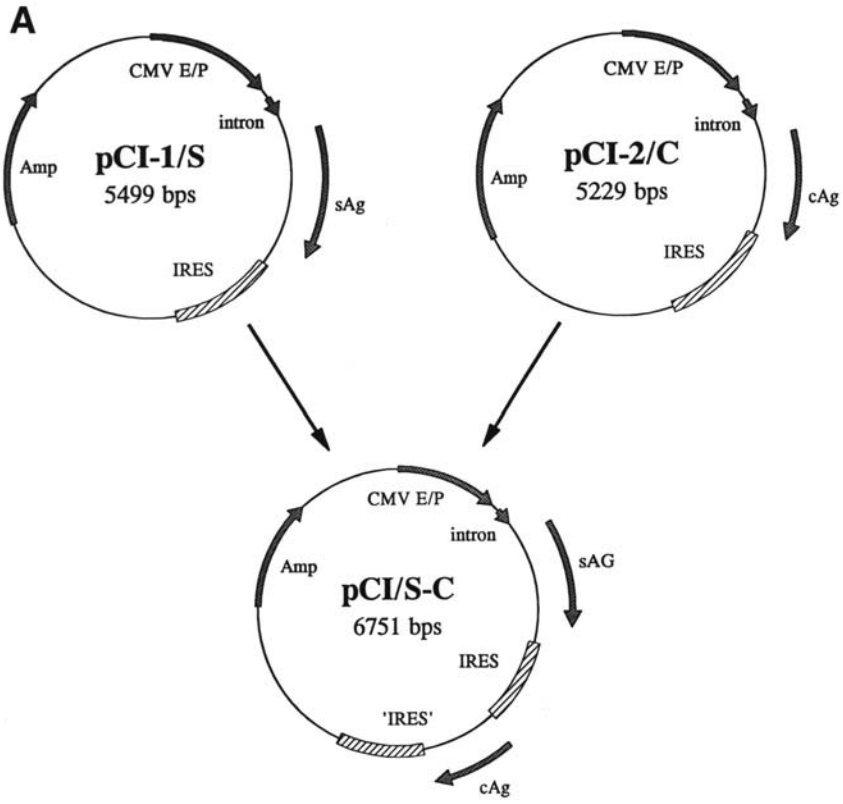

Fig. 2. [Construction of dicistronic expression plasmids encoding surface (HBsAg) and core (HBcAg) antigen of HBV.] **(A)** The dicistronic plasmid pCI/S-C was constructed by inserting the HBcAg/IRES-containing *Eag*I fragment from pCI-2/C into the *Not*I-digested pCI-1/S plasmid.

2. Transfect 1 µg plasmid DNA per dish using the $CaPO_4$-method.
3. Test transient expression of HBsAg and HBcAg by [^{35}S]-methionine labeling of cells at 48-72 h post transfection.
4. Lyse cells and remove cell debris by centrifugation for 30 min, 20,000*g* at 4°C.
5. Immunoprecipitate HBsAg by adding 5 µg of a polyclonal rabbit anti-HBsAg antiserum. Precipitate HBcAg by adding 5 µg of a polyclonal rabbit anti-HBcAg and 50 µL protein-A dissolved in PBS.
6. Process the immunoprecipitates for SDS-PAGE and fluorography.

3.2. Preparation of Plasmid DNA Used for DNA-based Vaccination

Plasmid DNA to be used for DNA vaccination is purified by anion exchange chromatography.

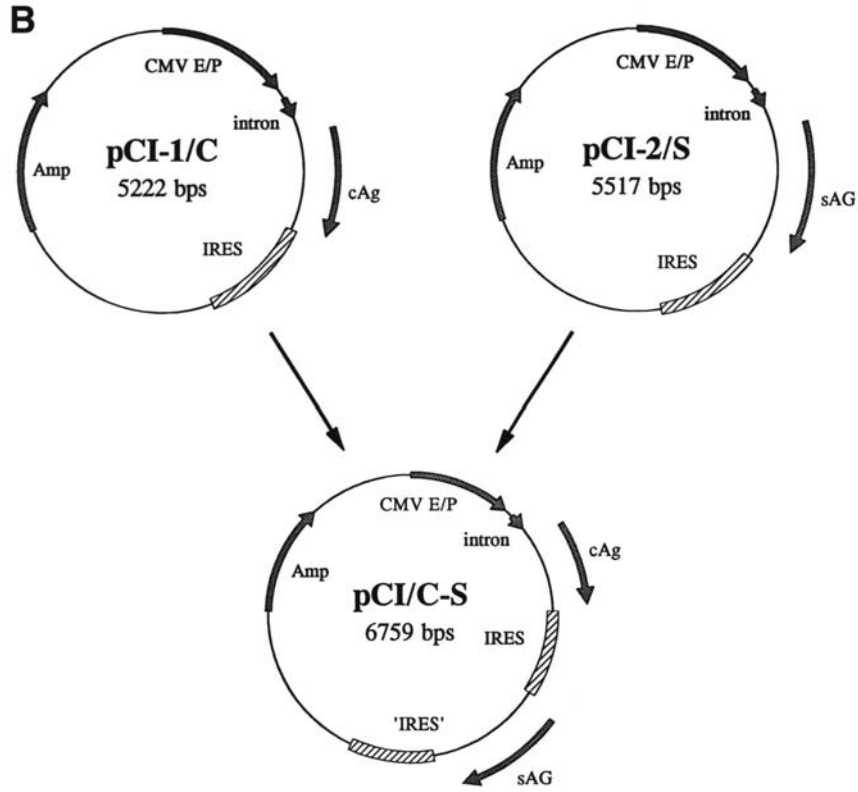

Fig. 2. [Construction of dicistronic expression plasmids encoding surface (HBsAg) and core (HBcAg) antigen of HBV.] **(B)** The dicistronic plasmid pCI/C-S was constructed by inserting the HBsAg/IRES-containing *Eag*I fragment from pCI-2/C into the *Not*I-digested pCI-1/C plasmid.

1. Harvest about 60 g of wet weight of biomass from 5 L cultures of cloned *E. coli* DH5 cells harboring the plasmid DNA; submit the bacteria to alkaline lysis.
2. Isolate about 100 mg plasmid DNA from the lysate by use of ultrapure anion exchange columns.
3. Suspend plasmid DNA at 10 µg/µL in 10x TE buffer (100 m*M* Tris-HCl, 10 m*M* EDTA, pH 7.4) and store at –20°C (*see* **Note 7**).
4. Dilute this solution 1:10 with Ca^{++}/Mg^{++} -free PBS, within 30 min before injection, to obtain a 1 µg/µL DNA solution.

3.3. Intramuscular Nucleic Acid Inoculation

1. Inject 100 µL of a 0.01 m*M* cardiotoxin solution into each tibialis anterior or quadriceps muscle of the mice.

2. Five days later, inject 50 µL of the 1 µg/µL DNA solution into each regenerating tibialis anterior or quadriceps muscle.
3. Use non-injected mice or mice injected with plasmid DNA without insert as negative controls.

3.4. Cytotoxic T Cell Assay (see Note 8)

1. Obtain spleens from DNA-vaccinated BALB/c (H-2^d) mice.
2. Prepare single cell suspensions from these spleens in α-MEM tissue culture medium supplemented with 10 m*M* HEPES buffer, 5×10^{-5} *M* 2-mercaptoethanol, antibiotics and 10% (v/v) of a selected batch of fetal calf serum (FCS).
3. Co-culture 3×10^7cells with 1.5×10^6 irradiated (20,000 rad) stimulator cells in a mixed tumor cell-lymphocyte culture (MTLC). Maintain 10 mL volume aliquots in 25 cm^2 upright tissue culture flasks in a humidified atmosphere of 5% (v/v) CO_2 at 37°C. Use stimulator cells taken from syngeneic P815 mastocytoma transfectants expressing either the small hepatitis B antigen (HBsAg), or the hepatitis core antigen (HBcAg).
4. Harvest in vivo-primed and in vitro-restimulated spleen cell populations from the 5-d MTLC. Wash the cells twice and coculture in 200 µL round-bottom wells for 4 h at 37°C at titrated densities with 2×10^3 [^{51}Cr]-labeled targets. Use effector/target ratios in the range of 1–20.
5. Collect 100 µL of the supernatant for γ-radiation counting. Calculate the percent specific release as [(experimental release – spontaneous release)/(total release – spontaneous release)] × 100. Measure total counts by resuspending target cells. Measure spontaneously released counts using target cell cultures without cytolytic effector cell populations. Plot the data as the mean of triplicate cultures. The SEM of triplicate data was usually less than 10% of the mean. **Figs. 3C,D** show representative examples of the anti-HBsAg and anti-HBcAg cytotoxic T lymphocyte (CTL) response of BALB/c (H-2^d) mice immunized with mono- or dicistronic expression plasmid DNA.

3.5. Serum Antibody Assays

Use the IMxAUSAB test to measure anti-HBsAg titers and the CORE kit to measure anti-HBcAg in sera of immunized mice at different time points post-vaccination as follows below.

3.5.1. Serum Antibodies Against HBsAg

1. Mix microparticles coated with HBsAg with mouse serum samples to allow specific binding of anti-HBsAg antibodies.
2. Bind the microparticles coated with antigen-antibody complexes to the glass fibre matrix.
3. Add biotinylated recombinant HBsAg.
4. Quantify the binding using an alkaline phosphatase-conjugated anti-biotin antibody followed by the substrate 4-methyl-lumbelliferyl-phosphate.

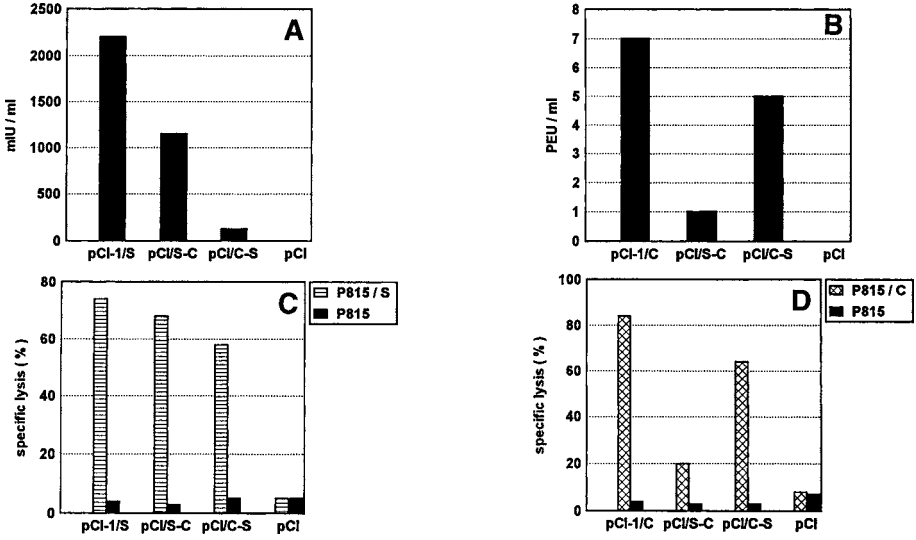

Fig. 3. [Polyvalent induction of humoral and cellular immune responses using dicistronic plasmid DNA.] BALB/c mice were intramuscularly injected once with 100 µg monocistronic pCI-1/C or pCI-1/S plasmid DNA, dicistronic pCI/S-C plasmid DNA, dicistronic pCI/C-S plasmid DNA, or pCI plasmid DNA without insert. Five wk post-vaccination, the serum antibody titers against HBsAg **(A)** and HBcAg **(B)**, and the MHC-I-restricted cytotoxic T lymphocyte (CTL) response against HBsAg **(C)** and HBcAg **(D)** were measured.

5. Quantify the antibody levels using six standard serum dilutions (0–1000 mIU/mL); dilute the test sera so that the measured OD values fall within the standard curve, and calculate values as mIU/mL.

3.5.2. Serum Antibodies Against HBcAg

1. Follow the instructions supplied with the CORE kit.
2. Standardize the concentrations of anti-HBc against the reference sample from the Paul-Ehrlich Institute (Langen, Germany) and express the results as Paul-Ehrlich units (PEU)/mL.

Figs. 3A and **B** show representative examples of anti-HBsAg and anti-HBcAg serum antibody responses of BALB/c (H-2^d) mice immunized with the mono- or dicistronic expression plasmid DNA described.

4. Notes

1. The use of IRES elements allows the co-expression of two or more cistrons from one promoter. Tricistronic expression has been successful (R. Schirmbeck, unpublished results). The promoter/enhancer element defines the strength of

expression, which also depends on the cell type in which the genes are to be expressed. The use of a promoter that is active in most cell types, such as the HCMV promoter in pCI, is advisable.

2. Different IRES elements are available today. However, only few of them show strong translational enhancement in different tissues. Currently, the IRES element from the poliovirus (as it is used in this work), and the IRES element from Encephalomyelocarditis Virus (EMCV), are the most universal and strongest sequences known. Since the promoter/enhancer, as well as the IRES element, have an influence on the expression of the individual cistrons in the tissue, the strength and ratio of expression might vary to some extent. This has to be considered if experiments carried out in primary or continuous cell lines are transferred to animals in particular, as it is not known in which tissue the immunogenic response is initiated.
3. In using IRES elements, the following points have to be considered: (i) The first cistron, usually a cDNA, should not have a poly (A) signal. Otherwise, premature termination would result in monocistronic expression; and (ii) In using the polio virus IRES element, the distance between the IRES element and the AUG of the second cistron is variable. However, in using the EMCV IRES, the AUG has to be exactly positioned in order to get strong translational re-initiation.
4. Coexpression of two or more distinct gene products at stochiometrically defined ratios from a single transcript within the same APC allows novel options in DNA-based vaccination. Polycistronic expression plasmids can deliver a multivalent vaccine with a single injection, i.e., it can stimulate the generation of specific immune reactivities against two or more antigens from the same or different pathogens. Coexpression of different antigens by the same APC is expected to provide an immune response-enhancing "helper" effect. We have demonstrated this using HBsAg and HBcAg *(7)*: (i) polycistronic expression plasmids can codeliver an antigen and a cytokine to the same APC; and (ii) polycistronic expression constructs allow the coexpression of the antigen and an inducible suicide gene in the *in situ* transfected cell. This can restrict the presence of in vivo transfected cells in the vaccinated organism to a limited period of time (sufficient to prime an immune response). Subsequent elimination of all transfected (biologically relevant or bystander) cells can be achieved by delivering an external stimulus. This excludes persistent genetic alteration of the immunized host as a result of DNA-based vaccination.
5. Major objectives in the design of DNA vaccines are the enhancement of the immunogenicity of antigens expressed from expression plasmids, and the selective modulation of the spectrum of effector functions that the elicited T cell responses mediate. Two major observations stress the relevance of these objectives: (i) Only particular functional subclasses of T cells are able to confer protective immunity against certain pathogens while others mediate the immunopathology accompanying persisting infections. To prime protective responses, and to avoid complications of immunopathology, DNA vaccination will have to achieve efficient, selective, and stable priming of the appropriate T cell reactivity; and (ii)

DNA vaccination may be useful to convert an established inefficient, potentially pathogenic T cell response into a protective immune response. This is relevant in the immunotherapy of cancer and allergy, and in the design of protective, therapeutic vaccines for chronic persistent infections. Here DNA vaccination will have to overcome an established, inappropriately polarized T cell response and convert it into T cell reactivity with an alternative functional phenotype.

6. Adjuvants have played a prominent role in formulating vaccines. 'Naked,' nonmethylated plasmid DNA has strong adjuvanticity for T-cell responses shifting them towards the TH^1 phenotype as has been known for a long time for poly I:C [reviewed in ***(8)***]. The mechanism of action of adjuvants is supposed to be mediated by inducing and/or amplifying the *in situ* cytokine milieu. A rational approach to adjuvants would be the codelivery of selected cytokines with antigen. Codelivery of cytokines with antigen in DNA-based vaccination has been achieved by different means. Current strategies in DNA-based vaccinations use the following approaches: (i) vaccination with a mixture of expression plasmids that encode either an antigen, or a cytokine; (ii) vaccination with complex expression plasmids that express the antigen and a cytokine under separate promoter controls; (iii) vaccination with expression plasmids encoding a fusion protein composed of the antigen (or an immunogenic domain of the antigen) and a cytokine; and (iv) vaccination with polycistronic expression constructs encoding the antigen and cytokine(s).

   We have described the latter strategy. The potential of this approach, and the advantages and disadvantages of this approach relative to alternative strategies, will have to be investigated in different animal models and different antigen systems.
7. Quality controls showed that this plasmid DNA typically contains <100 endotoxin units/mg DNA, >90% supercoiled DNA, and <1% (w/w) residual protein content.
8. T-cell responses have a "strength" and a "functional phenotype." A specific T cell reactivity can be "strong" or "weak" reflecting (among other parameters), the number of T cell clones specifically activated and their avidity for antigen. T-cell responses are usually polarized, i.e., the primed T cell clones mediate distinct sets of effector functions. The main functional subclasses of $CD4^+$ T cells designated Th^1 and Th^2 show differences in the profile of secreted cytokines, in the susceptibility to 'activation-induced cell death,' in CD95/CD95L-dependent cytolytic reactivity, and in cell contact-dependent APC activation. Furthermore, Tc1 and Tc2 subsets have been identified in primed $CD8^+$ cytotoxic T lymphocyte (CTL) populations that are less well characterized.

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A Nonviral Cytoplasmic T7 Autogene System and Its Applications in DNA Vaccination

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1. Introduction

The use of DNA vectors to elicit an immune response has produced a lot of interest. Unfortunately, one of the limiting factors has been the problem of gene expression. In order to obtain a strong expression of the vaccinating gene, several steps are necessary. The vector has to be delivered in such a way that it is not being degraded by the immune nor by the hepatic system; it has also to enter efficiently the targeted cells; and it must be expressed in the appropriate compartment of the cells at a high level. For these reasons, we have developed a gene expression vector that contains a T7 autogene and is being expressed in the cytoplasm of the cells ***(1,2)***. We will describe this system and two possible applications: infectious disease vaccination and tumor ablation. The latter application may be combined with DNA vaccination against cancer cells.

1.1. Description of the T7 Autogene System

Traditionally, a nonviral mammalian gene expression vector consists of a circular plasmid DNA encoding a gene driven by a eukaryotic promoter. Since the eukaryotic promoter functions only in the nuclei of mammalian cells, the plasmid DNA has to be transported into the nuclei for gene expression. In contrast, the T7 vector has a cytoplasmic expression because it contains a bacteriophage T7 promoter that is neither recognized nor used by the mammalian transcriptional machinery. This T7 promoter is functional in the cytoplasm of the transfected mammalian cells when coinjected with T7 RNA polymerase (RNAP) because the T7 RNAP is a bacterial protein and it localizes in the cytoplasm when introduced into mammalian cells ***(3)***. The T7 vector has been

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

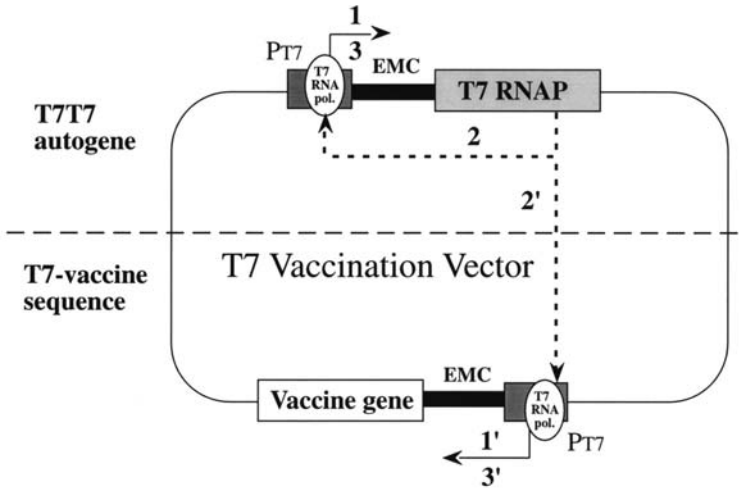

Fig. 1. Schematic structure of the T7 vaccination vector. A general T7 vaccination gene expression vector contains a T7 autogene (a T7 RNAP gene driven by a T7 promoter (P_{T7})) as shown in the top half of the vector, and a vaccine gene (in this study, it can be either a HA, or a HIV gp120 genes) driven by a second P_{T7} as shown in the bottom half of the vector. EMC sequences provide ribosome internal entry sites (IRES) for efficient translation of uncapped mRNAs generated in the cytoplasm, 1 and 1'. When the vector is cotransfected with T7 RNAP into a target cell, the T7 RNAP initiates the transcription from the two P_{T7} in the cytoplasm of the transfected cells. 2 and 2', cytoplasmically transcribed T7 RNAP mRNA will be translated into T7 RNAP protein which bind to the two P_{T7} for 3 and 3' more transcriptions of both the T7 RNAP and vaccine genes. The ratio between T7 autogene and the vaccine gene can be further adjusted by separating the two sequences into two plasmids (pT7T7 and pT7-vaccine), as in the case for the pT7gag.

constructed to express high levels of mRNA and proteins by including a T7 autogene. The T7 autogene is defined as a T7 RNAP gene driven by its own cognate T7 promoter ***(1,4)***. With such a genetic arrangement, a positive feedback loop is formed. The T7 RNAP enzyme produced from the T7 RNAP gene can return to the T7 promoter, making more and more T7 transcripts ***(1)*** (**Fig. 1**). Another T7 promoter leading the expression of the gene of interest can benefit from this strong T7 RNAP production and allow for the expression of this gene. This T7 autogene system have been found to rapidly express high levels of proteins in cultured cells ***(1,2)*** and in various animal tissues ***(2)***.

1.2. Humoral Immunity Induced by the T7 System

When the pT7T7/T7HA, a plasmid vector containing both a T7 autogene and an influenza viral hemagglutinin gene driven by a second T7 promoter,

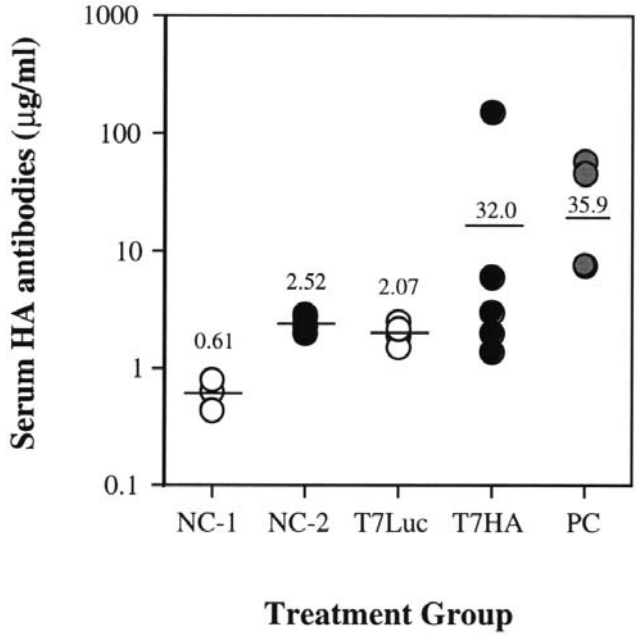

Fig. 2. Detection of anti-HA antibodies in BALB/c mice immunized with pT7T7/T7HA. Immunocompetent BALB/c mice were immunized with pT7T7/T7HA as described in Methods. ELISA results from the second bleeding were shown in this figure. NC-1 = sera from three uninjected mice from the site where the ELISA was performed; NC-2 = sera from 5 uninjected mice from the site where the plasmid injections were performed; T7Luc = sera from 5 mice injected with pT7T7/T7Luc, a luciferase- (irrelavant-) gene encoding plasmid; T7HA = sera from 5 mice injected with pT7T7/T7HA; and PC = sera from three mice infected with influenza virus PR8.

was repeatedly coinjected with T7 RNAP into muscles and tail connective tissues of immunocompetent BALB/c mice, specific anti-HA antibodies were detected in three out of the five injected mice (**Fig. 2**).

1.3. Cellular Response Induced by the T7 System

Specific CTL response against HIV-1 p55gag was induced by intramuscular immunization with a T7 vaccine system (**Table 1**) ***(5)***. The T7 vaccine system consists of three components: a pT7gag plasmid encoding a p55gag gene driven by a T7 promoter, a second plasmid pT7T7, and T7 RNAP. This result demonstrates for the first time that regulated expression of vaccine genes in vivo could be accomplished using this T7 system. This system may be useful in vaccine settings where short-term cytoplasmic expression of protein in antigen presenting cells is desirable.

Table 1
HIV-1 p24gag-specific CTL Activity in Mice Immunized with T7 Vaccine System

				Percent specific lysis of targets		
				SV Balb		MC57
pT7 gag[a]	pT7T7	T7 RNAP	E:T ratio[b]	Untreated	p24(199–207)	p24(199–207)
50 μg	50 μg	2,500 U	50:1	<1[c]	13	<1
			10:1	<1	8	2
			2:1	<1	3	2
			50:1	<1	51	2
			10:1	<1	27	1
			2:1	<1	17	<1
			50:1	<1	30	<1
			10:1	<1	16	<1
			2:1	<1	6	<1
50 μg	50 μg	10,000 U	50:1	<1	31	<1
			10:1	1	25	<1
			2:1	2	11	<1
			50:1	3	38	1
			10:1	3	19	2
			2:1	2	19	2
			50:1	4	44	5
			10:1	3	27	3
			2:1	1	13	2

[a]Balb/c mice were immunized i.m. with pT7gag, pT7T7, and T7 RNAP in DOTAP. Results are shown for 3 individual mice per group. Results are representative of two replicate experiments.

[b]E:T ratio is the effector:target cell ratio.

[c]Values represent percent specific 51Cr release in a 4 h assay.

1.4. Tumor Ablation

DNA vaccination against cancer cells is a tantalizing application of DNA vaccination. The existence of tumor-specific antigens combined with the use of cytokines to enhance the immune response of the patient seems to be an appealing path for treating metastatic cancers. Although the T7 vector has not been used yet to induce a vaccination against cancer cells, it has been used to ablate tumors grafted in nude mice ***(6)***. A human osteosarcoma cell line (143B)

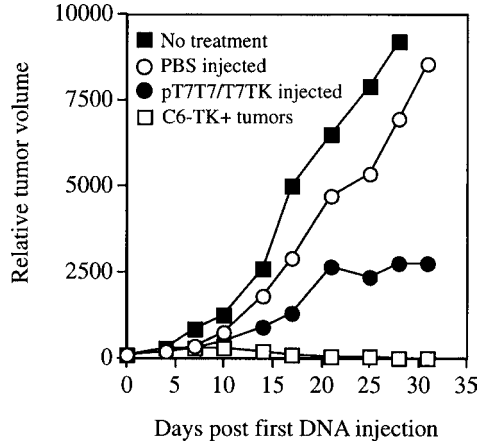

Fig. 3. Tumor growth inhibition by direct tumor injections of pT7T7/T7TK DNA. 143B tumors are generated in the flank of nude mice by intradermal injection of 10^6 cells. Ten days after the tumor cells injection, the tumors are being injected with 20 μg of DNA prebound with 1000 units of T7 RNAP with tuberculin syringes mounted with 28G needles. The tumor injections are performed every three days for 21 d. GCV is injected i.p. twice a day for 28 d. The tumor sizes are monitored with a caliper twice a week. The tumor growths are represented in relative volumes with the initial tumor volumes of all treatment groups at d 0 (the day the tumor-bearing nude mice started to receive T7 DNA and GCV injections) assigned as 100.

was grafted in the flank of nude mice. The tumors were thereafter injected with a T7 vector bearing the thymidine kinase gene. The prodrug ganciclovir was injected intraperitoneally and tumor sizes were monitored with a caliper. The tumors treated with the T7-TK vectors exhibited a much slower growth pattern (–66%) compared to the control animals (**Fig. 3**).

2. Materials

2.1. Plasmids and Humoral Immunity

2.1.1. Plasmid Constructions

1. *Escherichia coli* strains: HMS174(DE3)pLysE from Novagen (Madison, WI) for propagation of T7 autogene-containing plasmids, and DH5α (Gibco-BRL, Gaithersburg, MD) for plasmids that do not contain the T7 autogene (*see* **Note 1**).
2. Restriction enzymes and T7 RNAP (New England BioLabs, Beverly, MA).
3. LipofectAMINE as transfection agent (Gibco-BRL).

2.1.2. Immunochemical Staining and Hemadsorption

1. C6 cells or any other adherent mammalian cells that can be easily transfected by LipofectAMINE.

2. Slide chambers for transfection (Nunc International, Rochester, NY).
3. Anti-HA antibodies obtained from Dr. Walter Gerhard of Wistar Institute, University of Pennsylvania, Philadelphia.
4. Vectastain Elite ABC Ig G kit and peroxidase AEC kit from Vector Laboratories (Burlingame, CA) for immunochemical staining.
5. 1–5 cc human red blood cells.
6. PBS for hemadsorption.

2.1.3. DNA Injections (**see Note 2**)

1. Five milliliter sterile polystyrene tubes, P20, P200, and P1000 pipets, sterile tips, and a tube-holding rack and PBS for preparing the DNA solution.
2. One cubic centimeter tuberculin plastic syringes and 28G needles for injection.
3. High quality (Cesium chloride or Qiagen purified) pT7T7/T7HA DNA resuspended in endotoxin-free water.
4. T7 RNAP (1000 units/μL) from New England BioLabs.
5. Ice bucket for temporary storage of DNA and T7 RNAP.

2.2. Cellular Immunity (**see Note 3**)

2.2.1. Plasmids, Synthetic Peptides, and Recombinant Proteins

1. Plasmids pT7gag and pT7T7, T7 RNAP as three components of the T7 vaccine.
2. Synthetic epitope peptide HIV-1 p24gag (p24(199-207)) synthesized with free amine N-terminal and free acid C-terminal using Fmoc solid phase methods by Research Genetics (Huntsville, AL), and p24 gag protein purified from yeast cells.

2.2.2. Animals and Immunizations

1. Balb/c (H-2d) 4–6 wk-old mice from the Charles River Laboratories, the National Institute of Health)
2. Injection materials are the same as in **Subheading 2.1.3.**

2.2.3. Lymphocyte Cultures

1. 24-Well cell culture plates.
2. RPMI 1640 and α-MEM media (Gibco-BRL), fetal calf serum, mercaptoethanol, antibiotics.
3. IL-2 (Rat T-Stim, Collaborative Biomedical Products, Bedford, MA).

2.2.4. Cytotoxic Cell Assays

1. Radioisotope ^{51}Cr (1 Ci = 37 Gbq).
2. 96-Well round bottom tissue culture plates.

2.3. Tumor Ablation

1. High quality pT7-TK plasmid DNA.
2. The human osteosarcoma 143B cell line can be obtained from the American Tissue Culture Collection (ATCC).

3. DMEM plus 10% fetal bovine serum, trypsin, and PBS.
4. Female inbred Nu-nu mice obtained from Charles River Laboratories.
5. Injection materials for DNA: 28G needles and tuberculin syringes.
6. Injection materials for GCV: 28G needles and tuberculin syringes.
7. Stock solution of ganciclovir (Cytovene, Syntex Laboratories, Palo Alto, CA).
8. Anesthetic solution.
9. Caliper.

3. Methods

3.1. Humoral Immunity

3.1.1. Construction of the T7 Vaccination Vector

In order to evaluate the T7 system in DNA vaccination application, a human hemagglutinin (HA) gene from influenza virus strain Mount Sinai PR8 ***(7)*** was chosen as a model system for humoral immunity study. First, the HA gene was removed from the original plasmid vector pT3PR8HA ***(8)*** using *Nhe*I/*Xba*I restrictions. The 1.8 kb HA fragment was then inserted into the SpeI site of pT7-2, a derivative of pTM-1 ***(1)***. Then, for efficient gene expression in the T7 vector, both of the 5' and 3' untranslated regions of the HA gene were deleted using a phagemid mutagenesis approach ***(9)***. The resultant plasmid vector was named as pT7HA. To explore a single T7 vector system, a *Not*I DNA fragment containing both the T7 promoter and the HA gene was removed from the pT7HA and inserted into the *Not*I site of pT7T7-N, a derivative of pT7T7 ***(1)***. The resultant final plasmid was designated pT7T7/T7HA. The correct deletions and the junction sequences between the internal ribosome entry site sequence EMC ***(1)***, the 5' end of the HA gene, and between the 3' end of the HA gene and the T7 termination sequence ***(1)***, were verified by DNA sequencing analysis ***(10)***.

3.1.2. Hemadsorption and Immunochemical Staining of Cells Transfected with pT7T7/T7HA Vector

To test whether the vector pT7T7/T7HA was functional and the HA protein could be produced, properly processed, and presented on the cell surface, pT7T7/T7HA was introduced into rat glioma C6 cells with T7 RNAP by lipofection ***(1,2)***. Twenty-four hours after transfection, the transfected cells were analyzed for the expression of the HA genes by either hemadsorption using human red blood cells (RBC) or immunochemical staining using anti-HA antibodies. The immunochemical staining revealed that as high as 30% of the transfected C6-Bu cells expressed the HA protein and the hemadsorption assay showed that the HA protein molecules were properly processed by the transfected C6 cells and presented on the cell surface as demonstrated by the strong hemadsportion of human RBC on a proportion of transfected C6 cells.

3.1.3. Induction of Humoral Immune Response Against HA in Mice by Injection of pT7T7/T7HA Vector

1. Once the functionality of the gene expression vector pT7T7/T7HA was confirmed by assays using cultured cells, an animal study was carried out to test if pT7T7/T7HA could induce the humoral immune response in animals.
2. BALB/c 2–4 mo-old mice, 5 per group, were injected with 20 μg of pT7T7/T7HA plus 1,000 units of T7 RNAP in PBS solution in a total volume of 50 μL into the mouse tail base (connective tissue) and leg muscle, 25 μL for each tail and muscle, respectively. The pT7T7/T7HA DNA and T7 RNAP were first mixed in a 5 mL sterile polystyrene tube at room temperature for 10 min, then transferred to a 1 cc tuberculin plastic syringe with an attached 28 G needle. The DNA was then slowly (~10–20 s) injected into the target tissue.
3. As a control group for the baseline reading, another group of mice were identically injected with 20 μg of pT7T7/T7Luc DNA plus 1,000 units of T7 RNAP. The pT7T7/T7luc is a luciferase-encoding plasmid ***(1)*** constructed similarly to the pT7T7/T7HA. The DNA injections were performed three times at two wk intervals. The leg muscles were injected in an alternate fashion (left–right–left). The injected mice were bled 3 d before and 1 wk after the third injections.
4. The presence of the anti-HA antibodies in the mouse serum was detected by ELISA using purified virus as solid phase. Each serum sample was tested in triplicate at dilutions of $^1/_{20}$, $^1/_{100}$, $^1/_{500}$, and $^1/_{2500}$. The assay was standardized with a purified anti-HA mAb (H36-4). Sera from BALB/c mice at 7, 14, and 21 d after pulmonary infection with influenza virus strain PR8, were used as positive controls.

3.2. Cell Mediated Immunity

3.2.1. Plasmid Construction and Preparation

pT7gag was constructed by insertion of a p55gag gene, PCR amplified from a HIV-1 SF2 genomic clone, into pTM-1 ***(1)***. The plasmid pT7T7 ***(1)*** is the same as described in **Subheading 3.3.1.** Plasmids pT7gag and pT7T7 were transformed into *E. coli* strains DH5α and HMS174(DE3)pLysE, respectively. The plasmid DNA were prepared using Qiagen Maxiprep kits.

3.2.2. Animal Immunization

Mice were infected i.p. with 1×10^7 pfu of vTF7-3. At 2 and 18 h later, pT7gag and pT7T7 (**Table 1**) were mixed with T7 RNAP in PBS, and injected into the tibialis anterior muscle. Control mice were infected intraperitoneally with 1×10^7 pfu of the VVgag/pol viruses. Spleen cells pooled from 2–3 mice per group were assessed 4–6 wk later for HIV-1 gag-specific CTL activity.

3.2.3. Lymphocyte Culture and Cytotoxic Cell Assay

1. Spleen cells from the immunized mice were cultured in 24-well dishes at 5×10^6 cells/well. Of those cells, 1×10^6 were sensitized with synthetic epitope peptide p24 gag protein at a concentration of 1 μ*M* for 1 h at 37°C, washed, and co-cultured with the remaining 4×10^6 untreated spleen cells in 2 mL of culture medium (50% RPMI 1640 and 50% α-MEM, supplemented with 10% heat-inactivated FCS, 5×10^{-5} *M* 2-mercaptoethanol, antibiotics, and 2% IL-2). Cells were fed with 1 mL of fresh medium on d 3 and 5, and cytotoxic activity was assessed on d 6.
2. Histocompatible SVBalb (H-2^d) and histoincompatible MC57 (H-2^b) target cells used in the ^{51}Cr release assays express class I but not class II MHC molecules. Approximately 1×10^6 target cells were incubated in 200 μL containing 50 μCi of ^{51}Cr and synthetic HIV-1 or HSV-2 peptides (1 μM) for 60 min and washed three times. Effector (E) cells were cultured with 5×10^3 target (T) cells at various E:T ratios in 200 μL of culture medium in 96-well plates for 4 h. The average cpm from duplicate wells was used to calculate percent specific release ***(11)***.

3.3. Tumor Ablation

1. One million cells from the human osteosarcoma cell line (143B) are grafted intradermally in the flank of *nude* mice (*see* **Note 4**).
2. Seven to 10 d after grafting, when the tumor volumes are between 50 to 150 mm^3, the tumors are injected with 20 μg of pT7T7/T7TK DNA prebound with 1000 units of T7 RNAP, every 3 d for 21 d. The intratumoral injections are performed slowly (~20 s) with 28G needles on anesthetized animals (*see* **Notes 5** and **6**).
3. Twenty-five mg/kg body weight of ganciclovir are injected I.P. twice a day for 28 d.
4. Tumor sizes are measured with a caliper twice a week during the entire experiment. The formula used to calculate the tumor volume is: Vol. = $L \times H \times W \times 0.52$.

4. Notes

1. Based on our experience with the T7 plasmids, the success of an experiment with the T7 autogene containing vectors depends primarily on the quality of the T7 plasmids produced. Because of the extraordinary efficiency of the T7 autogene, the *E. coli* host cell that harbors a T7 autogene-containing plasmid will be killed by the toxicity generated by the T7 autogene if appropriate protective measures are not taken ***(1,4)***. Those clones that managed to grow up have been found to harbor mutated and inactivated plasmids (unpublished observations). In order to produce fully functional T7 autogene-containing plasmids, the T7 autogene-containing plasmids have to be transformed into and propagated in a specific strain of *E. coli* HMS174(DE3)pLysE which can be purchased from Novagen. This *E. coli* host provides a protective plasmid pLysE encoding a T7 lysozyme protein that inhibits T7 RNAP activity in the bacteria ***(12)***. Since the T7 lysozyme also weakens the *E. coli* cell membrane, *E. coli* hosts with pLysE plasmid tend to lyse after long period of shaking incubation. In order to produce high yield of T7

autogene-containing plasmids in large scale preparations, the shaking incubations were usually done at ^{2}150 rpm, and the incubation time should be shorter than ^{2}17 h (most often = 16 h).

2. Although the repeated pT7T7/T7HA injections generated the anti-HA antibodies at a level comparable to that generated by direct viral infection (**Fig. 2**), the Ab levels in each individual injected mice are found more variable than those in the virus infected mice (**Fig. 2**). One reason for this variability is that the mechanism for DNA uptake by the animal cells is largely unknown. However, we found that, to increase consistency of gene expression levels, a ^{3} 10 min room temperature incubation of T7 DNA and the T7 RNAP in PBS was important. Secondly, consistency should be maintained for injection site, depth, and speed.
3. CTL activity specific to p55gag can be induced with the T7 vaccine system when the vaccine is delivered intramuscularly, with or without a cationic lipid such as DOTAP. In contrast, DOTAP will be strictly required for CTL priming when pT7gag and T7 RNAP are delivered i.p. into mice infected with vTF7-3 by the same route. CTL response specific to p55gag can be induced by even a two-component T7 vaccine system: either (pT7gag + T7 RNAP), or (pT7gag + pT7T7) (5). Similar CTL response against HIV-1 gp120 was observed when a pT7gp120 plasmid was used in the T7 vaccine system ***(5)***.
4. Reproducibility in tumor volumes seems to be a critical element in this kind of experiment. We suggest to get used to the intradermal injections by practicing on extra animals before the experiment begins and by checking tumor volume variability thereafter.
5. A reliable anesthesia is also a key element of this approach. Because mouse movements may provoke DNA mishandling, anesthesia is recommended during DNA injections.
6. The DNA/T7 RNAP prebinding must be performed at room temperature in order for the prebinding to be completed in about 10 min. Prebinding on ice may result in low expression rates.

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Immunization with Naked DNA Coexpressing Antigen and Cytokine via IRES

Jochen Heinrich, Bettina Strack, Michael Nawrath, Jovan Pavlovic, and Karin Moelling

1. Introduction

Inoculation of plasmid DNA vectors encoding immunogenic proteins induce humoral as well as cell-mediated immune responses. Protection against challenge with pathogens has provided protective immunity in several instances in animal models. ***(1,2)***. DNA vaccines allow the simultaneous expression of antigens and immune-stimulatory cytokines via an internal ribosomal entry site (IRES). Here we describe the construction of a DNA vaccine against malignant melanomas using: (i) the tumor-associated antigen gp100 (or pmel17), known to be over-expressed in many malignant melanomas ***(3,4)***, and (ii) the granulocyte macrophage stimulating factor (GM-CSF) which has been shown to have a stimulatory effect on humoral and cellular immune responses ***(5)***.

Vectors for DNA immunization in a clinical context should allow high levels of expression of the target protein in mammalian cells but should not be able to replicate or to integrate into the host chromosome. Therefore vectors are not allowed to contain: (i) origins of replication for plasmids in mammalian cells, e.g., from SV40 DNA, because proteins that are able to functionally replace the T antigen of SV40 might be active in the recipient cells; (ii) retroviral long terminal repeats as promoter/enhancer sequences, because they might lead to integration; and (iii) an Ampicillin-resistance gene for selection in bacteria, because this might confer resistance to antibiotics that are used therapeutically. Estimates of integration frequencies indicate about 1 in 10^{-16} per DNA molecule to affect gene expression by an integration event. However 200 μg of a 6-kb DNA plasmid contains approximately 3×10^{13} molecules, which is therefore about 1000-fold below the safety margin ***(6)***.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

For DNA vaccines, we use the vector pVR1012 (**Fig. 1A**) which originated from Vical Co., San Diego. pVR1012 is very similar to the vector V1J constructed by Montgomery et al. ***(7)***, but contains the Km-resistance gene instead of the Ampicillin resistance gene for selection in bacteria. The origin of replication of the high copy-number plasmid pUC18 in bacteria allows the production of large amounts of pVR1012 DNA in *Escherichia coli*. The expression of the target gene is directed by a combination of the human cytomegalovirus (CMV)-immediate-early (IE-1) promoter/enhancer region, including the intron A sequence which increases mRNA stability, with the bovine growth hormone (BGH) polyadenylation signal. This combination turned out to be very efficient for gene expression in mouse muscle cells ***(7,8)***.

In order to clone two genes in a way that allows both genes to be under the control of the same promoter, and are translated from a bicistronic mRNA, both genes are cloned together with an internal ribosomal entry site (IRES) yielding the structure: promoter-gene A-IRES-gene B. For that purpose plasmid pIRES 2 (**Fig. 1B**) is used, which contains 650nt of the IRES of encephalomyocarditis virus (EMCV) inserted into a pBSKS derivative. At least 450 nt of the IRES sequence are required to form at the RNA level, the secondary structures that encompass some less-known recognition motifs. Only one of several start codons, namely the 11th ATG codon (**Fig. 1B**) in EMCV strain R is used for cap-independent initiation of translation ***(9,10)***. Therefore it is important to insert the start codon of gene B in this ATG codon. The original IRES sequence has been modified in order to create a *Nco*I site CCATGG overlapping the 11th start codon, thereby leading to increased initiation of translation due to the higher homology to the Kozak sequence ***(11)***. Genes harboring a corresponding *Nco*I site can directly be fused to the IRES. However, usually the gene of interest does not possess such an *Nco*I site. In this case either the *Nco*I site, or a second restriction site recognized by *Bsm*FI localized 20 nucleotides upstream of *Nco*I, can be used together with oligodeoxynucleotide linkers, especially when the second amino acid of the gene of interest must not be changed. Alternatively, an *Nco*I site overlapping the start codon of the gene of interest might be created by oligonucleotide directed mutagenesis or by PCR amplification using a modified upstream primer harboring the *Nco*I site.

The construction of the DNA plasmids pVR1012.gp100, pVR1012.mGM-CSF, and pVR1012.gp100-IRES-mGM-CSF for the expression of gp100/pmel17, mGM-CSF and of both genes, respectively, is shown in **Fig. 1C**. In the Methods section we describe how oligodeoxynucleotides are used in order to fuse correctly the IRES sequence with the mGM-CSF sequence in the intermediate plasmid pVR1012.IRES-mGM-CSF. For one tumor model, K1735-M2 melanoma cells are stably transfected with plasmid pVR1012.gp100 in

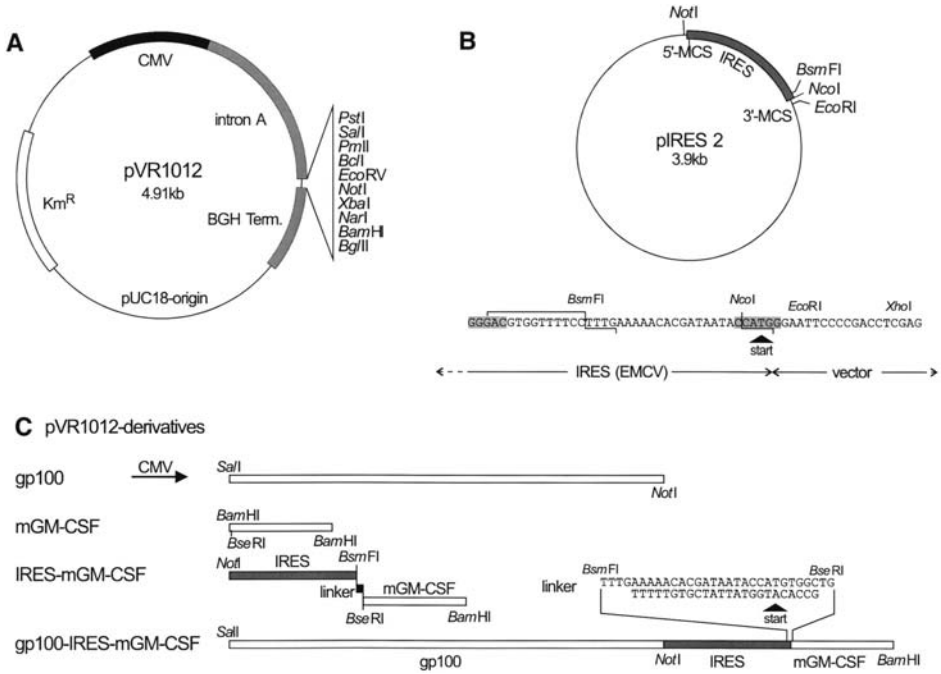

Fig. 1. Construction of vectors for DNA vaccination. **(A)** Plasmid pVR1012 contains the CMV immediate early promoter, an intron A sequence, a multicloning site, the terminator of bovine growth hormone (BGH Term.), and a kanamycin resistance gene (Km^R) on a pUC 18 origin of replication. **(B)** Plasmid pIRES 2 is a derivative of plasmid pBSKS.IRES (kindly provided by Silvio Hemmi), the multicloning sites of which have been modified to fit to the multicloning site of the retroviral pBABE vectors. It served as a source of an IRES sequence that originated from encephalomyocarditis virus (EMCV). **(C)** Construction of DNA vaccine plasmids. The human gp100/pmel17 cDNA and the murine mGM-CSF cDNA were inserted into the *Sal*I/*Not*I and *Bam*HI sites of the pVR1012 multiple cloning site, yielding plasmids pVR1012.gp100 and pVR1012.mGM-CSF, respectively. The intermediate plasmid pVR1012.IRES-mGM-CSF results from the ligation of the IRES sequence as a *Not*I/*Bsm*FI-fragment and the indicated oligodeoxynucleotide linker into the *Bse*RI/*Not*I-digested pVR1012.mGM-CSF. This plasmid was used to clone the gp100/pmel17 gene as a *Sal*I/*Not*I fragment in front of the IRES-mGM-CSF cassette. The expression of gp100/pmel17 and mGM-CSF is verified in cell-culture experiments by indirect immunofluorescence analysis, using the monoclonal antibody HMB45 directed against gp100/pmel17 (kindly provided by S. Wagner, Essen, Germany ***[4]***), and a commercially available ELISA for mGM-CSF.

order to overexpress gp100/pmel17. Mice are vaccinated following established protocols ***(12)*** by intramuscular (i.m.) injection of 2×25 µg of each plasmid DNA into the right and left quadriceps. Usually 5–10 animals per point are tested. The mice are boosted one time 3 wk after the initial immunization. Tumor challenge is performed after an additional 2 wk by subcutaneous (s.c.) injection of 1×10^6 syngeneic melanoma cells: (i) the murine melanoma cell-line B16-F0 and its subclone B16-F10, both expressing high levels of the murine homologue of gp100/pmel17, are used in syngeneic C57B1/6 mice ***(13)***, and (ii) K1735-M2/gp100$^+$ cells are used in C3H mice syngeneic to the parental K1735-M2 cells ***(14)***.

2. Materials

2.1. Generation of the IRES-mGM-CSF Fusion Using an Oligodeoxynucleotide Linker

1. Plasmid DNAs: pVR1012.mGM-CSF, pIRES 2 (*see* **Fig. 1**).
2. Oligodeoxynucleotide-linkers are available from Microsynth (Balgach, Switzerland): (IRES-mGM-CSF) 5'-TTTGAAAAACACGATAATACCATGTGGCTG-3' (IRES-mGM-CSF reverse) 5'-GCCACATGGTATTATCGTGTTTTTC-3'
3. 10X annealing buffer: 10 m*M* Tris-HCl pH 7.8, 10 m*M* $MgCl_2$, 50 m*M* NaCl.
4. T4-DNA ligase and 5X ligation buffer are available from Gibco-BRL.
5. Kanamycin sulfate: 40 mg/mL stock in H_2O, sterile-filtered. Store at –20°C. At 4°C the solution is stable for several weeks.
6. Standard cloning material: Restriction endonucleases, 10X reaction buffers, agarose and low-melting agarose are available from many suppliers. A gel extraction kit and plasmid purification kits are available from Qiagen (Chatsworth, CA).

2.2. Cotransfection of K1735-M2 Cells

1. Plasmids: pVR1012.gp100 and pX343(kindly provided by H. Diggelmann).
2. Murine malignant melanoma cells K1735-M2.
3. Stock cell culture reagents: Dulbecco's modified Eagle medium containing 0.11 g/L sodium-pyruvate and pyridoxine (DMEM), 200 m*M* L-glutamine, penicillin-streptomycin containing 10,000 IU/mL penicillin and 10,000 µg/mL streptomycin are available from Gibco-BRL; fetal calf serum (FCS) is available from Sera-Tech Zellbiologische Produkte GmbH (Aidenbach, Germany); trypsin EDTA Solution B 0.25% in Puck's saline A is available from Biological Industries (Kibbutz Beit Haemek, Israel).
4. Lipofectamine reagent, 2 mg/mL is available from Gibco BRL.
5. Hygromycin B is available from Boehringer Mannheim (Mannheim, Germany).
6. Growth medium: DMEM supplemented with 0.1 vol FCS, 0.01 vol glutamine, and 0.01 vol penicillin/streptomycin.
7. Transfection medium: DMEM supplemented with 0.01 vol glutamine.
8. Selection medium: growth medium supplemented with 200 µg/mL hygromycin.

2.3. Vaccination of Mice and Tumor Challenge

1. Mice (6–8-wk-old with an avarage body weight of 30 g).
2. Syngeneic tumor cell line (here we used B16-F0, B16-F10 or K1735-M2/gp100$^+$).
3. 1.1mg purified DNA of each plasmid: pVR1012, pVR1012.gp100, pVR1012.mGM-CSF, pVR1012.gp100-IRES-mGM-CSF.
4. Dulbecco's PBS without calcium and magnesium, and without bicarbonate, is available from Gibco-BRL.
5. Metofane is available from Mallinckrodt Veterinary, Inc. (Mundelein, IL).
6. 1-litre vessel, or similar size, for narcotizing mice.
7. Paper towels.
8. Precision Glide 0.5-mL tuberculin syringes with 27G1/2 needles for i.m. injection of DNA are available from Becton Dickinson (Franklin Lakes, NJ).
9. 1-mL syringes (Plastipak) with 25G5/8 needles (Microlance 3) for s.c. injection of tumor cells are available from Becton Dickinson.
10. Neubauer cell-counting chamber.

3. Methods

3.1. Generation of the IRES-mGM-CSF Fusion Using an Oligodeoxynucleotide Linker

1. For the vector DNA, digest 5 µg pVR1012mGM-CSF with *Bse*RI and *Not*I, and for the insert DNA, digest 5 µg of the plasmid pIRES2 with *Bsm*FI (at 65°C) and *Not*I (*see* **Note 1**). Do not dephosphorylate the vector (*see* **Note 2**). Separate the DNA fragments by 1.2% agarose-gelelectrophoresis, using low-melting agarose and purify the vector- and insert-fragments using the QIAquick gel extraction kit. Elute the DNA with 30 µL of twice-distilled H_2O (*see* **Note 3**).
2. For the annealing, mix both oligodeoxynucleotide linkers (*see* **Note 4**) to a final concentration of 10 µM each in 20 µL 1X annealing buffer. Denature for 3 min at 95°C and then let equilibrate to RT during 2 min to 3 h (*see* **Note 5**).
3. Mix 2.5 µL vector pVR1012mGM-CSF(*Bse*RI/*Not*I) with 5 µL *Not*I/*Bsm*FI-fragment (pIRES2), 0.5 µL of the annealed oligodeoxynucleotide-linkers, 3 µL 5X ligation buffer, and 1 µL T4-DNA ligase to a final volume of 15 µL, and incubate overnight at 16°C for ligation (*see* **Note 6**).
4. Transform competent bacteria and select transformants on LB-plates containing 50 µg/mL kanamycin. From 10 single colonies, grow 2.5 mL over night cultures. Use 1.5 mL for analytical preparations of the plasmid DNA and test for correct recombination by restriction analysis. Use the remaining 1 mL to inoculate over night cultures for glycerol stocks and preparation of the plasmid DNA. Confirm the structure of the plasmid by dideoxy sequencing.

3.2. Cotransfection of K1735-M2 Cells

1. Plate K1735-M2 cells in growth medium on a 10cm dish and grow them for 2 d at 37°C, in an atmosphere of 5% CO_2 and 90% humidity, so that the cells will have a confluency of 70% at the time of transfection (*see* **Note 7**).

2. Prepare: (i) in a 1.5-mL Eppendorf tube a DNA solution made by mixing 5 µg pVR1012.gp100, 3 µg pX343 (*see* **Note 8**), and 800 µL transfection medium; (ii) in a 2.2-mL Eppendorf tube prepare a lipofectamine solution by mixing 30 µL lipofectamine and 800 µL transfection medium; and (iii) place 6.4 mL of transfection medium in a 15-mL Falcon tube.
3. Pipet the DNA solution into the lipofectamine solution, mix by inverting the tube several times, then let the tube sit at room temperature for 45 min..
4. Pipet the DNA/lipofectamine mixture with a serological 2-mL pipet into the Falcon tube and mix gently by inverting the tube three to four times.
5. Remove the growth medium from the cells and carefully pipet or pour the transfection mixture onto the cells.
6. Incubate the cells for 16 h.
7. Replace the transfection mixture with growth medium and incubate the cells for additional 24 h.
8. Split the cells 1:10 on nine 10-cm dishes and one 6-cm dish and let them grow for 12–16 h (*see* **Note 9**).
9. Use the 6-cm dish to test for the expression of gp100/pmel17 by immunofluorescence analysis using the monoclonal antibody HMB45 (*see* **Note 10**).
10. Use the 10-cm dishes for the selection of stably transfected cells as follows. Replace the growth medium by selection medium. After approximately 2 d, massive cell death occurs. As long as many cells die and detach from the surface, change the medium every day. After several days, the number of dying cells decreases. Then change the medium every 3 or 4 d until the surviving cells form visible colonies and cells no longer die.
11. Use a sterile scalpel to scratch single colonies along with a thin layer of the plastic from the surface of the dish and transfer each into a well of a 24-well plate that has been pre-filled with 1 mL of selection medium. Pipet the content of each well 5–6 times with a pasteur pipet up and down in order to rinse cells from the surface of the transferred plastic (*see* **Note 11**).
12. Let the cells grow in selection medium until they can be split for: (i) further cultivation; (ii) analysis of the protein expression; (iii) cryo-conservation of cells expressing the protein; and (iv) tumor challenge.

3.3. Vaccination of Mice and Tumor Challenge

1. When vaccinating, for each 10 animals, prepare DNA solutions by mixing 550 µg plasmid DNA with PBS to a final volume of 550 µL.
2. Prepare to narcotize the mice by putting 3–5 drops of metofane on a paper towel in a suitable vessel (*see* **Note 12**). Place a second dry paper towel on top of the first one in order to protect the mice from direct contact to metofane. Cover the vessel with a lid to generate a saturated atmosphere, but allow air supply.
3. Set one mouse into the vessel for a few min until it is anaesthesized. This is when the mouse no longer moves and breathing becomes calm. Then take the mouse out and lay it on its back.

4. Inject 25 µL of the DNA solution into the left, and 25 µL into the right quadriceps muscle (*see* **Note 13**).
5. After injection, mark the animal, and proceed with the next mouse with steps 2–4.
6. 21 d after the initial immunization, boost the mice once by repeating steps 1–5.
7. After a further 14 d, use the mice for tumor challenge.
8. Grow K1735/pVR1012.gp100 cells on 10-cm dishes, so that they will have a confluency of approximately 90% ($1–2 \times 10^7$ cells/10-cm dish) at the day of the tumor challenge. Remove the growth medium and wash the cells once with 1.5 mL of trypsin EDTA solution for a few sec. Incubate the plate for 3 min. at 37°C and resuspend the cells in 5 mL of growth medium by pipetting up and down 5–10 times. Count the cells using a Neubauer chamber.
9. Centrifuge the cell suspension for 5 min at 150*g*. Aspirate the supernatant and resuspend the cells in 5mL DMEM. Adjust the suspension to a final cell density of 3.3×10^6/mL. Store the cells on ice for up to 2 h.
10. Anaesthetize mice as described above and inject 300µL of the cell suspension (1×10^6 cells) subcutanously in the right flank using a 1mL syringe with a 25 gauge needle (*see* **Note 14**).
11. Monitor the growth of tumors by determining the tumor volume ***(16)*** every fifth day. Measure the largest diameter (d_1) and the perpendicular diameter (d_2) and calculate the tumor volume, according to the formula $V = d_1 \times d_2^2 \times 0.5$.

4. Notes

1. The oligodeoxynucleotides are designed to create the fusion between the *Bsm*FI-generated 3'end of IRES and a unique *Bse*RI site in pVR1012.mGM-CSF localized at the extreme 5'-end of the mGM-CSF coding sequence (*see* **Fig. 1**). This allows the ligation of the IRES sequence as a *Not*I/*Bsm*FI-fragment into *Bse*RI/*Not*I-digested pVR1012.mGM-CSF via the linker which is compatible to the ends generated by *Bsm*FI and *Bse*RI.
2. The oligonucleotides are not phosphorylated, to avoid multimerization that might be difficult to detect by restriction analysis of the recombinant plasmid products. Therefore, the vector must not be dephosphorylated. In order to limit oligomerization of vector- and insert-DNA-fragments, an excess of oligodeoxynucleotides is used in the ligation reaction, which contains vector:insert:linker in a molar ratio of approx 1:3:40.

   The molarity of the purified fragments is calculated according to the formula: x = plasmid [µg]*M*/(elution volume [µL] × plasmid length [bp] × 660), e.g., here for the vector, x = 47n*M*.
3. We usually check for the presence of the correct DNA fragments after their purification by subjecting 0.1–0.2 vol of the eluted DNA to agarose-gel electrophoresis. The concentrations of the fragments can be roughly estimated from the intensities of the bands in order to set up the ligation with a correct ratio of vector and insert.
4. In the case of longer oligonucleotides (>30 nucleotides), it is recommended to use gel-purified oligodeoxynucleotides.

5. The annealing can be done in several ways. Usually the oligodeoxynucleotides are denatured in a waterbath at 90–95° and then the tube is transfered together with 10–20 mL of the hot water into a small vessel to cool slowly down during 10–15 min. Alternatively, a thermoblock might be switched off to cool slowly for about 3 h. The buffer conditions are not stringent. For the annealing, some salt should be included, but avoid using high salt concentrations, since T4 DNA ligase requires low salt conditions.
6. The ligation can be monitored by agarose gel electrophoresis. For small insert DNA fragments, the ligation products are slightly shifted, compared to a non-ligated control fragment. Alternatively, it is possible to set up a positive control with all components, but using 0.5 μg of DNA-fragments that have ends that are non-compatible to the linker (e.g., ΦX174-HaeIII blunt-ended fragments), and to test for ligated products. Furthermore, the incorporation of the linker is often indicated by the inhibition of the re-ligation of DNA fragments compared to a control without linker.
7. In our experience, the efficacy of transfection depends highly on the confluency of the cells, which should be 60–80%. Furthermore transfection might be more efficient when cells have grown for 2 d rather than only overnight. It is a good idea to test the efficacy of transfection using a reporter plasmid, expressing for example *LacZ*, in order to optimize the transfection procedure.
8. Plasmid pVR1012.gp100 does not confer any resistance to mammalian cells. Therefore a cotransfection is performed using plasmid pX343, which harbors a hygromycin B resistance gene under the control of the SV40 early promoter, to allow the selection of stably transfected cells. It is important to determine the sensitivity of the cells against hygromycin B before transfection. This is done by growing the cells in 6 wells of a 6-well plate to a confluency of 80%. Subsequently the growth medium is changed and hygromycin B is added to wells in a range of concentrations, e.g., 20, 50, 100, 200, 400, 800 μg/mL. After a lag phase of 1–2 d, cells will die dependent on the concentration of hygromycin B in the growth medium. For the selection of transfectants use the lowest concentration of hygromycin B that causes complete elimination of all non-transfected cells.
9. The cells are split 1:10 in order to have single cells instead of a compact cell layer (since single cells are more sensitive to hygromycin B), and to limit the final number of colonies on one dish. When the cells are not split, the selection procedure will last longer and might select for resistance to hygromycin in some non-transfected cells. It can be helpful to make a range of cell dilutions, e.g., 1:20, 1:10, 1:5, 1:2.5.
10. Protein expression should be detected by immunofluorescence analysis in order to estimate the ratio of transfected and non-transfected cells. The result allows estimation of how many cells will die or survive during the selection procedure.
11. There are several methods for subcloning described in the literature, but this method worked best in our hands. Make certain that the scalpels and pipets have equilibrated to room temperature before use. Additionally, the cells of one colony can be trypsinized by putting a 2–5 μL spot of trypsin EDTA solution for 20–60 s

onto the colony immediately before it is transfered to the 24-well plate. Usually a fraction of the colonies will not grow further after the transfer procedure. Therefore it is recommended to pick about 50 colonies to finally have at least 10 independent clones.

12. A simple way to narcotize the mice is to use a 1-L vessel, which can be covered by aluminium foil. However, a glass cylinder (depth = 10 cm, height = 10 cm) with a glass lid is more comfortable. Use 1 drop of metofane for approximately 200 cm^3. When the vessel stays covered, every 10 min add the same amount of metofane to maintain the saturated atmosphere.
13. At the flexed hind leg, start to prick directly above the patella parallel to the femur and penetrate through the whole muscle. Then start to inject. During the injection, very slowly pull the syringe back in order to distribute the DNA along the whole muscle. Repeat this procedure with the other quadriceps.
14. For s.c. injection, take the skin with two fingers and lift it up. The needle is directed parallel to the surface of the body so that it penetrates the lifted skin. Stop penetrating after resistance is lost. Then inject the cell suspension all at once (bolus injection). Transiently (3–5 min), the injected suspension forms a visible bolus under the skin.

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Genetic Subunit Vaccines

A Novel Approach for Genetic Immunization

I. Frank Ciernik and David P. Carbone

1. Introduction

Induction of epitope-specific cytotoxic T lymphocyte (CTL) responses can improve the outcome of disease in a variety of model systems. However, natural immunity or that arising by immunization with whole proteins or organisms with multiple potential epitopes, may not always result in the desired effect. For example, antigenic variation of CTL epitopes can result in immune escape in HIV disease ***(1,2)***, and malaria ***(3)***. This could potentially be avoided if it were possible to direct the immunity to selected regions of proteins that are highly conserved and required for function. The immunization with specific CTL epitopes rather than whole proteins also avoids induction of unwanted responses such as enhancing antibodies in HIV, which may actually promote infection ***(4)***.

By analogy to the use of purified proteins and mixtures of proteins, genetic immunization has been shown to allow induction of cellular as well humoral immune responses ***(5,6)***. There is increasing evidence that genetic immunization might be even more efficient than regular immune approaches using protein in adjuvant ***(7)***. We have explored the ability of genetic immunization to specifically target defined CTL epitopes. Such epitope-targeted immunity may be especially desirable when the epitope is altered "self," as when targeting the mutant site of endogenous oncoproteins. The ability to direct a cellular immune response to a defined epitope allows increased precision in studies of cell-mediated immunity and immunotherapy.

Synthetic peptides are a useful approach to the induction of epitope-specific immunity. The use of peptides as immunogens is complicated, however, by

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

their weak inherent immunogenicity and variable chemical and physical properties. Some peptides are nearly impossible to synthesize for chemical reasons. A variety of strategies have been employed to enhance the immunological efficacy of peptide-based vaccines, including chemical, bacterial, and mammalian cell-based adjuvants. The chemical and physical problems of protein or peptide-based vaccines can be avoided by the use of "genetic vaccines." Several studies have shown that plasmid DNA expression vectors encoding the entire cloned open reading frame of proteins may generate substantial humoral and cellular immunity when directly introduced into living animals *(5,8–11)*.

Strategies for increasing the efficacy of genetic immunization are in constant demand. For instance, the presence of immunomodulatory sequences, such as CpG sequences, in the expression vector may dramatically improve immunogenicity *(12)*. Others have observed enhanced effective immunity using multigene DNA immunization *(13)*. Additional co-expression with immunomodulatory cytokines may further increase the efficacy of genetic vaccines *(14)*.

We have designed a vector using the adenovirus E3 leader sequence, which facilitates the transport of a single epitope encoded in a short open reading frame into the endoplasmic reticulum (ER). This leader is designed to enhance the concentration of the peptide in the ER and thus binding to class I MHC molecules, and to bypass the need for transport by the TAP transporter. We show that this leader sequence can improve CTL induction and lead to enhanced protective immunity. **Fig. 1** indicates the strategy used to obtain the appropriate expression vector containing a short open reading frame driven by a CMV promoter coding for a model peptide derived from mutant p53.

2. Materials

1. Special equipment: Gene Pulser, available from Bio-Rad (Hercules, CA); PE Biosystems (Foster City, CA) 430A peptide synthesizer; harvesting system, available from Skatron (Lier, Norway); gamma counter, available from LKB (Gaithersburg, MD).
2. Purchase female BALB/c mice (age: 10–16 wk) from Harlan Inc. (Indianapolis, IN).
3. Obtain BALB/c 3T3 ($H2^d$) fibroblasts and DBA/2 P815 ($H2^d$) mastocytoma cells from ATCC (Rockville, MD). Grow cells in flasks with 10% (v/v) FCS in RPMI 1630 medium supplemented with gentamycin and penicillin in a 5% CO_2 incubator at 37°C.
4. Obtain the pRc/CMV expression vector from Invitrogen (San Diego, CA). This 5.4 Kb plasmid includes a multiple cloning site flanked by a T7 and SP6 promoter. A human cytomegalovirus promoter/enhancer element allows expression of the cloned sequence in mammalian cells.
5. DH5α competent cells™ are available from Life Technologies, Inc. (Gaithersburg, MD) (Cat. No. 18265-017).

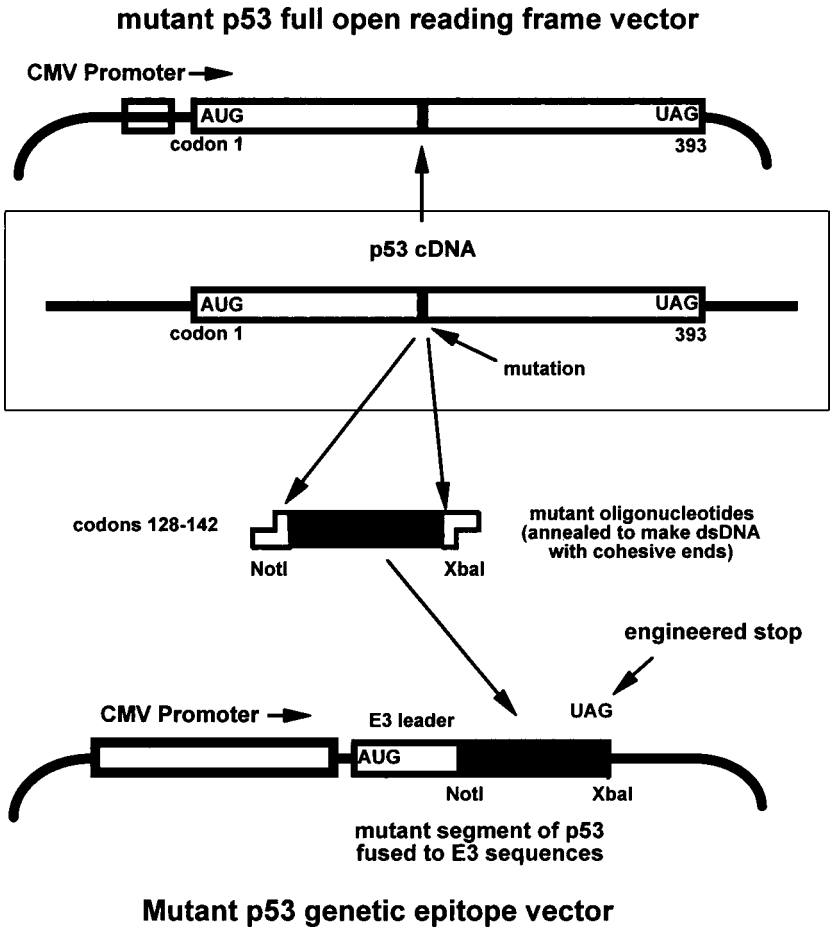

Fig. 1. Structure of the whole open reading frame and genetic epitope expression vectors. The cassette vector allows convenient insertion of synthetic oligonucleotide sequences in-frame with an ER targeting sequence in an expression vector. See **Subheading 3** for details of construction.

6. Oligonucleotides are purchased from vendors specializing in their synthesis (*see* **Note 1**).
7. Prepare synthetic peptides using t-Boc solid-phase peptide synthesis on an peptide synthesizer. Custom made peptides can also be obtained by commercial means. Store the peptides at 70°C after dilution to 100 µM final concentration in RPMI 1640.
8. Obtain monoclonal antibody 53–6.7 (rat anti-mouse CD8 IgG2a) and H129.19 (rat anti-mouse CD4 IgG2a) from Boehringer Mannheim Biochemicals (Indianapolis, IN).
9. Obtain Rat-T Stim lacking concanavalin A from Collaborative Biomedical Products (Bedford, MA).

3. Methods

3.1. Gene Construction

1. Amplify the full open reading frame of the p53 mRNA by cDNA PCR using RNA from the human non-small cell lung cancer T1272. The amino acid sequence of this protein contains the point mutation C to Y at position 135.
2. Clone the cDNA between *Hind*III and *Xba*I sites in the mammalian expression vector pRc/CMV using standard materials and molecular biology techniques.
3. Sequence the full open reading frame in both directions to exclude artifactual polymerase chain reaction-derived mutations.

3.2. Construction of the Minigene Expression Vector

1. Verify the purity of the oligonucleotides on a conventional sequencing gel (*see* **Note 2**).
2. The oligonucleotide AGCTA TGAGG TACAT GATTT TAGGC TTGCT CGCCC TTGCG GCAGTC TGCAG CGC coding for the E3 leader sequence of Adenovirus (including the ATG start codon) is inserted between the restriction sites *Hind*III and *Not*I. Then the oligonucleotide GGCCC TCAAC AAGAT GTTTT ACCAA CTGGC CAAGA CCTGC CCTGT GCAGCT coding for the amino acids 128–145 of p53, with a tyrosine at codon 135, is inserted between *Not*I and *Xba*I following the E3 leader sequence, or is inserted as AGCTA TGCTC AACAA GATGT TTTAC CAACT GGCCA AGACC TGCCC TGT GCAGCT into pRc/CMV between *Hind*III and *Xba*I without the E3 leader sequence. The vector *Xba*I site generates an engineered stop codon.
3. As a control, insert the oligonucleotide GGCCC TCAAC AAGAT GTTTT GCCAA CTGGC CAAGA CCTGC CCTGT GCAGCT from amino acids 128–145 of p53 coding for the wild-type sequence between *Not*I and *Xba*I following the E3 leader sequence.
4. Insert a scrambled Adenovirus E3-leader sequence AGCTA TGTGC CTTGC AAGGG GCGCC TACGC GATGT TGATT AGCTT AGCAG TCGC between the restriction sites *Hind*III and *Not*I, followed by the oligonucleotide coding for the amino acids 128–145 of p53, with a tyrosine at codon 135 between *Not*I and *Xba*I.
5. Insert the sequence coding for the T cell epitope p18IIIB of HIV gp160 between *Hind*III and *Xba*I, without the E3 leader sequence (AGTCA TGCGT ATACA ACGCG GGCAA GGTCG CGCGT TCGTA ACGAT AGGTA AGTAG), or following the Adenovirus E3 leader sequence (GGCCC GTATA CAACG CGGAC CAGGT CGCGC TTTCG TAACG ATAGG TAAG TAG).
6. After ligation, transform the vectors into DH5α competent cells.

3.3. Generation of Tumor Cells

1. Grow BALB/c 3T3 fibroblasts and DBA/2 P815 in RPMI 1640 medium supplemented with 10% (v/v) FCS, gentamycin and penicillin in a 5% CO_2 incubator at 37°C.

2. Suspend P815 cells in serum-free RPMI 1640. Electroporate the plasmid DNA into the cells using a Bio-Rad Gene Pulser using conditions recommended by the manufacturer.
3. Grow the cells under G418 selection (600 μg/mL) and use the surviving, stably transfected cells for experiments.

3.4. Construction of the Genetic Vaccine

1. Construct expression vectors coding only for the p53 or HIV epitopes with or without the E3 leader sequence by ligating double-stranded oligonucleotides into the pRc/CMV vectors.
2. Generate the double-stranded oligonucleotides by annealing the oligonucleotides with their complements with longer or shorter ends as needed to generate the appropriate restriction site overhang (5' *Hind*III or *Not*I and 3' *Not*I or *Xba*I). See **Fig. 1**.
3. Digest 1 μL of a stock of vector at a concentration of 1 mg/mL with *Hind*III and *Not*I in a reaction volume of 10 μL at 37°C for 1 h. Use 1–3 μL in the ligation reaction.
4. Determine the concentration of double-stranded oligonucleotide using the absorption of UV light at a wavelength of 260 (OD260). The extinction coefficient needs to be adjusted for the length of the oligonucleotide in question.
5. Dissolve the complementary oligonucleotides in TE buffer (pH 8.0) and anneal at a molar ratio of 1:1 in a water bath gradually cooling from 60°C to 4°C.
6. Test for proper annealing on a high percentage agarose gel.
7. Ligate the annealed oligonucleotide into the digested vector under conditions recommended by the manufacturer.
8. Thaw competent cells on wet ice. Add 1 μL of the ligation reaction to 50–100 μL of cells and incubate on ice for 30 min. Heat-shock the cells for 20–60 s at 37–42°. Put them back on ice for 2 min. Add bacterial growth medium, such as TB or LB without Ampicillin and shake the culture gently for 1 h at 37°C. Plate the cells on ampicillin-containing plates overnight.
9. Pick 6–12 colonies and make minipreps of plasmid DNA.
10. Perform a restriction digest using restriction enzymes that cut in the insert and not in the original vector to screen for candidate clones.
11. Completely sequence all of the inserts to verify their structure (*see* **Note 3**). Use the UBS sequencing kit. Denature the plasmid and anneal with T7 and SP6 primers; sequence in both directions. Perform sequencing on an 8% acrylamide gel. Use a CsCl gradient for plasmid purification. Alternatively, use Qiagen columns. Both methods yield plasmid suitable for genetic immunization.

3.5. Gene Gun-based Immunization

1. Prepare the DNA for ballistic gene transfer (*see* **Note 4**) as follows. Suspend 2 mg of gold beads in H_2O to obtain a concentration of 2 mg/mL and store at –20°C in 1.5 mL Eppendorf tubes.

2. Attach the DNA to micron-sized gold particles with calcium and spermidine (10 µg DNA / 5 µL gold bead suspension at 2 µg microparticles per µL). Dilute DNA with 2.5 *M* $CaCl_2$ to yield a final concentration of at least 1 *M* $CaCl_2$. Attach DNA to the gold particles with $^1/_{10}$ of the volume 1 *M* spermidine (Sigma, St. Louis, MO) (*see* **Note 5**).
3. Wash the plasmid DNA and gold particles with 70% ethanol first and again with 100% ethanol in a volume of 0.5 mL.
4. Resuspend the gold beads in 40 µL of 100% ethanol and load 8 µL onto a macrocarrier that is appropriate for the gun used (Kaptan disk). The macrocarrier is a thin plastic disk onto which the DNA-loaded gold particles are coated. Prepare five disks for each mouse to be vaccinated.
5. Load the disk onto the gene gun and shoot at 1200–2000 psi into the dorsal part of the ears of anesthetized mice.

3.6. Restimulation of CTL

1. Use peptide T1272 (TSPALNKMFYQLAKTCPVQL) for in vitro restimulation of effector cells at a concentration of 1 to 5 µ*M* (*see* **Note 6**).
2. Pulse target cells with peptide V10 (FYQLAKTCPV) for CTL assays.
3. Use the wild-type counterpart (wtV10) (FCQLAKTCPV) or peptide VSV-8 (RGYVYQGL) as controls for the pulsing of target cells.
4. Restimulate 3×10^6 immune spleen cells in vitro for 5–6 d three wk after immunizing mice in a 50:50 mixture of IMDM and Eagle-Hanks amino acid medium containing 10% (v/v) fetal calf serum, 2 m*M* L-glutamine, 5×10^{-5} *M* ß-mercaptoethanol, and antibiotics.
5. Restimulate with or without T1272 peptide (5 µ*M*) in 10% Rat-T Stim lacking concanavalin A.

3.7. CTL Phenotype and MHC Restriction

1. Use monoclonal rat anti-mouse CD8 and rat anti-mouse CD4 at 30 µg/mL in the presence of 1:5 diluted rabbit complement for 1 h at 37°C.
2. For the CTL assay, pulse target cells with peptide V10.
3. As controls for the pulsing of target cells, use the wild-type counterpart wtV10 or peptide VSV-8.
4. Incubate peptide at 1 µM with 1 to 5×10^6 cells for 1 h at 37°C. Wash unbound peptide twice using RPMI 1640 with 2% FCS in polyethylene 15 mL blue-cap tubes.
5. Spin at 1200–200 rpm for 5–10 min.

3.8. CTL Assay

1. Use round-bottom 96-well plates with covers.
2. Plate cells in a volume of 100 µL in duplicates at different concentrations, starting with a concentration of 10^6 cells /mL. Serially diluted at least four times with an equal volume of cell-free medium.
3. Label target cells with ^{51}Cr in a small volume, e.g., 10^6 cells in 0.3 mL, in order to allow adequate uptake of ^{51}Cr. Use 15 mL blue-cap Falcon tubes. Slight shak-

ing during incubation at 37°C is preferable, but regular resuspension of the cells every 10–20 min is adequate. Incubate for 1–2 h. Remove unbound radioactivity by washing with RPMI 1640 containing 2% (v/v) FCS.

4. Bring targets to a concentration of 10^4/mL and overlay the target cell suspension onto CTL loaded wells in 100 µL.
5. To allow optimal contact between target cells and effector cells, spin the plates at room temperature at 1500*g* in a tabletop centrifuge.
6. Incubate the plates at 37°C for 4–6 h.
7. Harvest the supernatant with a Skatron Harvesting System. Count the radioactivity in a gamma counter.
8. Analyze each animal independently. Average the results within groups and determine the standard error. Calculate percent specific lysis using the equation: $100 \times$ [(experimental release – spontaneous release) / (maximal release – spontaneous release)].

3.9. Tumor Protection

1. Immunize mice with 20 µg of DNA on the back of their ears (each ear, 10 µg) using the ballistic apparatus.
2. Challenge mice with 9×10^5 P815 mastocytoma cells expressing a minigene coding for the appropriate T-cell epitope.
3. For tumor protection experiments with the HIV-gp160 p18IIIB epitope, use fibrosarcomas expressing gp160. Generate these cells by transfection of murine fibroblasts that endogenously express gp160 of isolate IIIB with EJ-ras and c-myc ***(15)***. These cells express HIV gp160 and have been shown to significantly present the epitope to p18IIIB specific CTL ***(16)***.
4. Immunize mice as described above for protection experiments targeting the mutant p53 epitope and challenge with subcutaneous tumor 14 d later. Measure tumor-protective immunity and p53-specific CTL ***(17)***.

4. Notes

1. When designing oligonucleotide encoding the desired epitopes, keep in mind the redundancy of the genetic code. You can design a restriction site without changing the translation product.
2. Before using the oligonucleotide, it should be run on a high percentage agarose gel to better estimate the purity, or preferably an 8% acrylamide gel.
3. Characterization of the constructs should be done with 2–3 restriction enzymes before sequencing to save time.
4. We use a custom-built helium-driven ballistic device. The Kaptan disk loaded with the DNA-coated microparticles is placed in a vacuum chamber facing a stopping screen. The skin of the mouse seals an outlet. A membrane restrains pressure built up with helium against the vacuum chamber. The membrane is subsequently ruptured by a lance and allows the disk to be propelled toward the target tissue by the resulting shock wave. The stopping screen stops the disk while the microparticles continue to penetrate the tissue. Commercial units work on similar principles.

5. Addition of the spermidine is done while keeping the suspension of gold particles on the vortex in order to prevent aggregation of the beads.
6. Avoid peptide concentrations over 5 μ*M* for restimulation. Certain peptides are only slightly soluble at low or high pH making them difficult to handle and potentially toxic to cells.

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32

Antigen Engineering in DNA Immunization

Shan Lu, Steve Manning, and James Arthos

1. Introduction

J. A. Wolff and colleagues demonstrated in 1990 ***(1)*** that naked, non-infectious DNA plasmids can be transferred into muscle cells of living mice to express the encoded protein in vivo. Further studies have shown that proteins expressed in vivo via such a "DNA inoculation" process can serve as immunogens to effectively stimulate the body's immune system to produce potentially strong, long-lasting, antigen-specific, humoral and cell-mediated immune responses ***(2–8)***.

The technique of DNA immunization is simple. It can be used to develop DNA-based vaccines, and it can also serve as a tool to generate animal antisera as research reagents. DNA immunization approach is quicker, less expensive, and more likely to preserve a better antigen conformation than the recombinant protein vaccine approach. In addition, DNA immunization allows in vivo packaging of newly synthesized proteins, which may be critical for antigens with multiple subunits. DNA immunization is an extension of the original subunit immunization approach, but with its unique advantages.

However, it is frequently neglected that the overall immunogenicity of any given protein antigen still plays an important role in DNA immunization even though the antigen is now delivered in the form of a DNA plasmid. If a particular protein antigen is not immunogenic, it is unlikely that immunization with DNA vaccine encoding for this antigen will induce high immune responses. But DNA immunization is an ideal approach to modify the sequences of protein antigens so that the immunogenicity of protein antigens can be improved. The conventional recombinant subunit approach requires additional steps for in vitro protein expression and purification while DNA immunization can test the immunogenicity of modified protein antigens with direct inoculation of antigen-expressing

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

DNA plasmids. Therefore, a well-thought protein antigen engineering process may improve the chance to achieve a successful DNA immunization.

Human immunodeficiency virus-1 (HIV-1) DNA vaccines are good examples of how the principles of protein chemistry, cell biology and virology have to be considered to design an effective AIDS DNA vaccine. In this chapter, some of the HIV-1 DNA vaccines are analyzed to demonstrate the key conceptual components of antigen engineering for each of these DNA vaccines. Currently most biomedical research laboratories are capable of doing the routine molecular biology and protein chemistry experiments, technical details in this chapter are provided only for steps unique to DNA immunization. The emphasis in this chapter is to avoid the potential pitfalls of DNA immunization.

2. Materials

2.1. HIV-1 DNA Templates

1. HIV-1 molecular clone NL4-3 (GenBank Accession no.: M19921).
2. HIV-1 molecular clone HXB2 (GenBank Accession no.: K03455, M38432).
3. pSVIIIenv encoding for HIV-1 envelope protein with major deletions in V1/2 and V3 domains ***(9)***.

2.2. Expression Vectors

1. pBC12/CMV/IL-2 vector, provided by Dr. B. Cullen, Duke University Medical Center, Durham, NC, USA ***(10)***.
2. pJW4303 vector, provided by Dr. J. Mullins' laboratory, University of Washington, Seattle, WA, USA ***(11–13)***.

2.3. PCR Oligonucleotide Primers

1. JApcr503: (containing an *Xba*I site, TCTAGA).
   5'-GTCGCTCCTCTAGATTGTGGGTCACAGTCTATTATGGGTACC-3'.
2. JApcr502: (containing a *Bam*HI site, GGATCC , and a stop codon, TTA).
   5'-CGACGGATCCttaTGTTATGTCAAACCAATTCCAC-3'.
3. JApcr504: (containing a *Bam*HI site, GGATCC , and a stop codon, TTA).
   5'-GGTCGGATCCttaCTGCACCACTCTTCTCTTTGCC-3'.

2.4. Special Reagents and Equipment

1. Qiagen's QIAEX II kit (Cat no. 20021) is available from Qiagen Inc. (Santa Clarita, CA).
2. T4 DNA ligase is available from Boehringer Mannheim (Indianapolis, IN). (1 U/μL, Cat. no. 4812201 or 5 U/μL, Cat. no. 799009).
3. Gene Pulser Electroporator is available from Bio-Rad Laboratories (Hercules, CA).
4. Electroporation cuvettes (Cat. no.165-2088) is available from Bio-Rad Laboratories.
5. TEN buffer (0.1 *M* NaCl, 10 m*M* Tris-HCl/pH 8.0, 1 m*M* EDTA/pH 8.0).
6. Helios Gene Gun System (Cat. no. 165-2431) is available from Bio-Rad Laboratories.
7. Protein concentration spin columns, selected on the basis of volume and molecular weight cut-off, are available from Amicon, Inc. (Beverly, MA).

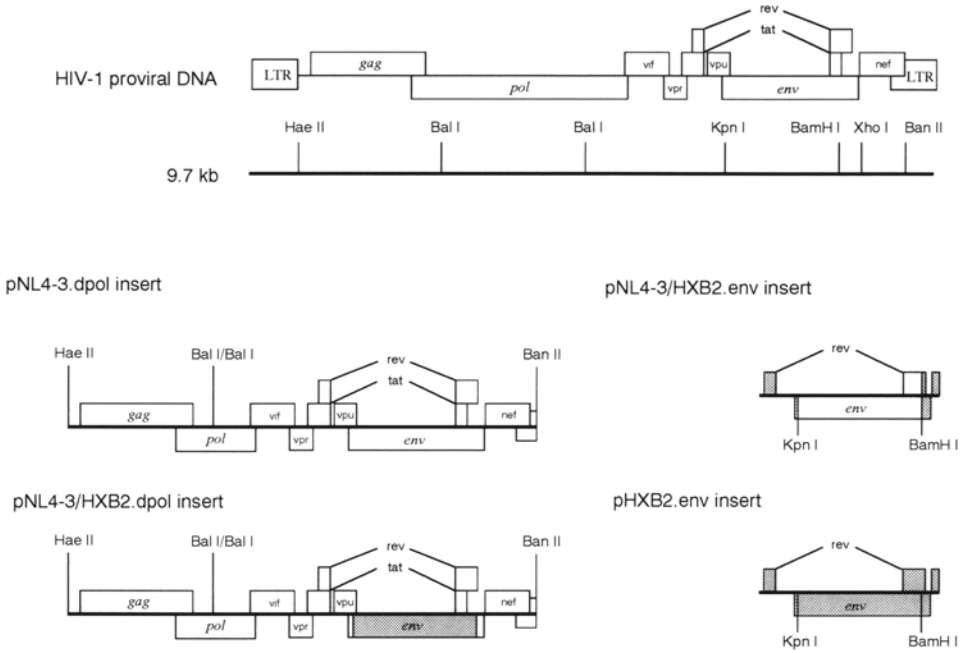

Fig. 1. HIV-1 DNA vaccine inserts expressing full-length envelope proteins.

3. Methods

3.1. Construction of HIV-1 env*-Expressing DNA Vaccine Inserts*

3.1.1. pNL4-3.dpol and pNL4-3/HXB2.dpol DNA Vaccines

1. Digest HIV-1 NL4-3 (9709 bp) with *Bal*I (=*Msc*I) at sites 2621 and 4553 (*see* **ref. *14*** and **Fig. 1**) (*see* **Note 1**). Remove the smaller 1932-bp fragment (including most of the pol gene) and re-ligate the remaining NL4-3 sequences to produce a new single *Bal*I site at 2621.
2. Digest the NL4-3 sequence at the *Hae*II (641) and the *Ban*II site following the *nef* region (now at nt 7556 after *pol* deletion) to remove both LTRs. This produces the final NL4-3.dpol insert with a total length of 6915 bp.
3. Subclone the NL4-3.dpol insert into the pBC/CMV/IL-2 (-*Kpn*) vector (*see* **Subheading 3.2.**) at the *Hind*III (756) and *Bam*HI (1439) sites to give a pNL4-3.dpol DNA vaccine construct of 10,962 bp.
4. Construct the chimeric pNL4-3/HXB2.dopl DNA vaccine plasmid. Replace the *Kpn*I (4532)–*Bam*HI (6650) sequences in pNL4-3.dpol DNA vaccine with a DNA fragment spanning from *Kpn* I (6350) to *Bam*HI (8474) in the HXB2 genome. The resulting construct will express the chimeric HXB2/NL4-3 Env on an otherwise mainly NL4-3-based viral particles (**Fig. 1**).

3.1.2. pHXB2.env and pNL4-3.env DNA Vaccines

pHXB2.env ***(15)*** (**Fig. 1**)

1. Digest the full-length HIV-1 HXB2 cDNA with *Sal*I (5787) and *Xho*I (8898) to produce a 3111-bp fragment that encodes both the *rev* and *env* genes (*see* **Note 2**).
2. Subclone the fragment into pBC/CMV/IL-2 (-*Kpn*) vector at the *Hind*III (756) and *Bam*HI (1439) sites to give a pHXB2.env DNA vaccine of 7158 bp.

3.1.3. pNL4-3/HXB2.env

1. Generate a 2118-bp, *Kpn*I (6347) to *Bam*HI (8465) fragment from the full-length HIV-1 NL4-3 genome (*see* **ref. *14*** and **Fig. 1**).
2. Use the foregoing fragment to substitute the *Kpn*I (1323)-*Bam*HI (3447) sequences within the pHXB2.env construct (*see* **step 1**). The resulting pNL4-3/HXB2.env DNA vaccine expresses a chimeric HIV-1 Env with the most of the *env* sequences coming from the NL4-3 strain.

3.1.4. pHXB2. gp140

1. Produce a 1929 bp of HXB2 DNA sequences (nt 6323-8251) encoding for a gp140 form of *env* by PCR using primers JApcr503 and JApcr502 and HIV-1 HXB2 genome DNA as templates (*see* **refs. *15–17*** and **Fig. 2**) (*see* **Note 3**). The 5' primer JApcr503 contains an *Xba*I site to clone the fragment in frame with the tPA leader sequences in vector pJW4303. The 3' antisense primer JApcr502 introduced a stop codon preceding the transmembrane region of gp41. There is a *Bam*HI site at the 3' end for use in cloning with the pJW4303 vector.
2. Cut the vector pJW4303 at the *Nhe*I site (nt 2102) and *Bam*HI site (nt 2119).
3. Discard the small fragment and insert the gp140-expressing segment into the *Nhe*I/*Bam*HI sites to produce a 7051 bp pHXB2.gp140 DNA vaccine construct.

3.1.5. pHXB2. gp120

1. Produce a 1422 bp of HXB2 DNA sequences (nt 6323-7744) encoding for a gp120 form of *env* by PCR using primers JApcr503 and JApcr504 and HIV-1 HXB2 genome DNA as template (*see* **refs. *15–17*** and **Fig. 2**). The 5' primer JApcr503 contains an *Xba*I site to clone the fragment in frame with the tPA leader sequences in the vector pJW4303. The 3' antisense primer JApcr504 introduced a stop codon near the junction of gp120 and gp41. There is a *Bam*HI site at the 3' end for use in cloning with the pJW4303 vector.
2. Cut the vector pJW4303 at the *Nhe*I site (2102) and *Bam*HI site (2119).
3. Discard the small fragment and insert the gp120-expressing segment into the *Nhe*I/*Bam*HI sites to produce a 6544 bp pHXB2.gp120 DNA vaccine construct.

3.1.6. pHXB2.dV123.gp140 and pHXB2.dV123.gp120

1. Produce the pHXB2.dV123.gp140 and pHXB2.dV123.gp120 plasmids in the same way as for pHXB2.gp140 and pHXB2.gp120 using the pSVIIIenv plasmid ***(6)*** as template for the PCR reactions (*see* **ref. *15*** and **Fig. 2**) (*see* **Notes 4** and **5**).

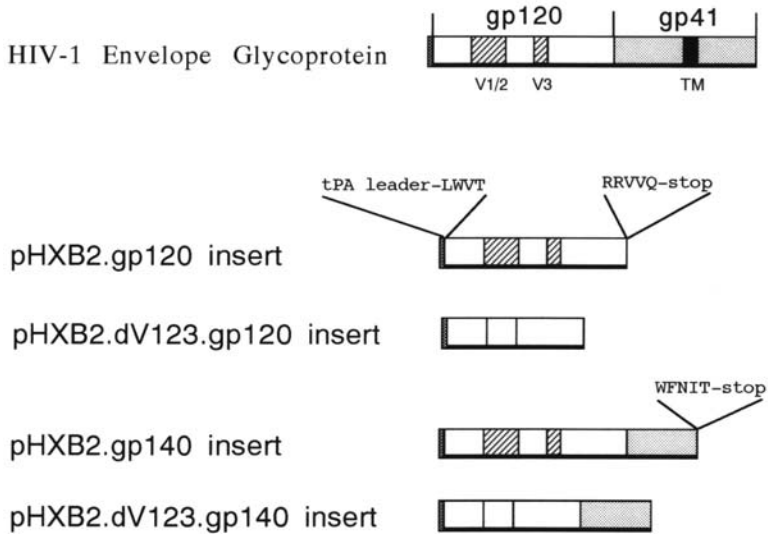

Fig. 2. HIV-1 DNA vaccine inserts expressing gp120, gp140, and the variable domain-deleted forms of envelope proteins.

The pSVIIIenv plasmid encodes the HIV-1 Env proteins with deletions of the major variable regions V1/2 (amino acids 121–203) and V3 (amino acids 297–329). A 3-amino acid sequence (Gly-Ala-Gly) replaced each of the two deletions.

2. Produce the pHXB2.dV123.gp140 insert PCR primers JApcr503 and JApcr502.
3. Produce the pHXB2.dV123.gp120 insert with PCR primers JApcr503 and JApcr504.
4. Subclone the inserts into the pJW4303 vector at the *Nhe*I and *Bam*HI sites as for the pHXB2.gp120 and pHXB2.gp140 DNA vaccines (*see* **Subheadings 3.1.3.** and **3.1.4.**).

3.2. Subcloning of DNA Vaccine Inserts into Expression Vectors

3.2.1. pBC12/CMV Vector

The pBC12/CMV/IL-2 (4732 bp) vector (**Fig. 3A**) contains the following elements (*see* **Note 6**):

bp 1-755: CMV immediate early promoter
bp 756-1439: human IL-2 sequences (to be replaced by antigen inserts)
bp 1440-2407: rat preproinsulin II gene
bp 1631-2113: intron
bp 2313: polyA site
bp 2408-4619: pXF3 backbone (*amp R*)
bp 4620-4732: SV40 *Ori*

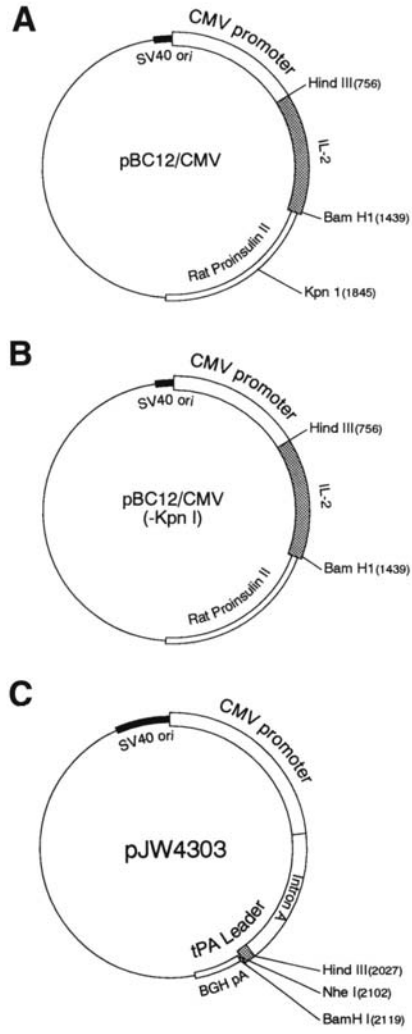

Fig. 3. DNA vaccine vectors: **(A)** the original pBC12/CMV, **(B)** modified pBC12/CMV with the removal of *Kpn*I site, and **(C)** pJW4303 with the additional CMV intron A and the tissue plasminogen activator sequences.

Use the pBC12/CMV/IL-2 vector to subclone DNA vaccine inserts as follows:

1. Digest the parental pBC12/CMV/IL-2 at *Hind*III (756) and *Bam*HI (1439) sites. Two fragments will be generated: the 685 bp human IL-2 insert and the 4047 bp pBC12/CMV vector.
2. Purify the 4047 bp pBC12/CMV vector (e.g., Qiagen's QIAEX II kit, West Sussex, UK). Discard the 685-bp human IL-2 fragment.

3. (Optional) Blunt one or both of the two ends on pBC12/CMV vector by Klenow treatment if the ends of DNA vaccine insert do not match these two sites.
4. Ligate a DNA vaccine insert into the pBC12/CMV vector.

3.2.2. pBC12/CMV (–Kpn *I*) Vector

Remove the single-*Kpn*I site (nt 1845) in pBC12/CMV/IL-2 vector (**Fig. 3B**) to produce a pBC12/CMV (–*Kpn*) vector. This allows for the easy use of *Kpn*I site(s) in antigen inserts. This was done in some of our HIV-1 DNA vaccines to facilitate the HIV-1 *env* cloning among different strains of HIV-1 virus:

1. Digest the parental pBC12/CMV/IL-2 with *Kpn*I to generate a linear form of pBC12/CMV/IL-2.
2. Treat this linear form with T4 DNA polymerase to blunt the 3' overhangs generated by *Kpn*I digestion. This removes nt 1841–1844 (GTAC).
3. Re-ligate the plasmid to produce the pBC12/CMV/IL-2 (-*Kpn*) plasmid vector.
4. Subclone DNA vaccine inserts into this vector at the *Hind*III and *Bam*HI sites, as described for the pBC12/CMV/IL-2 vector in **Subheading 3.2.1.**

3.2.3. The pJW4303 Vector

The pJW4303 vector (5139 bp) (**Fig. 3C**) (*see* **Note 7**) contains the following:

bp 1- 1194: CMV IE (immediate early) promoter
bp 1195-2027: CMV IE promoter intron A
bp 2027-2102: tissue plasminogen activator (tPA)
bp 2119-2419: BGH (bovine growth hormone) polyA
bp 2419-4794: pBR vector backbone (*amp R*)
bp 4794-5139: SV40 *Ori*

The complete nucleotide sequences of pJW4303 has recently been confirmed. Due to the wide distribution of pJW4303, its sequences and the list of its restriction endonuclease sites are included in Appendices A and B. Subclone DNA vaccine inserts into pJW4303 vector by either of the following two ways.

To use *Hind*III and *Bam*HI as the cloning sites:

1. Digest the pJW4303 with *Hind*III (2027) and *Bam*HI (2119). Purify the large fragment (5047 bp) and discard the small one (92 bp).
2. (Optional) Blunt one or both of these two ends by Klenow treatment if the ends on DNA vaccine insert do not match these two sites in pJW4303.
3. Ligate the DNA vaccine insert into the pJW4303 vector.

To use *Nhe*I and *Bam*HI as the cloning sites: Sequences between *Hind*III and *Nhe*I sites in pJW4303 encode for the leader sequences from tissue plasminogen activator (tPA). Using the *Nhe*I site would require an in-frame subcloning of the DNA vaccine insert to utilize the tPA leader, therefore the original leader sequences from the encoded protein antigen must be removed from the DNA insert. By careful design of an oligo primer, an *Nhe*I site at the 5' end of the vaccine insert can be created by polymerase chain reactions (PCR).

The subcloning steps are similar to those listed in the *Hind*III - *Bam*HI approach (see above) except the *Nhe*I site is used instead of the *Hind*III site:

1. Digest pJW4303 at *Nhe*I (2102) and *Bam*HI (2119) sites.
2. Purify the large fragment (5122 bp) and discard the small one (17 bp).
3. (Optional) Blunt one or both of these two ends by Klenow treatment if the ends of the DNA vaccine insert do not match these two sites.
4. Ligate the DNA vaccine insert into the pJW4303 vector.

3.3. Verification of Antigen Expression in COS Cells

1. Grow COS cells (*see* **Notes 8–11**) two to three passages from frozen cells in RPMI medium and 10% fetal bovine serum in 100 mm culture dishes.
2. Split the cells 1:2 into new dishes the night before transfection when cells reach 90% confluence.
3. The next day, discard the tissue culture media. Collect the cell layer by treating the dish with 5 mL of Trypsin-EDTA solution, and incubating it for 5–10 min until the cells become round.
4. Collect the cell suspension, and pellet the cells at 1500 rpm for 5 min; discard the supernatant.
5. Resuspend the cells in 0.5–1 mL of RPMI medium without fetal bovine serum. Count the cells and resuspend the cells at 2×10^6 cells/mL.
6. Transfer 0.7–0.8 mL of cell suspension into a special cuvette for electroporation.
7. Add 2–10 mg plasmid DNA to the cuvette and mix it with the cells.
8. Tap the cuvette to suspend the cells before electroporation.
9. Place the cuvette in the electroporation chamber and expose it in the applied electric field: 250 V and 960 mF when using the Bio-Rad Gene Pulser II.
10. Keep the cells in the cuvette for about 10 min.
11. Transfer the cells to a 100-mm tissue culture dish, add 6–10 mL medium and incubate for 24–78 h at 37°C in 5% (v/v) CO_2.
12. Harvest the transfected cells in 48–72 h as follows. Collect the supernatant; treat the confluent cells with TEN buffer for 5–10 min; collect the cells and microfuge them at 1500 rpm for 5 min; separate the pellet from the supernatant.
13. Store the supernatant at –70°C for future use or concentrate it by using protein concentration spin columns.
14. Lyse the cells as follows. Resuspend the cell pellet in 100–200 µL of cell lysis buffer with selected protease inhibitors and incubate for 5–10 minutes on ice; centrifuge the lysed cell suspension for 1 h at 12000 rpm in a cold room; collect the supernatant and discard the pellet; store the cell lysis at –70°C for future use.

3.4. Animal Immunization (*see* **Notes 12,13**)

3.4.1. Routes and Doses

Intramuscular route (*see* **Notes 14–16**) with a DNA concentration of ~1 mg/mL:

Balb/C mice: 50–100 µg for each of the quadriceps
NZW rabbits: ~100 µg for each of the quadriceps
Macaques: 100–250 µg for each of the quadriceps

Gene gun (*see* **Note 17**):

Balb/C mice: 0.2–0.5 μg per gun shot, 4–6 shots per animal,
NZW rabbits: 0.5 μg per gun shot, 6–36 shots per animal.
Macaques: 0.5–7.2 μg per gun shot, 4–20 shots per animal

3.4.2. Immunization Schedule

The immunogenicity of encoded antigens dictates the need and the number of boost immunizations (*see* **Note 18**). In general, a series of two to three immunizations separated by 4–8 wk is a reasonable initial trial.

4. Notes

4.1. Construction of HIV-1 Env-Expressing DNA Vaccine Inserts

1. Removal of the gene sequence for *pol* from the full length HIV viral genome produced non-replicating viral particles encoded by ".dpol-like" constructs. Env proteins are present on the surface of viral particles, therefore mimicking the actual viruses ***(14)***. Virus-like particles can also include more than one viral protein. One caveat is that the effective dose for Env in this type of plasmid may be less than in those DNA vaccines encoding for only one single Env protein if equal masses of these two types of DNA plasmid constructs are used for immunization.
2. Full length Env-expressing DNA vaccines produce a complete envelope glycoprotein. Once a basic Env plasmid is constructed, DNA sequences encoding for Env from different strains of HIV-1 can be exchanged into this construct at *Kpn*I and *Bam*HI sites to produce many chimeric Env-expressing plasmids. This may be a quick way to test the antigen specificity of Env sequences from the different HIV-1 patient isolates. However, the full-length gp160-expressing DNA vaccine was shown to be less immunogenic than the gp140 and gp120-expressing ones in raising anti-Env antibody responses ***(15)***.
3. HIV-1 gp120- and gp140-expressing constructs further demonstrate the potential of DNA immunization to make subunit-based vaccines. The gp120 form is the natural proteolytic product of the HIV-1 Env gp160 precursor protein. It is highly immunogenic, presumably due to its monomer conformation and a high level of secretion of gp120 from the cells. The gp140 form of env encodes for the entire extracellular portion of Env, including gp120 plus the ectodomain of gp41. This construct is used to produce a soluble form of Env yet maintaining Env's oligomeric conformation.
4. Removal of the variable domains V1, V2, and V3 from the Env protein would further expose certain hidden antigenic determinants to improve the overall immunogenicity of Env. The ease of the DNA immunization technique offers a unique opportunity to eventually find an Env design that is very immunogenic yet preserves the critical conformation of its original protein structure.
5. These constructs demonstrate that one antigenic protein (such as the HIV-1 envelope) can be expressed in many forms. The overall goal is to find the most immunogenic ones that also have a conformation most closely resembling the native

antigens. Judging from the results of expressing antigens ranging from nearly complete viral particles down to only certain segments of one viral protein, the above model constructs suggested that it is extremely important to design the right DNA inserts for the right purpose. If a protein is highly hydrophobic or many of its epitopes are inaccessible due to a complex protein conformation, careful antigen engineering is essential.

4.2. Subcloning of DNA Vaccine Inserts into Expression Vectors

6. Except for a few very immunogenic antigens, a high in vivo protein antigen expression is required for most DNA vaccine inserts. Therefore, it is critical to select mammalian expression vectors that have high efficiency and also match with the design of DNA vaccine inserts. Many DNA vaccine studies have employed the CMV immediate early promoter as the key component in vectors, similar to the two described in this chapter. For beginners in DNA immunization, it is wise to try vectors that are known to be effective, rather than to simply put DNA vaccine inserts into any available plasmid vector in the initial trial.
7. In comparison with the classical pBC12/CMV vector, the pJW4303 vector has two major additions: the intron A sequences just downstream of the CMV promoter and a tPA leader sequences. By including the intron A sequences in the vector, the overall expression level was improved ***(13)***. The tPA leader sequences in pJW4303 provide the choice of making secreted protein antigens ***(13)***. It is desirable to use the tPA leader sequences to produce soluble proteins. On the other hand, the tPA leader may become less helpful if the goal is to have a high expression of mainly intracellular proteins, such as to mainly induce the cytotoxic lymphocyte responses.

4.3. Verification of Antigen Expression in COS Cells

8. Verification of the authenticity for a particular DNA vaccine is necessary before great effort is placed on the immunization process. The in vitro transient expression in tissue cultured cells is a quick and reliable quality control step to detect the encoded antigens in DNA vaccines.
9. COS cells are described here as an example. The most important element for a successful transfection is the condition of cells. Only healthy cells in logarithmic growth phase can produce high expression.
10. Electroporation and the liposome transfection reagents are equally effective. Electroporation is simple but needs special equipment (such as one described in this chapter). The settings of electroporation need to be fine-tuned for each type of target cell. Ideally, a "killing curve" should be established to find the highest electrical pulsing under which 50–80% cells can still recover and grow in 2–3 days.
11. Enzyme-linked immunosorbent assay (ELISA), Western blot, or indirect immunofluorescent staining of the transfected cells can be used to detect the expression of encoded antigens, if there is a specific serum or antibody available to identify the antigen. If there is no antibody available, a "tag" sequence can be added to the vaccine insert sequences, and then an antiserum against the tag can

be used to detect the expression of the whole fusion proteins. In the worst situation, the expression study can be postponed until post-immunization. Sera from immunized animals can be used at that time to verify antigen expression in transfected cells.

4.4. Animal Immunization

12. Ideally, it will be most useful to demonstrate in vivo expression of DNA vaccines. However, due to the minute amount of protein expressed in the local tissue of a living animal, antigen expression may frequently fall below the level of detection in an ELISA assay. One alternative way is to do immunohistochemistry staining on tissue sections. Taking biopsy samples at ~24 h post-immunization approximately coincides with the peak antigen expression at the site of inoculation.
13. Using a DNA vaccine encoding for a marker protein, such as luciferase ***(18)***, may increase the sensitivity of detecting antigen in vivo, but can only be used as an indirect reference for the expression of specific antigens.
14. Intramuscular inoculation with a conventional syringe and epidermal inoculation using the gene gun have become the dominant methods of delivering DNA vaccines. However, the following routes can also be used to administer DNA vaccines: intravenous, intradermal, subcutaneous, or peritoneal routes by a syringe, or via mucosal surface by a gene gun ***(5,19–20)***.
15. Both intramuscular and gene gun routes are effective in delivering DNA vaccines. From our experience, for a given antigen insert, gene gun delivery may require less DNA and may induce a more homogenous response for a group of animals than the intramuscular route. On the other hand, the gene gun approach is more expensive due to the cost of gene gun, gold beads, and other ancillary equipment associated with gene gun use.
16. The DNA doses described in this chapter can only be used as a general reference. There is great variation in the immunogenicity of different antigens. For beginners in DNA immunization, a higher dose of DNA plasmids can be used at first to at least induce some level of desired immune responses before eventually titrating down the dose-response curve.
17. With the marketing of commercial Helios Gene Gun, a more standardized gene gun use is anticipated and the manufacturer will provide post-sale support including a detailed protocol. However, two critical points are emphasized here for interested users: (i) Always give enough time to dry the plastic tubing with nitrogen gas so that the potential moisture can be removed; (ii) Always test the delivery depth of a gene gun before starting on a new animal species. This can be achieved by taking a biopsy sample from gene gun-inoculated skin to examine the gold bead distribution in H & E histology sections.
18. The number of required immunizations is dependent on individual antigens and may also be dependent on animal species. Generally, a very close-spaced immunization schedule is not helpful. Spacing the immunizations to have at least 4 wk resting period is recommended. For some antigens, one immunization is sufficient and the antibody responses may take 8–10 wk to peak ***(11)***.

Appendix A. pJW4303 DNA Sequences

pJW4303 Sequences

```
          *          *          *          *          *          *          *  70
CTCGTCTCCGACGTCGCGGCCGCTCTAGGCCTCCAAAAAAGCCTCCTCACTACTTCTGGAATAGCTCAGA
          *          *          *          *          *          *          * 140
GGCCGAGGCGGCCTCGGCCTCTGCATAAATAAAAAAAATTAGTCAGCCATGCATGGGGCGGAGAATGGGC
          *          *          *          *          *          *          * 210
GGAACTGGGCGGAGTTAGGGGCGGGATGGGCGGAGTTAGGGGCGGGACTATGGTTGCTGACTAATTGAGA
          *          *          *          *          *          *          * 280
TGCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACACCTGGTTGCTGACTAATTGA
          *          *          *          *          *          *          * 350
GATGCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACACCCTAACTGACACACATT
          *          *          *          *          *          *          * 420
CCACAGAATTAATTCCCGGGGATCGATCCGGTCGACAATATTGGCTATTGGCCATTGCATACGTTGTATC
          *          *          *          *          *          *          * 490
TATATCATAATATGTACATTTATATTGGCTCATGTCCAATATGACCGCCATGTTGACATTGATTATTGAC
          *          *          *          *          *          *          * 560
TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAA
          *          *          *          *          *          *          * 630
CTTACGGTAAATGGCCCGCCTCGTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGT
          *          *          *          *          *          *          * 700
TCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCAC
          *          *          *          *          *          *          * 770
TTGGCAGTACATCAAGTGTATCATATGCCAAGTCCGGCCCCCTATTGACGTCAATGACGGTAAATGGCCC
          *          *          *          *          *          *          * 840
GCCTGGCATTATGCCCAGTACATGACCTTACGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCA
          *          *          *          *          *          *          * 910
TCGCTATTACCATGGTGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGACTCACGGGG
          *          *          *          *          *          *          * 980
ATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCA
          *          *          *          *          *          *          *1050
AAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAA
          *          *          *          *          *          *          *1120
GCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAG
          *          *          *          *          *          *          *1190
ACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCAAGAGT
          *          *          *          *          *          *          *1260
GACGTAAGTACCGCCTATAGACTCTATAGGCACACCCCTTTGGCTCTTATGCATGCTATACTGTTTTTGG
          *          *          *          *          *          *          *1330
CTTGGGGCCTATACACCCCCGCTCCTTATGCTATAGGTGATGGTATAGCTTAGCCTATAGGTGTGGGTTA
          *          *          *          *          *          *          *1400
TTGACCATTATTGACCACTCCCCTATTGGTGACGATACTTTCCATTACTAATCCATAACATGGCTCTTTG
          *          *          *          *          *          *          *1470
CCACAACTATCTCTATTGGCTATATGCCAATACTCTGTCCTTCAGAGACTGACACGGACTCTGTATTTTT
          *          *          *          *          *          *          *1540
ACAGGATGGGGTCCCATTTATTATTTACAAATTCACATATACAACAACGCCGTCCCCCGTGCCCGCAGTT
          *          *          *          *          *          *          *1610
TTTATTAAACATAGCGTGGGATCTCCACGCGAATCTCGGGTACGTGTTCCGGACATGGGCTCTTCTCCGG
          *          *          *          *          *          *          *1680
TAGCGGCGGAGCTTCCACATCCGAGCCCTGGTCCCATGCCTCCAGCGGCTCATGGTCGCTCGGCAGCTCC
          *          *          *          *          *          *          *1750
TTGCTCCTAACAGTGGAGGCCAGACTTAGGCACAGCACAATGCCCACCACCACCAGTGTGCCGCACAAGG
          *          *          *          *          *          *          *1820
CCGTGGCGGTAGGGTATGTGTCTGAAAATGAGCTCGGAGATTGGGCTCGCACCGTGACGCAGATGGAAGA
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        *         *         *         *         *         *        *1890
CTTAAGGCAGCGGCAGAAGAAGATGCAGGCAGCTGAGTTGTTGTATTCTGATAAGAGTCAGAGGTAACTC
        *         *         *         *         *         *        *1960
CCGTTGCGGTGCTGTTAACGGTGGAGGGCAGTGTAGTCTGAGCAGTACTCGTTGCTGCCGCGCGCGCCAC
        *         *         *         *         *         *        *2030
CAGACATAATAGCTGACAGACTAACAGACTGTTCCTTTCCATGGGTCTTTTCTGCAGTCACCGTCCAAGC
        *         *         *         *         *         *        *2100
TTGCAATCATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTC
        *         *         *         *         *         *        *2170
GGCTAGCCCGGGACGCGTGGATCCTCGCAATCCCTAGGAGGATTAGGCAAGGGCTTGAGCTCACGCTCTT
        *         *         *         *         *         *        *2240
GTGAGGGACAGAAATACAATCAGGGGCAGTATATGAATACTCCATGGAGAAACCCAGATCTACGTATGAT
        *         *         *         *         *         *        *2310
CAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCT
        *         *         *         *         *         *        *2380
GGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGT
        *         *         *         *         *         *        *2450
CATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATG
        *         *         *         *         *         *        *2520
CTGGGGATGCGGTGGGCTCTATGGAACCAGCTGGGGCTCGACAGGACCGCTATCAGGACATAGCGTTGGC
        *         *         *         *         *         *        *2590
TACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCC
        *         *         *         *         *         *        *2660
GCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTT
        *         *         *         *         *         *        *2730
CGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAA
        *         *         *         *         *         *        *2800
AGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGG
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        *         *         *         *         *         *        *2940
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        *         *         *         *         *         *        *3150
TAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGT
        *         *         *         *         *         *        *3220
GCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTC
        *         *         *         *         *         *        *3290
CTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTA
        *         *         *         *         *         *        *3360
ATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCA
        *         *         *         *         *         *        *3430
GGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAA
        *         *         *         *         *         *        *3500
TCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGC
        *         *         *         *         *         *        *3570
TCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAA
        *         *         *         *         *         *        *3640
GCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGG
        *         *         *         *         *         *        *3710
CTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAAC
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         *         *         *         *         *         *         *3780
CCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAG
         *         *         *         *         *         *         *3850
GCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTG
         *         *         *         *         *         *         *3920
CGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCT
         *         *         *         *         *         *         *3990
GGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTT
         *         *         *         *         *         *         *4060
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         *         *         *         *         *         *         *4130
ATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATAT
         *         *         *         *         *         *         *4200
GAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTC
         *         *         *         *         *         *         *4270
GTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCC
         *         *         *         *         *         *         *4340
CAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCC
         *         *         *         *         *         *         *4410
GGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGG
         *         *         *         *         *         *         *4480
AAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGT
         *         *         *         *         *         *         *4550
GTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCC
         *         *         *         *         *         *         *4620
CCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAG
         *         *         *         *         *         *         *4690
TGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC
         *         *         *         *         *         *         *4760
TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCG
         *         *         *         *         *         *         *4830
GCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTT
         *         *         *         *         *         *         *4900
CGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAA
         *         *         *         *         *         *         *4970
CTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCA
         *         *         *         *         *         *         *5040
AAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCA
         *         *         *         *         *         *         *5110
TTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGT
         *         *       5139
TCCGCGCACATTTCCCCGAAAAGTGCCAC
```

Enzyme	#	Cuts/Positions
AatII	5	14 614 667 751 937
AccI	1	382
AciI	70	16 20 78 128 139 149 161 170 182 465 548 576 587 601 769 860 893 997 1018 1142 1144 1169 1201 1279 1533 1613 1616 1655 1741 1756 1830 1896 1948 2459 2497 2547 2563 2589 2645 2714 2717 2783 2953 2957 3021 3124 3180 3190 3214 3257 3264 3285 3376 3404 3531 3550 3671 3781 3916 3925 4287 4378 4569 4615 4736 4780 4857 4966 5065 5112
AflII	1	1821
AflIII	3	1582 2113 3331
AhdI	1	4224
AluI	24	64 1056 1308 1621 1676 1782 1852 1972 2029 2159 2480 2542 2833 2974 2996 3091 3155 3273 3499 3635 3892 4413 4513 4576
AlwI	18	369 378 1126 1567 2114 2127 2766 2795 2955 2956 3892 3978 3980 4076 4077 4540 4857 4861
Alw26I	6	9 924 1079 1439 4285 5061
AlwNI	2	1448 3747
ApaLI	2	3645 4891
ApoI	2	1499 2868
AseI	5	359 497 3102 3161 4396
AvaI	3	365 1575 2107
AvaII	6	1127 1480 1640 2494 4362 4584
AvrII	1	2133
BamHI	1	2119
BanI	4	955 2315 3075 4172
BanII	9	1058 1601 1637 1784 1797 2061 2161 2468 2488
BbsI	4	1124 1823 2083 2439
BbvI	17	1685 1839 1861 1931 2058 2061 2614 2842 3142 3223 3241 3660 3750 3753 3959 4262 4653
BclI	1	2237
BfaI	9	25 490 2103 2134 2259 2903 3826 4079 4414
BglI	5	78 701 773 1144 4344
BglII	1	2226
BlpI	1	1309
BpmI	5	1103 1635 2761 2818 4294
BsaI	1	4285
BsaAI	3	830 1583 2233
BsaHI	9	11 611 664 748 934 1088 2680 2759 4761
BsaJI	17	73 82 244 316 365 366 850 1142 1636 1751 1999 2107 2133 2212 2307 3070 3491
BsaWI	6	377 1588 1606 3537 3684 4515
BseRI	1	34
BsiEI	8	20 382 1148 2957 3247 3671 4594 4743
BsiHKAI	6	1058 1784 2161 3649 4810 4895
BsiI	16	123 539 1148 1389 1595 1637 1692 1706 2000 2503 2784 3179 3353 3371 3537 3816

Enzyme	#	Cuts/Positions
BsmI	1	2898
BsmBI	2	9 1079
BsmFI	14	198 262 334 664 816 984 1140 1466 1507 1626 2124 2189 2661 2770
BsoBI	3	365 1575 2107
BsoFI	37	17 20 79 1145 1614 1656 1674 1741 1828 1831 1850 1945 1948 2072 2075 2548 2589 2603 2717 2831 2954 2957 3156 3237 3255 3258 3376 3531 3674 3739 3742 3948 4276 4615 4642 4737 4966
Bsp1286I	13	1058 1533 1601 1637 1784 1797 2061 2161 2468 2488 3649 4810 4895
BspDI	1	373
BspEI	1	1588
BspHI	2	4051 5059
BspMI	1	2696
BsrI	12	149 785 1733 3131 3739 3752 3864 4270 4388 4431 4700 4870
BsrBI	7	22 1281 2591 2645 3023 3264 5065
BsrDI	4	402 2052 4285 4459
BsrFI	2	2761 4304
BsrGI	1	433
BssHII	2	1950 1952
BssKI	21	243 260 315 364 365 771 1080 1123 1147 1636 2106 2107 2307 2753 3069 3357 3478 3491 3709 4405 4756
BssSI	5	580 2570 2696 3504 4888
BstBI	1	2660
BstNI	11	245 262 317 773 1082 1638 2309 3071 3359 3480 3493
BstUI	19	16 550 1144 1169 1569 1950 1952 1954 2115 2783 2953 2959 3178 3180 3378 3959 4289 4782 5114
BstYI	10	1559 2119 2226 2787 3972 3983 4069 4081 4849 4866
Cac8I	25	214 233 286 305 576 769 1243 1533 1797 1847 1952 1954 2031 2104 2267 2444 2448 2763 3124 3153 3262 3348 3385 3945 4336
ClaI	1	373
Csp6I	10	434 707 788 821 872 1029 1198 1580 1935 4703
DdeI	11	65 1309 1705 1853 1928 2369 2641 3606 4015 4181 4721
DpnI	25	372 376 1077 1133 1561 2121 2228 2239 2773 2789 2950 2962 3899 3974 3985 3993 4071 4083 4188 4529 4547 4593 4851 4868 4904
DpnII	25	370 374 1075 1131 1559 2119 2226 2237 2771 2787 2948 2960 3897 3972 3983 3991 4069 4081 4186 4527 4545 4591 4849 4866 4902
DraI	3	4090 4109 4801
DrdI	1	3439
EaeI	6	17 399 1145 2954 3170 4612
EagI	3	17 1145 2954
EarI	5	1606 2045 2532 3215 5019
Ecl136II	3	1056 1782 2159

Enzyme	#Cuts	Positions
Eco57I	4	1425 2556 3879 4891
EcoO109I	2	1265 1480
FokI	10	177 1078 1487 1614 2054 2468 2781 4190 4371 4658
FspI	1	4446
HaeII	2	3209 3579
HaeIII	22	19 29 72 81 87 401 574 737 767 1147 1267 1699 1750 2956 3172 3346 3357 3375 3809 4267 4347 4614
HgaI	9	1016 1096 1815 2121 2688 2767 3442 4020 4750
HhaI	18	1952 1954 1956 2607 2783 3115 3180 3208 3241 3511 3578 3678 3852 3961 4354 4447 4784 5116
HinP1I	18	1950 1952 1954 2605 2781 3113 3178 3206 3239 3509 3576 3676 3850 3959 4352 4445 4782 5114
HincII	4	383 474 1006 1906
HindIII	1	2027
HinfI	15	900 1172 1210 1457 1571 1875 2597 2649 2707 2744 3166 3231 3306 3702 4219
HpaI	1	1906
HpaII	20	366 378 734 1124 1148 1589 1607 2108 2754 2762 3049 3538 3685 3711 3901 4305 4339 4406 4516 4758
HphI	9	866 1308 1370 2010 4067 4294 4710 4916 4951
MboI	25	370 374 1075 1131 1559 2119 2226 2237 2771 2787 2948 2960 3897 3972 3983 3991 4069 4081 4186 4527 4545 4591 4849 4866 4902
MboII	17	1129 1593 1828 1848 1851 2062 2083 2444 2549 2629 2796 3202 3993 4064 4819 4897 5006
MluI	1	2113
MnlI	34	40 52 55 62 68 92 98 589 1031 1119 1149 1659 1689 1874 1907 2048 2131 2133 2166 2254 2296 2341 2416 2579 2785 3180 3230 3439 3513 3763 4163 4244 4391 4597
MscI	1	401
MseI	15	359 497 1545 1822 1905 3102 3161 4037 4089 4094 4108 4161 4396 4435 4800
MslI	5	855 1735 4476 4635 4994
MspI	20	366 378 734 1124 1148 1589 1607 2108 2754 2762 3049 3538 3685 3711 3901 4305 4339 4406 4516 4758
MspA1I	9	1144 1655 1830 1852 2480 3155 3673 3918 4859
MwoI	23	78 84 167 218 290 701 773 866 890 1096 1144 1287 1953 2112 2456 2688 3075 3119 3203 3270 3384 3956 4344
NaeI	1	2763
NciI	10	366 367 1125 1149 2108 2109 2755 3711 4407 4758
NcoI	3	850 1999 2212
NdeI	1	723
NgoMI	1	2761
NheI	1	2102
NlaIII	27	121 125 216 288 454 472 794 854 1245 1392 1597 1648 1664 2003 2041 2216 2450 2796 2944 2989 3335 4055 4546 4556 4634 4670 5063
NlaIV	20	242 314 738 957 1128 1266 1481 1482 1642 2121 2317 2475 3077 3363 3402 4174 4268 4309 4520 5110
NotI	2	17 2954
NsiI	4	123 214 286 1243
PflMI	1	1389
PleI	8	894 1204 1451 1883 2643 3225 3710 4213
Ppu10I	4	119 210 282 1239
PpuMI	1	1480
Psp1406I	2	4450 4823
PstI	1	2016
PvuI	1	4594
PvuII	3	1852 2480 3155
RsaI	10	435 708 789 822 873 1030 1199 1581 1936 4704
SacI	3	1058 1784 2161
SacII	1	1145
SalI	1	381
SapI	3	1606 2532 3215
Sau96I	12	573 736 766 1127 1265 1480 1640 2494 4266 4345 4362 4584
Sau3AI	25	370 374 1075 1131 1559 2119 2226 2237 2771 2787 2948 2960 3897 3972 3983 3991 4069 4081 4186 4527 4545 4591 4849 4866 4902
ScaI	2	1936 4704
ScrFI	21	245 262 317 366 367 773 1082 1125 1149 1638 2108 2109 2309 2755 3071 3359 3480 3493 3711 4407 4758
SfaNI	13	199 271 847 1832 2032 2366 2446 2616 2870 3428 4480 4671 4920
SfcI	8	1205 1214 1291 1315 2012 3596 3787 4465
SfiI	1	78
SmaI	2	367 2109
SnaBI	2	830 2233
SpeI		1 489
SphI	4	216 288 1245 2450
SspI	2	389 5028
StuI	1	29
StyI	4	850 1999 2133 2212
TaqI	9	373 382 2246 2488 2660 2705 2968 3431 4875
TfiI	7	1172 1571 2597 2707 2744 3166 3306
TseI	17	1673 1827 1849 1944 2071 2074 2602 2830 3155 3236 3254 3673 3738 3741 3947 4275 4641
Tsp45I	7	582 1188 1358 1803 2016 4480 4691
Tsp509I	15	106 202 274 356 360 508 1499 2352 2868 3011 3028 3103 4091 4397 4652
TspRI	7	1697 1740 1925 4023 4172 4277 4624
XcmI	1	1352
XmaI	2	365 2107
XmnI	2	360 4823

Appendix B. The Potential Enzyme Sites on pJW4303

Enzyme	#Cuts	Positions
AatII	5	14 614667 751 937
AccI	1	382
AciI	70	16 20 78 128 139 149 161 170 182 465 548 576 587 601 769 860 893 997 1018 1142 1144 1169 1201 1279 1533 1613 1616 1655 1741 1756 1830 1896 1948 2459 2497 2547 2563 2589 2645 2714 2717 2783 2953 2957 3021 3124 3180 3190 3214 3257 3264 3285 3376 3404 3531 3550 3671 3781 3916 3925 4287 4378 4569 4615 4736 4780 4857 4966 5065 5112
AflII	1	1821
AflIII	3	1582 2113 3331
AhdI	1	4224
AluI	24	64 1056 1308 1621 1676 1782 1852 1972 2029 2159 2480 2542 2833 2974 2996 3091 3155 3273 3499 3635 3892 4413 4513 4576
AlwI	18	369 378 1126 1567 2114 2127 2766 2795 2955 2956 3892 3978 3980 4076 4077 4540 4857 4861
Alw26I	6	9 924 1079 1439 4285 5061
AlwNI	2	1448 3747
ApaLI	2	3645 4891
ApoI	2	1499 2868
AseI	5	359 497 3102 3161 4396
AvaI	3	365 1575 2107
AvaII	6	1127 1480 1640 2494 4362 4584
AvrII	1	2133
BamHI	1	2119
BanI	4	955 2315 3075 4172
BanII	9	1058 1601 1637 1784 1797 2061 2161 2468 2488
BbsI	4	1124 1823 2083 2439
BbvI	17	1685 1839 1861 1931 2058 2061 2614 2842 3142 3223 3241 3660 3750 3753 3959 4262 4653
BclI	1	2237
BfaI	9	25 490 2103 2134 2259 2903 3826 4079 4414
BglI	5	78 701 773 1144 4344
BglII	1	2226
BlpI	1	1309
BpmI	5	1103 1635 2761 2818 4294
BsaI	1	4285
BsaAI	3	830 1583 2233
BsaHI	9	11 611 664 748 934 1088 2680 2759 4761
BsaJI	17	73 82 244 316 365 366 850 1142 1636 1751 1999 2107 2133 2212 2307 3070 3491
BsaWI	6	377 1588 1606 3537 3684 4515
BseRI	1	34
BsiEI	8	20 382 1148 2957 3247 3671 4594 4743
BsiHKAI	6	1058 1784 2161 3649 4810 4895
BslI	16	123 539 1148 1389 1595 1637 1692 1706 2000 2503 2784 3179 3353 3371 3537 3816
BsmI	1	2898
BsmBI	2	9 1079
BsmFI	14	198 262 334 664 816 984 1140 1466 1507 1626 2124 2189 2661 2770
BsoBI	3	365 1575 2107
BsoFI	37	17 20 79 1145 1614 1656 1674 1741 1828 1831 1850 1945 1948 2072 2075 2548 2589 2603 2717 2831 2954 2957 3156 3237 3255 3258 3376 3531 3674 3739 3742 3948 4276 4615 4642 4737 4966
Bsp1286I	13	1058 1533 1601 1637 1784 1797 2061 2161 2468 2488 3649 4810 4895
BspDI	1	373
Enzyme	**#Cuts**	**Positions**
BspEI	1	1588
BspHI	2	4051 5059
BspMI	1	2696
BsrI	12	149 785 1733 3131 3739 3752 3864 4270 4388 4431 4700 4870
BsrBI	7	22 1281 2591 2645 3023 3264 5065
BsrDI	4	402 2052 4285 4459
BsrFI	2	2761 4304
BsrGI	1	433
BssHII	2	1950 1952
BssKI	21	243 260 315 364 365 771 1080 1123 1147 1636 2106 2107 2307 2753 3069 3357 3478 3491 3709 4405 4756
BssSI	5	580 2570 2696 3504 4888
BstBI	1	2660
BstNI	11	245 262 317 773 1082 1638 2309 3071 3359 3480 3493
BstUI	19	16 550 1144 1169 1569 1950 1952 1954 2115 2783 2953 2959 3178 3180 3378 3959 4289 4782 5114
BstYI	10	1559 2119 2226 2787 3972 3983 4069 4081 4849 4866
Cac8I	25	214 233 286 305 576 769 1243 1533 1797 1847 1952 1954 2031 2104 2267 2444 2448 2763 3124 3153 3262 3348 3385 3945 4336
ClaI	1	373
Csp6I	10	434 707 788 821 872 1029 1198 1580 1935 4703
DdeI	11	65 1309 1705 1853 1928 2369 2641 3606 4015 4181 4721
DpnI	25	372 376 1077 1133 1561 2121 2228 2239 2773 2789 2950 2962 3899 3974 3985 3993 4071 4083 4188 4529 4547 4593 4851 4868 4904
DpnII	25	370 374 1075 1131 1559 2119 2226 2237 2771 2787 2948 2960 3897 3972 3983 3991 4069 4081 4186 4527 4545 4591 4849 4866 4902
DraI	3	4090 4109 4801
DrdI	1	3439
EaeI	6	17 399 1145 2954 3170 4612
EagI	3	17 1145 2954
EarI	5	1606 2045 2532 3215 5019
Ecl136II	3	1056 1782 2159
Eco57I	4	1425 2556 3879 4891
EcoO109I	2	1265 1480
FokI	10	177 1078 1487 1614 2054 2468 2781 4190 4371 4658
FspI	1	4446
HaeII	2	3209 3579
HaeIII	22	19 29 72 81 87 401 574 737 767 1147 1267 1699

		1750 2956 3172 3346 3357 3375 3809 4267 4347 4614
HgaI	9	1016 1096 1815 2121 2688 2767 3442 4020 4750
HhaI	18	1952 1954 1956 2607 2783 3115 3180 3208 3241 3511 3578 3678 3852 3961 4354 4447 4784 5116
HinP1I	18	1950 1952 1954 2605 2781 3113 3178 3206 3239 3509 3576 3676 3850 3959 4352 4445 4782 5114
HincII	4	383 474 1006 1906
HindIII	1	2027
HinfI	15	900 1172 1210 1457 1571 1875 2597 2649 2707 2744 3166 3231 3306 3702 4219
HpaI	1	1906
HpaII	20	366 378 734 1124 1148 1589 1607 2108 2754 2762 3049 3538 3685 3711 3901 4305 4339 4406 4516 4758
Enzyme	**#Cuts**	**Positions**
HphI	9	866 1308 1370 2010 4067 4294 4710 4916 4951
MboI	25	370 374 1075 1131 1559 2119 2226 2237 2771 2787 2948 2960 3897 3972 3983 3991 4069 4081 4186 4527 4545 4591 4849 4866 4902
MboII	17	1129 1593 1828 1848 1851 2062 2083 2444 2549 2629 2796 3202 3993 4064 4819 4897 5006
MluI	1	2113
MnlI	34	40 52 55 62 68 92 98 589 1031 1119 1149 1659 1689 1874 1907 2048 2131 2133 2166 2254 2296 2341 2416 2579 2785 3180 3230 3439 3513 3763 4163 4244 4391 4597
MscI	1	401
MseI	15	359 497 1545 1822 1905 3102 3161 4037 4089 4094 4108 4161 4396 4435 4800
MslI	5	855 1735 4476 4635 4994
MspI	20	366 378 734 1124 1148 1589 1607 2108 2754 2762 3049 3538 3685 3711 3901 4305 4339 4406 4516 4758
MspA1I	9	1144 1655 1830 1852 2480 3155 3673 3918 4859
MwoI	23	78 84 167 218 290 701 773 866 890 1096 1144 1287 1953 2112 2456 2688 3075 3119 3203 3270 3384 3956 4344
NaeI	1	2763
NciI	10	366 367 1125 1149 2108 2109 2755 3711 4407 4758
NcoI	3	850 1999 2212
NdeI	1	723
NgoMI	1	2761
NheI	1	2102
NlaIII	27	121 125 216 288 454 472 794 854 1245 1392 1597 1648 1664 2003 2041 2216 2450 2796 2944 2989 3335 4055 4546 4556 4634 4670 5063
NlaIV	20	242 314 738 957 1128 1266 1481 1482 1642 2121 2317 2475 3077 3363 3402 4174 4268 4309 4520 5110
NotI	2	17 2954
NsiI	4	123 214 286 1243
PflMI	1	1389
PleI	8	894 1204 1451 1883 2643 3225 3710 4213
Ppu10I	4	119 210 282 1239
PpuMI	1	1480
Psp1406I	2	4450 4823
PstI	1	2016
PvuI	1	4594
PvuII	3	1852 2480 3155
RsaI	10	435 708 789 822 873 1030 1199 1581 1936 4704
SacI	3	1058 1784 2161
SacII	1	1145
SalI	1	381
SapI	3	1606 2532 3215
Sau96I	12	573 736 766 1127 1265 1480 1640 2494 4266 4345 4362 4584
Sau3AI	25	370 374 1075 1131 1559 2119 2226 2237 2771 2787 2948 2960 3897 3972 3983 3991 4069 4081 4186 4527 4545 4591 4849 4866 4902
ScaI	2	1936 4704
ScrFI	21	262 317 366 367 773 1082 1125 1149 1638 2108 2109 2309 2755 3071 3359 3480 3493 3711 4407 4758
SfaNI	13	199 271 847 1832 2032 2366 2446 2616 2870 3428 4480 4671 4920
Enzyme	**#Cuts**	**Positions**
SfcI	8	1205 1214 1291 1315 2012 3596 3787 4465
SfiI	1	78
SmaI	2	367 2109
SnaBI	2	830 2233
SpeI	1	489
SphI	4	216 288 1245 2450
SspI	2	389 5028
StuI	1	29
StyI	4	850 1999 2133 2212
TaqI	9	373 382 2246 2488 2660 2705 2968 3431 4875
TfiI	7	1172 1571 2597 2707 2744 3166 3306
TseI	17	1673 1827 1849 1944 2071 2074 2602 2830 3155 3236 3254 3673 3738 3741 3947 4275 4641
Tsp45I	7	582 1188 1358 1803 2016 4480 4691
Tsp509I	15	106 202 274 356 360 508 1499 2352 2868 3011 3028 3103 4091 4397 4652
TspRI	7	1697 1740 1925 4023 4172 4277 4624
XcmI	1	1352
XmaI	2	365 2107
XmnI	2	360 4823

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Genetic Vaccination Targeting T-Cell Receptors

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1. Introduction

T-cell antigen receptor (TCR) genes (which consist of variable (V), diversity (D), joining (J) and constant (C) segments) undergo rearrangement during T-cell development and result in the expression of a disulfide linked heterodimer (α and ß chains) on the surface of mature T-cells ***(1,2)***. The TCR confers specificity to each T-cell for antigen recognition (in the context of major histocompatibility (MHC) molecules ***(1,3)***. Clonal TCR-ß chain gene rearrangements have been demonstrated in DNA samples derived from cutaneous tumors, peripheral blood lymphocytes and lymph nodes of patients with cutaneous T cell lymphomas (CTCL) ***(4–6)***. Together with immunohistologic data ***(7)***, these findings indicate that CTCL is a clonal disease of malignant T cells that express α/ß-TCR.

The TCR, like surface immunoglobulin (sIg) on B-cells, is a cell specific idiotype and can serve as a target for anti-idiotype therapy. Anti-idiotype therapy ***(8)*** has been reported in B-cell malignancies ***(9)*** and T-cell mediated autoimmune disease ***(10–12)***. A variety of approaches have been attempted in murine and human B-cell lymphomas and include: monoclonal antibody (mAb) directed at surface Ig ***(13)***, immunization with sIg protein ***(9)*** and sIg conjugated to cytokine/growth factors ***(14)***. In the rodent model of multiple sclerosis-experimental autoimmune encephalomyelitis (EAE), reports demonstrate that the disease can be modulated by immunizing the animals to TCR peptides on pathogenic $CD4^+$ T-cell clones reactive to myelin basic protein and responsible for inducing EAE ***(10–12)***.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

Recently, direct in vivo inoculation with plasmid DNA has been shown to result in the intracellular synthesis of plasmid encoded protein which, in turn, produces a targeted immune response (DNA vaccination) ***(15–17)***. DNA constructs encoding T-cell receptor (TCR) and surface immunoglobulin (sIg) have been utilized in vaccination protocols, producing protective immunity in murine models of T-cell mediated autoimmune disease and B-cell lymphoma ***(18,19)***.

B and T cell neoplasms provide a likely target for genetic immunization in that each of these tumors comes with its own tumor antigen in the form of a unique antigen receptor. Recombinant DNA technology allows us to "borrow" from the antigen receptor the coding sequence for the variable region. This region contains the structures most unique to the molecule. Here we focus on the beta chain of the T-cell receptor (TCR) as the source of our immunogen, although analogous strategies could be useful for antibody based immunogens, and thus B-cells.

The application of this technology to tumor immunity may confer the unique advantage that although the product immunogen protein derives from native structures, the synthetic nature of the product may be subject to novel immunological surveillance.

1.1. Identification and Cloning of Pathological TCR

The experiments performed to date have involved cloning particular T cell receptor variable region sequences with no regard to the clonotype. In other words, we are vaccinating with a variable region coding sequence representing the family of the pathogenic clone but not necessarily the idiotype. Ultimately, we would like to use vaccines that would generate a specific response to the TCR idiotype (third hypervariable region) as well as specificity for the entire family. The successful immunization against T-cell receptors may eventually allow us to not only eliminate the obvious target of T-cell derived cancers but also to combat T cell-mediated autoimmunity.

Our experience has been with a chronic T-cell lymphoma. Two methods were used to determine the TCR family of the tumor. First, the patients peripheral blood lymphocytes were stained with a battery of antibodies specific for the various TCR V-beta family gene products. Second, cDNA reverse transcribed from total cellular RNA of peripheral blood mononuclear cells (PBMC) was subjected to a semi-quantitative PCR method which utilizes a battery of TCR beta specific primers. These two independent methods should expose a dominant signal for one TCR-V beta gene. Due to space limitations, neither of these techniques can be discussed here in detail.

Once the particular family of V beta gene is identified, we can specifically amplify a clone bearing such a segment. The assumption that the clone amplified in these experiments adequately represents the lymphoma is reasonable for two reasons: first, the clone in question is likely to have derived from a dominant clone (tumor) within the original sample cell population, and second, if a non-tumor clonotype from the correct family is used, it should provide immunity to the entire T-cell repertoire derived from V genes of that family. The amplified coding sequence spanning the beta chain variable region through the second beta constant region is then subcloned into our expression vector. The resultant clone is sequenced to confirm a plausible open reading frame of the correct V beta gene family.

2. Materials

2.1. Cloning Vß13.1 T Cell Receptor (TCR) Variable Regions and DNA Inoculation (see Note 1)

2.1.1. PBMC Isolation

1. Ficoll-Hypaque is available from Pharmacia Biotech (Uppsala, Sweden).
2. Dulbecco's Phosphate Buffered Saline, Cat. No. 14040-059, is available from Life Technologies Inc. (Gaithersburg, MD).
3. Sterile Eppendorf tubes.
4. Freezing medium: 90% (v/v) human AB serum, Cat. No. H2520, which is available from Sigma (St. Louis, MO) and 10% (v/v) dimethylsulfoxide (DMSO), Cat. No. D5879, Sigma.
5. Cryogenic vials: Disposable Sterile Vials, Cat. No., 25704, are available from Corning (Corning, NY).
6. Propanol bath: Cryo Freezing Container, Cat. No. 5100-001, is available from Nalgene (Rochester, NY).

2.1.2. RNA Isolation and Reverse Transcription

1. RNAse-free pipet tips.
2. RNAse-free microfuge tubes.
3. TRIzol™ reagent is available from Life Technologies.
4. 70 % (v/v) ethanol.
5. Maloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) RT and 5X RT buffer are available from Life Technologies.
6. Random hexamer RT primers.
7. dNTPs, 10 m*M*: Available from Boehringer Mannheim Biochemicals (Indianapolis, IN), mix 20 µL of each 100 m*M* stock (G, A, T, C) into 120 µL of sterile water.

2.1.3. PCR and Subcloning

1. Special equipment: Thermocycler.
2. Vß13.1 5'/*Not*I primer (gagaggcggccgcaATGAGCATCGGCCTCCTGTG).

3. Cß2/*Mlu*I primer (cgtctcacgcgtCTATGGGAACACGTTTTTCAGGTCCTC).
4. *Taq* polymerase is available from Boehringer Mannheim.
5. dNTPs, 1.25 m*M*: Mix 12.5 µL of each 100 m*M* stock (G,A,T,C) into 950 µL of sterile water.
6. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl pH 8.3, 15 m*M* $MgCl_2$, 0.1% (w/v) Gelatin.
7. SeaPlaque low-melting-temperature agarose is available from FMC Inc. (Rockland, ME).
8. Calf intestinal alkaline phosphatase is available from Boehringer Mannheim Biochemicals.
9. Dye Terminator Cycle Sequencing Kit is available from Perkin-Elmer/Cetus (Norwalk, CT).
10. T4 Ligase is available from Life Technologies.
11. APL400-004 vector is proprietary material; contact Apollon, Inc. (Malvern, PA).

2.1.4. In Vitro Transformation and RNA Expression

1. Lipofectamine is available from Gibco-BRL (Gaithersburg, MD).
2. Ultraspec Reagent is available from Biotecx (Houston, TX).
3. DNAase is available from Worthington (Freehold, NJ).
4. Megaprime Kit, Cat. No. RPN 1606, and Hyperfilm-MP are available from Amersham Life Sciences (Arlington Heights, IL).

2.2 Immunization

1. 1 cm^3 Syringe and 25 gauge needles are available from Becton Dickinson (Franklin Lakes, NJ).

2.3. Immune Response Assessment

2.3.1. Proliferation Assays

1. B95-8 cell line producing the cell-transforming Epstein-Barr virus (EBV).
2. Complete medium: RPMI 1640 with penicillin/streptomycin, L-glutamine, sodium pyruvate, non-essential amino acids, 10 mM HEPES buffer pH 8.0, 5×10^{-5} *M* 2-mercaptoethanol with either 4% or 10% (v/v) human serum (HS).
3. Round-bottom 96-well tissue culture plates, Falcon Cat. No. 3077, are available from Becton Dickinson.
4. Tritiated thymidine is available from NEN Life Science Products (Boston, MA).
5. Peptides: CDR I, CDR II, CDR III, CDR IV dissolved at 1 mg/mL in deionized distilled water. We had a set of peptides made which represent the complementarity determining regions of our particular V-beta gene. These were designed to each incorporate one of the surface hypervariable loops (complementarity determining regions or CDR) of the targeted TCR beta chain.
6. Peptide mixture: Mix equal µg amounts of CDR I, CDR II, CDR III, CDR IV and dilute in 10% (v/v) HS for use.
7. Sample bags, Cat. No. 1450-432, are available from Wallac (Turku, Finland).

2.3.2. CTL Assays

1. Complete medium containing 4% or 10% (v/v) HS.
2. B95-8 EBV transforming cell line.
3. Peptides: CDR I, CDR II, CDR III, CDR IV dissolved at 1 mg/mL in deionized distilled water.
4. Peptide mixture: Mix equal microgram amounts of CDR I, CDR II, CDR III, CDR IV and dilute in medium.
5. Round-bottom 96-well tissue culture plates, Falcon Cat. No. 3077, are available from Becton Dickinson.
6. Apoptosis lysis buffer (10 m*M* EDTA/50 m*M* Tris-HCl, pH 8.0/0.5% [w/v] Sarkosyl [Sigma]) ***(20)***.
7. Optiphase Supermix, 96-well sampler plate and sealing tape, Cat. No. 1450-461, are available from Wallac.
8. ^{51}Cr-Na_2CrO_4 (NEN).

2.3.3. DNA Enzyme-Linked Immunosorbent Assay (ELISA)

Microplate reader: Molecular Devices E max Precision Microplate Reader MR5000, is available from Dynatech (Chantilly, VA).

1. 2M H_2SO_4.
2. TMB, T-3405, is available from Sigma.
3. Wash buffer: PBS containing 0.05% (v/v) Tween-20.
4. Peroxidase-conjugated anti-human IgG, Cat. No. A-8400, is available from Sigma.
5. Blocking buffer: Phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween-20 and 2% (w/v) bovine serum albumin (BSA).
6. CBC buffer: 0.1*M* carbonate-bicarbonate buffer.
7. Microtiter plates, Cat. No. 0110103455, are available from Dynatech.
8. Protamine sulfate: 0.1% (w/v) stock. Stable for 30 d at 4°C.
9. DNA diluent: 15 m*M* Na Citrate, 150 m*M* NaCl, 0.02% (w/v) NaN_3, pH 8.0).
10. Triethanolamine-buffered saline (TBS): 50 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.5.
11. Substrate buffer: dissolve one capsule of phosphate-citrate buffer with sodium perborate in 100 mL deionized water to yield a 0.05*M* buffer pH 5.0 / 0.03% (w/v) sodium perborate.

2.3.4. Anti-V Beta ELISA

1. 50 mM NH_4HCO_3
2. Blocking buffer: PBS containing 0.05% (v/v) Tween-20 and 2% (w/v) BSA.
3. Biotin-conjugated anti-human IgG1, 2, 3, 4, or IgM, with respective Cat. Nos. B-6775, B-3398, B-3523, B-3523, B-3648 and B-1265, are available from Sigma. Dilute them in blocking buffer as follows: anti-H IgG1, 1:1,000; anti-H IgG2, 1:20,000; anti-H IgG3, 1:3,000; anti-H IgG4, 1:10,000; anti-H IgM, 1:10,000.
4. Avidin-HRP, 5 mg/mL, Cat. No. 29994, is available from Pierce (Rockford, IL). Dilute in blocking buffer to 10 mg/mL. Store 1 mL aliquots as stocks.

3. Methods

3.1. Cloning Vß13.1 T Cell Receptor (TCR) Variable Regions and DNA Inoculation

Obtain peripheral blood mononuclear cells (PBMCs) from a patient with previously documented Vß13-expressing CTCL by Ficoll-Hypaque gradient separation. Extract RNA from the PBMCs and reverse transcribe it. Amplify the Vß13.1 segment with specific PCR primers, digest it with restriction enzymes and subclone it into the vaccine vector. Sequence the vector and produce milligram quantities of the vaccine. Formulate it with 0.25% (w/v) Bupivicane and saline and administer. The steps of vaccine production are presented in **Fig. 1**.

3.1.1. PBMC Isolation

1. Collect blood into green cap (heparinized) tubes and dilute it 1:3 with Dulbecco's PBS (DPBS) (*see* **Note 2**).
2. Layer 30 mL of a diluted blood sample over 15 mL of Ficoll-Hypaque in a sterile 50-mL conical centrifuge tube at room temperature (*see* **Note 3**).
3. Centrifuge at 360*g* at room temperature in a swinging-bucket centrifuge for 20 min with no brake (*see* **Note 4**).
4. Collect the lymphocytes at the interface of the PBS and Ficoll then wash them in 30 mL DPBS, count the cells and centrifuge the suspension at 280*g* (*see* **Note 5**).
5. Wash the cells once more in DPBS and resuspend them in freezing medium (*see* **Note 6**).
6. Prepare aliquots of the cells in cryogenic vials at a concentration of 1 x 10^7 cells per vial (*see* **Note 7**).
7. Place the vials in a room-temperature propanol bath, transfer it to the –80°C freezer for more than 4 h and finally place the vials in liquid nitrogen (*see* **Note 8**).

3.1.2. RNA Isolation and Reverse Transcription

Extract RNA from the PBMCs using TRIzol® reagent and reverse transcribe the total cellular RNA with nonspecific random-hexamer priming.

1. Suspend each 5×10^6 cells in 500 µL of TRIzol reagent without allowing air bubbles to form (*see* **Note 9**).
2. Transfer the suspension to an RNAse free microfuge tube and vortex it to ensure complete mixing (*see* **Note 10**).
3. Let stand for 5 min at room temperature.
4. Add 100 µL chloroform for every 500 µL TRIzol used and vortex thoroughly.
5. Microfuge at <12,000*g* for 15 min at 4°C.
6. Transfer the aqueous phase to a fresh RNAse-free microfuge tube and precipitate the RNA by adding 250 µL isopropanol for each 500 µL TRIzol used, vortexing and allowing to stand for 10 min at room temperature.
7. Microfuge at <12,000*g* for 15 min at 4°C.

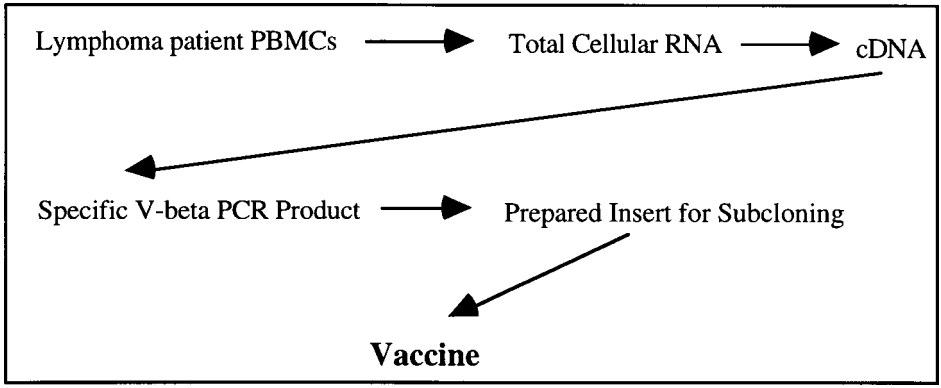


Fig. 1. Steps in vaccine development.

8. Carefully remove the supernatant without disturbing the pellet of RNA and then wash with 1 mL ice cold 75% (v/v) ethanol.
9. Microfuge at <7,500*g* for 5 min at 4°C.
10. Air dry the RNA for 10 min and redissolve in the RNA in 24 µL RNAse-free water (*see* **Note 11**).
11. Set up reverse transcription reactions. Each reaction contains 10 µL of total cellular RNA, 0.5 µL of placental RNAse inhibitor, 2 µL random hexamers, 5 µL of 10 m*M* dNTPs, 5 µL of 5X RT buffer, and 2 µL M-MLV RT (*see* **Note 12**).
12. Incubate the mixture for 1 h at 37°C.
13. Store the cDNA (i.e., the completed RT reactions) at –20°C.

3.1.3. PCR and Fragment Preparation

Amplify the particular Vß gene segment of interest with specific primers using a high stringency PCR program (*see* **Note 13**). Design the primers to introduce restriction sites into the product to facilitate cloning (*see* **Note 14**). Digest the product and purify it on a gel.

1. Set up the PCR reaction with V-beta specific primers (in our case Vß13 5'/Not I and Cß2 3'/Mlu I primers). Each reaction contains 5 µL of cDNA,16 µL dNTPs, 10 µL 10X PCR buffer, 63 µL double-distilled water, 5 µL of each primer and 1 µL of *Taq* polymerase
2. Cover the aqueous reaction mixture with mineral oil and place it in a thermocycler (40 cycles of 95°C × 1 min 55°C × 1 min 72°C × 1 min) ***(21–23)*** (*see* **Note 15**).
3. Treat the PCR products with proteinase K ***(24)*** to avoid interference of the *Taq* polymerase with restriction digestion. Combine 80 µL of PCR reaction (without oil) with 10 µL 10X PK buffer and 10 µL of 5 mg/mL proteinase K.
4. Heat inactivate the proteinase K for 10 min at 68°C (*see* **Note 16**).
5. Extract with phenol:chloroform:isoamyl alcohol (25:24:1), then with chloroform: isoamyl (24:1) (*see* **Note 17**).

6. Precipitate final aqueous phase by adding one-tenth volume of 3M sodium acetate, pH 5.0, and 2 volumes of ice-cold 100% (v/v) ethanol (*see* **Note 18**). Redissolve the DNA in 50 µL deionized distilled water.
7. Restriction digest with *Not*I and *Mlu*I: digest 50 µL of PK-treated PCR product with 30 units (3 µL) of each restriction enzyme (RE) and 6 µL of 10X RE buffer (*see* **Note 19**).
8. At the same time, digest 10 µg of the vector using at least 10 units of each enzyme.
9. Dephosphorylate the vector (not the insert Vß) with calf intestinal alkaline phosphatase (CIAP, *see* **Note 20**). Add 1 µL of CIAP and one-tenth volume of 10 CIAP buffer to the completed restriction digestion and incubate the mixture at 37°C for 15 min.
10. Load the entirety of the digestion products and insert on a 1% (w/v) low-melting temperature agarose slab gel containing 100 ng/mL ethidium bromide (*see* **Note 21**).
11. Run the gel at 75 V for at least 2 h to resolve the individual fragments (*see* **Note 22**).
12. Visualize the fragments with a handheld UV light source and excise them with a single-edged razor blade (*see* **Note 23**). The gel fragments can be stored for several days at 4°C before use.
13. Completely melt the fragments containing bands at 68°C for 5–15 min and extract the DNA extracted with phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and then chloroform:isoamyl (24:1) (*see* **Notes 17** and **24**).
14. Add one-tenth volume of 2*M* sodium acetate pH 5.0 and 2 vol of ice-cold 100% (v/v) ethanol to precipitate the aqueous phase from the final extraction (*see* **Note 25**).
15. Incubate these precipitations at –80°C or on dry ice for at least 20 min and microfuge to pellet the DNA (*see* **Note 26**).
16. Wash the pellet with 500 µL ice cold 70% (v/v) ethanol to remove salt and air dry the pellet (*see* **Note 27**).
17. Redissolve the pellets in 20 µL deionized water.
18. Perform fragment quantitation by loading 2 µL of each product onto a 1% (w/v) agarose gel along with 500 ng lambda *Hind*III markers. Comparison of the ethidium bromide staining intensities and the knowledge of the size (thus intensity) distribution in the ladder will allow a rough quantitation of the ng amount of product in 2 µL (*see* **Note 28**).

3.1.4. Ligation, Transformation and Sequencing

1. For each target molecule, combine 50 ng of dephosphorylated vector with 0.3, 1, or 3M equivalents of insert and 1 µL T4 ligase and 2 µL 5X ligase buffer (*see* **Note 29**).
2. Also set up a vector only reaction containing no insert (*see* **Note 30**).
3. Incubate the ligation reactions at 16°C overnight and store for less than 1 wk at 4°C (*see* **Note 31**).
4. Transform 2 µL each ligation into subcloning efficiency DH5-α *E. coli* and plate on LB agar continuing the appropriate antibiotic or selecting agent. Follow the manufacturer's directions or a standard protocol.

5. Pick a reasonable number (>10) of colonies from the plate(s) and grow a 3 mL overnight culture (*see* **Note 32**). Freeze 450 µL of each culture with 50 µL glycerol in a sterile microfuge tube and store at –80°C.
6. Perform Qiagen minipreps on the clonal cultures following the manufacturer's protocol.
7. Digest each DNA with cloning restriction enzymes to confirm insert ligation.
8. Sequence clones with correct insert with appropriate primer(s) using the Perkin-Elmer/Cetus Dye Terminator Cycle Sequencing Kit to confirm an open reading frame from the expected V-beta gene family (follow manufacturer's protocol).
9. Large-scale preparation of vaccine for human use is beyond the scope of this chapter, and requires the availability of a guanosine monophosphate (GMP) facility with extensive safety profiles, shelf stability, and other evaluations. Vaccines destined for use in mice are prepared with a Qiagen tip-500 kit (Qiagen, Valencia, CA).

An example of a TCR DNA vaccine is shown in **Fig. 2**.

3.1.5. Assessment of Expression by Northern Blot

An example of expression analysis by Northern Blot is shown in **Fig. 3**.

1. Transfect RD cells ***(25)*** with plasmid DNA using lipofectamine according to the manufacturer's protocol.
2. Harvest the cells 48 h post-transfection and isolate RNA using Ultra-Spec Reagent according to the manufacturer's protocol (Biotecx Laboratories, Houston, TX).
3. Digest 20 µg of RNA with DNAse and analyze it by Northern blot analysis using the formaldehyde-gel procedure of Maniatis et al. ***(26)***.
4. Label a probe comprised of the cloned TCR-Vß13.1 insert (excised by restriction digestion and low melting temperature gel purification) with ^{32}P using the Megaprime Kit according to the instructions; hybridize the blot at 65°C for 16 h.
5. Wash the blots as described ***(26)*** and expose them to Hyperfilm-MP (Amersham-Pharmacia Biotech, Piscataway, NJ) for 2–24 h.

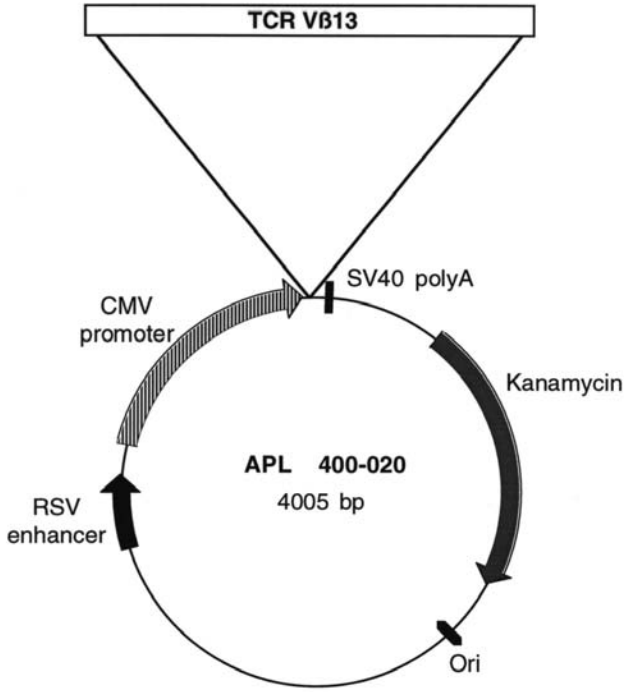
3.2. Immunization

1. Inject clinical-trial patients intramuscularly every 2–4 wk with DNA vaccine formulated in buffer with bupivacaine (*see* **Note 33**).

3.3 Immune Response Assessment

3.3.1. Proliferation Assay for TCR DNA Vaccine

To characterize the lymphocyte proliferation response induced by genetic vaccination, use an assay with PBMCs collected by the Ficoll-Hypaque purification protocol (Pharmacia, Piscataway, NJ). Expose PBMCs to homologous EBV-transformed B cells that have been loaded with various peptides. These peptides represent particular epitopes found in the specific V-beta antigen. Assess proliferation in response to these epitopes by incorporation of tritiated thymidine.




```
10/1                                                    40/11
ATG AGC ATC GGC CTC CTG TGC TGT GCA GCC TTG TCT CTC CTG TGG GCA GgT CCA GTG AAT
Met ser ile gly leu leu cys cys ala ala leu ser leu leu trp ala gly pro val asn
70/21                                                   100/31
GCT GGT GTC ACT CAG ACC CCA AAA TTC CAG GTC CTG AAG ACA GGA CAG AGC ATG ACA CTG
ala gly val thr gln thr pro lys phe gln val leu lys thr gly gln ser met thr leu
130/41                                                  160/51
CAG TGT GCC CAG GAT ATG AAC CAT GAA TAC ATG TCC TGG TAT CGA CAA GAC CCA GGC ATG
gln cys ala gln asp met asn his glu tyr met ser trp tyr arg gln asp pro gly met
190/61                                                  220/71
GGG CTG AGG CTG ATT CAT TAC TCA GTT GGT GCT GGT ATC ACT GAC CAA GGA GAA GTC CCC
gly leu arg leu ile his tyr ser val gly ala gly ile thr asp gln gly glu val pro
250/81                                                  280/91
AAT GGC TAC AAT GTC TCC AGA TCA ACC ACA GAG GAT TTC CCG CTC AGG CTG CTG TCG GCT
asn gly tyr asn val ser arg ser thr thr glu asp phe pro leu arg leu leu ser ala
310/101                                                 340/111
GCT CCC TCC CAG ACA TCT GTG TAC TTC TGT GCC AGC AGT TTC CCC CGG CAG CCG TCC TAC
ala pro ser gln thr ser val tyr phe cys ala ser ser phe pro arg gln pro ser tyr
370/121                                                 400/131
AAT GAG CAG TTC TTC GGG CCA GGG ACA CGG CTC ACC GTG CTA GAG GAC CTG AAA AAC GTG
asn glu gln phe phe gly pro gly thr arg leu thr val leu glu asp leu lys asn val
430/141
TTC CCA TAG
phe pro AMB
```

Fig. 2. Design of the GENEVAX™-TCR-Vß13.1 vector. The sequence of the Vß13.1 insert cloned from patient JS is shown below the diagram of the vector.

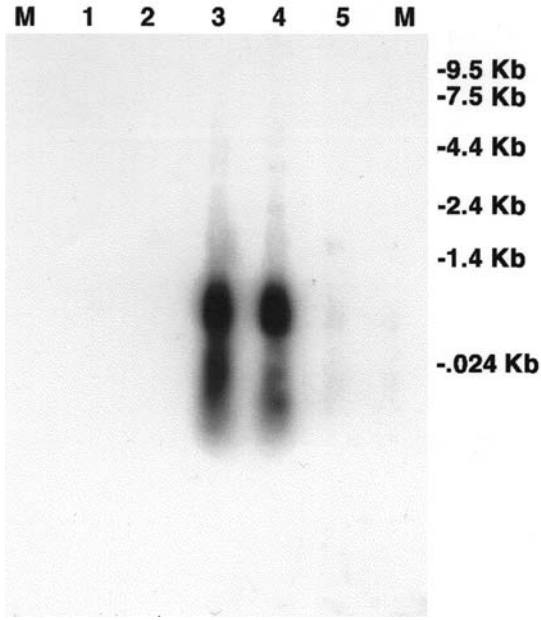


Fig. 3. Detection of Vß13 mRNA in GENEVAX™-TCR-Vß13.1 transfected cells. Northern blot analysis was performed on RNA isolated from RD cells transfected with GENEVAX™-TCR-Vß3 (lanes 1 and 2), GENEVAX™-TCR-Vß13.1 (lanes 3 and 4), or mock transfected (lane 5). The positions of the RNA molecular weight markers are shown. The RNA was hybridized to a Vß13 internal probe.

1. Prepare EBV-immortalized B cells from the patient and maintain them for several weeks before doing the cellular immune response assays (*see* **Note 34**). Resuspend 5×10^6 PBMCs in 5 mL medium and an equal volume of cell-free supernatant from B95-8 cells. Maintain this culture until cell growth is apparent; feed the cells with medium alone through at least three passages. Resuspend these transformed B cells (XX EBV) at 7.5×10^6/mL and incubate them with 100 µg/mL of the peptide mixture, or with individual peptides, or no peptide for 4–6 h. Irradiate with 10,000 rads (*see* **Note 35**).
2. Isolate PBMCs by Ficoll-Hypaque separation as described and freeze them in 10% (v/v) DMSO/HS in liquid nitrogen until needed.
3. Thaw the PBMCs, wash them 2 times with 4% (v/v) HS, resuspend them at a concentration of 10^6/mL and prepare aliquots of 100 µL/well in a 96-well round-bottom plate (*see* **Note 36**).
4. Prepare aliquots of EBV-immortalized B cells (EBV B cells) at 10^6/mL at 100 µL/well in 96-well round-bottom plate then transfer them to the PBMC plate. For negative and positive controls, add 100 µL/well of medium or 20 U/mL IL2/4 respectively.

5. Incubate plates for 3 d in a CO_2 incubator at 37°C and add 1 µCi ^{3}H-thymidine in 20 µL 10% (v/v) HS to each well (*see* **Note 37**). Incubate the plates overnight (18–20 h).
6. Harvest the plates the next morning with an automatic 96-well harvester and measure the amount of incorporated tritiated thymidine in a liquid scintillation counter (*see* **Note 38**).

3.3.2. Cytotoxic T-Lymphocyte Assay for TCR DNA Vaccine Clinical Trial

Characterize the cytotoxic T lymphocyte (CTL) response induced by genetic vaccination using a CTL assay with PBMCs collected by Ficoll-Hypaque gradient centrifugation and previously derived EBV-transformed B cells from the patient. Use a set of peptides to assess CTL activity against the V ß gene. An example of data resulting from CTL assays is shown in **Fig. 4**.

3.3.2.1. Preparation of Effector Cells

1. Isolate PBMC by Ficoll-Hypaque gradient centrifugation as above (**Subheading 3.1.1.**) and freeze them in 10% (v/v) DMSO/HS in liquid nitrogen.
2. Rapidly thaw the effector cells (~2×10^7 PBMCs) in a 37°C water bath, wash them twice with 10 mL medium to remove DMSO, recount the cells with a hemacytometer and culture them at 5×10^5/mL with 5 µg/mL PHA for 48 h (*see* **Note 39**).
3. Prepare Stimulators: on day two, after PBMCs are thawed, incubate EBV-immortalized B cells from the patient (XX EBV) at 7.5×10^6/mL with 100 µg/mL of peptide mixture for 4–6 h, then irradiate with 10,000 rads before using them as stimulator cells (*see* **Note 40**). Count the cells in the effector culture also.
4. Wash the effector cells twice with medium and resuspend in medium containing 10 U/mL IL2 and 10 µg/mL peptide mixture. Mix the stimulators with the effectors at a ratio of 1:20 (stimulator:effector) and incubate the mixture for about 10 d and then count the cells. Expand the cells by feeding them with medium containing 10 U/mL IL2 and 10 µg/mL peptide mixture until sufficient cells are available for the lysis reactions. Separate the healthy restimulated cells by Ficoll-Hypaque centrifugation, count them and then use in a CTL assay (*see* **Note 41**).

3.3.2.2. Lysis Reactions

1. In the morning of the day on which the lysis reaction will be run, resuspend XX EBV cells in 7.5×10^6/mL containing 100 µg/mL of a peptide mixture, or individual peptides, or no peptide, and incubate at 37°C for 2 h. Spin down the cells, pour off the supernatant and resuspend the cells by gentle pipetting up and down. (Do not exceed a volume of 500 µL at this point). Add 20 µCi of ^{51}Cr to the cells, incubate another 1.5 h at room temperature, wash 3 times, and resuspend the ^{51}Cr-loaded target cells at 10^5/mL in medium (*see* **Note 42**). Do not wash these target cells until it is time to use them.

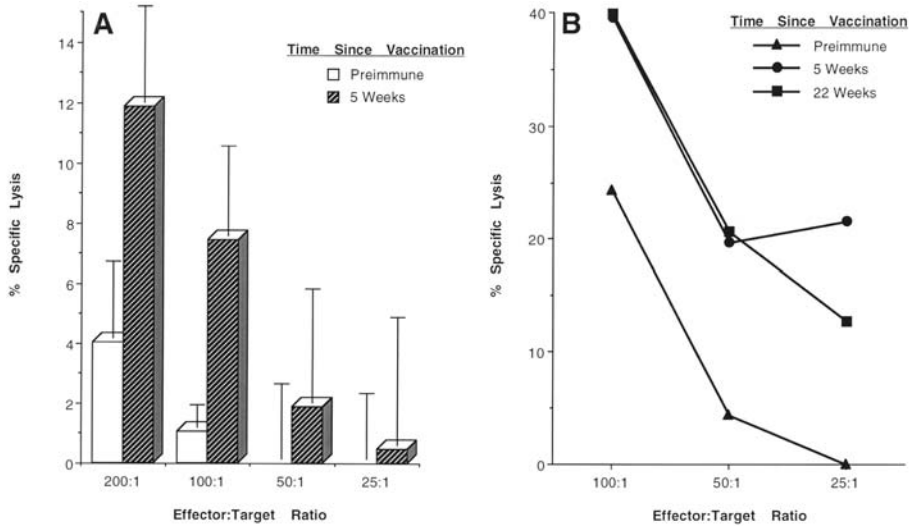


Fig. 4. Induction of cytotoxic T-cells by DNA-based vaccination. The % specific lysis (mean ± SD of triplicate wells) is shown for several effector:target ratios. **(A)** Preimmune PBMC are compared with those obtained at week 5 following the initial vaccination. The targets were EBV-APC coated with a mixture of all four Vß13-derived peptides. **(B)** Preimmune PBMC are compared with those obtained at week 5 and week 22 following the initial vaccination. The targets were EBV-APC coated with the CDR III peptide.

2. Resuspend the effector cells at a concentration of 1×10^7/mL and put 100 µL medium into columns 4–12 of a 96-well round-bottom plate (*see* **Note 43**).
3. Put 100 µL aliquots of cells in columns 1-6 effecting a 2-fold dilution of cells in columns 4,5 and 6; wells 1–3, 4–6, 7–9, and 10–12 are triplicates (*see* **Note 44**). Each row contains different effector samples. Devote one row for each type of target cell for estimates of minimum spontaneous release and maximum release. To measure minimum release, add 100 µL of media to wells 1–6 of one row. To measure maximum release, add 100 µL of apoptosis lysis buffer to wells 7–12 of that row (*see* **Note 45**).
4. Make subsequent 1:2 dilutions of effectors by mixing columns 4-6 and transferring 100 µL to columns 7-9. Avoid the minimum/maximum row in these effector dilution steps.
5. Repeat step 4 from wells 7–9 to 10–12 and then discard 100 µL from the mixed wells 10–12. Thus, all wells have a volume of 100 µL.
6. Add 100 µL of target cells (10^5/mL) to all wells including those for minimum and maximum release estimates. The resultant effector:target ratios are 100:1, 50:1, 25:1, 12.5:1. Centrifuge the plate at 800 rpm (160–200*g*) for 2 min (*see* **Note 46**).
7. Incubate the effector and target cells at 37°C in a CO_2 incubator, remove a 50 µL aliquot of supernatant at the time-points 5 h and 16 h respectively (*see* **Note 47**).

8. Mix 150 µL of Optiphase Supermix (Wallac, UK) with 50 µL supernatant in a Wallac 96-well sample plate and cover the plate with sealing tape. Count the plate(s) in a liquid scintillation counter (Wallac Inc., Gaithersburg, MD) (*see* **Note 48**).
9. Percent specific lysis equals 100 × [(experimental release—spontaneous release)/(maximum release—spontaneous release)].

3.3.3. Anti-DNA ELISA

To characterize the immune response to DNA induced by genetic vaccination, collect the serum samples from subjects inoculated with Vß-expressing DNA plasmids and analyze anti-DNA antibodies by ELISA.

1. Treat microtiter plates at room temperature for 90 min with 200 µL/well of 0.0001% (w/v) protamine sulfate solution. Prepare this solution just before use by dilution from a 0.1% (w/v) stock into double-distilled H_2O. Wash 3X under the deionized water tap and empty and slap dry on paper towel (*see* **Note 49**).
2. Coat microtiter plates with 50 µL/well of plasmid DNA at a concentration of 3 µg/mL in DNA diluent. Coat a plate with 1% (w/v) BSA in DNA diluent as a negative control. Place the plates at 4°C in a sealed moist container at least overnight or until ready for use (*see* **Note 50**). Wash the plates 3 times with TBS-0.05% (v/v) Tween-20.
3. Add blocking buffer, 200 µL per well (*see* **Note 51**). Incubate at 37°C for 1 h. Empty and slap dry on a paper towel.
4. Add 50 µL of diluted serum (diluted in blocking buffer) to each well (*see* **Note 52**). Incubate at 4°C O/N (or at 37°C for 1 h). Wash plate 6 times with TBS-0.05% (v/v) Tween-20.
5. Add 50 µL/well of anti-human Ig conjugated to peroxidase diluted 1:8,000 in blocking buffer. Incubate at 37°C for 1.5 h. Wash plate 8 times with TBS-0.05% (v/v) Tween-20.
6. Dissolve 1 mg of TMB in 10 mL of substrate buffer. Place 100 µL aliquots in each well and incubate in the dark at room temperature until the blue color develops (*see* **Note 53**).
7. Add 20 µL of 2M H_2SO_4 to each well to stop the reaction (*see* **Note 54**) and read the plate at 450 nm on a microplate reader.

3.3.4. V-Beta ELISA

To characterize the immune response induced by genetic vaccination, perform enzyme-linked immunoassay analysis (ELISA) of sera samples collected from subjects inoculated with Vß-expressing DNA plasmids. An example of data resulting from anti-Vß ELISA analysis is shown in **Fig. 5**.

1. Coat microtiter plates with 50 µL/well of peptide antigen at a concentration of 10 µg/mL in 50 m*M* NH_4HCO_3. Coat one plate with 50 mM NH_4HCO_3 as a control (*see* **Note 55**). Place the plates in a 37°C dry incubator to dry for 4 h or overnight.

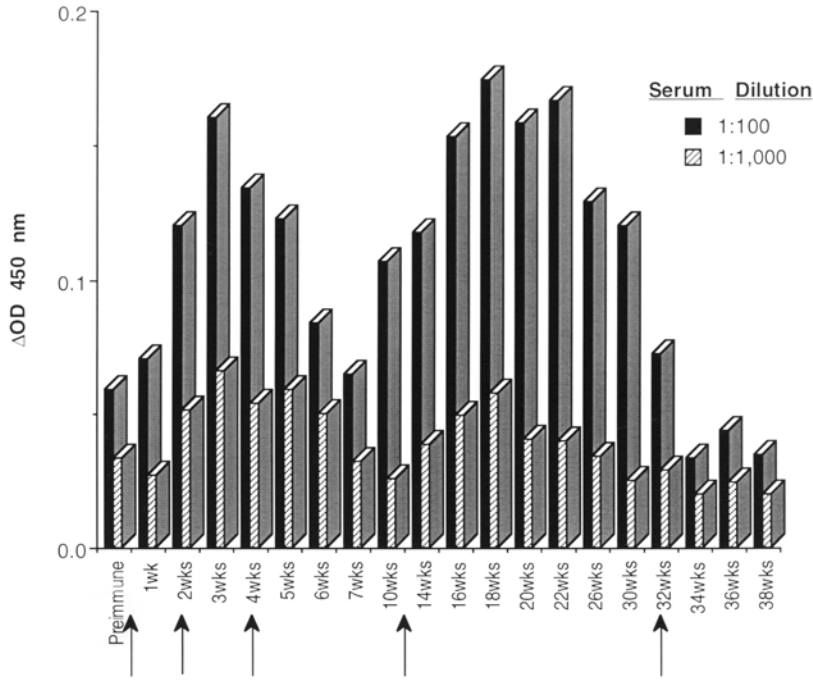


Fig. 5. Humoral response to DNA-based vaccination. ELISA wells were coated with peptides spanning the CDR1 region of the Vß13 T-cell receptor. Serum was assayed for binding to this peptide by ELISA assay. The OD 450 nm refers to the binding to the peptide on ELISA minus the binding to control wells with no peptide (mean of duplicate wells). The times of vaccination are indicated with arrows. One of several assays, all showing similar trends, is shown.

2. Add 200 µL per well of blocking buffer. Incubate the plate at 37°C for 1 h. Pour off the blocking buffer.
3. Add 50 µL of diluted serum (diluted in blocking buffer) to each well (*see* **Note 56**). Incubate the plates at 4°C overnight (or at 37°C for 1 h). Wash the plates 6 times with PBS-0.05% (v/v) Tween-20 (*see* **Note 57**).
4. Add 100 µL/well of peroxidase-conjugated anti-human IgG diluted 1:8,000 in blocking buffer as recommended. Incubate the plates at 37°C for 1 h. Wash the plates 8 times with PBS-0.05% (v/v) Tween-20.
5. For isotype-specific responses add 100 µL/well of biotin-conjugated anti-human IgG1, 2, 3, 4, or IgM diluted in blocking buffer. Incubate the plates at 37°C for 1 h then wash them 6 times with PBS-0.05% (v/v) Tween-20.
6. Assay of isotype-specific responses (skip this step for non-isotype-specific ELISA): add 100 µL/well of 0.1 µg/mL Avidin-HRP. Incubate the plates at 37°C for 1 h, wash them 8 times with PBS-0.05% (v/v) Tween-20.

7. Dissolve 1 mg of TMB in 10 mL of substrate buffer. Aliquot 100 µL to each well and incubate in the dark at room temperature until the blue color developed (*see* **Note 53**).
8. Add 20 µL of 2M H_2SO_4 to each well to stop the reaction (*see* **Note 54**) and read the plate at 450 nm on a microplate reader (Dynatech MR5000).

4. Notes

1. **Figs. 1** and **2** show the overall design of the DNA vaccine vector.
2. Blood dilution is necessary for good yields of PBMCs.
3. Ficoll-Hypaque is stored at 4°C but should be brought to room temperature prior to use. Overlaying should be done gently to avoid mixing with Ficoll. Underlaying of the Ficoll also works well. Do not agitate the tubes upon transport to and from the centrifuge as this may reduce the yield of PBMCs.
4. The centrifugation step must be done at room temperature to avoid cooling of the Ficoll. Take time to warm up a refrigerated unit if that is all that is available. ($RCF(g) = 1.12r\ (rpm/1000)^2$ where r is the radius of the rotor expressed in millimeters).
5. The layer of PBMCs at the gradient interface should be slowly and thoroughly pipetted out of the tube. If too great an amount of Ficoll is collected with the cells, the volume of the wash should be adjusted to insure pelleting of cells.
6. The treatment of cell samples at this step varies depending on their intended use. For cloning purposes, dry pellets of 5–10 million cells should be stored at –80°C.
7. For immune response assays cells should be resuspended at 10^7/mL in freezing media and frozen in 1 mL aliquots.
8. The propanol bath insulates the cryovials and thus facilitates a slow steady cooling to –80°C. This enhances cell viability upon thawing. The propanol bath should be left in the –80°C for at least 4 h and can be left at –80°C for up to 2 d and then transferred to liquid nitrogen.
9. Use enough cells! We find that 5×10^6 cells yield consistently high-quality cDNA. More are even better but be sure to scale up the RNA preparation reagents.
10. Use standard RNAse-free precautions. Mix completely to ensure that all cells are lysed. As with all viscous solutions of macromolecules, bubbles or foaming tend to diminish product yield.
11. Do not overdry the product RNA as it becomes very difficult to dissolve.
12. Add reverse transcriptase reagents in the order listed to protect the RNA from RNAse and to provide a buffered environment for the M-MLV-RT.
13. The polymerase chain reaction amplification of any particular DNA segment should be optimized to yield a product that contains minimal background amplification products but is also sufficient in quantity. Optimize the annealing temperature first as this affects the purity of the product greatly. We recommend trying 40, 45, 50, 55, 60, and 65°C. Higher temperatures generally yield less background amplification.
14. Primer design is based on simple principles using the leader peptide sequence or the mature protein amino terminus and going through to the end of the coding

region or (if the CDR III is going to be included), using the 5' end of the constant region. The complete sequence of the human Vß gene locus has been published and is available from GenBank ***(27)***. Another good source of sequence information comes from Kabat ***(28)***.

15. Mineral oil is required to prevent evaporation of the reaction during prolonged heating. Avoid collecting mineral oil with your aqueous PCR product by taking only 80 out of 100 µL and also by wiping the tip with a clean paper towel.
16. Heat inactivation protects the restriction enzymes that are used in the subsequent step from proteinase K.
17. Microfuge the phenol/chloroform/isoamyl alcohol extraction for 5 min at full speed. Only 1 min is required for the chloroform/isoamyl extraction.
18. Precipitation should be by incubation at –80°C for at least 20 min.
19. The restriction enzymes will vary depending on the vector used.
20. Dephosphorylation reduces the chance that incompletely digested compatible sticky ends on the vector will mediate self-ligation and contribute to a false positive signal following transformation.
21. Care should be taken when handing low-melting temperature (LMT) gels as they are more fragile than regular agarose gels. Allow more time for a LMT gel to solidify.
22. Do not exceed 75 V as the gel may be heated above its melting temperature.
23. Take precautions to avoid contaminating the gel slices. Use a face shield for UV protection. Long-wave UV is less damaging to DNA and molecular biologists than short wave.
24. Carefully inspect the tubes to insure that the slices are completely melted.
25. Measure the volume of the final aqueous phase with an appropriate pipet tip to calculate precipitating agents.
26. Orientate the tubes in the microfuge so that the pellet (if visible) will be expected at a certain spot within the tube. For example; the pellet will be found below the hinge of the tube if the hinge is oriented toward the outside in the rotor.
27. If solids are seen suspended in the wash it will be necessary to microfuge again before removing the wash fluid.
28. A nanogram quantity can be assigned to each of the ladder bands according to the fraction of the whole lambda molecule each embodies.
29. One molar equivalent of 50 ng of a 3000-bp vector is 16.66 ng of a 1000-bp insert. Approximations are acceptable here.
30. This vector-only reaction is an important control for the ligation/transformation experiment. The number of background colonies derived from this reaction upon ligation are subtracted from the number of colonies from an insert-containing ligation to determine on a coarse level whether the ligation/transformation worked.
31. Immediate transformation of the ligation reactions (i.e., within one day) seems to work best.
32. Ten to 20 colonies per target molecule should yield a suitable clone.
33. Too few human experiments are available to provide any useful hints. We always formulate our vaccines with bupivacaine as this enhances muscle cell uptake and

expression of the encoded gene. Injections may be delivered into the deltoid or gluteal muscles following swabbing the skin with an alcohol wipe.

34. EBV-immortalized B cells from the patient must be prepared several weeks before doing the cellular immune response assays. The sequences of the peptides we used were:

    CDR I: NAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSC
    CDR II: RQDPGMGLRLIHYSVGAGITDQGEVC
    HV IV: PNGYNVRSTTEDFPLRLLSAAPSQTSVC
    CDR III: YFCASSFPRQPSYNEQFFGPGTRLTVLC

    These peptides have an added C-terminal cysteine that is not part of the native sequence.
35. 10,000 rads will require different amounts of time depending on the irradiator source. Consult appropriate sources. If no irradiator is available, one can treat the cells (10^6/mL) with 25 μg/mL mitomycin C (Sigma Cat. No. M0503). Mitomycin must be completely washed away (3 washes) before the cells can be used.
36. Determine the number of plates needed and therefore the number of cells and all other reagent quantities before starting this experiment. Proper planning will make it much easier.
37. Add the tritiated thymidine carefully in a workable volume (20 μL) with a multichannel pipettor. Use proper safety techniques for handling ^{3}H-thymidine. Tritium contamination of the work area can only be detected by wipe tests followed by scintillation counting.
38. Harvesters and counters will vary.
39. When first attempting a CTL assay 2×10^7 of each PBMC sample should be thawed as effectors for each intended target. Rapidly remove the DMSO by washing because it is toxic to the cells.
40. Each stimulation culture will only require 3–4 million stimulator cells. When first doing the assay, use 3.75 million (0.5 mL) EBV-transformed cells with each peptide. Experience will allow you to reduce this amount and thus conserve peptide.
41. Removal of dead cells from the stimulator culture is necessary to prevent the dead stimulators from competing with targets for the interaction with cytotoxic effectors. It also makes counting much easier.
42. Calculate the number of target cells necessary for your assay and load 25–50% more cells than you need with peptide and ^{51}Cr. This will minimize the waste of peptide. Each sample of effector (PBMC) will be used for one row of a plate for each target used. Each full plate will get 9.6×10^5 targets.
43. Be certain that any cells to be aliquotted into a plate are thoroughly mixed. Minimum spontaneous release and maximum release should be done in triplicate or more. Often we do six wells of each as this takes one complete row of the plate.
44. We usually dilute the effectors in column 4 into column 7, 5 to 8, and 6 to 9 as a convention.
45. By the same convention dilute 7 to 10, 8 to 11, and 9 to 12 with the 8-channel pipettor.

46. Centrifugation enhances the requisite cell-cell contact. A 200:1 ratio may provide useful data if the available number of cells allows.
47. Sampling of the CTL supernatant must be done with a steady hand. The multichannel pipettor is held at a 45° angle and tips are immersed about halfway into the liquid in the well. Remove supernatants slowly. If air is "withdrawn" do not mix the wells by adding any of the solution back to the wells for a more even sampling. Note the bad sample and move on. Practice with non-radioactive water (200 µL/well).
48. The scintillation fluid is added to the supernatants and then mixed by shaking after the plate is covered with the sealing tape. Counting apparatus vary.
49. The protamine sulfate solution confers a positive charge to the plate for eventual DNA adhesion. Poly-L lysine is sometimes used instead.
50. DNA/BSA plates can be stored at 4°C for up to one month provided they do not dry out.
51. This step blocks nonspecific sites.
52. Dilution of sera can be done in the plate. Triplicates of dilution from 25: to 200:1 across the plate are often used.
53. Watch closely for the blue color to develop (usually 5–60 min).
54. If the blue color is faint, allow more time for its development. However, eventually additional incubation will obscure meaningful differences between wells.
55. The plate without peptide is a useful control to confirm the specificity of anti-Vbeta activity in the sera. This plate should be used in parallel with the specific peptide containing plate(s).
56. We find that dilutions ranging from 10:1 to 1000:1 work well in a screening assay. Endpoint titer is determined in a subsequent assay by serial two-fold dilutions starting at a dilution that gives a positive result in the screening assay.
57. Discarding material from ELISA plates is done with a flick of the wrist into a waste sink followed by several slaps of the plate into a paper towel. This procedure assures that subsequent addition to the plate will not be unduly diluted and thus increases the effectiveness of washes.

Acknowledgments

Dr. Lessin was supported by an NIH grant CA 55017 and VA career development award RA1710. Dr. Williams and this manuscript were supported by an FDA orphan grant FD-R-001149-01. We would like to acknowledge the excellent technical and intellectual support from Bernice Benoit, Wei Cai, Dan McCallus, Felicia Watson, Henry Klepser, Leslie Coney, and Apollon Inc.

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Immunity to T-Cell Receptor

Suppressive Vaccination with DNA Encoding a Variable Region Gene of the T-Cell Receptor

Ari Waisman

1. Introduction

A common theme found in studies with mouse models of autoimmune diseases is that pathogenic T cells are primarily responsible for the pathology. Such models include diabetes in non-obese diabetic (NOD) mice, experimental autoimmune encephalomyelitis (EAE)—a model of multiple sclerosis (MS), collagen II (CII)-induced arthritis (CIA)—a model of rheumatoid arthritis (RA). Pathogenic T cells in EAE utilize a restricted repertoire of genes encoding the T-cell receptor (TCR) ***(1)***. For example, upon immunization of H-2^u mice with either myelin basic protein, or its immunodominant fragment, peptide Ac1-20, the Vβ8.2 TCR gene product is expressed in the majority of pathogenic T cells ***(2–4)***. The restricted usage of the Vβ8.2 TCR gene product has also been found in rats in which EAE was induced by a peptide of myelin basic protein ***(5)***. Similarly, a restriction in the TCR usage was found also in CII-induced arthritis in mice ***(6,7)***, and in the TCR alpha chain usage in non-obese diabetic (NOD) mice ***(8)***.

Restriction in the TCR gene usage by T cells isolated from human MS patients has also been determined ***(9)***. To a lesser extent, some TCR beta genes were expressed in T cells implicated in pathogenesis of type 1 diabetes ***(10)*** and RA ***(11)***.

Two strategies have been utilized to therapeutically target these pathogenic T cells in a highly specific manner. The administration of monoclonal antibodies directed to pathogenic V gene products ***(3,12)*** and T cell vaccination with peptides from the second or third complementarity determining regions of the

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

pathogenic TCR V region have both proven successful in the therapy of EAE *(13,14)*. A similar approach has also proved to be successful in CII induced arthritis *(15,16)*.

We have described the prevention of EAE by injection of plasmid DNA encoding the Vβ8.2 region of a T-cell receptor that is critical for the pathogenesis of the disease *(17)*. This is a novel demonstration of "suppressive vaccination," an approach that is in contrast to what is normally desired when vaccinating for immunity to microbes. We have shown that the mechanism of DNA vaccination against the pathogenic TCR V gene segment, does not involve the depletion of these pathogenic T cells, nor does it push them into anergy. Instead, we found that DNA vaccination promoted a shift in the pattern of cytokines produced by the pathogenic T cells. The T cell populations no longer produced pathogenic cytokines like γ-interferon and IL-2, cytokines that define a Th1-type response *(18,19)*. Instead, the suppressive cytokine IL-4, which characterizes a Th2 response, was produced. This shift towards Th2 immunity had not been seen previously with DNA vaccination for a microbial protein *(20)*. This novel mechanism, induced by DNA vaccination to a gene encoding a TCR variable region polypeptide, may have potential effect for the treatment of autoimmune diseases triggered by specific Th1 T cells, such as diabetes and RA.

2. Materials

2.1. Plasmid Construction

1. We used pcDNA3 from Invitrogen (Carlsbad, CA). It is important that the vector has a strong non-specific promoter if the DNA is injected into muscle tissue. Alternatively, if a specific cell type is the target of the vaccination (e.g., B cells), a specific promoter can be employed (e.g., a promoter of a B cell specific gene). In addition, the vector should allow the transcription of a mature and stable mRNA, by providing polyadenylation and transcription termination signals.
2. Mega-prep kit (Qiagen, Chatsworth, CA).
3. Mini-prep kit (Qiagen).
4. QIAquick gel extraction kit (Qiagen).
5. Poly (dT) (Boehringer Mannheim, Mannheim, Germany).

2.2. DNA Vaccination

1. Dissolve one milligram of cardiotoxin (Sigma, St. Louis, MO) in 14.7 mL sterile saline (0.9% (w/v) NaCl solution), filter and store in aliquots at –20°C.
2. TRIzol: use according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD).
3. ABTS: use pre-prepared ABTS solution (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate) (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

2.3. Detection of Anti-TCR Antibodies

1. Monoclonal antibodies directed against the T-cell receptor conjugated to phenylephrine (PE) or fluorescein isothiocyanate (FITC) (Pharmingen, San Diego, CA).
2. Goat anti-mouse immunoglobulin conjugated to FITC (Jackson ImmunoResearch Laboratories, West Grove, PA).
3. PBS/BSA: PBS/0.1% (w/v) BSA/0.05% (w/v) azide.

2.4. Quantification of Cytokines

1. Enriched RPMI: RPMI 1640 medium supplemented with 2 m*M* glutamine, nonessential amino acids, 1mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone (BioLab, Jerusalem, Israel), 5×10^{-5}*M* β-mercaptoethanol (Fluka AG, Buchs, Switzerland) and 10 m*M* HEPES buffer (Sigma).
2. Cytokines levels: use antibody pairs from Pharmingen (La Jolla, CA).

2.5. Determining Antibodies Isotype

1. Maxisorp microtiter plates (Nunc, Naperville, IL).
2. Goat anti-murine IgG1 or IgG2a (Fc fragment specific) conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL).

3. Methods

3.1. Plasmid Construction

1. Isolate genomic DNA or cellular RNA from the spleen or peripheral blood lympho–cytes (*see* **Notes 1** and **2**). Synthesize first strand cDNA using poly(dT) as a primer.
2. Genomic DNA or cDNA serves as template for polymerase chain reaction (PCR) (*see* **Note 3**), with *Taq* polymerase and TCR specific primers (*see* **Note 4**).
3. Purify the PCR products from the gel, ligate it with the expression vector, and transform the bacteria (*see* **Notes 5** and **6**).
4. Sequence the DNA from a bacterial colony with an insert in the expected length, to verify the insertion of the right gene with an appropriate open reading frame.
5. Prepare large quantities of plasmid DNA with a Mega-prep kit.

3.2. DNA Injection

1. Inject into the tibialis anterior muscle of the mouse 50 μL of cardiotoxin solution per leg. The injection is done via the anterior surface of the muscle using a 27-gauge needle with a collar to limit penetration to 2 mm. One week later the mice are ready for DNA vaccination.
2. Inject the DNA three times with weekly intervals in the same manner as in step 1. Inject 50 μL DNA per leg (stock solution of 1 mg/mL DNA in sterile phosphate-buffered saline [PBS]).
3. Verify expression of the injected DNA: Remove the injected muscle, and extract RNA (*see* **Note 1**). Synthesize cDNA from the RNA, and use it as a template for PCR amplification (*see* **Note 3**). Sequence PCR products of the correct length, to confirm expression.

3.3. FACS Staining

In order to detect the presence of antibodies that recognize the product of the TCR used for immunization, cells that express the desired TCR can be used (*see* **Notes 7** and **8**).

1. Incubate the cells (5×10^5 cells/tube) with the sera of the immunized mice (diluted 1:100 in PBS/BSA). Keep the cells on ice and in the dark throughout the staining. A monoclonal antibody that recognizes the same TCR should be used as a positive control (*see* **Note 9**).
2. Pellet the cells and wash them with cold PBS.
3. Incubate the cells with goat anti-mouse Ig coupled to FITC for 30 min, wash and analyze them with a FACScan (Becton Dickinson, Rutherford, NJ).

3.4 Cytokines from Lymph Node Cells

1. Incubate lymph node cells (1×10^7) in enriched RPMI 1640 with 1% (v/v) syngeneic sera and 10 μg of the desired peptides (*see* **Note 10**).
2. Collect the medium 24 and 48 h later and test it for cytokines. Use the assay kits according to the manufacturer's instructions.

3.5. Antibody Isotype

IgG1 secretion is triggered by IL-4, whereas IgG2a secretion is induced by γ-interferon. These are the main cytokines of Th2 and Th1, respectively. It is therefore important to assess the cytokine function in vivo by determining the isotype of the antibodies produced by the mice as a response to immunization.

1. Bleed the mice about two weeks after antigen immunization. For example, we induced EAE by a peptide, and tested the antibodies directed to that peptide two weeks after immunization.
2. Coat Maxisorp microtiter plates with the antigen (*see* **Note 11**).
3. Wash and block over-night with 10% (v/v) FCS in PBS.
4. Incubate for 90 min with the sera of the mice, diluted serially in duplicates from 1:10 to 1:1000.
5. Wash and incubate for 75 min with goat anti-mouse IgG1 or IgG2a conjugated to alkaline phosphatase.
6. Wash and incubate with ABTS.
7. Read at 405 nm using an ELISA reader.

4. Notes

1. For extraction of genomic DNA or total RNA, we used TRIzol reagent. The reagent contains phenol, and therefore should be used under a chemical hood.
2. Care should be taken when selecting the mouse from which the DNA is to be prepared since some mouse strains contain large deletions in their chromosomes in the region that contains the TCR genes.

3. We amplified only the V gene-coding region, not including the D and the J elements. In case the whole variable region is needed, it is important to amplify the DNA from cDNA, and not from genomic DNA.
4. It is important to include an initiation codon in the 5' primer that will code for methionine, in frame with the TCR amplification product. Similarly, it is essential to introduce an in-frame termination codon in the 3' primer. In addition, restriction sites should be introduced in the same primers to simplify the subcloning process. The restriction enzyme sites chosen should exist in the expression vector to allow for cloning. In addition, it is important to verify that the selected sites are not found in the PCR product.

   As an example, we used the following primers for the TCR Vb8.2 cloning: 5'-CCGGAATTCATGGAGGCTGCAGTCACCCAAAGC-3' and 5'-TGCTCT-AGATTAGCTGGCACAGAAGTACACTGATGT-3'. These primers cover the complete V region (about 310 bp) and include *Eco*RI and *Xba*I sites used for cloning.
5. After PCR, the DNA product of the right length should be extracted from the agarose gel (we used the Qiagen QIAquick extraction kit). Prior to loading the preparative gel, cleave the DNA by the restriction enzymes selected. Cleave the DNA vector with the same enzymes, and then treat with alkaline phosphatase prior to gel purification. It is useful to treat the vector with CIP before loading the gel, since it saves a step of phenol extraction.
6. After ligation and transformation ***(21)*** grow colonies for mini-preparations of DNA. This step should be undertaken only if the background (i.e., the number of colonies after self-ligation of the vector) is very low. Otherwise it may be hard to find a positive colony.
7. Positive T-cell populations that express the desired TCR are sometimes hard to find. For example, we used a T-cell clone that was grown in the laboratory and that expresses TCR Vβ8.2. Another possibility is to isolate the desired cell population with a FACS sorter.
8. It is advisable to work with pure T cell populations, since B cells and macrophages will bind immunoglobulin in a non-specific manner through their Fc receptor.
9. When analyzing the sera, use normal mouse sera (from non-immunized mice) as a negative control. These sera may bind with low affinity to the T cells, and this binding should be considered as the background level.
10. Draining lymph node cells are easily visualized after immunization. In addition, spleen cells can be used. In some cases we have found that some cytokines (such as IL-4) are easier to detect from supernatants of activated spleen cells than from lymph node cells.
11. We used 10 μg/mL of peptide in PBS, and coated the plates for 90 min. Different peptides may adhere differently to the plate, and some will need to be conjugated to a carrier such as BSA before coating.

Acknowledgments

I would like to thank Dr. S. Casola and A. Gaur for comments on the manuscript. I especially would like to thank Prof. L. Steinman for his support.

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DNA Fusion Vaccines Against B-Cell Tumors

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1. Introduction

The ability of naked DNA to induce immune responses against encoded antigen has been clearly demonstrated for infectious diseases *(1)*. In many cases, the induced immunity is able to protect against infection, and can approach the efficacy of exogenous antigen *(2)*.

For cancer, the problems are greater since tumor antigens often represent small structural modifications of self proteins, and may therefore be poor at priming the immune system. Also, immunity has to be induced in patients already bearing tumor. Novel methods of presenting antigen in a potentially immunogenic manner must be devised, and DNA vaccines may be ideal for this. A further consideration is that long-term exposure to potential tumor antigens may have deleted or energized T cells able to recognize tumor *(3)*, and it is not clear if these can recover or be replaced. Inclusion of known epitopes to activate additional T-cell help may provide a way to circumvent this problem *(4)*. If these added epitopes are common to all patients' vaccines, the immune response against them can act as an indicator of immune status. This could be invaluable for patients who may have variable immune capacity following disease or treatment. Finally, cytokine genes can be used to promote and direct the immune response to attack tumor *(5)*.

For our DNA vaccines, we have focused on the idiotypic determinants of immunoglobulin that represent defined tumor antigens of neoplastic B cells *(6)*. These determinants are known to induce specific protective anti-idiotypic immunity in mouse lymphoma models when injected as IgM protein antigens with adjuvant *(7,8)*. However, they are individual to each tumor, and preparation of idiotypic IgM proteins for patient application is expensive and

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

difficult. This has given added impetus to the development of DNA vaccines since the variable region gene sequences (V_H and V_L) encoding the determinants can be readily identified by PCR ***(9)***. There are several options for assembing these genes in the vaccine plasmid; we have chosen the single chain Fv (scFv) format, which includes the minimal sequence known to fold into a conformation resembling that of the variable region of whole Ig ***(10)***. Folding is likely to be important in inducing protection against B-cell lymphoma, since anti-idiotypic antibody has been found to be a crucial component of protective immunity ***(1)***. Assembly as scFv is rapid, convenient and economical, and is therefore applicable to vaccine production on a patient-specific basis.

In this chapter we describe the preparation of scFv DNA vaccines from biopsy material of patients with B-cell tumors. In mouse models we have found that scFv sequence alone is capable of inducing only a low level of anti-idiotypic immunity. However, a small clinical trial of this preliminary design was undertaken in patients with advanced disease, largely to assess potential toxicity of DNA injection into muscle. Further vaccine development in mice showed that the anti-idiotypic response can be dramatically promoted by fusing a gene encoding the Fragment C (FrC) portion of tetanus toxin (TT) to the scFv gene sequence ***(4)***. The design and testing of this modified vaccine will be described. The fused TT sequence has the additional advantage of inducing an anti-TT response, which can be used as an indicator of immune capacity in patients. Although we have chosen idiotypic antigen as a target in B-cell tumors, the principles of vaccine design are applicable to other tumor antigens.

1.1. Identification of Tumor Variable Region Genes

Ideally, starting material should be a fresh biopsy, or frozen viable cells, so that RNA may be prepared. B cells can have two rearranged V_H genes, one of which will be non-functional, and tends to be transcribed at a lower level, therefore use of RNA favors identification of the functional allele. cDNA is then usually prepared by reverse transcription using an oligo dT primer, although constant region primers can be used ***(11)***. If fresh material is unavailable, genomic DNA can be used as a source, but the sequence must be carefully scanned for mutations or frameshifts in case the non-functional allele is amplified. The V_H and V_L genes used to encode the tumor idiotypic Ig are obtained by PCR using mixes of family-specific 5'-primers in the V-gene together with mixes of primers complementary to J_H sequences. The 5'-primers can be based in either the leader sequence or the first framework (FR1) region. The nucleotide sequences are shown in **Table 1**. The positions of the primers in the V_H sequence are shown in **Fig. 1** with the size of product expected. A similar PCR method is applied to obtain V_L product, with either κ or λ primers used depending on the phenotype of the tumor, which is usually known.

Table 1
Primers Used for Identification and Construction of scFv and svFv-Frag C Fusion[a]

Human VH leader primary PCR primers	
VH1 Ldr	5'-CAC ACC ATG CAC TGG ACC TGG AG-3'
VH2 Ldr	5'-ATG CAC ATA CTT TGT TCC ACC CTC-3'
VH3 Ldr	5'-CCA TGG AGT TTG GGC TGA OCT GG-3'
VH4 Ldr	5'-ACA TGA AAC AYC TGT CGT TCT TCC-3'
VH5 Ldr	5'-ATG GGG TCA ACC CCC ATC CTC G-3'
VH6 Ldr	5'-ATG TCT CTC TCC TTC CTC ATC TTC-3'
Human VH leader scFv assembly primers	
scVH1 Ldr	5'-TAT AAG CTT GCC GCC ACC ATG GAC TGG ACC TGG AG-3'
scVH2 Ldr	5'-TAT AAG CTT GCC GCC ACC ATG GAC ATA CTT TGT TCC-3'
scVH3 Ldr	5'-TAT AAG CIT GCC CCC ACC ATG GAC TTT GGG CTG AGC-3'
scVH4 Ldr	5'-TAT AAG CTT GCC GCC ACC ATG AAA CAY CTG TGG TTC-3'
scVH5 Ldr	5'-TAT AAG CTT GCC GCC ACC ATG GGG TCA ACC GCC ATC-3'
scVH6 Ldr	5'-TAT AAG CTT GCC GCC ACC ATG TCT GTC TCC TTC CTC-3'
scFv-Frag C fusion assembly primers	
A31 scVH Ldr	5'-TAT AAG CTT GCC GCC ACC ACC AAG TTG TGG CTG ACC-3'
A31-FCrev	5'-TTT CAT ACC TCC GGG TCC ACC TTT GAT CTC CAC CTT-3'
YJ-FCrev	5'-TTT CAT AGGTCC GGG TCC ACC TAG GAC CGT CAG CTT-3'
FCfor	5-CCA CCC GGACCT ATG AAA AAC CTT GAT TGT TG-3'
FCrev	5'-TAA TGC GGCCCC TGA GTC CTT GGT CCA ACC TTC-3'
FC (SacII)rev	5'-TTG TCC GGC AGG TCC CGG AAA G-3'

[a]Human VH FR1, Vκ/Vλ FR1, JH, and Jκ/Jλ primary and assembly primers have been described previously ***(9)***.

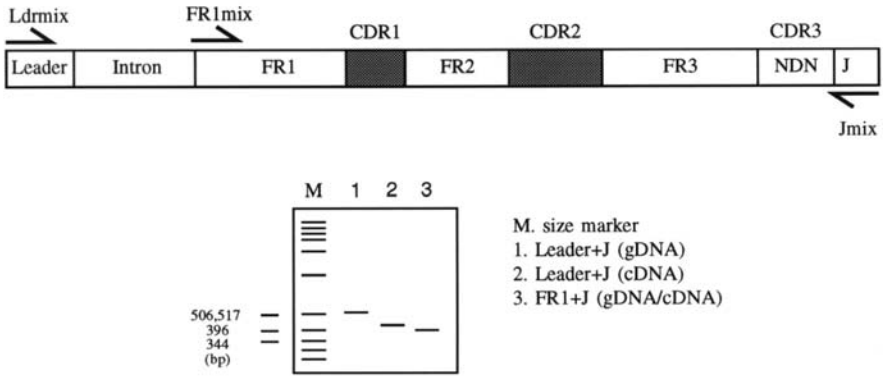


Fig. 1. Detection of tumor-related V_H-genes by PCR. Amplification of cDNA or genomic DNA from a tumor biopsy using the 5'-primers (Ldr mix or FR1 mix) together with 3'-Jmix primers is followed by separation of product by gel electrophoresis.

The amplified V-genes are then cloned into a pGEM-T vector and plasmids from ~10 randomly selected bacterial colonies are sequenced. Since the CDR3 sequence of V_H is unique to each B cell, identity of repeated similar CDR3 stretches in the cloned V_H product reveals the tumor-related sequence. In our hands the success rate in B-cell lymphoma is ~90%, with failure sometimes due to somatic mutations in the V-genes at the primer sites. Identity between repeated similar sequences can be confirmed by comparison of V_H with the closest germ line gene in the databases, which indicates sites of somatic mutation (**Fig. 2**). A similar approach is used to identify V_L sequence, which, although V_L has a less unique CDR3, is generally clearly revealed. In some B-cell tumors, such as follicular lymphoma, a degree of nucleotide variation between sequences which are clearly derived from the same original B cell may be evident ***(12)***. This does not affect construction of the scFv vaccine since there is usually a predominant sequence, and minor variation in amino acid sequence should be insufficient to allow escape from a polyclonal anti-idiotypic response ***(1)***.

1.2. Assembly and Cloning of ScFv Construct

The tumor-related V_H and V_L are then assembled as scFv by a two-step PCR procedure using overlapping primers (**Fig. 3**). A linker encoding 15 amino acids, (GlyGlyGlyGlySer) × 3 is incorporated, and the full length scFv is cut and cloned into the pcDNA3 vaccine vector for vaccination ***(9)***. When using the 5'-V_H leader sequence primer, the natural leader sequence of the scFv is included in the vaccine, and this is preferred. If the primer is based in the first framework region (FR1), a human V_H1 leader is used ***(9)***.

1.3. Assembly and Cloning of ScFv-Fragment C Fusion Construct

FrC is a non-toxic component of tetanus toxin which carries epitopes able to induce protective immunity against infection by *Clostridium tetani* ***(13)***. It has also been used in mice as a DNA vaccine construct, and found to be effective ***(14)***. The FrC sequence was amplified from pTech2 as template (kindly provided by Dr. Stephen Chatfield, Medeva plc, Leatherhead, Surrey, UK). For the first scFv-FrC construct, the whole FrC sequence was amplified and assembled with scFv by a two-step assembly process using the primers in **Table 1**. In order to minimize PCR error, subsequent scFv-FrC constructs were made using a 3'-primer close to the unique *Sac*II site near the 5'-end of FrC, so that only partial sequence was amplified (**Table 1**). The scFv-FrC partial sequence could then be cloned as the *Hind*III-*Sac*II fragment into the vaccine vector already containing the FrC gene, and digested with the same enzymes for cloning. The procedure introduces a peptide of GlyProGlyPro as a spacer between the scFv and FrC. For all constructs, sequences were checked after cloning to ensure fidelity. Constructs were again cloned into pcDNA3 (**Fig. 4**).

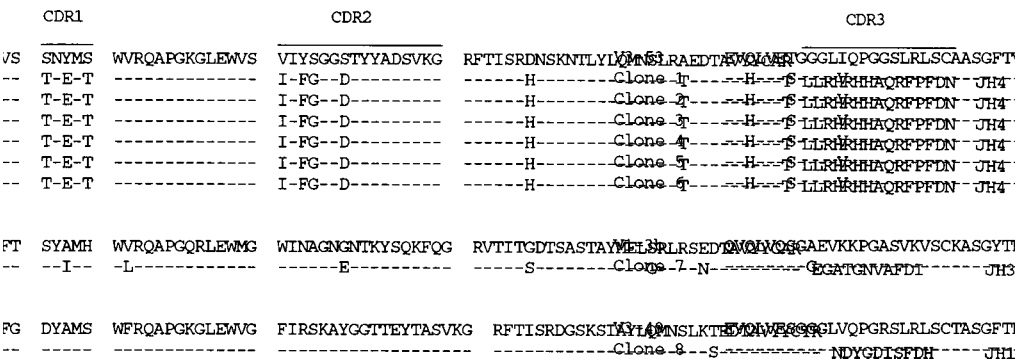


Fig. 2. Identification of tumor-related V_H sequences. Amplified V_H products are cloned in bacteria and random clones are sequenced. Deduced amino acid sequences are shown using the single letter code, and are compared with the closest germ line V_H gene. Dashes indicate identity, and amino acid replacements are identified. Repeated sequences with identical or closely similar CDR3s are likely to be derived from tumor cells. Further individual sequences are from normal B cells in the biopsy.

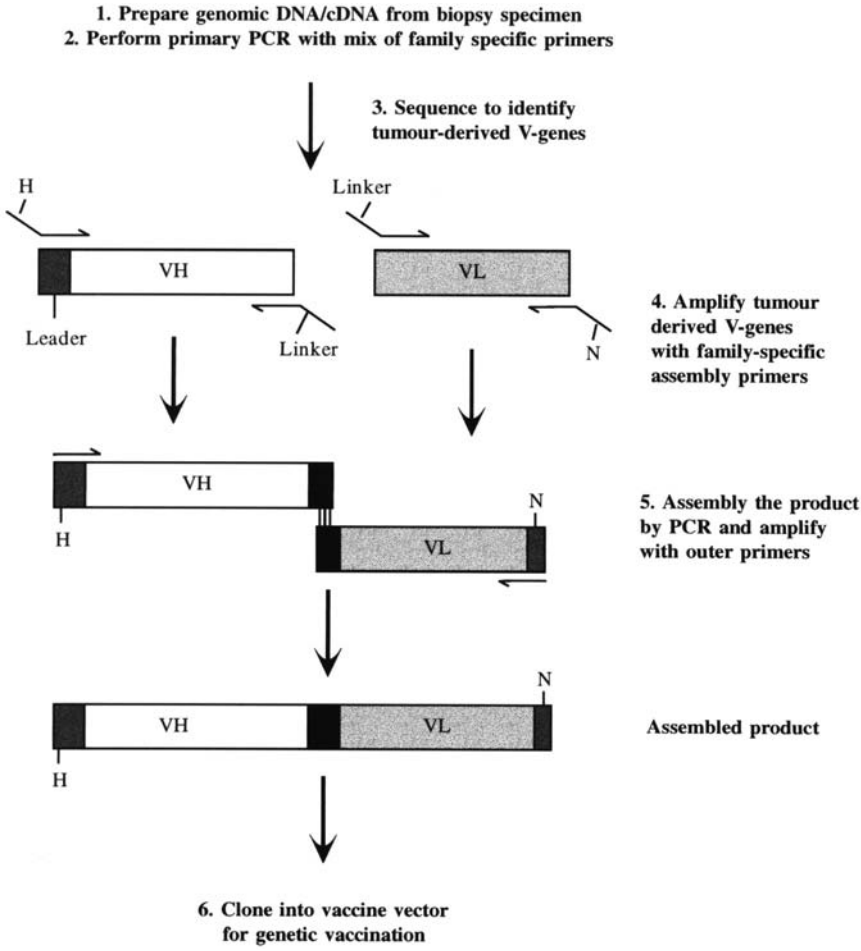


Fig. 3. Assembly procedure for scFv. Tumor-related V_H and V_L sequences are linked by a sequence encoding (GlyGlyGlyGlySer) × 3 prior to cloning into the vaccine vector.

1.4. Expression of ScFv or Fragment C Genes In Vitro

Although the sequences are always checked for mutations, we also assess the ability of each set of encoded genes in the vaccine vectors to express protein *in vitro*. This is especially important for the large scFv-FrC constructs where sequencing is more difficult. We have found that the simplest expression method is Promega's TNT^R (Madison, WI) coupled *in vitro* system, which uses rabbit reticulocyte lysate to incorporate biotinylated lysine residues into protein, with detection by streptavidin-labeled alkaline phosphatase, following fractionation by SDS-PAGE.

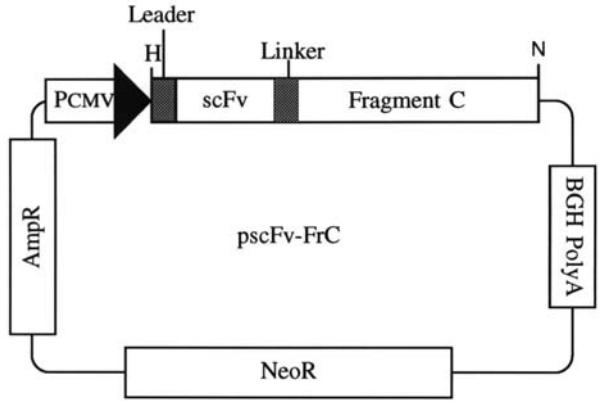


Fig. 4. DNA plasmid designed for vaccination. The pcDNA3-based vector contains fused scFv-FrC genes with expression driven from the CMV promoter.

1.5. Plasmid Preparation

For large scale purification of vaccine plasmids, the Qiagen Plasmid Giga Kit (Qiagen, Chatsworth, CA) is used. Aliquots can then be stored at –20°C as ethanol precipitates. Before use, the DNA precipitate is washed with 70% ethanol and redissolved in normal saline at 1 mg/mL. Vaccination procedure for mouse models will be described. We have done one small clinical trial in patients using an earlier plasmid containing only scFv, with the RSV LTR to drive gene expression. The protocol for this has been published ***(15)***. Subsequently we decided on the basis of our own unpublished data, and on that from other groups ***(16)***, that the CMV promoter in pcDNA3 was superior, and the new vaccines all use this vector.

1.6. Vaccination Protocol

In order to establish the procedure for optimizing the immune response to a scFv DNA vaccine, we first used a human scFv sequence either alone, or fused to the FrC gene ***(4)***. However, preliminary experiments showed that scFv alone was a weak immunogen ***(9)***, and all further experiments to optimize protocols have been carried out with the scFv-FrC fusion construct. This construct allows assessment of response against both scFv and FrC. For scFv, the objective has been to induce antibody against the patient's idiotypic IgM, the molecule expressed by the tumor cells. By using IgM as the test molecule, only the therapeutically relevant antibodies, which recognize idiotypic determinants expressed in a manner similar to those on tumor cells, are being measured.

Generally, we have used the intramuscular route for vaccination. With our vaccines, we have found that intramuscular and intradermal sites induce rather

similar immune responses, although we have not explored the use of the gene gun. Mice are injected in two sites in the quadriceps muscles with a total dose of 50 μg of plasmid DNA in 100 μL saline. We have found that a total of three injections of DNA vaccine, spaced at 3 wk intervals, induces high levels of antibodies against both FrC and tumor IgM (see below). Attempts to boost antibody levels by increasing the amount of DNA, or by a further injection after d 63, did not significantly improve antibody levels. Similar protocols appear applicable in both C57BL/6 and BALB/c mice.

1.7. Assessment of Effect of Co-injection of Cytokine Gene Plasmids

To assess the effects of co-delivery of cytokine genes with the vaccine vector, we use a minimal dose (10 μg) of DNA vaccine containing scFv-FrC fusion vector assembled from a patient YJ with lymphoma ***(4)***. A pVAC.CYTO vector (50 μg) containing the gene for murine GM-CSF, with expression driven by the CMV promoter (kind gift of Professor R. E. Hawkins, University of Bristol, Bristol, UK), is mixed with the vaccine vector and co-injected ***(5)***. The protocol is as for the vaccine vector, with co-injection of cytokine vector at each boost. As a control for the effect of injecting additional DNA, a vaccine plasmid with no scFv incorporated is used at the same dose as the cytokine vector. Outcome is measured as antibody levels against the two encoded proteins at d 63 (*see* **Subheading 1.8.**). Other cytokine genes can be investigated by the same procedure.

1.8. Measurement of Antibody Tesponses

Bleeds are taken at intervals by tail tipping, or from the cardiac site at termination of the experiment. The serial bleeds are taken one day prior to injection. Measurement of antibody against FrC is by ELISA using purified recombinant FrC (kind gift from Dr. Stephen Chatfield, Medeva plc). Antibodies against idiotypic IgM are measured also by ELISA using IgM obtained from heterohybridoma "rescue" fusions between tumor cells and the mouse myeloma cell line OURI-A, a subline of the X63-Ag8.653 line ***(17)***. Purification of IgM proteins is by precipitation of euglobulin following extensive dialysis against water ***(18)***.

In preparation for application to patients, several human scFv constructs have been tested in mice, primarily to assess the ability of a range of patients' scFv molecules to induce anti-idiotypic antibodies when fused with FrC. Specificity of antibodies is tested by measuring reactivity of sera with autologous and control idiotypic IgM proteins, using ELISA. In three out of three cases, induced antibodies were largely directed against autologous IgM, indicative of efficient folding of the encoded scFv in the fusion protein. The ability of antibodies to recognize idiotypic IgM on the surface of tumor cells is assessed by FACS-SCAN.

The antibody levels obtained using a scFv gene from patient YJ in fusion with the FrC gene, in C57BL/6 mice, are shown in **Fig. 5**. The effects of vaccinating with two different doses of DNA, and of repeated booster doses of DNA have been investigated. In general, responses against the two encoded antigens, scFv and FrC, are fairly similar, and allow conclusions to be drawn for planning protocols. It is clear that 10 μg of DNA is insufficient to obtain a strong antibody response, and that 50 μg improves both levels and consistency of antibody production. However, there is considerable heterogeneity among mice in the levels attained, and increasing the dose to 100 μg does not appear to raise the level further or reduce heterogeneity (data not shown). At least two injections of DNA are required, and we have found that a third injection improves response (**Fig. 5**). However, further boosts do not appear to increase the antibody titre significantly (data not shown).

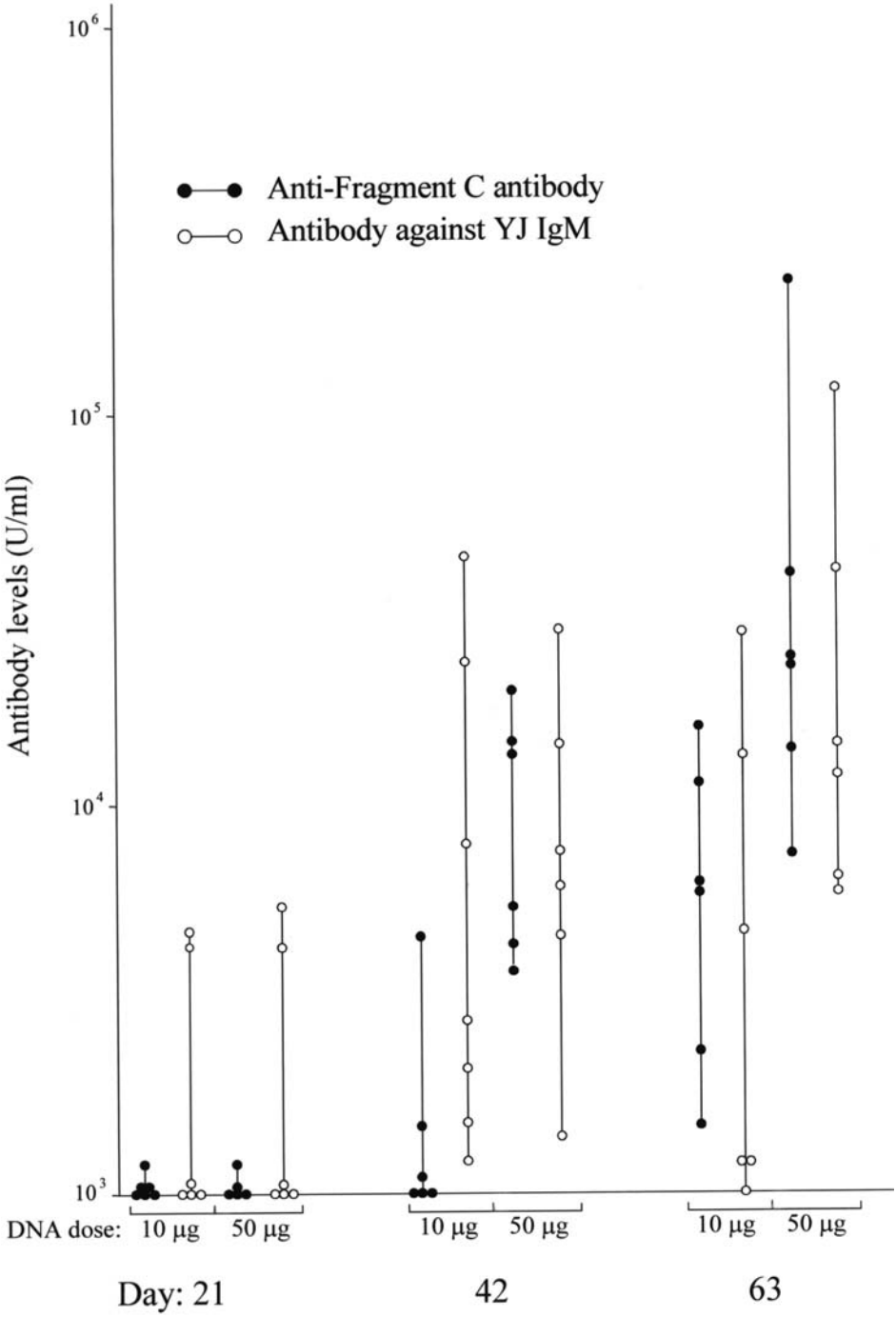
Co-injection of a GM-CSF-encoding vector together with the 10 μg dose of scFv-FrC vaccine from patient YJ significantly increased the levels of antibodies against both proteins (**Fig. 6**). Interestingly, empty vector DNA also increased the response to the low dose of vaccine vector, presumably by the immunostimulatory effects of bacterial DNA ***(19)***. This effect emphasizes the need to include this control in all experiments, especially when using low doses of vaccine vector. No significant promotion of antibody responses above this background was detected using co-delivery of genes encoding IL-2, IL-4 or IFNg (data not shown).

1.9. Mouse Lymphoma Model

To assess the ability of scFv DNA constructs to induce immunity in a syngeneic model, and to allow testing of protection against tumor challenge, the A31 murine lymphoma is being used ***(20)***. The V_H and V_L genes of mouse tumors can be identified by PCR/cloning as for human genes, using primers listed in **Table 1**. Assembly can be carried out similarly and the vaccination protocol is as for the human scFv. For measurement of antibodies induced by the mouse scFv, the IgM "rescued" from the A31 tumor can be used. Results are similar to those obtained with human scFv constructs, in that the scFv gene alone is ineffective as a vaccine, but that fusion with FrC markedly promotes the antibody response. The immunity generated by the fusion vaccine is also able to protect against tumor challenge ***(21)***.

2. Materials

Special equipment needed throughout this work: thermal cycler, agarose gel electrophoresis apparatus, DNA sequencing equipment, and suitable power supplies. Materials for preparing agarose and polyacrylamide sequencing gels and for doing ELISA tests are required.


Anti-Fragment C antibody
Antibody against YJ IgM
Antibody levels (U/ml)
10^6
10^5
10^4
10^3
DNA dose: 10 μg 50 μg 10 μg 50 μg 10 μg 50 μg
Day: 21 42 63

2.1. Identification of Tumor-Related VH and VL Genes

1. Oligonucleotide primers for PCR amplification are listed in Table 1. All oligonucleotides are diluted to a working concentration of 10 pmol/μL.
2. RNAzol™B: Cinna Biotecxlabs, Houston, Texas.
3. cDNA synthesis: First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden).
4. *Taq* DNA polymerase, Boehringer Mannheim, Mannheim, Germany.
5. *Taq* buffer: 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, 50 m*M* KCl (pH 8.3).
6. K buffer: 1X *Taq* buffer plus 0.5% Tween 20.
7. Proteinase K: make a stock solution at 20 mg/mL and store at –20°C. Use within 6 mo.
8. GENECLEAN II kit: BIO 101, Vista, CA.
9. pGEM-T vector: Promega, Madison, WI.
10. DNA sequencing: T7 Sequenase version 2.0 DNA sequencing kit, Amersham Life Science, Buckinghamshire, UK.

2.2 . Assembly and Cloning of scFv and scFv-Fragment C Fusion Construct

1. Primer list: *see* **Table 1**.
2. *Pfu* DNA polymerase: Stratagene, La Jolla, CA.
3. *Pfu* Pol buffer: 10 m*M* KCl, 10 m*M* $(NH_4)_2SO_4$, 20 m*M* Tris-Cl (pH 8.75), 2 m*M* $MgSO_4$, 1% Triton X-100, 100 mg/mL BSA.
4. Vectors (pcDNA3): Invitrogen BV, The Netherlands.
5. PCR template for FrC amplification: pTech2 plasmid containing the fragment C gene, kindly provided by Medeva plc.

3. Methods

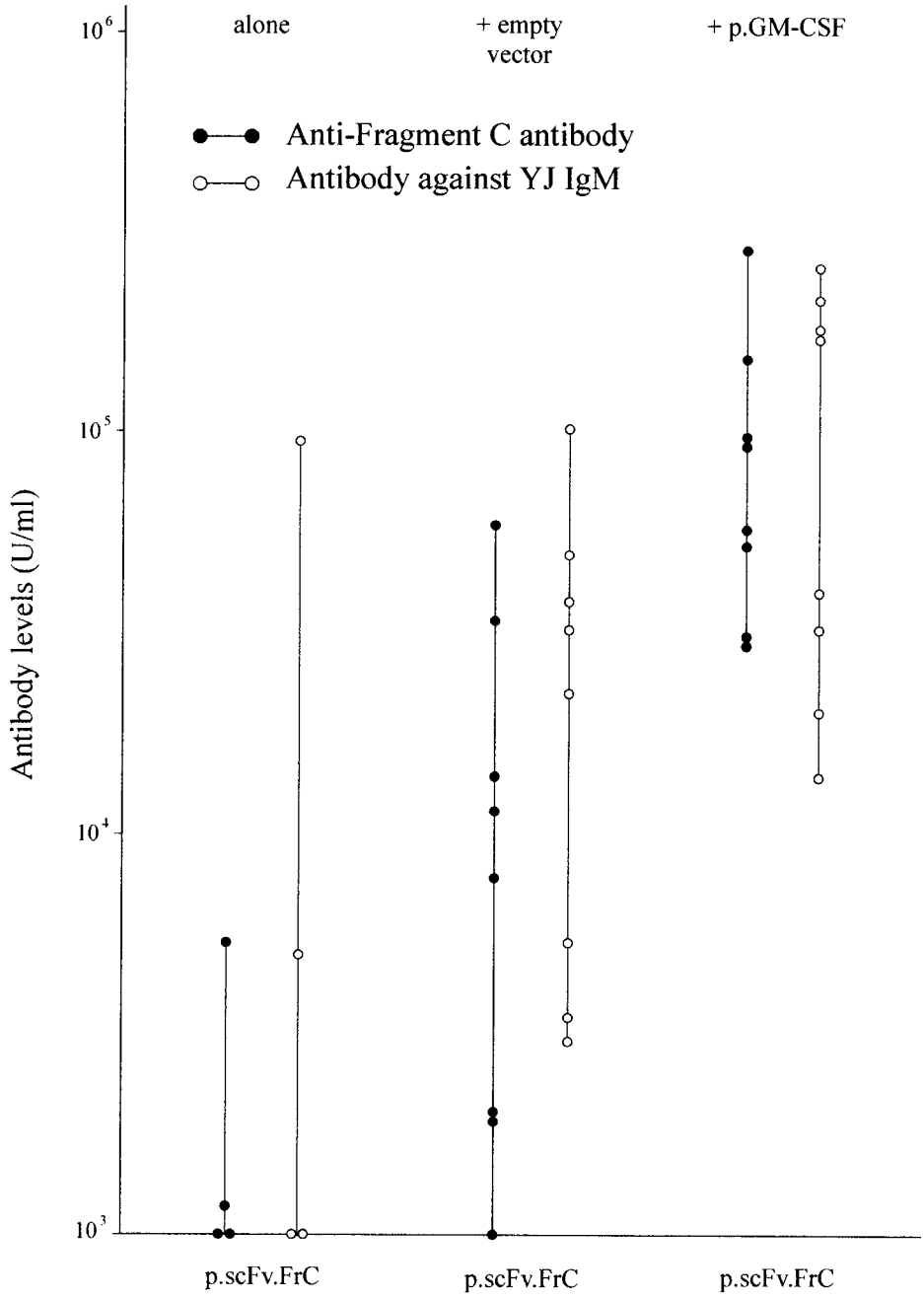
A number of commercial reagent kits are used throughout this work. Refer to the manufacturers' manuals for instructions. For general molecular biology techniques, see Sambrook et al. ***(22)***.

3.1. Identification of Tumor-Related VH and VL Genes

3.1.1. Isolation of Genomic (g) DNA (*see* **Note 1**)

1. Wash 10^6 cells (peripheral blood mononuclear cells or lymphoid tissues) with 1 mL of PBS, and then 1 mL of 1 X *Taq* buffer.
2. Resuspend the cells in 1 mL K buffer.
3. Add 5 μL of Proteinase K and incubate at 56°C for 1 h.

Fig. 5. Induction of antibodies by intramuscular injections of DNA vaccine plasmid containing the scFv-FrC fusion gene. ScFv sequence was derived from tumor cells of patient YJ. Serum antibodies recognizing YJ tumor IgM or FrC were measured by ELISA following vaccination of mice at day 0, 21 and 42, using two different doses of DNA. Each point represents the antibody level in a single mouse.


alone
+ empty vector
+ p.GM-CSF
Anti-Fragment C antibody
Antibody against YJ IgM
Antibody levels (U/ml)
10^6
10^5
10^4
10^3
p.scFv.FrC
p.scFv.FrC
p.scFv.FrC

4. Inactivate Proteinase K by heating to 95°C for 15–30 min.
5. Centrifuge for 5 min in a microfuge at maximum speed (13,000 rpm).
6. Transfer the supernatant to a fresh tube. Store the gDNA at –20°C if not used immediately.

3.1.2. Isolation of Total RNA

1. To lyse cells, add 0.2 mL of RNAzolB per 10^6 cells and mix by inverting the tube several times (*see* **Note 2**).
2. Add 1/10 vol. of chloroform and shake vigorously for 15 s. Leave on ice for 5 min.
3. Centrifuge for 15 min in a microfuge at maximum speed.
4. Carefully transfer the upper aqueous phase containing RNA in a fresh tube.
5. Add 1 vol. of isopropanol, mix well and leave on ice for 15 min.
6. Centrifuge at maximum speed for 15 min.
7. Wash the pellet with 0.5 mL of 75% ethanol by vortexing and subsequent centrifugation for 8 min.
8. Dry the pellet for 10 min at room temperature and redissolve in RNase-free water.

3.1.3. Preparation of cDNA

1. Heat 1–5 μg total RNA in 20 μL of water at 65°C for 5 min to denature RNA. Chill the tube on ice.
2. Add 1 μL of DTT, 1 μL of oligo $(dT)_{18}$ (0.2 μg) and 11 μL of Bulk First Strand Reaction Mix containing RNase inhibitor, dNTPs and M-MuLV reverse transriptase.
3. Incubate at 37°C for 1 h.

3.1.4. PCR Amplification of VH and VL Genes

1. Prepare a PCR reaction mixture that contains all the necessary components for the reaction except for the enzyme (*see* **Note 3**). If there is more than one sample, a master mix without DNA and enzyme should be made to reduce the steps and errors of pipetting. A typical reaction contains:

10X *Taq* Pol buffer	5 μL
2.5 m*M* dNTPs	5 μL
VH or Vk/Vλ primer	2 μL
JH or Jκ/Jλ primer	2 μL
Genomic DNA	1–5 μL (~100 ng)
or cDNA	1–5 μL
H_2O	to 49.5 μL

Fig. 6. Effect of co-delivery of a plasmid containing the gene encoding GM-CSF on induction of antibodies against a low dose of DNA scFv-FrC vaccine. Mice were injected with 10 μg of vaccine plasmid either alone, with added 50 μg empty vector, or with added 50 μg of GM-CSF plasmid. ScFv sequence was as for **Fig. 5**, and sera were taken at day 63 for ELISA.

2. Perform the amplification in a thermal cycler as follows: Incubate for 5 min at 94°C. Add 0.5 µL (2.5 units) of *Taq* DNA polymerase and then repeat 30 cycles of 30 s at 94°C, 30 s at 65°C, and 45 s at 72°C. Finally, incubate for 7 min at 72°C.
3. Analyze the PCR reaction by electrophoresis on an 1.5% agarose gel alongside a molecular weight size standard.
4. Excise and purify the bands of appropriate sizes ( ).
5. Clone the amplified V genes into the pGEM-T vector.
6. Isolate plasmid from randomly selected bacterial colonies.
7. Sequence 6–10 positive clones.
8. Identify tumor-related V_H and V_L genes.

3.2. Assembly and Cloning of scFv

The tumor V_H and V_L genes are assembled as a single-chain Fv (scFv) by a two-step PCR procedure as illustrated in **Fig. 3**. The scFv assembly primers are listed in **Table 1** (*see* **Note 4**). In the first step, the V_H and V_L genes are amplified separately from the plasmids containing tumor-related V_H and V_L genes with the appropriate assembly primers to incorporate the restriction sites and the (GlyGlyGlyGlySer) × 3 scFv linker. In the second step, the secondary PCR products are combined and the full-length scFv is assembled by PCR using the outer primers only. The fully assembled scFv is cut and cloned into a vaccine vector for genetic vaccination.

3.2.1. Secondary PCR

1. Prepare two PCR reaction mixes (*see* **Note 5**) by adding:

	Tube A (V_H)	**Tube B (V_L)**
10X *Pfu* Pol buffer	5 µL	5 µL
2.5 m*M* dNTPs	5 µL	5 µL
scVH primer	2 µL	
scJH	2 µL	
scVκ/Vλ		2 µL
scJκ/Jλ		2 µL
VH plasmid	1 µL (100ng)	
VL plasmid		1 µL (100ng)
H_2O	34.5 µL	34.5 µL

2. Carry out the amplification in a thermal cycler as follows: Incubate for 5 min at 94°C. Add 0.5 µL (2.5 units) of *Pfu* DNA polymerase, repeat 5 cycles of 30 s at 94°C, 30 s at 45°C, and 1.5 min at 72°C, and then 15 cycles of 30 s at 94°C, 30 s at 65°C, and 1.5 min at 72°C. Finally, incubate for 7 min at 72°C.
3. Analyze the PCR reaction on an 1.5% agarose gel with a molecular weight size standard.
4. Excise and purify the bands of appropriate sizes.
5. Resuspend the DNA fragments in 25 µL of sterile ddH_2O as scV_H and scV_L.

3.2.2. Assembly PCR

1. Prepare a PCR reaction mix by adding:

10X *Pfu* Pol buffer	5 µL
2.5 m*M* dNTPs	5 µL
scVH primer	2 µL
scJκ/Jλ	2 µL
scVH	5 µL
scVL	5 µL
H_2O	25.5 µL

2. Carry out the amplification in a thermal cycler as in the secondary PCR, except with a longer extension step of 3 min at 72°C.
3. Analyze the PCR reaction on an 1.5% agarose gel with a molecular weight size standard.
4. Excise and purify the band of ~800bp.
5. Digest the purified scFv fragment with appropriate enzymes (*see* **Note 6**).
6. Clone the scFv into the pcDNA3 expression vector.

3.3. Construction of scFv-Fragment C Fusion

3.3.1. Pre-assembly PCR

1. Prepare 2 PCR reaction mixes by adding:

	Tube A (scFv)	**Tube B (Frag C)**
10 X *Pfu* Pol buffer	5 µL	5 µL
2.5 m*M* dNTPs	5 µL	5 µL
scVH leader primer	2 µL	
scFv-FrCrev	2 µL	
FrCfor		2 µL
FrCrev		2 µL
scFv plasmid	1 µL (100ng)	
pTech 2 plasmid		1 µL (100ng)
H_2O	34.5 µL	34.5 µL

2. Carry out the amplification in a thermal cycler as follows: Incubate for 5 min at 94°C. Add 0.5 µL (2.5 units) of *Pfu* DNA polymerase, repeat 5 cycles of 30 s at 94°C, 30 s at 45°C, and 3 min at 72°C, and then 15 cycles of 30 s at 94°C, 30 s at 65°C, and 3 min at 72°C. Finally, incubate for 10 min at 72°C.
3. Analyze the PCR reaction on an 1.2% agarose gel alongside a molecular weight size standard.
4. Excise and purify the bands of appropriate sizes.
5. Resuspend the DNA fragments in 25 µL of water as scFv and FrC.

3.3.2. Assembly PCR

1. Prepare a PCR reaction mix by adding:

10X *Pfu* Pol buffer	5 µL
2.5 m*M* dNTPs	5 µL
scVH leader primer	2 µL
FrCrev primer	2 µL
scFv	5 µL
FrC	5 µL
H_2O	25.5 µL

2. Carry out the amplification in a thermal cycler as in the pre-assembly PCR, except with a longer extension step of 5 min at 72°C.
3. Analyze the PCR reaction on an 1.2% agarose gel alongside a molecular weight size standard.
4. Excise and purify the scFv-FrC band (~2.2 kb).
5. Digest the purified scFv-FrC fragment with *Hind*III and *Not*I restriction enzymes.
6. Clone the scFv-FrC into the pcDNA3 expression vector.

3.4. Assessment of Antibody Production

Antibodies induced following injection of the DNA vaccines are measured by ELISA. Antibodies raised via encoded scFv molecules can be detected by reactivity with the tumor-derived IgM proteins, thus ensuring that they are directed against idiotypic determinants expressed by the tumor. For this analysis, IgM has to be obtained from heterohybridomas between tumor cells and a mouse myeloma line.

3.4.1. Preparation of Idiotypic IgM

1. Fuse tumor cells from each patient (or mouse donor) with the mouse myeloma cell line OURI-A, an ouabain-resistant subline of the X63-Ag8.653 line, using PEG ***(17)***.
2. Following fusion, assess production of IgM by ELISA and clone, with re-selection for IgM of tumor light chain type.
3. Sequence the V-genes of secreting clones to ensure that the tumor cells have been rescued.
4. Prepare IgM from culture supernatants by euglobulin precipitation following dialysis into distilled water ***(18)***. Check purity by gel electrophoresis.

3.4.2. Measurement of Serum Antibody Levels

Antibody levels in sera of vaccinated mice are measured by ELISA.

3.4.2.1. Anti-fragment C Antibodies

1. Coat ELISA plates overnight at 4°C with recombinant Fragment C at 1 µg/mL in PBS. Treat washed plates then with PBS/0.5% BSA to block non-specific binding sites.

2. After washing, dilute test sera in PBS/TWEEN20 containing 0.1% BSA and incubate in the coated plates for 1.5 h at 37°C.
3. After washing, detect bound mouse IgG by incubation with HRP-goat anti-mouse Fcg (Serotec, Kidlington, UK; 1/1000 dilution) for 1 h at 37°C. Standardize the assay for comparative purposes by preparing a pool of positive sera, and assigning to it an arbitrary value of 200U/mL.

3.4.2.2. Anti-scFv Antibodies

1. Coat ELISA plates overnight at 4°C with purified tumor-derived idiotypic IgM at 0.5µg/mL in sodium carbonate buffer at pH 9.5. Block washed plates with PBS/0.5% BSA as above.
2. After incubation with diluted control or test sera, bound mouse IgG is detected as for anti-FrC.

3.4.2.3. FACS Analysis to Test Reactivity of Sera with Tumor Cells

1. Mononuclear cells from patients' blood samples or lymphoid tissue are prepared by centrifugation through a Ficoll-Hypaque gradient.
2. After washing, cells are incubated with diluted control or test sera for 20 min on ice. After washing, bound IgG is then detected following incubation with FITC-labeled goat anti-mouse Fcg, using the FACS-SCAN with LYSISII software.

4. Notes

1. The DNA can usually be used directly for PCR amplification. However, DNA can be further purified by phenol extraction and ethanol precipitation to give cleaner PCR products. Amplification of a house-keeping gene, such as ß-actin, can also be included as a control for the template quality.
2. Various RNA isolation kits which are commercially available also can be used.
3. Care must be taken to minimize contamination when performing PCR amplification, and a negative control reaction in which no DNA template has been added should always be included.
4. The scV_H primers contain a *Hind*III (Leader) or an *Sfi*I (FR1) restriction site and hybridize to the original set of V_H primers. The scJ_H and scVκ/Vλ primers hybridize to their respective initial primers, but also include the scFv linker sequences complementary to each other to allow production of an scFv. The scJκ/Jλ primers similarly hybridize to their respective initial primers, but also include the *Not*I restriction site.
5. In the secondary and assembly PCR reactions, the high-fidelity *Pfu* DNA polymerase is used to minimize possible PCR errors. Due to the low processivity of the enzyme, the extention step of the PCR reactions has been prolonged. Refer to the manufacturer's instructions for full description of the enzyme.
6. If an scFv leader primer is used, digest the scFv fragment with *Hind*III and *Not*I enzymes. If an scFv FR1 primer is used, digest the scFv fragment with *Sfi*I and *Not*I and then clone into the vector already containing a human V_H 1 leader and *Sfi*I site ***(9)***.

7. For scFv, the 5'-primer is the V_H leader scFv assembly primer. For the 3' end, a Jκ/Jλ reverse primer which also contains the GlyProGlyPro (anti-sense) linker sequence is used. The 5'-FrC primer also contains the GlyProGlyPro (sense) linker sequence and the 3'-primer incorporates a *NotI* site.

Acknowledgments

This work was supported by the Leukaemia Research Fund, the Cancer Research Campaign and Tenovus, UK.

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DNA-Based Vaccination Primes Tumor-Rejecting T-Cell Responses

Waltraud Böhm, Martin Schleef, Stefan Thoma, Reinhold Schirmbeck, and Jörg Reimann

Introduction

DNA-based vaccination efficiently primes MHC-restricted T-cell responses. This technique specifically stimulates MHC-II-restricted $CD4^+$ T-cell responses and MHC-I-restricted $CD8^+$ T-cell responses against "strong" (immunodominant) or "weak" (subdominant or cryptic) epitopes of intracellular, secreted or membrane-associated protein antigens. In many experimental systems, T-cell-mediated effector functions have the potential to control tumor growth. In particular MHC-I-restricted cytotoxic T lymphocytes (CTL) can reject tumors. This has been shown using either defined tumor-associated antigens (TAA), or viral antigens containing well-defined, MHC-binding and CTL-stimulating epitopes that are expressed by transfected tumor cells.

1.1. T-Cells Primed By DNA Vaccination Have the Potential to Reject Tumor Cells

We have studied two murine tumor models, i.e. the mastocytoma cell line P815 (H-2^d) and the melanoma cell line B16.F10 (H-2^b). We have generated stable transfectants of these tumorigenic cell lines that express either the small hepatitis B surface antigen (HBsAg), or the large tumor antigen (T-Ag) of simian virus 40. Cells of the non-transfected parental line and the transfected sublines form aggressively growing tumors when 10^3 to 10^4 cells of these lines are subcutaneously injected into adoptive, syngeneic hosts. Both tumor cell lines are deficient in T-cell co-stimulating activity. P815 cells constitutively express high levels of MHC-Ia (K^d, D^d, L^d) molecules but no MHC-II molecules on the cell surface. B16 cells constitutively express only low levels of MHC-Ia (K^b,

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

D^b) molecules on the cell surface, but MHC-I expression is strikingly upregulated and MHC-II expression is induced on the surface of these cells in response to interferon-γ (IFN-γ) stimulation. A fraction (10–40%) of B16 cells express the TNF-like CD95L (FasL) on the surface, which is supposed to paralyze potentially rejecting effector CTL.

DNA-based vaccination efficiently primes potent MHC-I-restricted CTL responses to HBsAg or T-Ag in high and low responder (H-2^d or H-2^b) mouse strains ***(1–3)***. The MHC-I-restricted, HBsAg-specific or T-Ag-specific CTL primed by DNA vaccination can reject P815 or B16 tumor cell grafts expressing the respective viral antigens when 100–fold higher numbers than the minimal tumorigenic graft size are transferred (***4***, data not shown). Representative examples are shown in **Fig. 1**. The T cell-mediated, specific rejection of B16 melanomas was thus not overridden by CD95L expression of the target cell. The time point of DNA vaccination relative to tumor cell engraftment is critical. Inoculation of antigen-encoding plasmid DNA up to 4 d before tumor cell transfer confers protection. In contrast, immunizations 1 to 3 d before, at the same time of, or after tumor cell engraftment are not protective. Alternative treatment protocols were therefore required to target rejecting CTL to growing tumors.

1.2. Targeting Anti-viral CTL Reactivity to a Growing Tumor Facilitates its Rejection, and Cross-primes Rejecting Immune Responses Against TAA

We found an effective targeting protocol that involved the following three steps: A potent anti-viral CTL reactivity was induced by DNA-based vaccination; A subcutaneously growing tumor was established; The established CTL reactivity was targeted to the tumor by repeated intra-tumor injections of DNA expression constructs that encode the relevant antigen. Using this protocol, we obtained stable rejection of the P815 mastocytoma tumors in 50-70% of the treated, tumor-bearing H-2^d DBA/2 mice (***4***, **Fig. 2**). Most encouraging was the observation that all mice that had rejected the mastocytoma following this therapeutic protocol showed stable resistance against a challenge with non-modified tumor cell grafts for >4 months post-rejection. Hence, the tumor rejection process initiated by in vivo restimulation of primed MHC-I-restricted, anti-viral CTL within the tumor (following intra-tumor DNA injections) facilitated cross-priming of a TAA-specific, rejecting immune response. This rejecting immune response could not control metastases because regression of a treated P815 mastocytoma on one flank of an adoptive host did not coincide with regression of a contralateral, non-treated, progressively growing P815 mastocytoma in the same host (*unpublished data*).

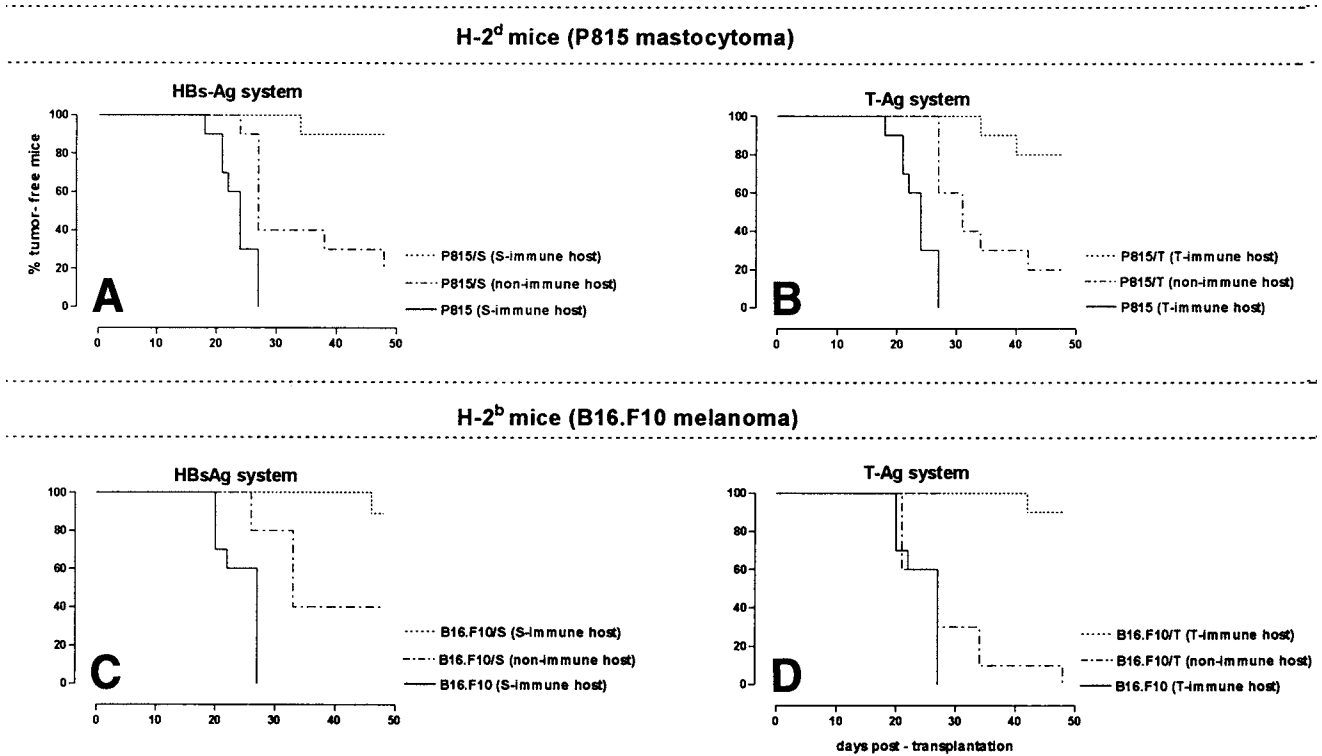

Fig. 1. T-cell responses to different viral antigens elicited by DNA-based vaccination can protect mice against growth of different types of malignant tumors. DBA/2 (H-2^d) or C57BL/6 (H-2^b) mice were vaccinated with HBsAg- or T-Ag-encoding DNA constructs (S-immune or T-immune host). Vaccinated mice or non-pretreated control mice (non-immune host) were subcutaneously transplanted with 10^6 tumor cells (10 mice/group); the tumor cells were non-transfected (P815 or B16.F10), or transfected expressing the HBsAg (P815/S, B16.F10/S) or the T-Ag (P815/T, B16.F10/T). The fraction of tumor-free mice was monitored for 50 d.

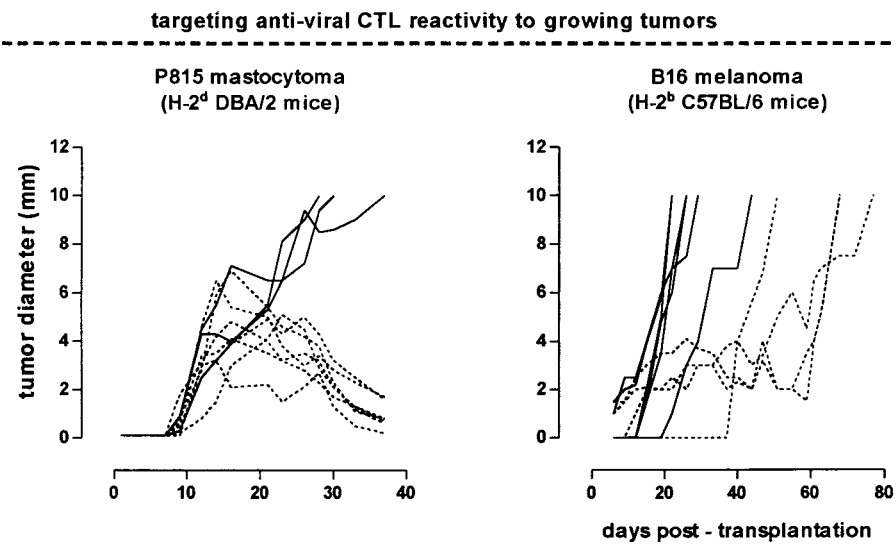

Fig. 2. Targeting anti-viral T-cell reactivity to growing tumors can facilitate their rejection. DBA/2 or C57BL/6 mice were immunized against HBsAg using DNA-based vaccination. The mice were subcutaneously transplanted with 5×10^5 non-transfected tumor cells (P815 or B16.F10) 4 weeks post-vaccination. When subcutaneous tumor nodules reached a diameter of >2 mm, HBsAg-encoding DNA was injected into the tumors every 2–3 d for a maximum of 3 wk. The size of the tumor was measured every 3–4 d.

In contrast to the P815 mastocytoma model, we observed only transient control of B16 melanoma growth for 3 to 8 wk in 10–30% of tumor-bearing H-2^b C57BL/6 mice using this protocol (**Fig. 2**, Waltraud Böhm, unpublished observations). In this model, restimulation of a potent, primed T-cell response to an immunodominant viral antigen within the tumor apparently did not facilitate cross-priming of a rejecting, TAA-specific immune response under the experimental conditions applied.

1.3. Potential for Specific Immunotherapy of Cancer Using DNA Vaccines

Because DNA-based vaccination is one of the most potent, currently available techniques to prime T-cell responses, it is of interest to test in vivo the tumor-rejecting potential of T-cell reactivities inducible by this novel type of vaccination. Our data set provides both encouraging and discouraging findings concerning the specific immunotherapy of cancer based on DNA vaccination.

1.3.1. T-Cells Primed by DNA Vaccination Can Potentially Control Growth of Solid Malignant Tumors

We used two viral model antigens (surface antigen HBsAg of HBV, and the nucleoprotein T-Ag of the papovavirus SV40) that are immunogenic for MHC-I-restricted, murine CTL precursors, eliciting CTL responses of well-characterized epitope and restriction specificities. T-cell responses against these antigens elicited by DNA-based vaccination could protect a syngeneic host against aggressively growing tumors (**Fig. 1**). The specific immune response conferred resistance against an up to 100-fold tumorigenic dose of tumor cells, but failed at higher doses. Tumors escaping after transfer of $>10^6$ transfected tumor cells into the immune host still expressed and presented the viral antigen, i.e. tumor growth was not the result of selection of "antigen loss" escape variants. Hence, there seems to be a limit to the tumor cell burden with which an efficient T-cell response can cope. This limit is well below average tumor cell burdens present during aggressive growth of tumors in vivo. Neither the type of antigen, nor the histotype of the tumor seemed to have a major influence on the T-cell-mediated rejection inducible by DNA-based vaccination. In H-2^d mice, HBsAg is a "strong" antigen and T-Ag is a "weak" antigen for CTL; the inverse is true for H-2^b mice, in which HBsAg is a "weak" antigen and T-Ag is a "strong" antigen for CTL. In both strains, both anti-viral CTL responses apparently mediate comparable levels of CTL-mediated protection against tumors. Mastocytomas and melanomas are very different types of tumors. The DNA vaccination-induced T cell responses conferred comparable levels of protection against both types of tumors in the two antigen systems.

A subset of B16 melanoma cells expresses CD95L (FasL) that has been shown to paralyze or to eliminate cytotoxic effector cells. Anti-viral CTL induced by DNA vaccination could apparently override this 'protective' mechanism of tumor cells against immune attack.

1.3.2. DNA Vaccination Protocols Can Be Designed to Facilitate Cross-Priming of Rejecting, TAA-Specific T-Cell Responses

Once a tumor (expressing a viral antigen) is growing in vivo, it seems largely resistant to attack by CTL. We therefore designed the targeting technique to: facilitate cross-priming of CTL precursors to TAA, and to deliver cytokines to tumors *in situ* to recruit rejecting, specific and non-specific immune mechanisms. This protocol was unexpectedly successful with P815 mastocytomas but controlled only transiently the growth of B16 melanomas in vivo (**Fig. 2**). The mechanism of protection against P815 cells stimulated by the CTL targeting protocol remains to be elucidated. Rejecting mice are stably resistant against challenge with non-modified P815 cells, and harbor P815-specific CTL reactivity. The generation of TAA-specific CTL against this tumor may there-

fore play a prominent role in this tumor-specific resistance. If this is the case, it would be a high priority to elucidate the mechanism that facilitates CTL cross-priming to TAA in this protocol.

2. Materials

2.1. Expression Vectors Used for DNA-based Vaccination

The CMV promoter controls the expression of the small HBsAg (subtype ayw) in plasmid pCI/S (**Fig. 3A**) and the SV40 large tumor antigen (T-Ag) in the plasmid pCMV-1/T (**Fig. 3B**). The construction of these expression plasmids has been described ***(3,4)***.

1. Plasmid pTKTHBV2: A gift of Dr. M. Meyer, Munich Germany; contains the complete HBV genome (subtype ayw).
2. Plasmid pEARLY: Provided by Dr. von Hoyninger-Huene, Institute for Virology, University of Würzburg, Germany; encodes the complete wild-type T-Ag sequence of SV40 ***(5)***. A *Bgl*II site replaces the *Hind*III site at position 5147 of the SV40 genome.
3. pCI vector, Catalogue No. E1731, is available from promega (Mannheim, Germany).
4. 3T3 fibrolasts, CCL-92, are available from ATCC.
5. Dulbecco's modified Eagle medium (DMEM), Catalogue No. Q41-01885, is available from Gibco-BRL (Eggenstein, Germany).
6. HBS buffer: 40 m*M* HEPES, 280 m*M* NaCl, 1.5 m*M* $Na_2HPO_4 \times 2H_2O$, pH 7.1.

2.2. Expression of Viral Antigens from Constructs Used for DNA Vaccination

1. [^{35}S]-methionine, Catalogue No. SJ1015, is available from Amersham (Braunschweig, Germany).
2. Methionine-free RPMI-1640 medium, Catalogue No. F1243, is available from Seromed (Berlin, Germany).
3. Polyclonal rabbit anti-HBsAg antiserum: may be available as a gift of the Behring AG (Marburg, Germany).
4. Monoclonal anti-T-Ag antibody PAb108, directed against the N-terminal extremity of the protein: a gift of Dr. W. Deppert, Hamburg, Germany.
5. Protein-A Sepharose, Catalogue No. 17-0780-01, is available from Pharmacia (Freiburg, Germany).
6. Fetal calf serum (FCS), amino acid-free: dialyze FCS against a large volume of Ca^{++}/Mg^{++}-free PBS for 2 d.
7. Lysis buffer: 120 m*M* NaCl, 1% (w/v) aprotinin (Trasylol, Catalogue No. 48764; Bayer, Leverkusen, Germany), 50 µ*M* leupeptin, 0.5% (v/v) Nonidet P-40 (NP-40), 10% (v/v) glycerol, 50 mM Tris/HCl, pH 8.0.
8. Wash buffer: 0.5 *M* LiCl, 1% (v/v) NP-40 and 0.1 *M* Tris/HCl, pH 9.0.
9. Elution buffer: 1.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol (2-ME) and 7 m*M* Tris/HCl, pH 6.8.

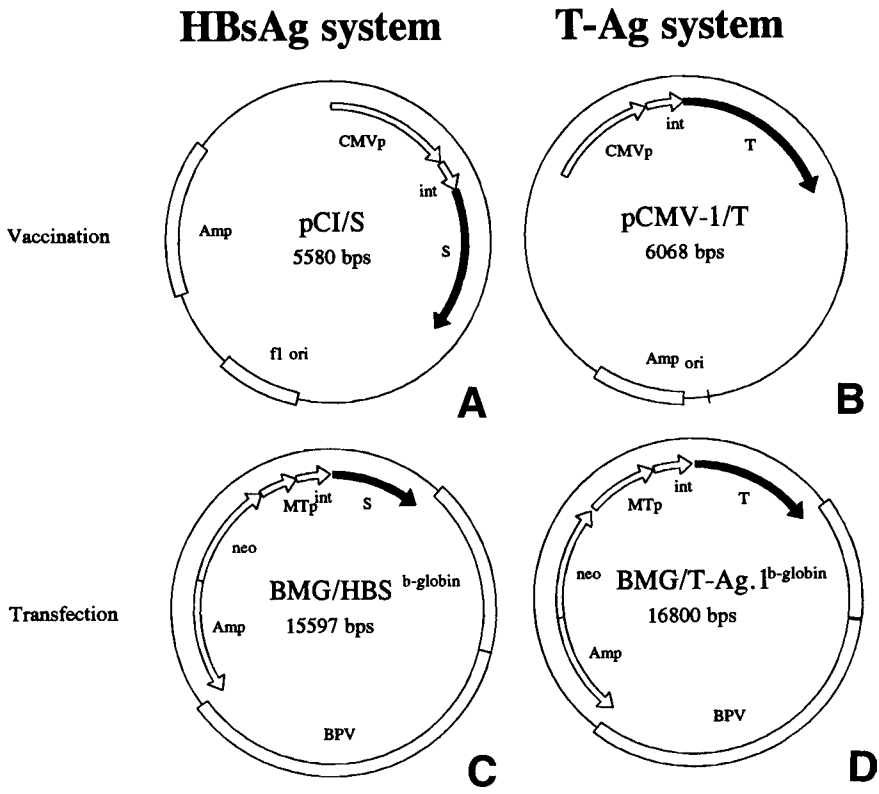

Fig. 3. Maps of the plasmids described.

2.3. Preparation of Plasmid DNA Used for Immunization

1. Ultrapure 100 anion exchange chromatography columns are available from Qiagen, (Hilden, Germany).
2. Endo-free buffer system is available from Qiagen.

2.4. Tumor Cell Lines

1. The B16 melanoma cell line originated in an H-2^b C57BL/6 mouse, the B16 sublines B16.F0 and B16.F1 lines are available from the ATCC as Catalogue Nos. CRL-6322 and CRL-6323. In most experiments we used the B16.F10 line, which we obtained from Dr. P. Antonsson, Lund, Sweden. The mastocytoma cell line P815 (TIB64) originated in an H-2^d DBA/2 mouse.
2. Bovine papilloma virus-based vector BMG*neo*: a gift from Drs. Y. Karasuyama and F. Melchers (Basel, Switzerland). This vector was used to construct the BMG/HBS expression plasmid and the BMG/T-Ag.1 expression plasmid (**Fig. 3C,D**).

3. DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate): liposomal transfection reagent, Catalogue No. 1202375, is available from Boehringer-Mannheim (Mannheim, Germany).
4. RPMI-1640/Clicks medium, Catalogue No. 900053, is available from Serva, Boehringer-Ingelheim.
5. G418 sulfate (Geneticin), Catalogue No. 11811-064, is available from Gibco-BRL. A stock solution of 125 mg/mL in PBS was stored at –20°C.

2.5. Nucleic Acid Immunization

1. Cardiotoxin, Catalogue No. L8102, is available from Latoxan (Rosans, France). Prepare a 0.01 m*M* stock solution in PBS.
2. Metofane is available from Janssen GmbH (Neuss, Germany).

2.6. Preparation of Tumor Cells for Transplantation

UltraCulture serum-free culture medium, Catalogue No. 12-725F, is available from Boehringer-Ingelheim.

2.7. Intratumor Injections of Recombinant DNA

Syringe needle, 0.4 × 19 mm 28G: Microlance 3, Catalogue No. E0896F07, is available from Becton-Dickinson (Heidelberg, Germany).

2.8. Cytotoxic T Cell Assay

MEM tissue culture medium, Catalogue No. 22561-021, is available from Gibco-BRL .

3. Methods

3.1. Expression Vectors Used for DNA-based Vaccination

3.1.1. Expression Plasmid pCI/S

1. Prepare the *Xho*I/*Bgl*II fragment (containing the small HBsAg coding region and the HBV 3' non-coding region and polyA signal) from pTKTHBV2.
2. Ligate the *Xho*I/*Bgl*II fragment into the pCI vector cut with *Xho*I/*Bam*HI to generate the plasmid pCI/S.

3.1.2. Expression Plasmid pCMV-1/T

1. Digest pEARLY with *Bgl*II and *Bam*HI.
2. Cut pCMV-1 ***(6)*** with *Bam*HI and treat it with shrimp alkaline phosphatase.
3. Clone the T-Ag encoding *Bgl*II/*Bam*HI fragment into the *Bam*HI-digested pCMV-1 plasmid to generate the plasmid pCMV-1/T.

3.1.3. Transient Transfection of Recombinant DNA

1. Grow 3T3 fibroblast cells to a density of 10^4–10^5 cells/mL in 100 mm tissue culture dishes in DMEM supplemented with 10% (v/v) FCS.
2. Introduce plasmid DNA (10 µg DNA/dish) into the cells by the $CaPO_4$-method.

3. Prepare Ca/DNA solution as follows: (i) pipet 62 µL of a 2 *M* $CaCl_2$ solution into a polystyrene vial; (ii) add 10 µg DNA; (iii) bring the final volume to 500 µL with H_2O; (iv) vortex the mixture gently and pipet it slowly into 500 µL 2× HBS buffer, which is being gently vortexed; (v) incubate the mixture for 90 s at room temperature.
4. Add the suspension (1 mL) to the cells in 5 mL of culture medium and incubate it for 16 h at 37°C.
5. Change the culture medium 16 h after the transfection and culture the cells for a further 48 h.
6. Test the cells for antigen expression.

3.2. Expression of Viral Antigens from the Constructs to be Used for DNA Vaccination

Expression of the viral proteins (HBsAg or T-Ag) is tested in metabolically labeled cells.

1. Label the transfected cells with 400 µCi [^{35}S]-methionine for 12–18 h at 37ºC in methionine-free RPMI-1640 medium supplemented with glutamine and 10% (v/v) amino acid-free FCS.
2. Wash the labeled cells twice in Ca^{2+}/Mg^{2+}-free PBS.
3. Lyse the cells with 1 mL lysis buffer for 30 min at 4°C.
4. Remove cell debris by centrifugation (30 min, 20.000*g*, 4°C).
5. Immunoprecipitate HBsAg by adding 5 µg of polyclonal rabbit anti-HBsAg antiserum.
6. Immunoprecipitate T-Ag protein using 5 µg of the monoclonal anti-T-Ag antibody PAb108.
7. Incubate the lysate/antibody mixtures for 2–4 h at 4ºC.
8. Dissolve 50 µL protein-A Sepharose in PBS, add it to the lysates, and incubate the mixture for 1 h at 4°C with gentle shaking.
9. Wash the immunoprecipitates four times in 1 mL wash buffer, two times in 1× PBS, and once in 0.1× PBS.
10. Resuspend the protein-A Sepharose pellets by extensive vortexing.
11. Recover the immunoprecipitates from the protein-A Sepharose by a 30-min incubation at 37°C in 400 µL elution buffer.
12. Lyophilize the SDS-denatured eluates and redissolve them in 30 µL aqueous solution of 7% (v/v) 2-ME, 10% (v/v) glycerol and bromophenol blue.
13. Boil the solution for 2 min.
14. Analyze 5-10 µL samples by SDS-PAGE (using the Laemmli buffer system).
15. Visualize the bands of labeled protein on X-ray film.

3.3. Preparation of the Plasmid DNA to be Used for Immunization

1. Transform plasmid DNA into *E. coli* DH5 cells and plate the cells under antibiotic selection *(7)*.
2. Select single colonies of transformants and grow up in 5 L fermentation cultures in modified LB medium at 37ºC overnight and pH 7.5, with pH control and maximum aeration (Schleef, M., unpublished data).

3. Harvest the cells and use 60 g of the wet weight biomass for alkaline lysis.
4. Isolate plasmid DNA using ultrapure 100 anion exchange chromatography columns. About 100 mg plasmid DNA can be obtained from a 5-L culture of transformants.
5. Remove endotoxin contamination using the Endo-Free buffer system.
6. Subject the DNA to quality controls to ascertain that it meets the appropriate quality criteria *(7)*. The preparations should contain <100 endotoxin units per 1 mg DNA, >90% (w/w) supercoiled plasmid DNA, and <1% (w/w) residual protein content. Measure the concentration and the UV absorbance spectrum by spectrophotometric analysis between 220 nm and 320 nm. These quality controls are described by Schorr et al. *(7)*.
7. Suspend the plasmid DNA at 10 µg/µL in 10× TE buffer (100 m*M* Tris-HCl, 10 m*M* EDTA, pH 7.4) and store it at –20ºC.
8. Dilute the DNA solution 1:10 with Ca^{2+}/Mg^{2+} -free PBS to obtain a 1 µg/µL DNA solution within the 30 min before injection of the plasmid DNA into mice.

3.4. Tumor Cell Transfectants

1. Construct the T Ag-expressing vector BMG/T-Ag.1 from the vector BMG*neo* *(8–10)* and the plasmid pEARLY.
2. Digest pEARLY with *Bgl*II and *Bam*HI.
3. Partially fill in the T Ag-encoding *Bgl*II/*Bam*HI fragment with A and G and fill in the *Xho*I-linearized vector BMG*neo* with C and T using Klenow DNA polymerase.
4. Ligate the compatible 5' ends to yield the T-Ag-expression vector BMG/T-Ag.1.
5. Construct the HBsAg-expressing vector BMG/HBS from the vector BMG*neo* and the plasmid TKTHBV2.
6. Digest BMG*neo* with the restriction enzymes XhoI and BamHI to delete the polyA signal of rabbit globin.
7. Ligate the *Xho*I/*Bgl*II-fragment of HBV with the *Xho*I/*Bam*HI digested BMGneo vector to yield the HBsAg-expression vector BMG/HBS.
8. Transfect BMG/HBS and BMG/T-Ag.1 vector DNA, or BMGneo vector DNA (control without insert), into B16.F10 or P815 cells using DOTAP following the manufacturer's instructions.
9. Culture the transfected cells in RPMI-1640/Clicks medium supplemented with penicillin and streptomycin, 5×10^{-5} *M* 2-ME, 5% (v/v) FCS and 2 m*M* glutamine.
10. Start selection 48 h after the transfection by adding 125 µg/mL G418.
11. Change the medium every 2–3 d. Slowly increase the G418 concentration to 1 mg/mL during a 4–6 wk selection period.
12. Test G418-resistant clones for stable expression of HBsAg or T-Ag as described in **Subheading 3.2.**

3.5. Nucleic Acid Immunization

1. Anesthetize mice with Metofane.
2. Shave their hind legs.
3. Inject 100 µL of the 0.01 m*M* cardiotoxin solution into each tibialis anterior muscle.

4. Inject 50 µL of 1 µg DNA/µL into each regenerating tibialis anterior muscle at 5 d after the cardiotoxin injection.
5. Use non-injected mice or mice injected with a plasmid DNA without insert as negative controls.

3.6. Preparation of Tumor Cells for Transplantation

To avoid "false" immunogenicity of in vitro cultured tumor cells presenting heterologous serum components from tissue culture medium, we either adapted transfected or non-transfected tumor cells to in vivo growth in mice, or grew tumor cells in serum-free medium.

3.6.1. Adaptation of Tumor Cell Lines to Growth in Mice

1. Inject P815 cells (non-transfected or transfected lines) intraperitoneally into immunodeficient C.B-17 *scid/scid* (SCID) mice using 10^5–10^6 cells per mouse.
2. Recover the cells 6 d later by rinsing the peritoneal cavity with 10 mL PBS.
3. Wash the cell suspensions three times in serum-free UltraCulture medium.
4. Use these cells directly for transplantation experiments.

3.6.2. Adaptation of Tumor Cells to Growth in Serum-Free Medium

B16 melanoma cells could not be recovered from the peritoneal cavity of SCID mice after injection because these mice efficiently reject allogeneic cells. These cells were adapted to growth in serum-free medium.

1. Transfer B16 cells and transfected sublines of this tumor cell line into serum-free UltraCulture medium.
2. Grow the cells for 2-3 wk prior to testing their in vivo immunogenicity.

3.7. Subcutaneous Transplantation of Tumor Cells into Mice

1. Subcutaneously inject titrated numbers of cells (10^2 to 10^6) in 50 µL PBS into the shaved left lateral flank of mice; use 5 or 10 animals per group.
2. Measure tumor growth every second or third day.
3. Kill mice bearing tumors with a diameter >1 cm.

3.8. Intratumor Injections of Recombinant DNA

1. Select mice bearing subcutaneous tumors with a diameter of 0.3–0.5 mm for treatment.
2. Add 25 µL DOTAP to 20 µg DNA dissolved in 25 µL PBS to generate DNA/DOTAP liposomes.
3. Use a 0.4 × 19 mm 28G needle to inject 50 µL PBS/DOTAP (control group) or 50 µL DNA/DOTAP (experimental group) into the tumors every second day.
4. Follow tumor growth until the tumor diameter reaches 1 cm or the tumor has regressed to a macroscopically undetectable size.

3.9. Cytotoxic T Cell Assay

1. Obtain spleens or lymph nodes from the DNA vaccinated mice.
2. Prepare single spleen cell or lymph node cell suspensions.
3. Suspend cells in MEM tissue culture medium supplemented with 10 m*M* Hepes buffer, 5×10^{-5} *M* 2-ME, antibiotics and 10% (v/v) of a selected batch of FCS and 2 m*M* glutamine.
4. Coculture responder or primed effector cells (3×10^7) with irradiated (20,000 rad) stimulator cells (1.5×10^6 cells) expressing the antigen of interest. Use 10 mL aliquots in upright 25 cm^2 tissue culture flasks in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C.
5. Harvest the in vivo-primed and in vitro-restimulated effector cell populations after 5 d of culture and wash twice.
6. Coculture serial dilutions of the effector cells with 2×10^3 of ^{51}Cr-labeled targets in 200 µL round-bottom wells for 4 h at 37°C. Use effector/target ratios in the range of 1–20.
7. Collect 50 µL of supernatant for γ-radiation counting following the 4-h assay period.
8. Calculate the percentage of specific release as [(experimental release-spontaneous release)/(total release-spontaneous release)] × 100. Measure the total radioactivity counts by resuspending the target cells; measure the spontaneously released counts using target cell cultures without cytolytic effector cell populations.
9. Plot the data as the mean of triplicate cultures. The standard deviation of triplicate data is usually less than 10% of the mean.

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Development of Female Contraceptive Vaccine Through DNA Inoculation of Human Chorionic Gonadotropin Beta Subunit (hCGß)

Fu-chun Zhang, Ni Wang, Dong-mei Liu, Youli Jian, You-zhen Chen, Xiao-zhou Shen, Yong-qing Cao, and Bin Wang

1. Introduction

Human chorionic gonadotropin (hCG) has been considered as a primary target molecule for a contraceptive vaccine by the World Health Organization because of its physiological and temporal specificity. hCG is an essential factor for the successful implantation and establishment of early pregnancy. For a decade, the most advanced works in development of a contraceptive vaccine have been concentrated on polypeptide based vaccines targeted directly against hCG. Although many studies have reached the stage of clinical testing using a number of prototype vaccines based on different parts of hCG molecule, their efficacy has been disappointing ***(1–4)***.

Nucleic acid vaccination is a novel technique that has many advantages over other methodologies. One of the advantages is to generate humoral responses as well as cell-mediated immune responses ***(5–8)***. Our laboratory has considered using this technology for development of a potent contraceptive vaccine. There are several reasons that hCG could be the good target for the DNA inoculation approach: (i) hCG is produced by the fertilized egg and is an essential factor for the implantation in the uterus, (ii) DNA immunization can generate specific antibody that could block the hCG function, and (iii) DNA immunization can generate specific cell mediated immunities that could attack the fertilized egg or expel the implanted egg.

Furthermore, hCG is a member of glycoprotein hormone family including lutenizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid stimulating hormone (TSH). They are genetically, biochemically, and immu-

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

nologically closely related. All four hormones are heterodimeric proteins that share the same alpha subunit but differ in their hormone-specific beta subunit, thus, the unique specificity to hCG can be determined by the hCG beta subunit (hCGß). hCGß vaccines have been shown to have a profound anti-implantation effect in clinical trials ***(2)***. In the methods described here, the specific hCGß subunit gene is selected as the target and cloned into the plasmid vector-pCMV4 to construct a prototype DNA vaccine ***(9)***. This prototype hCG vaccine can be examined in the animal system described and might be developed into a new and potent contraceptive vaccine.

2. Materials

2.1. Construction of pCMV-hCGß Plasmid DNA

1. Vector: pCMV4 ***(10)***.
2. Target gene: hCGß (pUC-hCGß).
3. Enzymes: *Hind*III, *Sma*I and T4 DNA ligase are available from Promega (Madison, WI).
4. 1.5% (w/v) low melting temperature (LMT) agarose gel.
5. TBE electrophoresis buffer, 10X stock solution: combine 108 g Tris (available from Bio-Rad, Hercules, CA), 55 g boric acid, 40 mL of 0.5*M* ethylenediaminetetraacetic acide (EDTA), pH 8.0, distilled water to 1000 mL.

2.2. Isolation and Purification of Plasmid

1. Host bacteria: *E. coli* DH5α.
2. LB-broth: combine 10 g Bacto-tryptone (Difco, Detroit, MI), 5 g Bacto-yeast extract (Difco), 10 g NaCl in 1000 mL distilled water; autoclave.
3. LB-broth/Agarose: 1.4 g agarose in 100 mL LB-broth medium.
4. Clean buffer 3.0: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 0.5% (v/v) Triton X-100, 3 *M* NaCl (Sino-American Co., Beijing, China).
5. TE buffer: 10 m*M* Tris-HCl, pH 7.5, 1 mM EDTA.
6. Lysozyme (Sino-American Co., Beijing): 10 mg/mL, freshly prepared in distilled water.
7. RNase (Promega): 10 mg/mL, prepared in distilled water, boiled for 15 min and stored at –20°C.

2.3. Transfection of pCMV-hCGß Using Lipofectamine

1. Lipofectamine reagent: 1 mL at 2 mg/mL (Gibco-BRL, Grand Island, NY).
2. DMEM culture medium (serum-free): combine 10.0 g DMEM (Gibco), 1 mL penicillin (100,000 U/mL; Northern China pharmaceutical factory, Shigiazhuang), 1 mL Streptomycin (100,000 µg/mL; Northern China pharmaceutical factory), 0.843 g HEPES (Sigma, St. Louis, MO), 1.2 g $NaHCO_3$, adjust the final volume to 1000 mL with distilled water and sterilize the medium by filtration using a 0.22-µm filter.
3. Cell line: maintain Hela cells in DMEM medium with 10% (v/v) fetal calf serum (FCS; available from Tian Jian Biochemical Products Factory, Beijing).

2.4. Analysis of Transient Expression of hCGß

1. 96-well cell culture plates are available from Nunc (Roskilde, Denmark).
2. DMEM + 20% (v/v) FCS: combine 20 mL FCS and 80 mL DMEM culture medium.
3. DMEM + 10% (v/v) FCS: combine 10 mL FCS and 90 mL DMEM culture medium.

2.5. DNA Immunization

1. Animals: pathogen-free BALB/C female mice, aged 8–10 wk, bodyweight about 20 g, are available from the Center of Experimental Animal, Institute of Genetics, the Chinese Academy of Sciences (CAS), Beijing. House the mice in a temperature controlled light-cycled room.
2. Adjuvant: 0.5% (w/v) bupivacaine-HCl (Injection USP) is available from Astra Pharmaceutical Products Inc. (Westborough, MA).
3. Vaccines: pCMV-hCGß at 1 mg/mL; pCMV4; hCG-TT protein vaccine is available from Prof. Liu Xue-Gao (Jinnan University, Shantou, China).
4. Saline solution: dissolve 0.9 g NaCl in 100 mL distilled water and autoclave the solution.

2.6. Antigen and Antibody Detection by ELISA

1. Mouse anti-hCGß monoclonal antibody is available from Sino-American.
2. Anti-hCGß rabbit serum and purified hCGb is available from the Laboratory of Endocrinology, Institute of Zoology, CAS.
3. Bicarbonate buffer capsules are available from Sigma (St. Louis, MO).
4. 0.01 *M* PBS (pH 7.4): 2.90 g $NaHPO_4 \cdot 12H_2O$, 0.30 g $NaH_2PO_4 \cdot 2H_2O$, 8.5 g NaCl, distilled water 1000 mL.
5. PBST (pH 7.4): add 0.5 mL Tween-20 (Merck) to 1000 mL of PBS.
6. Horseradish peroxidase-conjugated goat anti-mouse IgG: this is available from Boehringer-Mannheim (Indianapolis, IN).
7. Phosphate-citrate buffer with sodium perborate: capsules are available from Sigma.
8. TMB: 3,3',5,5'-tetramethylbenzidine dihydrochloride tablets, 10 mg, are available from Sigma.
9. 2 *M* H_2SO_4: slowly add 11 mL 98% (v/v) H_2SO_4 to 89 mL distilled water.
10. Microtiter plates are available from Corning, NY.
11. Plate reader: a suitable instrument is available from Bio-Rad (Hercules, CA).

2.7. Antigen-Specific Lymphocyte Proliferative Responses

1. RPMI-1640 culture medium: 10.4 g RPMI-1640 (Gibco-BRL), 5.95 g HEPES (Sigma), 3.4 mL 2-mercaptoethanol (Bio-Rad), 1 mL penicillin (100,000 U/mL; Northern China pharmaceutical factory), 1 mL Streptomycin (100,000 µg/mL; Northern China pharmaceutical factory), 2 g $NaHCO_3$, dissolved in distilled water to final volume of 1000 mL, sterilized with a 0.22-µm filter.
2. [^{3}H]-thymidine: 28 Ci/mmol, available from Amersham Ltd. (Piscataway, NJ).
3. Beckman LS Analyzer, Beckman Instruments (Fullerton, CA).

2.8. Analysis of Th1 and Th2 Immune Responses

2.8.1. Analysis of IgG1 and IgG2a

1. Anti-hCGß anti serum and hCGß are available from the Laboratory of Endocrinology, Institute of Zoology, CAS.
2. Bicarbonate buffer capsules (0.05 *M*) and phosphate-citrate buffer with sodium perborate capsules (0.05 *M*) are available from Sigma.
3. Corning microtiter plates.
4. Goat anti-mouse IgG1 and goat anti-mouse IgG2a (both horseradish peroxidase-labeled) are available from Southern Biotechnology Associates, Inc. (Birmingham, AL).
5. Mouse IgG1 and mouse IgG2a are available from Sigma.

2.8.2. Analysis of Cytokine Expression by RT-PCR

1. RNAgents® Total RNA Isolation System, AMV reverse transcriptase and dNTPs are available from Promega.
2. Bio-Rad Thermal Cycler.
3. 10 × PCR buffer: 15 m*M* $MgCl_2$, 100 m*M* Tris-HCl (pH 9.0), 500 m*M* KCl, 1% (v/v) Triton X-100.
4. Plasmid pPQRS ***(11)***.

2.9. Antifertility Trial

1. Animals: 8–10 wk old male and female Kunming mice are available from the Center of Experiment Animal, Institute of Zoology, CAS, Beijing.
2. Superovulation reagents: 5 IU hCG and 5 IU PMSG are available from the Laboratory of Endocrinology, Institute of Zoology, CAS.

3. Methods

3.1. The Construction and Transformation of pCMV-hCGß Plasmid DNA

3.1.1. The Cloning Strategy of pCMV-hCGß Plasmid DNA

1. Digest pCMV4 with *Hind*III.
2. Digest the complete hCGß gene fragment from pUC-hCGß with *Hind*III.
3. Isolate the resulting hCGß fragment and purify it on a 1.5% (w/v) LMT agarose gel.
4. Ligate the hCGb fragment and the *Hind*III-digested pCMV4 with T4 DNA ligase to yield pCMV-hCGß **(Fig. 1)**.

3.1.2. Transformation

1. Aliquot 100 µL of CaCl2-treated competent cells into a 1.5 mL pre-chilled sterile tube.
2. Add 5 µL of plasmid, swirl, and place the tube on ice for 25 min.
3. Heat-shock the cells by placing the tube tube in a 42°C water bath for 90 s, then place it on ice for 2 min.
4. Add 0.9 mL LB medium to each tube.

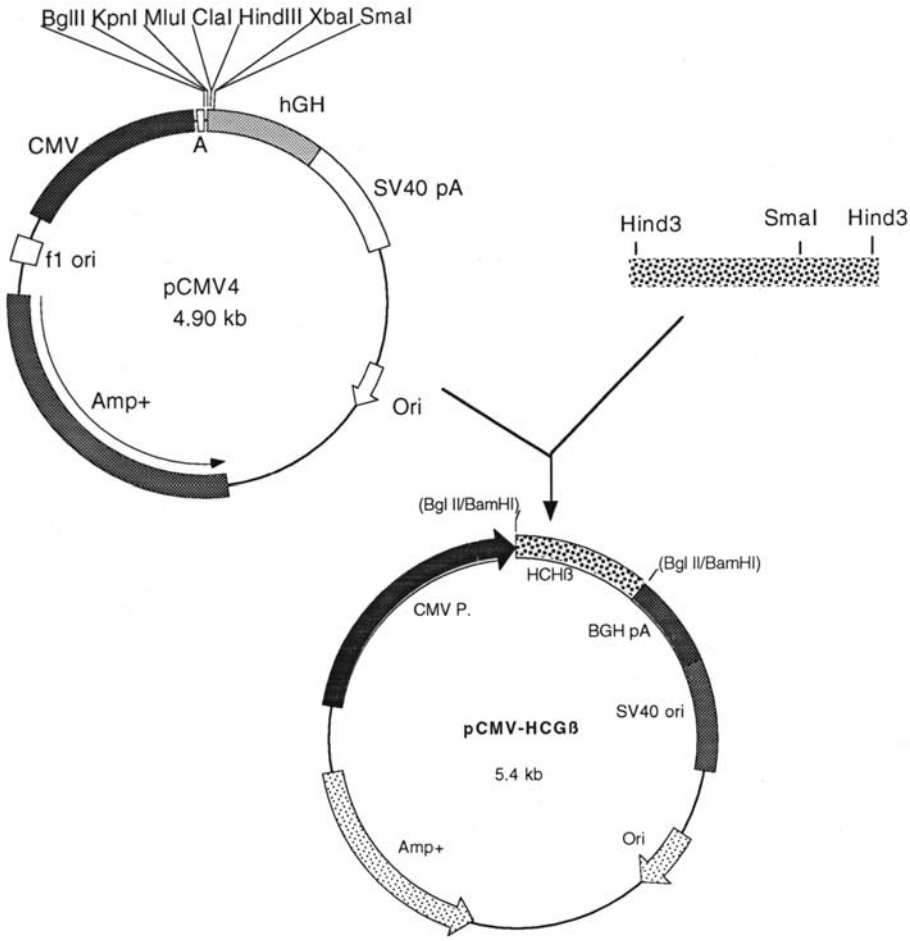

Fig. 1. The cloning strategy for HCGß subunit.

5. Incubate the tubes for1 h at 37°C on air-bath shaker (250 rpm).
6. Plate several dilutions on ampicillin-containing plates, and incubate 12–16 h at 37°C.

3.2. Isolation and Purification of Plasmid

3.2.1. Miniprep of Plasmid DNA by Alkaline Lysis

1. Transfer 1 mL of LB culture medium into each of two 1.5 mL Eppendorf tubes, microcentrifuge at top speed for 30 s, and discard the supernatant (*see* **ref. *12***).
2. Add 100 µL of prechilled solution I to each tube and vortex.
3. Add 200 µL of fresh solution II, mix, and place the tubes on ice for 5 min.
4. Add 150 of prechilled solution III, vortex the tubes for 2 sec., and place them on ice for 5 min.

5. Microcentrifuge at top speed for 5 min and transfer the supernatant to a new tube.
6. Add to the supernatant an equal volume of a 1:1 phenol-chloroform mixture, vortex, and microcentrifuge for 2 min. Transfer the supernatant into another tube.
7. Add 2 volumes of ethanol, vortex, and let the mixture sit for 2 min at room temperature, then place it on ice for 5 min.
8. Microcentrifuge at 12,000*g* for 10 min to precipitate plasmid DNA.
9. Wash the pellets twice with cold 70% (v/v) ethanol. Airdry the pellets for 15 min, redissolve the pellet in 50 µL of TE buffer and store the DNA solution at –20°C.
10. Digest 10 µL aliquots with appropriate restriction enzymes, and run the digested product on an agarose gel to analyze the recombinant plasmid.

3.2.2. Isolation and Large-Scale Plasmid Purification

1. Pick one colony from the plate or take 10 µL of frozen stock, inoculate 50 mL of LB and incubate it at 37°C overnight.
2. Centrifuge the overnight culture at 7,000 rpm for 5 min and discard the supernatant.
3. Resuspend the pellet with 10 mL of buffer 3.0.
4. Add 100 µL of lysozyme (freshly prepared at 10 mg/mL) and 20 µL of RNase (10 mg/mL).
5. Incubate the suspension at 50°C for 10 min or until bacterial lysis visible.
6. Centrifuge the cell lysate for 20 min at 15,000 rpm at 4°C.
7. Transfer the supernatant into a clean tube and mix it thoroughly with 0.6 vol of isopropanol.
8. Centrifuge the pellet for 10 min at 12,000*g* at 4°C and discard the supernatant.
9. Wash the pellet with 70% (v/v) ethanol.
10. Dry the pellet and add 1 mL of TE into the tube to dissolve the DNA.
11. Centrifuge to remove any undissolved material, transfer the supernatant into a new tube.
12. Determine the OD_{260}/OD_{280} ratio and perform gel analysis.

3.3. Transfection of pCMV-hCGß Using Lipofectamine™

1. Seed 10^5 Hela cells per well in 2 mL DMEM media plus 10% (v/v) FCS.
2. Incubate the cells at 37°C in a 5% (v/v) CO2 in air atmosphere until they reach 70% confluence.
3. Dilute 1–2 µg of the hCGß construct or the control vector DNA into 100 µL serum-free medium (solution A) and dilute 10 µL of lipofectamine reagent into a further 100 µL serum-free medium (solution B).
4. Combine the two solutions A and B, mix them gently, and incubate the mixture at room temperature for 30 min to allow DNA-liposome complexes to form.
5. Rinse the Hela cells once with 2 mL of serum-free medium.
6. For each transfection, add 0.8 mL of serum-free medium to the tube containing the complexes, mix gently and overlay the diluted solution onto the rinsed cells.
7. Incubate the cells with the complexes for 5 h at 37°C in a CO_2 incubator.
8. Following incubation, add 1 mL growth medium containing 20% (v/v) FCS without removing the transfection mixture.

3.4. Analysis of Transient Expression of hCGß Subunit

3.4.1. Sample Collection

1. Collect the culture medium from the transfected cells after 24 h.
2. Replace the DMEM medium with fresh medium containing 10% (v/v) FCS.
3. Continue to incubate the transfected cells for another 48 h, then collect the culture medium and cells.
4. Analyze the collected media and cell lysates for expression of the hCGß subunit gene (*see* **Note 1**).

3.4.2. ELISA for the Detection of hCGß Protein In Vitro.

1. Coat 96-well microtiter plates at 4°C overnight with 100 µL per well of a monoclonal antibody against native hCGß at a 1:500 dilution in 0.05*M* bicarbonate buffer (pH 9.6).
2. Block each well with 3% (w/v) BSA-PBST at 37°C for 1h.
3. Use twofold serial dilutions from 0.1 µg to 15 ng to establish a standard antigen curve.
4. Add 100 µL of culture medium from transfected cultures and the supernatant of cell lysates.
5. Incubate the plate for 1 h at 37°C.
6. Add rabbit anti-hCGß serum at a 1:500 dilution.
7. Incubate at 37°C for 1 h with a secondary goat anti-rabbit antiserum conjugated with horseradish peroxidase at 1:1000.
8. Dissolve a 10 mg TMB tablet in 0.025 *M* phosphate-citrate buffer and add the solution to each well for color development.
9. Stop the color development by adding 2*M* H_2SO_4.
10. Read the light absorption with a Bio-Rad plate reader at 450 and 655 nm.

3.5. DNA Immunization

1. Inject BALB/C mice in at least three sites of the leg muscles with 50 µL of 0.5% (w/v) bupivacaine-HCl per site using a syringe equipped with a 27-gauge needle (*see* **Note 2**).
2. After 24 h, inject 50 µg or 100 µg of the pCMV-hCGß construct into the same regions as the bupivacaine-HCl injections.
3. Boost the immune response with the same amount of DNA construct at wk 3 and 5.
4. Use the same amount of the pCMV4 vector as a negative control.
5. Inject 5 µg hCGß-TT protein as a positive immunization control.
6. Collect sera from the postorbital vein after each immunization.
7. Analyze the sera by ELISA ***(13–15)***.

3.6. Antibody Detection by ELISA

1. Coat 96-well microtiter plates overnight at 4°C using 100 µL per well of 2 µg/mL of hCGß antigen in 0.05 *M* bicarbonate buffer (pH 9.6).

2. Add to each well 100 µL of two-fold serial dilutions of sera starting with 1:100 from immunized mice (*see* **Note 3**); incubate at 37°C for 1 h.
3. Add horseradish-peroxidase conjugated goat anti-mouse IgG at 1:10000 in PBST; incubate at 37°C for 1 h.
4. Dissolve a 10 mg TMB tablet in 0.025 *M* phosphate-citrate buffer and add the solution to each well for color development.
5. Stop the color development by adding 2 *M* H_2SO_4.
6. Read the plates using a Bio-Rad plate reader at 450 and 655 nm.

3.7. Analysis of Th1 and Th2 Immune Responses

3.7.1. Analysis of IgG1 and IgG2a

Detection of IgG1 and IgG2a is performed using an ELISA as described in Subheading 3.6. The only difference is the use of secondary antibodies specific for IgG1 or IgG2a isotypes instead of against whole IgG molecules.

3.7.2. Analysis of Cytokines Expression by RT-PCR **(10)**

1. Extract total RNA from spleens of immunized or control BALB/C mice using the Promega Total RNA Isolation System (*see* **ref. *10***).
2. Homogenize tissue in denaturing solution for 5 min.
3. Add 2 *M* pH 4.0 sodium acetate and phenol:chloroform:isoamyl alcohol and keep the mixture on ice for 15 min.
4. Transfer the mixture to a fresh tube and centrifuge it for 20 min at a speed of 10,000 rpm.
5. Remove the aqueous phrase and precipitate it with isopropanol for 5 min.
6. Wash the pelleted RNA with ethanol and centrifuge it for 30 min at 10,000 rpm.
7. Dry the pellet and dissolve in nuclease-free water for 15 min.
8. Add 4 µg of total RNA in 10 µL of diethyl pyrocarbonate (DEPC)-treated water to 2 µL of random-hexamer primers (0.5 mg/mL), and 0.5 µL RNasin (Promega); heat the mixture at 65°C for 5 min.
9. Cool the tube on ice and spin it briefly in a microfuge at 4°C to bring the condensation water to the bottom of the tube.
10. Add 5 µL of 1.25 mM dNTPs, 4 µL of 5X RT buffer and 1 µL AMV reverse transcriptase; incubate the 20.5 µL reaction at 37°C for 1.5 h.
11. Stop the reaction by heating the tube to 95°C for 5 min.
12. Bring the volume to 200 µL using TE buffer (pH 8.0).
13. Add 10 µL of the diluted first-strand cDNA to a reaction containing 10 µL of 10X PCR buffer, 2 µL each of 20 µ*M* sense and anti-sense primers, 1 µL of 20 m*M* dNTPs, 74.5 µL of distilled water and 0.5 µL of *Taq* polymerase.
14. Amplify for 35 cycles in a thermal cycler using 94°C for 40 s, 60°C for 20 s, and 72°C for 40 s per cycle, with a final extension of 72°C for 10 min.
15. Use the polycompetitor pPQRS and first-strand cDNA to detect the mRNA expression level of different cytokines in immunized mice, including IL-2, IL-4, IL-5, IL-10, IL-12, IFN-γ, TNF-α, TNF-ß, iNOS and HPRT.

Table 1
Effects of pCMV-hCGβ DNA Vaccine on Fertility in Mice

Group I	OD450	No. of Embryos	Group II	OD450	No. of Embryos
I-1	0.138 ± 0.005	28	II-1	0.253 ± 0.036	0
I-2	0.097 ± 0.011	27	II-2	0.175 ± 0.010	18
I-3	0.159 ± 0.003	32	II-3	0.120 ± 0.019	24*
I-4	0.137 ± 0.003	33	II-4	0.205 ± 0.021	24*
I-5	0.217 ± 0.016	0	II-5	0.223 ± 0.011	7
I-6	0.191 ± 0.010	6	II-6	0.155 ± 0.020	32*
I-7	0.143 ± 0.005	23	II-7	0.126 ± 0.024	25
I-8	0.100 ± 0.005	36	II-8	0.103 ± 0.012	18
I-9	0.141 ± 0.004	19	II-9	0.146 ± 0.003	33*
I-10	0.121 ± 0.006	33	II-10	0.242 ± 0.003	0

Females in the control group contain 30-40 embryos per uterus. Each experimental group contains 10 mice numbered I-1 to I-10 and II-1 to II-10. Asterisks indicate the presence of dead embryos. The OD450 values represent the levels of antibodies to hCGß in sera diluted 1:100; the background value in the control groups is 0.049 ± 0.003.

3.8. Antigen-Specific Lymphocyte Proliferative Responses

1. Inject five mice per group intramuscularly with 100 µg/mL of pCMV-hCGß construct.
2. Boost some groups of mice at wk 3 and wk 5.
3. Prepare single splenocyte suspensions from immunized and non-immunized (control) mice one week after the last injection.
4. Grow the cells in RPMI 1640 culture medium.
5. Dilute an antigen in RPMI-1640 to the following concentrations: 10, 1, 0.1, and 0.01 µg/mL.
6. Add 30 µL of protein antigen or 100 µL for cellular antigens per well to 96-well flat-bottom microplates.
7. Use three replicate wells and include control wells with medium only.
8. Dilute splenocytes to 10^6 cells/mL with RPMI-1640.
9. Place 100 µL into each well and incubate the plates at 37 °C in 5% (v/v) CO_2 for 56 h.
10. Add 1 µCi of [^{3}H]-thymidine to each well and incubate the plates for additional 16 h.
11. Harvest cells and count the incorporated radioactivity in a Beckman LS analyzer.

3.9. Anti-fertility Trials

To examine the efficacy of pCMV-hCGß DNA vaccine in mice, perform anti-fertility trials as follows, *see* the results in **Table 1**.

3.9.1. Group I

1. Immunize female mice once with pCMV-hCGß DNA vaccine.
2. On d 3, treat the immunized mice with 5 IU of PMSG in 200 µL i.p. and 5 IU of hCG in 200 µL i.p. for 54 h to induce superovulation.

3. Mate the treated animals with male partners.
4. Examine the female mice for vaginal plugs the next morning to determine if they are pregnant (*see* **Note 4**).
5. Collect antisera on d 9 after pregnancy and analyze for the presence of specific anti-hCG antibody.

3.9.2. Group II

1. Immunize female mice once with pCMV-hCGß DNA vaccine.
2. On d 17, treat the immunized mice with 5 IU of PMSG intraperitoneally and 5 IU of hCG intraperitoneally for 54 h to induce superovulation.
3. Mate the treated animals with male partners.
4. Examine the female mice for vaginal plugs the next morning to determine if they are pregnant (*see* **Note 4**).
5. Collect antisera on d 9 after pregnancy and analyze for the presence of specific anti-hCG antibody.

3.9.3. Group Control

Use a group of non-immunized female mice.

4. Notes

1. The concentration of hCGß protein is 6.78 ng/mL and 15.2 ng/mL at 24 and 48 h after transfection, respectively. There is no detectable hCGß in cell lysates. No hCGß can be detected in the groups of mice receiving the control vector or the anti-sense pCMV-hCGb construct.
2. Multiple injection sites in the leg muscle is highly recommended.
3. We have found that mouse serum at less than 1:100 dilution reacts with TMB and creates a high background. Thus, we suggest that a 1:100 dilution should be the starting point for ELISA.
4. Mice with no sign of vaginal plugs should be excluded from the group in anti-fertility trials.

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Genetic Immunization for Allergy Immunotherapy

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1. Introduction

1.1. Allergy, A Manifestation of an Enhanced Th2 Response to Allergens

Allergic disorders are characterized by the prevalence of immunoglobulin (Ig) isotype E antibodies, and are considered to result from enhanced T helper type-2 (Th2) responses to allergens. A Th2 response is characterized by enhanced humoral responses and the production of IL-4 and IL-5 by $CD4^+$ T cells (Th2) in response to antigen ***(1,2)***. These "Th2 cytokines" enhance the allergic response by inducing B cell isotype switching to IgE ***(3–5)***, by inducing undifferentiated Th0 cells to further differentiate into Th2 cells, and by inducing eosinophil growth, and differentiation ***(5)***. In addition, the Th2 cytokines inhibit Th0 differentiation into Th1 cells, thereby reducing the recruitment of interferon gamma (IFN-γ) producing Th1 cells that could then down-regulate or modulate the Th2 responses ***(3,5)***. Consequently, the ability of a Th2 response to allergens to exert a positive feedback effect leads to a vicious cycle and may explain the exacerbation of allergic responses that follows continued exposure to allergens in atopic humans ***(2,6)***.

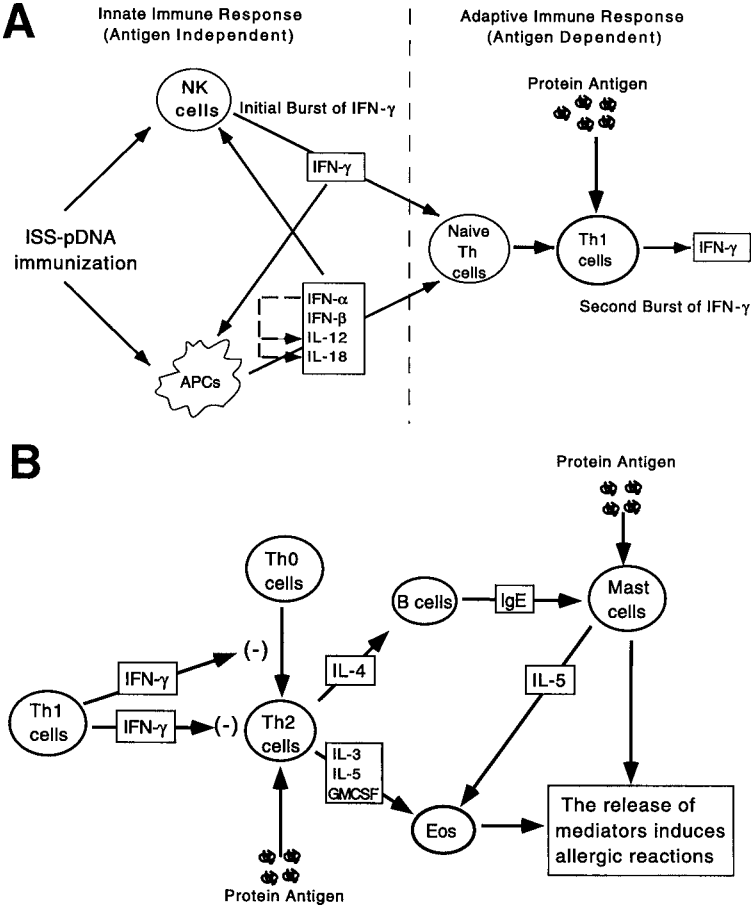
Since the beginning of the 20th century, allergic disorders have been treated by immunotherapy. This involves the subcutaneous injection of small, but gradually increasing amounts of allergen. The administration of low levels of allergen may result in induction of Th1 cells and high levels of IgG antibodies that block allergen binding to IgE ***(5)***. Another possible mechanism of action is that low levels of antigen induce tolerance (anergy) of the activated Th2 cells ***(5)***. Though partially successful, the injection of allergens involves the risk of anaphylaxis. Over the last 15 years the use of new pharmacological agents for

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

allergic disorders allowed the control of allergic symptoms at a lower risk to benefit ratio than was obtained with immunotherapy ***(7)***, which has led to a decline in the practice of immunotherapy.

The model above suggests that interventions that reduce or inhibit allergen-specific Th2 responses would be therapeutic in the treatment of allergies. As previously mentioned, immunotherapy most likely achieved this result by inducing partial anergy in the activated Th2 cells, or by blocking the allergens from binding to the IgE on the mast cells ***(5)***. A different approach for the treatment of allergies or asthma would be to induce antigen (allergen)-specific Th1 responses ***(8–13)*** that subsequently could inhibit the Th2 (allergic) cycle due to the inhibitory effects of IFN-γ ***(3,5)***. The induction of allergen-specific Th1 responses would enhance the differentiation of Th0 cells to Th1 cells, result in a B cell isotype switch to IgG2a (in the mouse), and the production of IFN-γ from Th1 cells upon exposure to allergen **(Fig. 1)**. The production of IFN-γ could then suppress Th2 and eosinophil cell recruitment, Th2 cytokine and IgE production, and subsequently mast cell degranulation. The induction of IgG2a antibodies could also act to neutralize allergen. Gene vaccination, either intradermal (i.d.), or intramuscular (i.m.) characteristically induces a Th1 type response. This antigen-specific Th1 response has been shown to result in both the prevention of IgE induction ***(8,9,13)***, the down-regulation of existing IgE antibody levels ***(8,10,13)***, and decreased eosinophil infiltration into a target organ (i.e. lung) ***(14,15)*** after allergen inhalation challenge. A proposed model for the induction of a Th1 immune response by pDNA immunization (Fig. 1) includes the induction of type 1 cytokines by immunostimulatory sequences (ISS) contained within the plasmid backbone ***(11,18,19)*** causing the Th1-based induction. In addition, immunostimulatory sequences can induce B-cell proliferation and isotype switching to IgM and IgG2a without IgE induction. The details of ISS effects are reviewed elsewhere in this book.

Fig. 1. *(facing page)* **(A)** Th1 induction by immunostimulatory sequence-containing plasmid DNA (ISS-pDNA) immunization. This is the proposed mechanism by which pDNA immunization induces a Th1-biased immune response. Note that in this system Th1-biased immunization may be achieved by vaccination with ISS-containing pDNA that encodes an antigen (allergen), or by coinjection of a protein antigen with ISS-pDNA or ISS-phosphorothioate oligo DNA. In this model, antigen-presenting cells (APCs) and natural killer (NK) cells produce type 1 cytokines in response to ISS as part of an innate immune response, which leads to the differentiation of Th0 cells to Th1 cells. APCs process and present antigen from gene expression of the pDNA or from phagocytosis of a protein antigen, resulting in the generation of antigen-specific Th1 cells. The subsequent exposure or challenge of these Th1 calls with protein antigen (allergen) results in the production and secretion of interferon gamma (IFN-γ), which is inhibitory to the



Th2 processes that mediate allergic reactions. Not shown, but relevant, is the ISS-stimulated proliferation of B cells with a concomitant antibody isotype switch to the IgG2a subtype. Increased levels of IgG2a antibodies help to block allergen contact with IgE on mast cells, thereby reducing allergic responses to allergen exposure. **(B)** The inhibitory effect of IFN-γ on Th2 cell recruitment and type 2 cytokine secretion, part of a positive feedback cycle in allergic reactions. IFN-γ produced as part of the innate immune response by NK cells and by antigen-specific Th1 cells stimulated with antigen acts to inhibit the differentiation of Th0 cells to Th2 cells, thereby inhibiting the recruitment of new antigen-specific Th2 cells. In addition, IFN-γ inhibits the secretion of type 2 cytokines, thereby inhibiting eosinophil growth and differentiation, as well as inhibiting the production of IgE from B cells. The inhibition of IgE and eosinophils ultimately results in reduced levels of inflammatory secretion (IL-4, IL-5, histamine, tryptase, leukotrienes, major basic protein, eosinophil cationic protein, and other mediators of inflammation) by mast cells and eosinophils on exposure to allergen. Taken together, induction of Th1 immune responses help to break the cycle of allergic reactions.

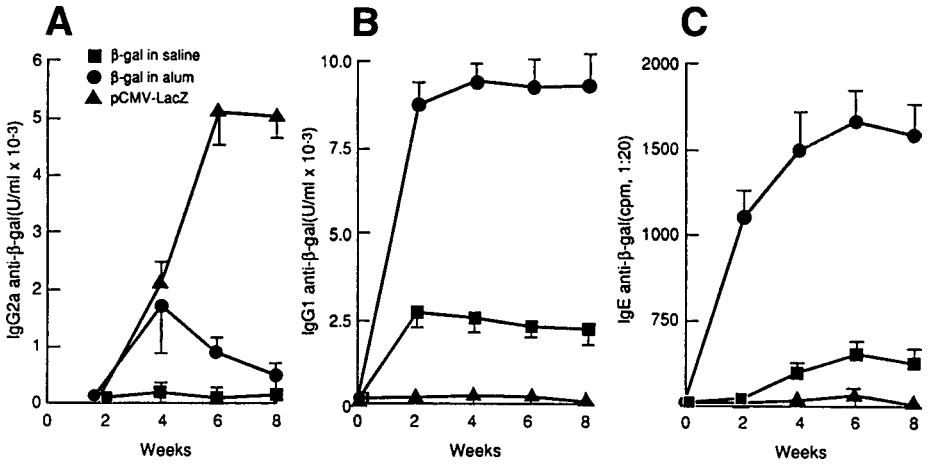


Fig. 2. Primary IgG subclass and IgE immune response of BALB/c mice after intradermal injection of 100 μg *LacZ*-pDNA (pCMV-*LacZ*) (▲), 10 μg ß-gal in saline (■) or 1 μg ß-gal in alum (●).

1.2. Gene Immunization: The Primary Immune Responses to Gene or Protein Immunizations

The immunization of mice (i.d. or i.m.) with plasmid (p) DNA encoding an antigen results in the induction of a Th1 immune response. This Th1 phenotype response is generally characterized by the induction of an IgG2a antibody response and IFN-γ production from $CD4^+$ splenocytes ***(8,16,17)***, whereas protein in alum (aluminum hydroxide) immunization mainly induces IgG1 and IgE antibody responses and IL-4 and IL-5 production from $CD4^+$ splenocytes ***(8,18)***. The different Ig isotypes induced by *E. coli* ß-galactosidase (ß-gal) plus alum immunization or gene immunization with pDNA expressing *LacZ* (*LacZ*-pDNA, plasmid-encoding β-gal) are illustrated in **Fig. 2**. The cytokine responses of splenocytes stimulation in vitro from these mice are shown in **Table 1**.

1.3. The Th1 Response Induced by Gene Vaccination Is Dominant over the Th2 Response Induced by Protein Vaccination

As mentioned above, protein in alum induces a Th2-type immune response. However, if mice were first immunized with pDNA encoding ß-gal, ß-gal/alum boosting resulted in an increase of the pre-existing IgG2a antibody response, an increase in the IFN-γ production in vitro by antigen stimulated splenocytes, and prevented the induction of antigen-specific IgE antibodies ***(8,9)***. When *LacZ*-pDNA immunized mice were boosted with an unrelated antigen such as hen egg ovalbumin (OVA) in alum, IgE antibodies to OVA were induced, thus demonstrating that the IgE antibody inhibition by pDNA-immunization is antigen-spe-

Table 1
Lymphokine Secretion by ß-Gal-Activated CD4+ Splenic T Cells from pDNA and/or Protein Immunized Mice

Exp.	Priming	Boosting	IFNγ pg/mL	IL-4 pg/mL	IL-5 Units/mL
A	None	None	<10	<2	<2
B	*LacZ*-pDNA	None	741±170	<2	<2
C	ß-Gal/saline	None	<10	12±2	15±4
D	ß-Gal/alum	None	<10	351±24	642±51
E	*LacZ*-pDNA	ß-Gal/alum	1050±314	4±2	46±10
F	ß-Gal/saline	ß-Gal/alum	53±48	126±43	165±54
G	ß-Gal/alum	*LacZ*-pDNA	730±193	307±128	183±70

Lymphokine secretion by ß-gal-activated CD4+ splenic T cells from pDNA and/or protein-immunized mice. The *LacZ*-pDNA mice were injected with 100 µg pDNA (AmpR) encoding ß-gal. The ß-gal/alum mice received 1 µg of ß-gal protein in 3 mg alum. The ß-gal in saline mice were injected with 10 µg ß-gal in normal saline. Cytokines were measured by ELISA using kits or reagents from Biosource (Camarillo, CA). Data represent mean ± SE of spleens of four mice per group.

cific. In contrast, when mice primed with ß-gal protein were boosted with ß-gal in alum or saline, the levels of both ß-gal-specific IgG1 and IgE increased.

Therefore, the Th1 immune response, which is initiated by gene vaccination, is boosted by a vaccination regimen that would induce a Th2 response in naive mice or protein-primed mice. The Th1 response to gene vaccination also prevents the subsequent induction of antigen-specific IgE antibodies ***(8,9)*** **(Fig. 3)**. These data demonstrate the potential of prophylactic immunization for the prevention of an allergic IgE response in susceptible individuals. This could be useful because the development of allergic disorders has a strong genetic component. Healthy individuals from families with a history of allergies and/or asthma could potentially be immunized with gene vaccines in order to initiate a protective Th1 response to allergens before the allergen could induce a Th2 response.

1.4. Down-Regulation of IgE Antibodies by pDNA Immunization

In order to investigate the potential of pDNA immunization for the treatment of an existing IgE response, we first induced anti-ß-gal IgE antibodies by immunization with ß-gal protein in alum. Six weeks after priming, these mice were immunized with a *LacZ*-pDNA. This resulted in a 75% reduction of anti-ß-gal IgE antibodies within 6 wk and in an increase in anti-ß-gal IgG2a antibodies ***(8)*** **(Fig. 4)**. The decrease in IgE antibody levels was antigen specific, as pDNA immunization with an irrelevant ovalbumin (Ova)-pDNA did not result in decreases in anti-ß-gal IgE.

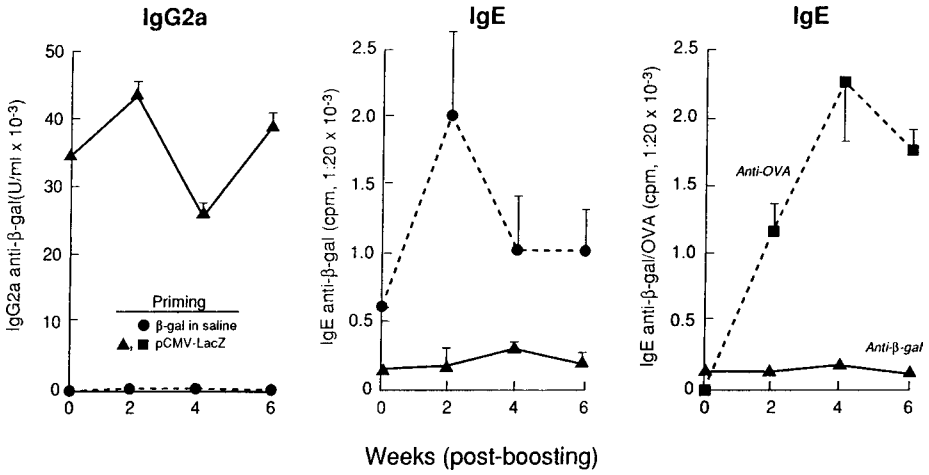


Fig. 3. IgG2a and IgE response of BALB/c mice primed with 100 µg *LacZ*-pDNA (pCMV-*LacZ*) and boosted with 1 µg ß-gal in alum (▲). Control mice were primed with 10 µg ß-gal in saline (●). For the antigen specificity control, mice primed with *LacZ*-pDNA were injected with 2 µg ovalbumin (Ova) in alum (■).

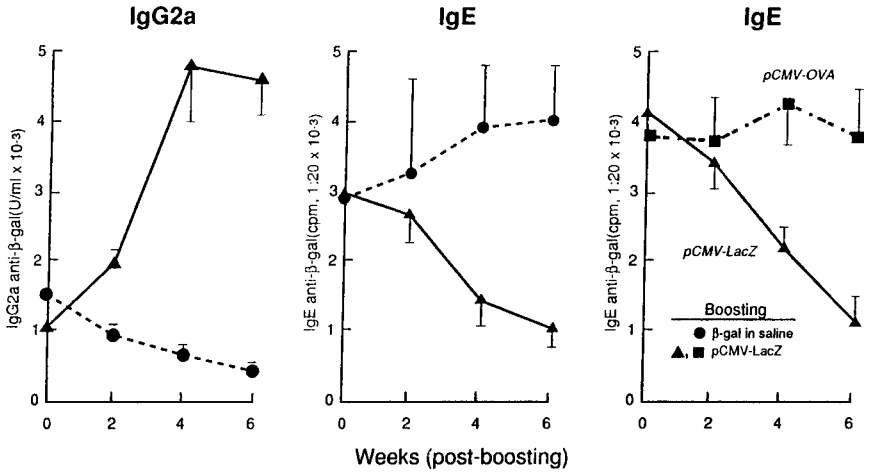


Fig. 4. IgG2a and IgE response of BALB/c mice primed with 1 µg ß-gal in alum and boosted with 100 µg *LacZ*-pDNA (pCMV-*LacZ*) (●). Control mice were primed with 1 µg ß-gal in saline (●). For antigen specificity control, mice were boosted with Ova-pDNA (■).

1.5. Gene Immunization Suppresses Antigen-Induced Pulmonary Eosinophilic Inflammation

The inhalation of allergens results in allergen binding to specific IgE antibodies attached via IgE receptors to the surface of mast cells and basophils. This binding results in the release of histamine, leukotrienes, IL-4, IL-5 and other mediators of inflammation. This IgE-mediated response is known as the immediate (hypersensitivity) reaction (within seconds to min. of allergen exposure) ***(6)***. The late-phase response occurs 4–24 h later, as a result of the initial mediator release, and is characterized by the infiltration of eosinophils into the site of allergen exposure. There, the eosinophils degranulate in response to immune complexes and mast cell mediators, resulting in the release of toxic substances such as major basic protein (MBP), which causes tissue damage ***(19)***. The release of proinflammatory mediators from both the immediate and late phase responses then leads to pulmonary hyperreactivity.

In order to generate a model for the development of pulmonary allergic inflammation, Balb/c mice were made hyper-reactive to ovalbumin by multiple injections of the antigen and alum, delivered either intraperitoneally or subcutaneously. Mice were then exposed to aerosolized protein within a chamber. This inhalation challenge induced eosinophil infiltration into the lung tissue and bronchioli, as well as increasing eosinophil numbers in the bone marrow and blood stream (indicative of production and trafficking of eosinophils to the lungs).

Using this model of an allergic reaction, we investigated the effect of gene immunization on the late phase reaction. We found that allergen-gene immunization not only inhibited IgE antibody formation in mice but also inhibited the eosinophil infiltration into the lung that occurred after Ova inhalation challenge ***(15)***. Mice immunized with Ova-pDNA and challenged by Ova inhalation showed a significantly reduced proportion of cells as eosinophils in the bronchial airway lavage (BAL) fluid (43% to 3.5%), in the lung tissue and also in the bone marrow (10.3% to 1.4%) as compared to control mice ***(15)*** **(Table 2)**. This demonstrated that gene vaccination could also inhibit the late phase allergic reaction. Splenocytes from these gene-vaccinated mice secreted IFN-γ but no IL-5 upon Ova stimulation, indicating that antigen-specific Th1 cells, rather than Th2 cells were generated by the pDNA immunization. Because IL-5 influences eosinophil differentiation and survival, it is likely that the observed decrease in eosinophil numbers mentioned above, was the result of the Th1 response induced by Ova-pDNA gene immunization, and occurred because of the lack of IL-5 secretion by antigen-specific Th cells **(Table 1)**.

Table 2
Percent Inhibition of Eosinophils by pDNA Immunization

Priming	Sensitization	Challenge	Blood	BM	BALF	Lung
Saline	+	+	5.0+2.4	10.2+1.8	39.5+5.0	40.3+15.8
Ova-pDNA	+	+	4.0+0.8	3.1+0.7	6.2+1.8	12.0+1.4
pUC19	+	+	8.1+0.7	8.4+0.5	32.2+7.2	38.5+1.7
Naive	-	-	7.4+2.0	3.7+0.5	0 +0	0 +0

Eosinophil counts in target organs: The numbers are the percent of eosinophils (Eos) present in various tissues after Ova inhalation. Ovalbumin gene immunization (50 μg Ova-pDNA administered intradermally) given prior to repeated Ova/alum administration (Corry protocol, ***[26]***), inhibited the subsequent Eos numbers in the bone marrow [BM] and blood (an indication of Eos growth), and inhibited the subsequent Eos numbers in the bronchial airway lavage fluid (BALF) and in the lung tissue (Lung) (an indication of Eos infiltration) after inhalation challenge with Ova. There were 8 mice per group. The Corry protocol was not applied on the naive animals. Eos counts were performed by a "blinded" investigator on slides coded with numbers. Five hundred WBCs were counted in the BALF of each mouse and 10 random microscope fields of lung sections were analyzed for determination of the Eos in the lung tissue. N/S denotes normal saline. Results are means ± S.E.

1.6. A Potential Mechanism of IgE Down-Regulation by pDNA-Immunization

Analysis of the lymphokine secretion profiles of $CD4^+$ splenic T cells stimulated in vitro with antigen gives us insight into what may be occurring in vivo. Splenocytes obtained after *LacZ*-pDNA immunization of either naive mice or mice that had been primed by vaccination with ß-gal in alum produced similar levels of IFN-γ after antigen stimulation in vitro **(Table 1)**. The lack of effect of pDNA immunization on IL-4 secretion by protein-in-alum primed mice indicates that the down-regulation of IgE levels caused by pDNA immunization was not mediated by a down-regulation of IL-4. It seems, therefore, that the IFN-γ production by the newly induced Th1 cells acts to inhibit B cell synthesis of IgE, and that this is responsible for the 75% decrease of the IgE antibody levels, despite the continued IL-4 production **(Fig. 3)**.

Plasmid DNA immunization results in prolonged antigen expression by the cells transfected in vivo ***(20)***. Therefore, Th1 stimulation and recruitment may be a continual process that leads to increasing amounts of Th1-B cell interactions **(Fig. 4)**. During this time there are increased levels of antigen-specific IgG2a antibodies. Increases in both protective IgG, and $CD4^+$ Th1 memory cells capable of secreting IFN-γ could explain the decreasing levels of anti-ß-gal IgE after injections of *LacZ*-pDNA. It remains to be shown whether a preexisting Th2 response and IgE antibody formation can be completely eliminated over a longer time period by multiple pDNA injections, and to what extent the IgE-down-regulation will have a therapeutic effect.

1.7. The Role of Immunostimulatory DNA Sequences (ISS) in the Induction of a Th1 Response to pDNA Immunization

As we were characterizing the immune responses induced by pDNA immunization, we found that the presence of specific DNA sequences contributed to the induction of a Th1 response *(21)*. The Th1-inducing effect of ISS-DNA occurred when these sequences were within pDNA expression vectors, within non-coding pDNA vectors co-injected with pDNA expression vectors, or when ISS-DNA (ISS-pDNA or ISS-oligonucleotides) were co-injected with protein antigens *(22)*. Details of the biological activities of the ISS are reviewed elsewhere in this book.

1.8. Conclusions

The recent findings described in this review suggest that allergen gene-vaccination has potential as a novel form of safe and effective immunotherapy. Gene vaccination does not result in inflammation when injected intradermally and has been shown to prevent *(8–13)* and down-regulate *(8)* existing antigen-specific IgE antibodies in mice and rats, and to result in the reduction of eosinophil infiltration of the lung in a model of the late phase allergic response *(14,15)*. The Th1 adjuvant effect of the ISS-containing DNA, whether within the pDNA expression vectors, or co-injected with pDNA expression vectors or protein, is important for the induction of IFN-γ and further promotion of Th1 cell differentiation. This response results in inhibition of IL-5 and IgE production.

To date, all in vivo experiments showing IgE inhibition have been performed in mice and rats. However, pDNA is taken up by human skin cells transplanted onto nude mice *(23)*, and ISS-containing DNA has been shown to induce Th1-promoting cytokines from human PBMCs *(22)*. This suggests that the ISS-DNA induced Th1 responses observed in rodents are likely to also occur in human. It has yet to be determined what regimen of genetic vaccination or ISS-DNA/protein mixture immunotherapy will be needed to decrease IgE levels and to reduce the numbers of allergen-specific Th2 cells to achieve clinical efficacy in humans.

As for safety considerations, in vitro data indicate that pDNA-transfected cells secrete low levels of antigen that are unlikely to induce the anaphylactic reactions observed in traditional immunotherapy. Furthermore, allergens encoded by pDNA constructs can be designed to include a transmembrane domain and anchor, thereby preventing the secretion of allergen and the possible induction of anaphylactic reactions. It is likely that allergen gene vaccination can be performed with a lower frequency of immunizations than the current mode of immunotherapy, because of the longevity of gene expression, the Th1 adjuvant effect of ISS-DNA, and a mechanism of action different from

allergen desensitization. In addition, ISS-DNA/protein co-immunization should be effective at low doses of allergens, without the necessity for increasing amounts as in desensitization immunotherapy.

2. Materials

2.1. General Materials

1. Triton X-114; available from Sigma Chemical Co. (St. Louis, MO).
2. QCL-1000 LAL Test kit, 50-648U; available from BioWhittaker Inc. (Walkersville, MD).
3. LAL water, W50-500; BioWhittaker Inc.
4. Endo Free Plasmid Maxi kit, 12362; available from Qiagen, Inc. (Valencia, CA).
5. Ficoll 400, Bromophenol blue, B 392; available from Fisher Scientific (Fairlawn, NJ).
6. RNase A; from Sigma.
7. 10X Tris borate EDTA buffer (TBE), 1666-703; available from Boehringer Mannheim (Indianapolis, IN).
8. Magic cloth, 475855; available from CalBiochem (San Diego, CA).

2.2. Vaccination with a Tyne Device

1. Allergy skin test applicator (Tyne device), Connaught Laboratories (Toronto, Canada).
2. NaOH, 0.1 *M*.
3. Sodium laurel sulfate (SDS), 0.5% (w/v); Sigma (St. Louis, MO).
4. Normal saline, (hospital grade, 0.9% (w/v) NaCl in sterile water).
5. Hair trimmer (animal or other).
6. pDNA (prepared as in **Subheading 3.2.**).
7. Aluminum hydroxide (alum).

2.3. ELISA for Antigen-Specific Antibodies

1. Carbonate buffer (for 1 L): 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, pH to 9.6.
2. Tween-20; Sigma.
3. Phosphate-buffered saline (PBS)-1% (w/v) bovine serum albumin (BSA), pH 7.4.
4. BSA, grade 5, A-9647; Sigma.
5. Nonfat milk (any supermarket brand).
6. 96-Well flat bottom ELISA plates, EIR/RIA, #3690; available from Corning Costar Corp. (Cambridge, MA).
7. Alkaline phosphate labeled goat anti-mouse IgG1, IgG2a, IgG2b, or IgG3; available from Southern Biotechnology Associates (Birmingham, AL).
8. p-Nitrophenyl phosphate, pNPP tablets N-2770; Sigma.

2.4 Radio-AllergoSorbent test (RAST) for Specific-IgE Antibodies

1. PBS, 1/10 dilution of 10X DPBS, 17-515F; BioWhittaker Inc.
2. Sample to be iodinated in PBS, pH 7.0-7.2 at a concentration of 1-2 mg/mL (no thiocyanate or azide).

3. 0.025*M* phosphate buffer pH 7.0-7.4 (NaH_2PO_4/Na_2HPO_4, pH 7.0, no chloride).
4. Freshly prepared: chloramine-T at 1 mg/mL in 0.25*M* PO_4 buffer.
5. Freshly prepared: sodium metabisulfite 1 mg/mL in 0.25*M* PO_4 buffer.
6. ^{125}I-Na, carrier-free (ICN #63037; usual specific activity 17 Ci/mg I, 10 mCi in 100 μL, i.e., 1 mCi/10 μL), ICN Biochemicals (Costa Mesa, CA).
7. 1% (w/v) BSA-PBS or PBS-5% (w/v) nonfat milk.
8. 12 × 75 mm polypropylene tubes with cap.
9. Dialysis tubing (appropriate to retain protein antigen), tied and tested for leaks.
10. Dialysis tube closures.
11. Gloves (use double gloves for ^{125}I work).
12. Plastic disposable 1 mL pipets.
13. Adjustable pipettors (designated for radioactive use) and pipet tips.
14. Disposable plastic 1 L bottle with a cap, filled with PBS and with a magnetic stirrer.
15. Scissors and forceps.
16. Borate-buffered saline (BBS), 0.1 *M*, pH 8.4 (18 liters): 171.65 g $Na_2B_4O_7 \times 10$ H_2O (0.025 *M*), 111.31 g H_3BO_3 (0.1 *M*), 78.91 g NaCl (0.075 *M*).
17. Blotto stock (5X) (1 L): 50 g non-fat dry milk (any supermarket brand), 0.1 mL antifoam A, (Sigma), 10 mL of 1% (w/v) thimerosal (as a preservative) (*see* Note 1). Bring the volume up to 1 L with BBS. Dilute 1: 5 with BBS for working stock.
18. Monoclonal rat anti-mouse IgE; available from Pharmingen (San Diego, CA).
19. Monoclonal mouse IgE anti-dinitrophenol (DNP) antibody IGELa2; available from American Type Culture Collection, Rockville, MD.
20. Goat polyclonal anti-mouse IgE antibodies; available from Nordic Immunological Labs (San Clemente, CA).
21. Purified antigen of interest for coating plates.
22. Protein-G Sepharose, #17-0618-02; Pharmacia (Piscataway, NJ).
23. Radioimmunoassay (RIA) plates, 96-well polyvinyl chloride (PVC); Dynatech (Chantilly, VA).

2.5. Induction of Antigen or Allergen Sensitization

1. Camco Quickstain-2 buffered differential Wright-Geimsa stain (VWR Scientific, San Diego, CA)).
2. PBS: 1/10 dilution of 10X DPBS, 17-515F; BioWhittaker Inc.
3. Sterile saline (hospital grade, 0.9% (w/v) NaCl).
4. Inhalation Chamber (4" × 8" plexiglass chamber fitted with inflow and outflow lines).
5. DeVilbiss UltraNeb-99 nebulizer; available from Sunrise Medical (Somerset, PA).
6. Cytospin apparatus; available from Shandon Southern Inc. (Pittsburgh, PA).
7. Tissue Tech OCT medium for tissue freezing; Sakura Finetek (available from VWR Scientific).
8. Freezing cryostat.
9. Chicken ovalbumin, grade 5, A-5503; Sigma.
10. Red blood cell lysing solution: potassium carbonate (100 m*M*, ammonium chloride, 1.5*M*).

11. 27-Gauge mm silicon tubing.
12. 23-Gauge 1 mL tuberculin syringes.
13. Aluminum hydroxide.
14. Purified antigen of interest.

3. Methods

3.1. Gene Vaccination, a General Introduction

pDNA used in gene vaccination should be endotoxin free. Plasmid preparations must be performed in such a manner to remove most endotoxin. Our procedure yields pDNA with endotoxin levels in the range of <1–2 ng/mg of pDNA. Slightly higher levels of endotoxin may be within the acceptable range for use, but care should be taken for endotoxin may induce IgG2 and IgE *(25)*.

3.2. Preparation of Low Endotoxin Plasmid DNA (Modified Qiagen Maxi Prep Protocol)

Plasmid DNA preparation or Maxiprep procedure may be performed as outlined in the Qiagen protocol by using Qiagen's low endotoxin kit (0.1–0.5 μg/mg DNA). This is generally sufficient for most applications. However, we find that our modified procedure (with 2 Triton X-114 steps), though more time consuming, generates plasmid DNA with endotoxin levels of <1–5 ng/mg DNA. Endotoxin may also be removed by Triton extraction (*see* **Subheading 3.3.,** Endotoxin Removal from pDNA preparation).

1. Streak plasmid-containing bacteria onto an L broth (LB) agar plate containing the appropriate antibiotic, i.e., 25 μg /mL kanamycin or 50 μg/mL ampicillin, in order to select single colonies, and allow colonies to grow overnight at 37°C.
2. Inoculate 5 mL LB with a single colony from the above plate, and incubate in a 37°C shaker for 6–8 h, in order to obtain a log phase culture.
3. Inoculate one liter of LB (containing appropriate antibiotic) using a 1:10,000 dilution of the 8-h log phase culture and then incubate it for16 h in a 37°C shaker.
4. Centrifuge the cultures at 40°C at 6000*g* for 15 min (6000 rpm in Sorvall GSA or GS3 rotors [Kendro Laboratory Products, Newtown, CT]).
5. Pour off the medium, resuspend the bacterial pellet in 50 mL P1 buffer (Qiagen) containing 200 μg/mL RNAase A (prepare by adding 2 mL of 100 mg/mL RNAase A to 1 L P1 buffer) and transfer the supernatant to 500 mL Nalgene bottles.
6. Add 50 mL P2 buffer (Qiagen), rotate the bottles gently 6–8 times to mix and incubate them at room temperature for no more than 5 min.
7. Add 50 mL P3 buffer (Qiagen), rotate the bottles gently 6–8 times to mix. Centrifuge the bottles for 30 min at 40°C at 20,000*g* (13,000 rpm in a Sorvall SS-34 rotor).
8. Filter the supernatant by pouring it through magic cloth in a funnel into clean 500 mL Nalgene bottles, and centrifuge them for 15 min at 10,000 rpm.
9. Pour the supernatant over magic cloth into clean 500 mL Nalgene bottles. Add 0.1 volumes 10% (v/v) Triton X-114, incubate the mixture on ice for 30 min.

10. Precipitate DNA by adding an equal volume of isopropanol. Centrifuge the bottles at 40°C for 30 min at 15,000*g* (11,000 rpm in a Sorvall SS-34 rotor [Kendro]).
11. Wash the DNA pellet with 70% (v/v) ethanol, dry it in air and redissolve it in 5 mL TE, pH 7.0 at room temperature.
12. Run 1.0 μL of the DNA solution on a 1% (w/v) agarose gel to check for genomic DNA or RNA contamination and estimate a plasmid concentration by comparing the plasmid band against a DNA mass ladder or known quantities of purified plasmid (determined from the optical density at 260 nm). Plasmid DNA contaminated with genomic DNA is discarded; plasmid DNA with RNA contamination can be treated with additional RNAase A.
13. After estimating the plasmid concentration, place 1 mg of plasmid (equivalent volume) into a 50-mL conical tube. Add an equal amount of 2X QBT (made 2X using Qiagen's recipe) and a subsequent volume of 1X QBT to obtain a final concentration of 41.6 μg /mL (1 mg/24 mL). Add 0.1 volumes (2.4 mL) of 10% (v/v) Triton X-114 and incubate the mixture on ice for 30 min.
14. Equilibrate a Qiagen 500-tip with 10 mL QBT (Qiagen).
15. Load 13.2 mL (or a 0.5 mg DNA equivalent) onto a Maxiprep column. Collect the flow-through in a 50 mL conical tube.
16. Wash the column twice with 30 mL of QC buffer (Qiagen).
17. Elute the plasmid with 15 mL of pyrogen-free QF (made using Qiagen recipe, using pyrogen-free water) into a 50-mL conical tube.
18. Re-equilibrate the column with 10 mL QBT.
19. Re-load the column with the flow-through and repeat steps 15 and 16.
20. Repeat steps 14–18 as needed.
21. Precipitate the plasmid DNA by adding 0.7 vol. of isopropanol. For maximal precipitation, place the mixture at –20°C for at least 1 h.
22. Centrifuge for 30 min at 40°C at 15,000*g* (11,000 rpm in a Sorvall SS-34 rotor [Kendro]).
23. Pour off the supernatant, add 10 mL 70% (v/v) ethanol, and swirl it gently to wash the plasmid DNA pellet.
24. Centrifuge for 30 min at 40°C at 15,000*g* (11,000 rpm in a Sorvall SS-34 rotor [Kendro]).
25. Pour off the supernatant and dry the pellet in air.
26. Dissolve the plasmid DNA pellet in 300 μL of pyrogen-free LAL water.
27. Quantitate the plasmid DNA by measuring the absorbance at 260 nm, and check for RNA or genomic DNA contamination by agarose gel electrophoresis.

3.3. Endotoxin Removal from pDNA Preparation

If plasmid endotoxin levels are still high after isolation (or if a different method of isolation was used) endotoxin may be removed by the following method.

1. Dilute the pDNA preparation up to 40 mL in a 50-mL Falcon tube (Falcon, Los Angeles, CA) with (limulus amebecyte lysate) LAL water; use one tube per liter of culture.

2. Add 10 µL of 100% (v/v) Triton X-114 per mL of diluted DNA prep (400 µL/40 mL).
3. Place the tubes on a rotator in a cold room for 15 min. Check for complete dissolution before continuing.
4. Incubate the tubes on ice for 20 min.
5. Place tubes in 55°C water bath for 25 min. A biphasic separation should occur with a large, cloudy top aqueous layer, and a clear lower Triton layer (approx 5% volume).
6. Centrifuge at 37°C for 20 min at 4000*g* (2000 rpm in a tabletop centrifuge, radius 4 in.).
7. Transfer the upper phase to a fresh 50-mL Falcon tube.
8. Repeat steps 2–7 two more times.
9. Transfer the DNA solution to a 50-mL Falcon tube. Put only 12 mL of solution in each tube to accommodate the EtOH/NaOAc precipitate mixture.
10. Add 0.1 volume of 3*M* sodium acetate, pH 5.2 and 2.5 vol. of 100% (v/v) EtOH.
11. Store over night at –20°C for complete precipitation.
12. Centrifuge the tubes at 4°C and 4000 rpm for 30 min.
13. Wash the pellet twice with 25 mL 70% (v/v) ethanol, then centrifuge it at 4000 rpm for 10 min.
14. Air dry the pellet and dissolve it in an appropriate volume of saline.

3.4. Limulus Amebocyte Lysate Assay

This assay may be performed using a Pyrotell® LAL kit or the BioWhittaker LAL kit. The latter has an increased sensitivity of endotoxin determination. Special care with plasmid preparation and endotoxin determination is required when experimental plasmids (or oligonucleotides) are being tested or compared for their ability to induce, enhance, or bias the immune response (contaminating endotoxin may induce artifactual immune responses) ***(25)***. We have found that synthetic oligonucleotides must also be tested for endotoxin in order to allow clear interpretations of their immunostimulatory effects, as we have found that an occasional oligo synthesis could have high endotoxin levels.

3.5. Animals

Pre-bleeds are taken prior to immunizations. Perhaps because of diet or other environmental factors, we have found that some mice show background titers of antibodies to common allergens or antigens. It is therefore advisable to screen animals prior to use in experiments where allergens will be used as experimental antigens. You can then choose truly naive animals for comparisons of IgE induction and inhibitions. Recommended vendors include Harlan Sprague Dawley (Indianapolis, IN) and Jackson Labs (Bar Harbor, ME). Adult mice (1–6 wk of age), sex-matched are used for immunizations.

3.6. Intradermal Immunizations

Vaccinations are performed by injecting 50 µL volumes (50 µg) of pDNAs or ODNs, in 0.9% (w/v) NaCl, intradermally at the base of the tail. For protein in saline, controls, or protein-DNA co-immunizations, 10–50 µg of protein are injected (depending on the antigen; we use 10 µg of ß-gal, and 50 µg of Ova), alone or with 50 µg of DNAs. During injection a bleb forms under the surface of the skin. Immunizations are generally given at 1–3 wk intervals. Mice are bled at 2-wk intervals, starting at 2-wk post-immunization. Injections are generally performed with tuberculin syringes with 25 gauge needles.

3.7. Immunization with Protein

In order to induce a primary IgE antibody response or boost a secondary response, mice are immunized either i.p. or s.c. with 1–25 µg of protein (depending on the antigen, some are less immunogenic and require higher doses) and 1–5 mg of alum in either 500 µL or 200 µL of saline (intraperitoneally and subcutaneously, respectively) (*see* **Note 2**). For boosting, 1–25 µg of protein in 0.5 mL of saline containing 3–10 mg of alum, is injected intraperitoneally.

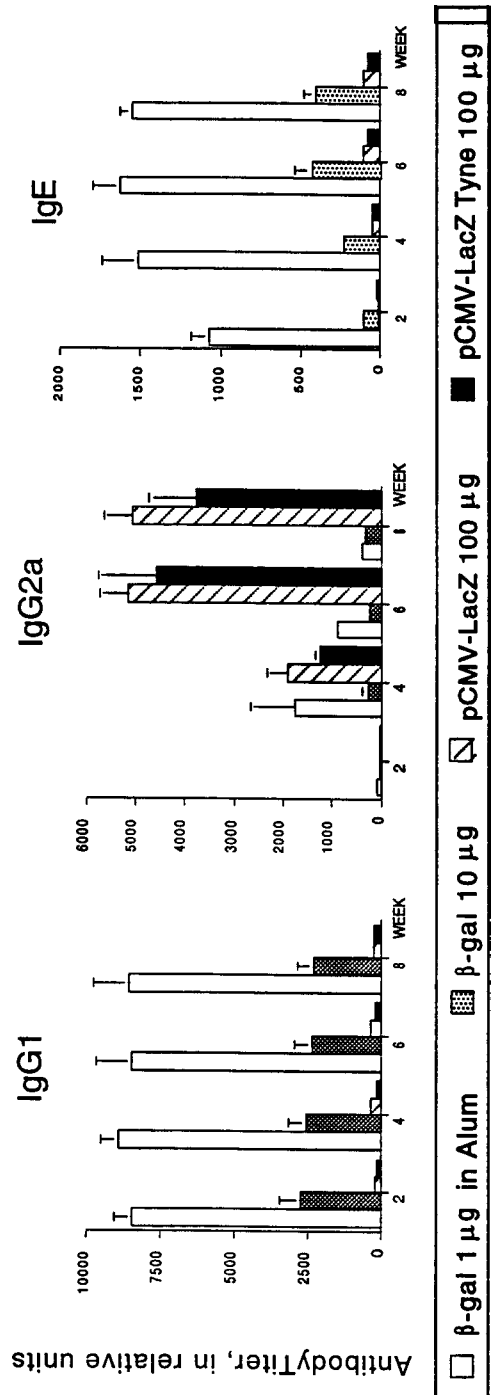
3.8. Vaccination with a Tyne Device

Intradermal immunization may be performed using a Tyne device (tuberculosis, or allergy skin test applicator, supplied by Connaught Laboratories) to deliver pDNA into the skin ***(9)***. This delivery, achieved by puncturing the shaved skin, results in the generation of immune responses comparable to those achieved by intradermal injection of pDNA at the base of the tail **(Fig. 5)**. At the time of our testing, the Tyne devices were not commercially available in the uncoated form.

1. Wash the Tyne applicators extensively in distilled water, soak them overnight in 0.5% (w/v) sodium dodecyl sulfate (SDS), rinse with water, soak overnight in 0.1*M* NaOH, rinse with water, and dry at 37°C for 8 h.
2. Dissolve 50 µg of pDNA in 6 µL of saline and drip the viscous solution onto the spikes of the Tyne applicator.
3. Immunize mice with two applications of the Tyne applicator (100 µg/immunization) onto the shaved lower back area.
4. Immunize once a week for 3 wk.

3.9. ELISA for Antigen-Specific Antibodies

1. Coat microtiter plates overnight or for 4 h with 5 µg of ß-gal per mL in carbonate buffer.
2. Wash the wells with BBS, 0.05% (v/v) Tween-20, and block non-specific binding sites with PBS with 1% (w/v) BSA or 5% (w/v) nonfat milk for a least 2 h.


IgG1
IgG2a
IgE
Antibody Titer, in relative units
WEEK
β-gal 1 µg in Alum
β-gal 10 µg
pCMV-LacZ 100 µg
pCMV-LacZ Tyne 100 µg

3. Wash the wells twice with BBS, 0.05% (v/v) Tween-20.
4. Dilute serum samples 1:40 and then 1:4 for 8 steps in PBS-1% (w/v) BSA, pH 7.4.
5. Use dilutions of a high-titer anti-ß-gal serum as positive controls.
6. Add 50 µL volumes to the wells, in duplicate, and incubate the plates overnight at 4°C.
7. Wash the plates with BBS, 0.05% (v/v) Tween-20 and incubate with alkaline phosphate-labeled goat anti-mouse IgG1, IgG2a, or IgG2b for 2 h at room temperature.
8. Wash the plates 3 times with BBS, 0.05% (v/v) Tween-20, and then incubate with developing buffer (1 mg/mL p-nitrophenyl phosphate).
9. Read the absorbency of the solutions in the wells at 405 nm on a Elisa reader after 1 h.
10. Express the results as units per milliliter, based on arbitrary units per milliliter of the standard serum.

3.10. Radioallergosorbent Test for Specific-IgE Antibodies

Specific IgE antibodies are measured by a radioallergosorbent assay (RAST) ***(15,21)***. Label proper antibody with ^{125}I by the chloramine-T method (*see* **Note 3**) at one week to one day before setting up the RAST. The percent uptake of ^{125}I should be greater than 30%.

3.10.1. Protein Iodination Using Chloramine-T

1. Place 10 µL of protein at 1–2 mg/mL concentration in a 12 × 75 mm polypropylene tube, stoppered and with a tape label.
2. Add 25 µL of 0.25*M* PO_4 buffer.
3. Using a pipettor designated for radioactive use, dispense 10 µL of Na ^{125}I (1 mCi).
4. Add 10 µL of chloramine-T solution. Stopper the tube and mix rapidly, but without splashing onto the wall of the tube.
5. Let the reaction proceed for 5 min with intermittent mixing.
6. Stop the reaction by adding 10 µL of metabisulfite solution.
7. Add 1.5 mL of PBS-1% (w/v) BSA (or PBS-NFM) with a 1-mL plastic pipette and transfer the contents of the tube to a dialysis bag, snap on the bag closure, transfer the tape label to the bag closure and put the bag in a 1-L bottle of PBS (the bottle must be capped).
8. Dialyze with stirring overnight at 4°C behind a lead shield to remove unbound ^{125}I.
9. Remove the iodinated sample from the dialysis bag and put it into a 15 mL conical tube using a 1-mL plastic pipet. Be careful to keep the outside of the tube free from iodinated material.

Fig. 5. IgG1, IgG2a and IgE antibody response to ß-gal encoding pDNA administered via intradermal injection, or by pressing the points of a pDNA-coated Tyne device into the skin. The magnitude and characteristic responses are similar with both intradermal administrations, illustrating that pDNA vaccinations may be performed easily by using skin test applicators.

10. Determine the ^{125}I uptake by counting a small aliquot in a gamma counter.
11. If desired, dilute the iodinated protein up to 5 mL with PBS-1% (w/v) BSA or PBS-NFM.
12. Store the iodinated protein at 4°C in a lead container or aliquoted and frozen at –20° to –70°C (*see* **Note 4**)).

3.10.2. RAST Method

1. Number the polyvinyl chloride plates on the bottom of the wells from right to left.
2. Coat the wells with 5 to 10 µg/mL of ß-gal (or other antigen of interest) in borate-buffered saline (BBS) (pH 8.4).
3. Incubate the plates for 1 h at room temperature.
4. Aspirate the solution and wash the plates 5 times with BBS 5% (w/v) non-fat milk (BBS-NFM).
5. Incubate for 1 h minimum or overnight at 4°C with BBS-NFM in order to block non-specific binding sites.
6. Aspirate the blocking buffer and pat the plates dry against paper towels.
7. For the best quantitation of specific IgE antibodies, pass the sera over protein G-Sepharose (Pharmacia) in order to remove essentially all of the IgG2a, IgG2b and IgG3, and about 90% of the IgG1 (*see* **Note 5**). Use a pool of 0.5 mL of 1:10 diluted serum of 4 mice per group. Add 100 µL of protein G slurry (50 µL of beads + 50 µL BBS), to 15 µL serum, and 85 µL BBS = 150 µL of 1:10 serum dilution + 50 µL beads. Rotate the beads and serum for 1–2 h at room temperature, then overnight at 4°C.
8. Dilute the serum samples 1:10 and 1:20 in BBS-NFM.
9. Add 100 µL of serum dilutions and standards to the wells in duplicate and incubate for 16 h or overnight at 4°C.
10. Wash the plates 5 times with BBS-NFM.
11. Add 100 µL of ^{125}I-radiolabeled purified goat anti-IgE antibodies (diluted to $4–6 \times 10^6$ cpm/100 µL) for 4 h at 4°C.
12. Wash the plates 5 times with BBS-NFM, and pat the plates dry.
13. Cut off the plastic wells into labeled plastic tubes and count the radioactivity with a gamma scintillation counter. Use appropriate precautions for the use of radioactivity and storage of radioactive waste.
14. Use serial dilutions made from anti-antigen-containing serum (from antigen/alum immunized mice) as a standard curve and positive control, and normalize the cpm from different plates (*see* **Note 6**).

3.11. RIA Method for Total IgE

The RIA for total IgE is performed identically to the aforementioned RIA IgE RAST assay except that the assay plates are coated with a monoclonal rat anti-mouse IgE antibody at 10 µg/mL and a mouse IgE myeloma protein is used for the standard curve ***(15)***. Make antigen standard solutions ranging from 0.098–200 ng/mL in BBS-NFM. The standard curve is usually linear in the 0.39 ng/mL to 100 ng/mL range.

3.12. Induction of Antigen or Allergen Sensitization (Model of Late-Phase Pulmonary Allergic Response)

3.12.1. Method of Antigen Sensitization: Corry Protocol (26)

For sensitization (induction of IgE antibodies) mice receive 4 s.c. injections of 25 μg protein antigen (ovalbumin) + 200 μL PBS + 1 mg aluminum hydroxide (alum) on d 1, 7, 14, and 21.

3.12.2. Method of Antigen Inhalation Challenge

The inhalation challenge consists of three 30-min inhalations in an inhalation chamber, separated by 30-min intervals. An antigen concentration of 10 mg/mL is used in the nebulizer, set up to aerosolize 80–100 mL of protein solution in the 30-min inhalation period. The outflow of the inhalation chamber is attached to a vacuum line, and adjusted to a minimal suction rate that is enough to prevent excessive condensation from occurring in the chamber.

3.13. Method for the Harvest of Tissues for Eosinophil Counts

At 24 h after the antigen inhalation challenge, kill the experimental mice by cervical dislocation.

3.13.1. Blood

1. Collect blood from the carotid artery.
2. Lyse red blood cells using a 1:10 solution of 100 m*M* potassium carbonate, 1.5*M* ammonium chloride.
3. Attach the cells from this fluid to microscope slides using a cytospin 3 min at 28*g* or 500 rpm (in a Shandon Cytospin 3 centrifuge) and dry the slides in air at room temperature.

3.13.2. Bronchial Airway Lavage Fluid (BALF)

1. Canulize the tracheas of the mice with 27 gauge mm silicon tubing attached to a 23-gauge needle on a 1-mL tuberculin syringe.
2. Pump 600 μL of sterile saline into the lungs.
3. After massaging the lungs, withdraw the fluid.
4. Attach the cells from this fluid to microscope slides using a cytospin 3 min. at 28*g* or 500 rpm (in a Shandon Cytospin 3 centrifuge) and let them dry in air (*see* **Note 7**).

3.13.3. Bone Marrow (BM)

1. Extract bone marrow cells from femurs and rinse twice in PBS.
2. Attach the cells to microscope slides by cytospin 28*g* or 500 rpm (in a Shandon Cytospin 3 centrifuge)and stain them with Wright-Geimsa stain for cell differential counts.

3.13.4. Lung

1. Freeze unfixed lung tissue in OCT medium at –80°C.
2. Prepare thin sections (10 μ*M*) of frozen lung tissue, attach them to a microscope slide, fix them with acetone for 5 min, and dry them in air.

3.14. Eosinophil Cell Staining for Differential Cell Counts

Evaluate the eosinophil numbers by staining and microscopic examination as follows.

1. Establish a key code for the groups of mice, and assign slides colors.
2. Label color-coded slides to designate mouse numbers within groups (i.e., 1–6).
3. Apply Camco quickstain-2 buffered differential Wright-Geimsa stain for 10 s.
4. Rinse the slides twice with distilled water and dry them in air.
5. Mount glass no. 1 coverslips onto the slides with permount
6. Examine the slides under high magnification, using someone unfamiliar with the key code.
7. Select random fields and count the eosinophils (cells containing large dark red-staining granules) *(27)* and the total cells until >500 cells have been counted.
8. Express eosinophil counts as a percent of total cells.

4. Notes

1. Do not use azide or thiocyanate.
2. Protein in saline gives only a transient increase in IgE in mice.
3. All steps involving use of ^{125}I are performed in fume hood with appropriate precautions.
4. ^{125}I-labeled antibodies can be used up to 3 wk.
5. Absorption of IgG from the sera eliminates competition for antigen on the plate, which may vary depending on the IgG titer, and it increases the cpm of the specific IgE antibodies.
6. To quantitate the relative amount of antibody, compare titration curves for individual sera to a standard curve on each plate using DeltaSOFT II v. 3.66 software (BioMetallics, Princeton, NJ).
7. If the BAL fluid is bloody, RBCs may be lysed before attaching the cells to the microscope slides.

Acknowledgment

Figures 2–4 are reproduced with the permission of *Proc. Natl. Acad. Sci. USA* (1996), **93,** 5141–5145. This work was supported by U.S. National Institutes of Health grants AI40682 and by Dynavax Technologies (Berkeley, CA)..

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Preclinical Safety of DNA Vaccines

A Method to Analyze the Distribution of Plasmid DNA in Animal Models

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1. Introduction

1.1. Advantages of DNA Vaccines

DNA or genetic vaccines are currently being evaluated for safety and efficacy in human clinical trials in the areas of infectious disease and cancer. Since DNA vaccines induce antibodies and cytotoxic T lymphocytes (CTLs), they are currently being evaluated in humans for both prevention and therapy of HSV-2, HIV-1, and HBV infections, for prevention of influenza and malaria, and therapy of cutaneous T-cell lymphoma (CTCL) and colorectal cancer.

DNA vaccines offer advantages over subunit vaccines, peptide vaccines and whole-killed vaccines in their ability to generate both humoral and cellular immune responses ***(1)***. DNA vaccines are also potentially safer than live-attenuated vaccine approaches, since they are non-replicating and present no danger of reversion to a pathogenic form. DNA vaccines also offer manufacturing advantages over other vaccine technologies, since they are manufactured in large quantities as plasmids in *Escherichia coli*, and different vaccine vectors can be made by the same process. Furthermore, since purified DNA is relatively stable in solution, and since it can be lyophilized, DNA vaccines offer stability advantages over live-virus vaccines. The enhanced stability of DNA vaccines may be particularly important for their distribution in developing countries.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

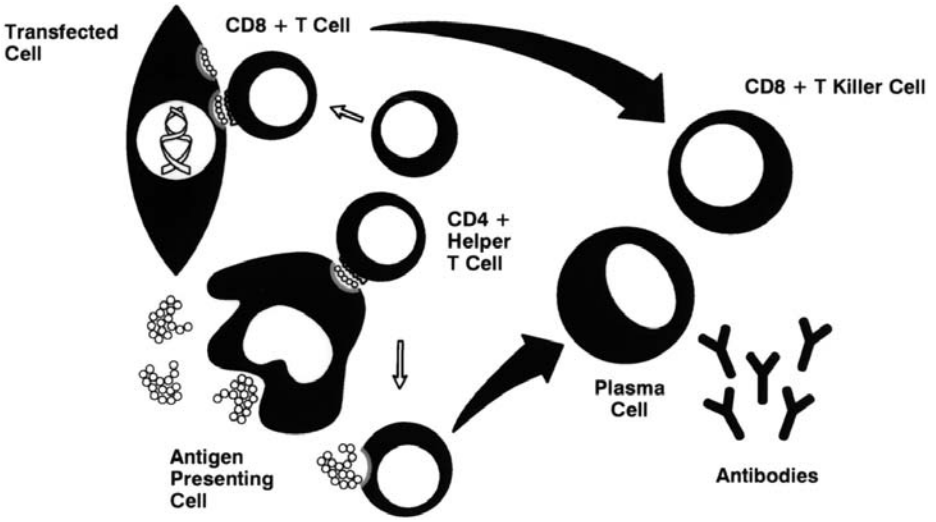

Fig. 1. Immunological consequences following introduction of DNA vaccines into muscle. Following administration, plasmid DNA is taken up by muscle cells and other cell types in the animal. The transfected cells serve as factories that produce encoded gene products. The immune response is initiated by the professional antigen-presenting cells (APCs) either through transfection by the administered DNA, or through the acquisition of the protein product. These APCs interact with CD4+ T cells in proximal lymphoid tissues, stimulating a cascade of events leading to an expansion of antigen specific CD4+ or CD8+ T cells and B cells (plasma cells) that serve as effectors of cellular or humoral immune responses.

1.2. Overview of DNA Vaccine Technology

A DNA vaccine is typically a double stranded supercoiled plasmid DNA produced in *E. coli* that is engineered for mammalian expression of genes from pathogens or tumors. The purified supercoiled DNA is formulated in isotonic saline, or with transfection facilitating components such as bupivacaine and cationic lipids. The DNA vaccine formulation stimulates cellular and humoral immune responses to the expressed proteins antigens ***(1)***. DNA vaccines have stimulated immune responses using several parenteral and mucosal routes of administration, with the intramuscular route being the most widely practiced. When plasmid DNA is delivered to muscle, transfected myofibers express the gene for plasmid-encoded antigen(s) transiently, with expression usually peaking at 7 to 14 d post-transfection as measured by reporter genes such as luciferase ***(2)***. Uptake of the plasmid by cells in muscle as measured by expression and the immune response to the encoded antigen can be enhanced by pre-

treatment with the local anesthetic bupivacaine, or alternatively by directly formulating the DNA with bupivacaine ***(3,4)***.

DNA vaccines introduced into muscle, stimulate both humoral and cellular components of the host immune response, as shown in **Fig. 1**. Myofiber expression of antigens is not required to generate immune responses, since other routes of delivery have also produced both antibodies and CTLs ***(5)***. This is primarily due to the role of professional antigen presenting cells (APC's) present in muscle and other tissues. In muscle, transfected cells produce plasmid encoded proteins and display their processed peptide fragments on the MHC-I complex. Although specific $CD8^+$ T-cells are capable of recognizing peptide-bound MHC-I complexes, muscle cells lack specific co-stimulatory molecules such as B7.1/B7.2 needed to generate a long-lasting memory CTL response. Therefore, antigen presenting cells (APCs) such as macrophages and dendritic cells trafficking in muscle are presumed to pick up fragments of the antigenic proteins, and process them by the MHC class II pathway. These APCs may then travel to draining lymph nodes. It is also likely that such APCs engulf and express plasmid DNA, and the expressed antigens are also processed through the MHC class I pathway in these cells. In addition, cytokines are released from T-helper cells which stimulate both T- and B-cell directed responses. In this manner both antigen specific antibodies and CTLs are produced ***(1,6)***. Immune responses generated by this approach have proven to be protective against live challenge by infectious agents in a number of mammals, including chimpanzees ***(3,7,8)***. The ability of DNA vaccines to induce CTL responses raises the possibility of their use in therapy as well as in protection. The utility of DNA vaccines for therapy was recently demonstrated in HIV-1 positive chimpanzees, which cleared HIV-1 virus after vaccination with a plasmid expressing gp160 ***(9)***.

Although DNA vaccines have been well tolerated and have produced robust immune responses in animal models, their safety, tolerability and efficacy in humans have only begun to be evaluated. In the first human trial of a DNA vaccine, in an HIV-1 seropositive patient population, a bupivacaine-formulated plasmid expressing the HIV-1 env and rev genes was well tolerated and increases in immune responses to gp 120 were observed ***(10)***. Recently, DNA vaccines for HIV-1, HSV-2, HBV, influenza, malaria, and cancer have been advanced to clinical trials.

Advancement of DNA vaccines to the clinic requires extensive preclinical research and development aimed at demonstrating both safety and immune responses in animal models. This article describes preclinical development studies necessary for advancement of these vaccines into humans, and presents a detailed method for one of these studies, the analysis of plasmid DNA distribution in animal tissues.

1.3. Preclinical Safety Studies for DNA Vaccines

1.3.1. General Considerations

Preclinical safety studies for DNA vaccines should provide enough information in the area of their intended clinical use to allow initiation of a phase I clinical study *(11)*. Preclinical studies are carried out with the clinical formulation in accordance with good laboratory practices (GLP). Design of the preclinical safety assessment plan should include the standard animal studies that are required of other types of vaccines, as well as studies for specific safety issues associated with DNA vaccines *(12)*. The specific safety studies should address development of anti-DNA antibodies, rescue of latent viruses, recombination with related sequences, oncogenesis resulting from expression of the antigen, and plasmid integration. Furthermore, DNA vaccines could potentially be used as therapeutics, either alone or in combination with other drug therapies. Preclinical support for the use of therapeutic DNA vaccines might require their assessment of safety and immune responses in a relevant animal model for the specific disease.

Standard safety studies to assess local site reactogenicity as well as systemic toxicity are carried out with DNA vaccine formulations in a similar manner to other types of vaccines. Local reactogenicity studies in well established models such as rabbit or pig should address both the method (e.g., needle and syringe) and the route (e.g., intramuscular) of vaccine administration. The DNA vaccine formulation should be administered at or above the intended clinical dosage. Tissues at or near the injection site(s) should be evaluated histologically. This evaluation should include determination of the local effects due to administration of DNA formulation (e.g., local irritation), as well as effects of foreign antigen expression and presentation to the immune system. Systemic toxicity studies should also be assessed in a well characterized animal model, such as rabbit. The intended clinical dose range and protocol should be addressed. The animal's general health and clinical pathology are evaluated, along with toxicity to tissues and target organs as determined by gross pathology and histopathology. Specific toxicological assessment of cells and organs of the circulatory and immune systems should also be evaluated.

In addition to standard toxicological studies, specific safety issues for DNA vaccines should be addressed. Such issues include: plasmid DNA tissue distribution and persistence, including the persistence of low levels of antigen expression leading to immune tolerance, or in the case of human proteins, adverse autoimmune responses; the development of anti-DNA antibodies, and immune responses to DNA or chromatin; and insertion of plasmid DNA sequences into genomic DNA (integration), leading to tumorigenesis *(11,12)*.

The potential development of anti-DNA antibodies and the immuno-stimulatory effects of bacterial DNA in humans has been the subject of a recent review ***(20)***. Although DNA is a substance of relatively low immunogenicity, preclinical safety studies in animals with specific plasmids should include an assessment of anti-DNA antibodies following multiple immunizations, in particular with the specific vaccine formulation to be used in humans. Impure DNA preparations or DNA associated with proteins might be more immunogenic than highly purified plasmid DNA. In one study, mice immunized with an SV40 T-antigen expressing plasmid developed antibodies against DNA and DNA-histone complexes. The antibodies presumably developed due to the DNA-bound T-antigen, since mutation of the DNA binding domain of the SV40 T-antigen abolished the anti-DNA antibody response ***(20)***.

Another safety issue associated with DNA vaccines is DNA integration. The probability of a DNA integration event being oncogenic has been estimated to be exceedingly low ***(15)***. DNA integration by a DNA vaccine is defined as random insertion (non-homologous recombination) of plasmid DNA sequences into the genomic DNA of host tissues. In theory, plasmid DNA could cause an insertional mutation leading to activation of an oncogene or disruption of a tumor suppressor gene. Even if the insertion event occurred in a cell type capable of propagating the mutation, this still would only be one step in the multistep process of tumorigenesis. In addition, the probability of a DNA insertion is decreased through the use of non-replicating plasmids and DNA transfection in terminally differentiated, non-replicating cells. Although the probability of a DNA insertion event leading to tumorigenesis has been predicted to be below the background mutation frequency ***(14)***, it is prudent to evaluate the possibility of plasmid integration in preclinical animal models.

1.3.2. Persistence of DNA, Antigens, and Antibodies

In rodents, antibody responses have remained elevated for periods of at least one yr following intramuscular (i.m.) DNA injection. However, this has generally not been the case in primates ***(13)***. Longevity of the antibody response might be related to persistent expression of the DNA, which requires that the DNA itself remain in an intact and expressible state in tissues. Plasmid DNA persisting in tissues is likely to be only in an extrachromosomal (non-integrated) form. Persistent expression of plasmid encoded genes might also be the result of integration into the cellular genome (discussed in **Subheading 1.3.1.**). However, studies with a site-specific integrating adenovirus (Ad8) showed silencing of adenoviral genes following chromosomal insertion ***(21,22)***. In theory, cells expressing foreign antigen should be eventually elimi-

nated by a host immune response, so it is possible that persistence of antigen expression might also be due to delivery of the plasmid DNA to cells that are poorly recognized by the host immune system.

Early studies with reporter genes have indicated that gene expression could occur at low levels for periods of at least 18 mo after a single intramuscular injection in mice ***(16)***. In a recent study with a DNA vaccine vector expressing a secreted viral antigen (HBsAg) in mice, expression was maximum at 7 to 10 d, decreased through 30 d and was undetectable at 50 d. However, the presence of anti-HBsAg antibodies which developed in the serum of these animals complicates the analysis of expression in this study ***(17)***. Long term expression of antigens is a concern since it could possibly lead to an immunologically unresponsive state (tolerance), although this has yet to be observed experimentally ***(18)***.

To address the issue of plasmid DNA persistence, it is important to determine both the distribution and the kinetics of distribution following administration of the plasmid. It is also important to recognize that the persistence and distribution of the plasmid DNA is highly dependent on the DNA formulation and route of delivery. DNA plasmids formulated with bupivacaine and delivered intramuscularly have been cleared rapidly from the site of injection ***(19)***. Starting with initial doses of plasmid corresponding to 10^{13} molecules, plasmid was not detected at a sensitivity of 10^4 molecules (9 log below the initial dose) in 2–3 d in muscle and skin at the site of injection ***(19)***.

Although plasmid DNA was rapidly cleared from the site of injection, it was useful to analyze for a low number of copies of plasmid in a wide variety of tissues at later time points (mo). To achieve this goal, a highly sensitive, PCR-based method was developed and is described in the following sections. Using this method, genomic DNA from rabbit tissues was analyzed for the presence of DNA plasmid sequences thirty days following intramuscular administration of 0.4 mg of a DNA vaccine formulation designed to express the HIV-1 *gagpol* genes (**Fig. 2**). Total DNA was isolated from a wide variety of tissues collected from six injected rabbits, and was analyzed for the presence of the plasmid sequences. All the animals analyzed had PCR-positive plasmid-signals in DNA isolated from muscle and skin at the site of injection, spleen, thymus and draining lymph nodes (inguinal). Liver and bone marrow in some animals also was positive for plasmid sequences. However, other tissues were negative, including ovary, testes, semen, PBL, and kidney. In other experiments, the PBL fraction has been positive in some animals, which is consistent with the presence of plasmid signals in the draining nodes.

This experiment indicates that tissues beyond the site of injection, and in particular, tissues associated with the mammalian immune system, have plasmid associated with them at least 30 d post-injection, which might be related to the mechanism of long-term immune responses generated in animals by DNA

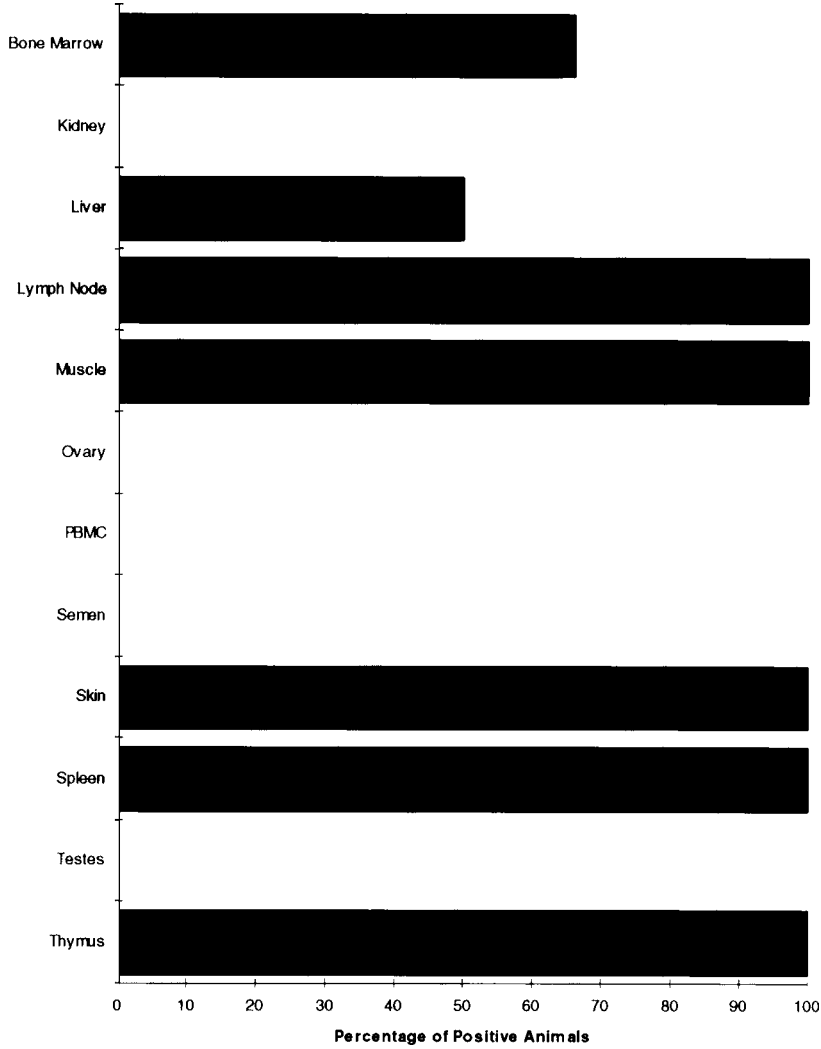

Fig. 2. Distribution of plasmid DNA in animal models. Tissue DNA was isolated and analyzed as described. Sensitivity of the PCR-based detection as determined by spike recovery was 10^2 copies of plasmid DNA in over 10^8 genome equivalents. The figure summarizes the studies with four separate study plasmids, with 6 rabbits/study (3 males and 3 females), 30 d following intramuscular administration of bupivacaine formulated plasmid SNA. Plasmid DNA was not detected in brain and lung tissues.

vaccines. However, this experiment cannot determine whether these plasmid-specific PCR signals indicate the presence of intact, expressible plasmid in these tissues.

2. Materials

2.1. Isolation of Total DNA from Tissues

2.1.1. Isolation of Total DNA from Tissues Using DNAzol™

2.1.1.1. Special Equipment

1. Mini food processor.
2. Light Phase Lock Gels™ (available from 5'→3', Boulder, CO).
3. Fine wire mesh strainers; Teflon microcentrifuge sample pestle.
4. 65°C and 37°C water baths.
5. General centrifuge with swinging bucket and fixed rotors.
6. Vacuum centrifuge; microcentrifuge.
7. Laminar flow hood.

2.1.1.2. Enzymes

1. Proteinase K: Prepare a stock solution of 20 mg/ml in sterile distilled water. Aliquot and store at –20°C.
2. RNase A: Prepare a stock solution of 50 mg/mL in sterile distilled water. Store in aliquots at –20°C.

2.1.1.3. Reagents

1. Buffer saturated phenol:chloroform (1:1): store in the dark at 4°C.
2. Sodium acetate, 3*M*, pH 5.2/acetate.
3. Ethanol and 70% ethanol in water (v/v).
4. SDS (10%)
5. CIP-200: Manufactured by Calgon-Vestal Corp. (St. Louis, MO). Prepare a 1% solution (v/v) using distilled water.
6. DNAZOL®: manufactured by Life Technologies (Grand Island, NY).

2.1.2. Isolation of Total DNA from Muscle and Skin

1. All equipment and reagents described in **Subheading 2.1.1.** except DNAzol™.
2. Digestion buffer: 25 m*M* EDTA; pH 8.0/NaOH, 10 m*M* Tris; pH 8.0/HCl, 100 m*M* NaCl, 0.5% SDS. Sterilize by filtration through a 0.45 μm filter. Store at room temperature.

2.2. Chromatographic Separation of Plasmid Sequences from Genomic DNA

2.2.1. Restriction Digestion

1. Restriction enzymes, selected as described in **Subheading 3.2.1.**
2. Proteinase K.
3. SDS (10%)
4. Buffer saturated phenol/chloroform (1:1): store in the dark at 4°C.

2.2.2. Column Chromatography

1. Special equipment: FPLC; Sephacryl S-1000 column (75 mL bed volume). Bed volumes of 40–150 mL can be used. Choice of the bed volume is dependent on

the amount and volume of the sample to be applied on the matrix. Optimal resolution, and minimal loss of an added marker DNA was obtained when 0.5 mg DNA in 0.5 mL was applied to a 40-mL bed.
2. Dulbecco's Phosphate Buffered Saline (DPBS)(pH 7.5): 0.2 g/L KCl, 0.2 g/L, KH_2PO_4, 8g/L NaCl, 1.15 g/L Na_2HPO_4.
3. Column buffer: 1 m*M* EDTA, pH 8.0, 0.1% SDS, 5% ethanol in DPBS.
4. Sodium acetate, 3.0*M*, pH 5.2/acetate.
5. Ethanol and 70% ethanol in water (v/v).

2.2.3. Regeneration of Column

1. Freshly prepared 1.0*M* sodium hydroxide
2. Column buffer.

2.3. Screening of Column Fractions by PCR

2.3.1. Preparation of Column Fractions for PCR

Ethanol and 70% ethanol in water (v/v).

2.3.2. PCR of Column Fractions

1. Special equipment: Thermal cycler; agarose gel electrophoresis equipment; suitable power supply; materials for the preparation and ethidium bromide staining of DNA agarose gels.
2. Oligonucleotide primers: selection of appropriate primers sets is described in **Subheading 3.3.** Prepare 100 μm stock solutions in sterile distilled water. Store at –20°C.
3. 10× PCR buffer: 500 m*M* KCl, 100 m*M* Tris, 15 m*M* $MgCl_2$, pH 8.3 / HCl , 0.01% (w/v) gelatin.
4. Deoxyribonucleotide triphosphate (dNTP) mixture: Prepare a stock solution mixture of dATP, dGTP, and dTTP, at 2.5 m*M* each. The mixture is stored at –20°C.
5. Dithiothreitol (DTT): Prepare a 100-m*M* stock solution in sterile distilled water and filter sterilize using a 0.22-μm filter. Aliquot and store at –20°C.
6. *Taq* polymerase is available from Perkin-Elmer/Cetus (Norwalk, CT).

3. Methods

3.1. Isolation of Total DNA from Tissues

High sensitivity of the assay requires requires decontamination of all work areas and equipment prior to tissue processing (*see* **Note 1**). All tissue manipulations are performed in a laminar flow hood to ensure elimination of airborne plasmid contamination. The method described is the preferred method for isolating DNA from all tissues types. However, DNA is isolated in low yield from certain tissues such as muscle and skin by this method and may contain inhibitors that affect latter steps of the assay. Therefore, we have modified the method to yield pure DNA from muscle and skin, and the modification is also described.

For both methods, frozen tissue must be fully thawed on ice, and its weight determined prior to processing. Purity of isolated DNA is essential to ensure complete digestion by restriction enzymes, while having the isolated DNA as a high-molecular-weight species (>20 kbp) ensures efficient separation of chromosomal and plasmid sequences during column chromatography. Optimal performance of both these steps is required to achieve the sensitivity that this method offers.

3.1.1. Isolation of Total DNA from Tissues Using DNAzol™

1. Place the whole tissue in a mini food processor, and mince at maximum setting until the tissue is completelyfully homogenized (*see* **Note 2**).
2. Transfer the homogenate into an appropriate size container (*see* **Note 3**), and add 25 mL of DNAzol™ per gram of tissue. Resuspend the tissue homogenate (*see* **Note 4**) and incubate it at room temperature for 45 min. Centrifuge to remove any undissolved tissue in a centrifuge at 5000 rpm.
3. Recover the aqueous DNAzol™ phase by pouring it into a new container through a fine wire mesh (*see* **Note 5**).
4. Add one-half volume of ice-cold ethanol to the recovered DNAzol™ phase to precipitate the DNA. Mix gently by inversion (*see* **Note 6**).
5. Pellet the precipitated DNA by centrifugation at 5000 rpm for 10 min and gently pour off the supernatant. Wash the pellet twice with 70% ethanol.
6. Dry the DNA pelletsample in a vacuum centrifuge, and dissolve in a minimal volume of sterile distilled water (*see* **Note 7**).
7. Add 50 µg RNase A per milliliter of DNA sample, mix by inversion. Incubate at 37°C for 1 h.
8. Add 100 µg proteinase K stock per milliliter of DNA sample, and SDS to 0.5%. Incubate at 65°C for 30 min.
9. Transfer the solution to a Phase-Lock™ tube (*see* **Note 8**), and add an equal volume of buffer-saturated phenol:choroform solution. Mix gently by inversion and centrifuge at 3500 rpm for 10 min to separate the phases.
10. Transfer the aqueous phase to a new container, and precipitate the DNA by adding one-tenth volume of 3.0 *M* sodium acetate and two volumes of ice-cold ethanol. Gently mix by inversion.
11. Pellet the DNA by centrifugation at 5000 rpm for 10 min. Pour off the supernatant, and wash the pellet twice with 70% ethanol.
12. Dry the DNA pellet in a vacuum centrifuge, and dissolve in a minimal volume of sterile distilled water (*see* **Note 7**).
13. Repeat steps 9–13 as needed (*see* **Note 9**).

3.1.2. Isolation of Total DNA from Muscle and Skin Samples

1. Place the whole muscle in a mini food processor and mince it until the muscle is fully homogenized (*see* **Note 2**).
2. Transfer homogenized muscle to an appropriate container (*see* **Note 3**), and suspend the muscle in preheated (65°C) digestion buffer (2.0 mL/g tissue) (*see* **Note 4**).

3. Add SDS (0.5%), and 100 µg of proteinase K stock per milliliter of homogenate. Incubate in a 65°C water bath for 1 h. Repeat proteinase K addition twice for a total incubation time of 3 h.
4. Recover the aqueous solution by pouring it into an appropriate container through a fine wire mesh strainer. Transfer the aqueous phase into a Phase-Lock™ tube, and add an equal volume of buffer-saturated phenol:chloroform (*see* **Note 8**). Gently mix by inversion. Centrifuge at 3500 rpm for 10 min. to separate the phases.
5. Transfer the aqueous phase to an appropriate container, and add one-tenth volume of 3.0*M* sodium acetate and two volumes of ice-cold ethanol to precipitate the DNA. Gently mix by inversion.
6. All further processing of the DNA is identical to that described for steps 6–13 in Section 3.1.1.

3.2. Chromatographic Separation of Plasmid Sequences Form Genomic DNA

3.2.1. Restriction Enzyme Digestion of Total Isolated Tissue DNA

Choice of the restriction enzyme is based on its ability to generate plasmid fragments of no more than 2500 base pairs in length while generating genomic DNA fragments that are larger than 10 kbp. Enzymes that are employed for pulse-field gel electrophoresis (PFGE) separation of high molecular weight DNA (e.g., *Not*I, *Nru*I, *Rsr*II) are suitable. If necessary, multiple restriction enzymes may be used in conjunction to generate plasmid fragments of less than 2500 bp in size (*see* **Note 10**). The appropriate restriction enzyme reaction conditions should be determined prior to sample DNA processing (*see* **Note 11**).

1. Digest the DNA sample isolated from tissue with an appropriate amount of restriction enzyme for the pre-determined time and temperature conditions.
2. Following digestion, add proteinase K to 100 µg/mL and SDS to 0.5%. Incubate at 65°C for 30 min.
3. Apply the sample to the column. The sample may be stored at –20°C. A portion of the sample is analyzed by Southern blot to determine the extent of digestion by restriction enzymes.

3.2.2. Gel Filtration Column Chromatography

1. Equilibrate the Sephacryl S-1000 column with at least two volumes of column buffer.
2. Apply 1 mL of the restriction enzyme digested DNA to the column. Elute the DNA using the column buffer at a flow rate of 1 mL/min.
3. Collect 40 2.0-mL fractions.

3.2.3. Regeneration of the Column Matrix

1. Remove residual DNA not eluted from the matrix by washing the column with one column volume of a 1.0*M* sodium hydroxide.

2. Wash the column with three volumes of sterile water and two volumes of column buffer. The column may be indefinitely stored in column buffer.

3.3. PCR Screening of Column Samples

Oligonucleotides primer pairs for amplification of plasmid DNA sequences should be designed to amplify each fragment of the plasmid generated by cleavage with the restriction enzyme chosen in **Subheading 3.2.** Use of multiple sets of PCR primers increases the probability of detection of plasmid fragments, and is therefore recommended (*see* **Note 12**).

3.3.1. Preparation of Column Fractions for PCR

1. Precipitate column fractions by adding two volumes of ethanol. Mix well.
2. Centrifuge at 10,000 rpm for 10 min to pellet the DNA. Gently pour off the supernatant, and wash the pellet twice with 1 mL of 70% ethanol.
3. Dry the DNA samples under vacuum in a vacuum centrifuge, and dissolve them in 100 mL of sterile water. Samples may be stored at –20°C until they are analyzed by PCR.

3.3.2. PCR of Column Fractions

1. Prepare a master mixture containing all necessary components for the reaction. The following recipe is for 20 PCR reactions; sterile distilled water (474 µL), 10X PCR buffer (100 µL), mixture of 2.5 m*M* each deoxyribonucleotide triphosphate (80 µL), 100 µ*M* oligo primer 1 (8 µL), 100 µ*M* oligo primer 2 (8 µL), 100 m*M* DTT (10 µL), and *Taq* polymerase (20 µL).
2. Add 35 µL of master PCR mixture to 15 µL of each sample, along with appropriate control reactions (*see* **Note 13**).
3. Carry out 25 cycles of amplification reaction in a thermal cycler as follows: 30 s at 94°C, 30 s at 55°C, 30 s at 72°C (*see* **Note 14**).
4. Analyze 20 µL of each reaction by agarose gel electrophoresis. Visualize the amplified DNA sequences by ethidium bromide (0.5 µg/mL) staining.

4. Notes

4.1. Isolation of Total DNA from Tissues

1. Decontamination of all equipment and work surfaces is accomplished through the use of a phosphoric acid detergent solution such as CIP-200 (Calgon-Vestal Corp.). A 10-min treatment with a 1% CIP-200 solution is sufficient to remove contaminating DNA from surfaces. However, routinely the surfaces should be soaked in the acid detergent for 1 h. It is recommended that filter pipette tips be utilized for pipetting manipulations to further decrease the potential of sample contamination, with plasmid DNA. Aerosol contaminated pipette barrels disseminate plasmid molecules, and these contaminate PCR samples.
2. Maximal recovery of DNA can be achieved by complete homogenization of tissues. The homogenization in a mini food processor, such as a Black & Decker Handy Chopper™ (Shelton, CT), should be performed at a continuous setting for the

required time (usually 1–2 min). Small tissues, such as lymph nodes, cannot be homogenized efficiently in a food processor. These tissues are homogenized in a microcentrifuge tube using a Teflon pestle. DNA can be isolated from skin by using a decontaminated razor blade, or a dissection knife, by separating skin layers and by cutting them into small pieces, prior to the addition of SDS and proteinase K.

3. The size of the tissue, and the amount of solution required, should be taken into account when selecting an appropriate container.
4. Complete suspension of the homogenate is required for maximal recovery of DNA. It is recommended that only a portion of the required re-suspension solution be used initially, by pipetting the homogenate up and down. Remaining tissue chunks in the homogenate should be small enough to be drawn through a wide aperture tip. Rapid re-suspension of the tissues in buffer minimizes nuclease action. To avoid coagulation problems, resuspend tissues such as liver and kidney immediately.
5. Recovering the aqueous phase through a wire mesh strainer effectively removes any tissue fragments from the sample. For small tissue samples, the aqueous phase may be recovered by pipetting.
6. Mixing by inversion is required for most steps to prevent shearing of genomic DNA. DNAzol™ and ethanol phases blend poorly, and multiple inversions are required for complete mixing.
7. Dissolution of the precipitated DNA sample is performed by pipetting with a wide bore pipette tip. Smaller samples can be dissolved in 500 µL of sterile water, while larger tissue samples such as liver may require up to 25 mL of sterile water. DNA pellets may also be dissolved at 4°C overnight. Avoid using harsh methods (e.g., vortexing) that could shear the genomic DNA. Overdrying the DNA pellet could make dissolution difficult.
8. For sample volumes up to 500 µL, microcentrifuge Phase-Lock™ tubes may be used. Up to 25 mL of sample my be used in 50 mL Phase-Lock™ tubes for phenol: chloroform extractions.
9. Proteinase K/SDS treatment should be performed until the DNA is pure. Usually two to three treatments are sufficient for smaller tissue samples. However larger tissues may require multiple rounds of digestion and extraction with phenol:chloroform. Purity of the DNA preparation is characterized by the ratio of its absorbance at 260 and 280 nm. Determination of the rate or the extent of digestion of the spiked plasmid are also used to characterize the purity of the DNA sample. Due to possible contamination of DNA preparations by nucleases, it is recommended that the DNA be stored as an ethanol precipitate at –20°C. Storage of genomic DNA solutions at –20◦C for extended periods of time (>2 wk) results in the appearance of significant amounts of degraded DNA. Quality of the genomic DNA preparation is also assessed by electrophoresis on agarose gels and visualized by ethidium bromide staining.

4.2. Chromatographic Separation of Plasmid Sequences from Genomic DNA

10. Pre-testing of the column fractionation process is performed to determine the effectiveness of the restriction enzyme(s) chosen to separate plasmid sequences

from the bulk of genomic DNA. Genomic DNA isolated from control tissue 'spiked' with a known amount of plasmid may be employed as a test sample. Most of genomic DNA should elute in early fractions, while plasmid sequences should elute in a few fractions toward the end of the column profile. Plasmid DNA is detected by PCR analysis of column fractions. Generation of a large amount of smaller molecular weight genomic fragments will shift the elution profile of genomic DNA to overlap that of plasmid DNA fragments. Also the use of multiple restriction enzymes for sample processing, the use of certain combinations of restriction enzymes, or nuclease contamination of the sample increases the possibility of an elution profile overlap. Co-elution of genomic and plasmid DNA decreases sensitivity of the PCR assay. An overlap of the elution profiles of genomic and plasmid sequences due to incomplete digestion by restriction enzymes, contaminating proteins or overloading the column could result in the detection of plasmid sequences in the entire bed volume of the column profile.

11. Digestion conditions must be optimized for the enzyme(s) chosen. Enzyme concentrations, time and temperature of incubation, enzyme stability during the digestion process, and the concentration of DNA are pre-tested to ensure complete digestion of plasmid in the presence of genomic DNA. Genomic DNA isolated from control tissue spiked with a known amount of plasmid is employed as a test sample. Completeness of plasmid digestion is best visualized by Southern blot analysis. Incomplete plasmid digestion will result in overlap of genomic and plasmid DNA elution profiles during column fractionation as described in Note 10. Poor restriction enzyme digestion of spiked plasmid DNA is indicative of exchangeable inhibitors in the genomic DNA preparation. These samples may be further treated with proteinase K/SDS, extracted with phenol:chloroform, and precipitated before use.

4.3. PCR Screening of Column Samples

12. The sensitivity of oligonucleotide primer sets should be determined by PCR analysis. Primer sets capable of detecting a minimum of 1500 copies of plasmid or less in the presence and absence of 1 μg genomic DNA using the PCR conditions described in Section 3.2.2. are suitable for analysis of fractions. An ideal primer set is characterized by its specificity in a genomic background, and its ability to produce visible quantities of product by staining of agarose gels with ethidium bromide. One oligo set for each plasmid fragment generated by restriction cleavage was chosen to analyze column fractions.
13. Control PCR reactions (no DNA control, 1 ng plasmid DNA control) should also be carried out for each primer set analyzed.
14. The PCR cycling conditions described are suitable for use with a Perkin Elmer (Branchburg, NJ) GeneAmp® 9600 PCR System employing thin-walled PCR tubes. For other thermocycler systems, cycling conditions of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C are recommended.

Acknowledgments

The authors wish to acknowledge the following individuals for additional intellectual and laboratory contributions to this work: Richard Carrano, Leslie Coney, Kathleen Herold, Terry Higgins, Larry Smith, Khushroo Shroff, Henry Klepser, Gary Gates, Maria Bamberger, Anthony Gayle, Edward Watson, Meiqing Lu, Donna Sosnoski, Dan McCallus, Malcolm Montgomery, James Semler, Lisa Borges, Linda Snyder, Robert Troutman, David Weiner, Dan Zurawski, and Vincent Zurawski.

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DNA Vaccination

Tolerance and Autoimmunity

Gil Mor and Mariel Eliza

1. DNA Vaccines: An Overview

Infectious diseases result in significant morbidity and mortality worldwide. Preventing these infections is both a public health priority and the primary goal of vaccine research. The discovery that cell-mediated and/or humoral immune responses against viruses, parasites, bacteria and tumor antigens can be induced by antigen-encoding DNA plasmids is revolutionizing the vaccine development field ***(1–3)***. DNA vaccines (also known as plasmid DNA or nucleic acid vaccines) have been proven to successfully prevent infection in a variety of animal models, and are currently undergoing clinical trials in humans for the prevention of a variety of infections, including HIV ***(1,4,5)***.

DNA vaccines consist of a plasmid DNA backbone containing an antigen-encoding gene and a strong mammalian promoter that controls its expression. When injected intramuscularly or intradermally ***(6,7)*** the antigen is transcribed, translated and presented to the immune system in the context of a self major histocompatibility complex (MHC) ***(8,9)***. More specifically, circulating lymphocytes encounter plasmid-encoded antigen in the muscle bed at the site of injection, initiate a humoral response in the draining lymph nodes and then seed distal lymphoid organs ***(9,10)***.

Although the ability of DNA vaccines to elicit strong and specific immune responses is well established, concerns have been raised regarding their safety. More specifically, their potential to induce deleterious immune responses, such as autoimmunity and the development of tolerance in response to the persistent expression of a foreign antigen.

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

1.1. Autoimmunity

The potential of DNA vaccines to result in the formation of anti-DNA antibodies is of special concern. This is important not only in healthy individuals, but also in those with autoimmune diseases, such as systemic lupus erythematosus (SLE) where antibodies against single- and double-stranded DNA, double-stranded RNA, polyribonucleotides, ribonucleoprotein, and ribosomes are produced.

The bacterial origin and intrinsic immunostimulatory activity of the plasmid backbone of DNA vaccines highlight concerns regarding the potential of DNA vaccines to induce deleterious immune responses. Specifically, DNA motifs composed of an unmethylated CpG dinucleotide flanked by two 5' purines (optimally a GpA) and two 3' pyrimidines (optimally a TpC or TpT) activate the innate immune system to produce a series of immunomodulatory cytokines such as interleukin-6, interferon-γ (IFN-γ), IL-12, and tumor necrosis factor-alpha (TNF-α), as well as immunoglobulin M (IgM) antibodies ***(11)***. Hexamers bearing this sequence motif are 20 times more common in microbial than mammalian DNA due to differences in the frequency of utilization and methylation pattern of CpG dinucleotides in prokaryotes versus eukaryotes. These immunomodulatory properties of bacterial DNA can result in immune activation, the production of IgG anti-DNA autoantibodies and the development of glomerulonephritis in mice. Bacterial DNA also has been shown to accelerate autoantibody production in lupus-prone (NZB X NZW)F1 mice ***(12)***.

An additional safety concern associated with the use of DNA vaccines is that myocytes that may take up the injected plasmid and express the encoded antigen could potentially become targets for antigen-specific T-cells or the developments of autoimmune myositis. In a series of studies in mice, DNA vaccination has been shown to trigger low level IgG anti-DNA, but not anti-muscle cell autoantibody production. In addition vaccination did not induce or accelerate the development of systemic or muscle cell-specific autoimmune disease **(13)**.

1.2. Tolerance

DNA vaccines (administered intramuscularly or intradermally) induce strong protective immune responses in adult animals. Yet, most vaccines intended for human use are administered to infants and children. Due to the immaturity of their immune system, newborns exposed to foreign antigens are at risk for developing tolerance rather than immunity.

A number of factors influence the development of neonatal tolerance, including the nature, concentration and mode of antigen presentation to the immune system, and the age of the host. Experimental evidence suggests that recognition of foreign determinants is acquired at distinct ages of maturation,

ranging from early gestation until days or weeks after birth ***(14)***. Since the protein encoded by a DNA vaccine is produced endogenously and is expressed in the context of self MHC, the potential exists for the neonatal immune system to recognize it as self, and therefore for the development of tolerance. This is confirmed by studies demonstrating that a plasmid vaccine encoding the circumsporozoite protein of the malaria parasite *Plasmodium yoelii* induces tolerance rather than immunity when administered to 2–5-d-old mice. Neonatally tolerized animals were unable to mount antibody, cytokine, or cytotoxic responses when rechallenged with the DNA vaccine *in vivo* or *in vitro*. This tolerance, however, appears to be specific for immunogenic epitopes expressed by the vaccine-encoded, endogenously produced antigen. Mice challenged with exogenous circumsporozoite protein produced antibodies against a different set of epitopes, and were not tolerized ***(15)***.

The nature and localization of the immune response elicited by DNA vaccination can be determined by sensitive and specific assays such as the enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot assay (ELISPOT). We hereby describe the application of these assays to the study of the following immune response parameters: (i) antigen-specific antibody production by B-cells, and (ii) cytokine production by lymphoid cells.

2. Materials

Use the following materials for standard ELISA and ELISPOT assays:

1. Polystyrene microtiter plates.
2. Inverted microscope.
3. ELISA reader.
4. Incubator.
5. ELISA plate washer (optional).
6. Multichannel pipette with disposable tips for 50–200 µL.
7. Micropipette with disposable tips for 200–1000 µL and 20–200 µL.

2.1. Reagents

1. 2-Amino-2-methyl-1-propanol alkaline buffer solution: Sigma, St. Louis, MO: 100 mL.
2. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP). Sigma, 500 mg.
3. SeaPlaque agarose. FMC Corp. (Fockland, ME): 25 g.
4. Streptavidin alkaline phosphatase (AP). Jackson ImmunoResearch Laboratories (West Grove, PA).
5. Phosphatase substrate kit. Kirkegaard-Perry Laboratories (Gaithersburg, MD).
6. Antibodies.

2.2. Buffers

1. Culture medium: RPMI 1640 containing 10% (v/v) fetal calf serum (FCS), 20 m*M* HEPES, 10 m*M* sodium pyruvate, 1.5 m*M* L-glutamine and supplemented with 100 U/mL penicillin /streptomycin.
2. PBS/BSA Stock solution: Add 50 g BSA to 500 mL 10X PBS. Dilute 1:10 before use.
3. Blocking buffer: add 25 g (5% (w/v)) BSA and 0.125 mL (0.025% (v/v)) of Tween-80 to 500 mL of 1X PBS. Sterilize by filtration.
4. PBS/Tween: Stock solution: 10X PBS and 0.25 % (v/v) Tween 80. Dilute 1:10 before use.
5. DDW-Tween: Double-distilled water (DDW) and 0.025% (v/v) Tween-80 (DDW-Tween).
6. Buffer for secondary antibody and alkaline phosphatase avidin D (SAB): 1X PBS, 5% (v/v) FCS and 0.05% (v/v) Tween.
7. BCIP Agarose mixture: Add 1 mL of water and 0.03 g of agarose to 4 mL of BCIP. Mix gently and melt the agarose by boiling in a microwave oven for 1–3 min depending on the volume.
8. Phosphatase substrate solution: Add 2 mL of diethanolamine buffer (5X) and two p-nitrophenyl tablets to 10 mL of water.

3. Methods

3.1. Newborn Immunization

1. Remove the mother mouse from the cage containing the pups and place her on a separate cage at the opposite side of the room.
2. Place the cage under a lamp to keep the pups warm.
3. Use a 32-gauge needle with a yellow tip on it to inject the pups (*see* **Note 1**). Leave ~2 mm of free needle at the end of the pipet tip to limit the depth of injection.
4. Inject 10 µL of plasmid on each of the pup's thighs while holding the pup between the index and middle fingers.
5. Clean all blood on the pup with warm water before returning the pup to the cage.
6. Hold the mother on your hand for at least 2–3 min before returning her to the cage with the pups.

3.2. Enzyme-Linked Immunosorbent Assay (*see* **Note 2**)

3.2.1. Antigen-Specific ELISA Assay

Use the antigen-specific ELISA assay for epitope mapping of specific antibodies in the serum of animals injected with plasmid DNA. Microtiter plates are coated with either synthetic peptides or the recombinant protein.

3.2.1.1. Coating Plates

1. Coat flat-bottom 96-well Immulon I (Dynatech Labs, Inc., Alexandria, VA) microtiter plates with purified protein or synthetic peptide (1–20 µg of antigen/mL) in 0.1*M* carbonate buffer (pH 9.6) (*see* **Note 3**). Cover with plastic wrap.
2. Incubate for 6 h at room temperature or overnight at 4°C.

3. Remove the wrap and discard the excess coating antigen.
4. Block the plates with blocking buffer for 2 h at room temperature.
5. Discard the blocking buffer and fill the wells with the washing solution PBS-Tween. Dip the plate in a container filled with washing solution to fill all the wells and let it sit for about 5 min. Wash plates the same way at least three times with PBS-Tween followed by 3 additional washings with DDW. Store the plates in the refrigerator for later use.
6. Add sample to the wells, diluting it in PBS as desired.
7. Incubate for 2 h at room temperature.
8. Wash plates as described in step 5 using DDW-Tween instead of PBS-Tween.
9. Add 1/1000 dilution of phosphatase conjugated anti-mouse IgG, IgM, IgG1 or IgG2a antibodies in PBS-BSA and incubate for 2 h at room temperature.

3.2.1.2. Detection

1. Wash the plates as in **step 5**, **Subheading 3.2.1.1.**
2. Add a 100-µL of fresh phosphatase substrate per well, cover the plates, and keep them in a dark place until you are ready to run the assay.
3. Develop the color for 15–30 min and read the optical densities at 405 nm (*see* **Note 4**).
4. The concentration of serum antibody bound to the plates is determined from the colorimetric reaction. Use a standard curve generated with known dilution of high-titer anti-serum.

3.3. Enzyme-Linked Immunospot Assays (ELIspot)

ELISPOT assay is the most appropriate technique for the detection and quantification of individual antibody or immunoglobulin-secreting cells in vitro. Based on the same principle as the enzyme-linked immunosorbent assay, the ELISPOT assay can be used to study antigen specific responses against both soluble (protein and polysaccharide) antigens and particulate antigens or peptides. In addition, this assay can be used not only for the detection of antibody secreting cells, but of any cell which secretes a specific product. In this capacity, the ELISPOT assay has proved to be a powerful technique for the study of cytokine-producing cells ***(16)***.

3.3.1. Cell Preparation

Prepare a single cell suspension from spleen or lymph nodes in RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS, 1.5 m*M* L-glutamine, and 100U/mL penicillin/streptomycin.

3.3.2. Antigen-Specific ELIspot Assay

3.3.2.1. Coating Plates

1. Coat flat-bottom Immulon II microtiter plates with 50 µL of the antigen (protein or peptide) in coating buffer. PBS (pH 7.4) and 0.1*M* carbonate buffer (pH 9.6) are the most frequently used coating buffers (*see* **Note 5**).
2. If the coating was carried out at 4°C , leave the plate at room temperature for 15 min to allow the temperature to equilibrate. Discard the excess coating buffer from the wells and add 200 µL blocking buffer for 1 h at room temperature.
3. Discard the blocking buffer and wash the plates with PBS-Tween 3 × 5 min and 5X with DDW as well to remove the Tween.
4. Add the spleen or lymph node cell suspension (1×10^6/well) to the antigen-coated plates in serial dilutions and incubate for 5 h at 37 °C in a humidified 5% (v/v) CO_2 environment. Do not move or shake the plates during this time.
5. Wash the plates as described in step 3.
6. Add 50 µL of the isotype-specific alkaline phosphatase-labeled secondary antibody in SAB buffer and incubate for 2 h at room temperature

3.3.2.2. Detection

1. Prepare the streptavidin AP in SAB buffer 30 min before adding it to the plates.
2. Wash the plates with DDW-Tween, 3 × 5 min. each.
3. Add 50 µL of the streptavidin AP per well (1:1000) and incubate the plates for 1 h at room temperature.
4. Wash the plates as in **step 2**, **Subheading 3.3.2.2.**
5. Add 50 µL of BCIP/0.6% (w/v) agarose mixture. Do not move the plates until the agarose solidifies.

3.3.2.3. Reading Plates

A colored precipitate will form to indicate the presence of antigen. After 24 h, count the spots using a dissecting microscope.

3.3.3. Cytokine-Specific ELISPOT

3.3.3.1. Coating Plates

1. Coat 96-well nitrocellulose-backed microtiter plates with 50 µL/well of 1–10 µg/mL of the desired anti-cytokine antibody in 0.1*M* carbonate buffer (pH 9.6). Cover the plates with a plastic lid/cover and incubate for 4 h. at room temperature.
2. Block the plates by adding 200 µL/well of blocking buffer for 2 h at room temperature (*see* **step 3**, **Subheading 2.2.**).
3. Wash 3 × 5 min with PBS-Tween and 3× with DDW.
4. Add serial two-fold dilutions of the single-cell suspension to the coated plates, starting with an initial concentration of 10^6 cells/well and incubate for 5h. at

37°C in a humidified 5% (v/v) CO2 environment.
5. Wash plates 3 × 5 min with DDW-Tween and 3× with DDW.
6. Add 50 µL of the biotinylated anti-cytokine antibody (1 µg/mL) in SAB and incubate overnight at 4°C.
7. Wash the plates as in **step 5**.
8. Using the same buffer as for the second antibody, treat the washed plates with a 1/2000 dilution of avidin-conjugated alkaline phosphatase for 1 h at room temperature. Longer incubations may be carried out at 4°C.
9. Wash the plates as in **step 5**.

3.3.3.2. Detection

1. Add 50 µL of BCIP/NBT per well and incubate for 10–20 min at room temperature.
2. Discard and wash 3 × 5 min with DDW.

3.3.3.3. Reading Plates

1. A colored precipitate, or ELISPOT, will form at sites were individual cells secreted the cytokine(s) of interest.
2. Select a cell dilution resulting in approximately 50 spots/well to calculate the total number of cytokine-secreting cells/sample.
3. Calculate the total number of cytokine-secreting cells per experimental animal as follows: (number of cytokine secreting cells/10^{-6} in each organ) × (number of cells/organ)/10^{-6}.

3.4. Assessing Renal Pathology and Proteinuria in Experimental Subjects

Autoantibody production may result in the deposition of antibody complexes in the glomeruli and therefore in the development of renal pathology. Antibody induced renal disease is manifested at the microscopic level by the visualization of antibody complexes deposited at the glomeruli and at the macroscopic level by clinically evident proteinuria.

3.4.1. Tissue Preservation and Staining

1. Remove organs at the time of sacrifice.
2. Fix one kidney in 10% (w/v) formalin, embed in paraffin and section for staining with hematoxylin and eosin.
3. Flash freeze the second kidney in liquid nitrogen, fix and section for staining with a fluorescein-conjugated goat anti-mouse IgG.

3.4.2. Renal Pathology and Proteinuria

1. Examine sections microscopically for evidence of immune complex deposition (*see* **Note 6**).
2. Monitor urine samples using Albustix (Bayer, Elkhart, IN) to establish the pres-

ence and monitor the progression of proteinuria.

4. Notes

1. Special care must be given to the handling of the pups when immunizing newborn mice to ensure that the mother will not reject the pups. Always wear gloves and a mask when handling newborn mice.
2. ELISA provides a safe and simple method of measuring antigen specific and total immunoglobulins. Engvall and Perlmann (1971) ***(17)*** and Schuurs and Weemen (1977) ***(18)*** first described this method. Various types of ELISA have been developed and described in the literature since then.
3. The protein should be titrated to determine the optimal coating concentration.
4. The degree of development attained correlates in a linear fashion with the time to reach the reaction plateau, therefore weak signals can be improved by longer developing times. In contrast, adding 50 µL/well of 0.2 *M* NaOH can stop a fast reaction. If an assay is overdeveloped, it can be re-read after washing with DDW-Tween, and DDW and then developing it again as in **steps 2** and **3**, **Subheading 3.2.1.2.**
5. It is important to determine which one is the optimal coating buffer for your experiment. Coating may be carried out for 6 h at room temperature or overnight at 4°C. Always cover the plates with plastic wrap to prevent evaporation of the antigen/buffer coating.
6. Quantitation may be possible on the basis of fluorescence intensity.

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Assuring the Quality, Safety, and Efficacy of DNA Vaccines

James S. Robertson and Elwyn Griffiths

1. Introduction

Scientists in academia whose research is aimed at the development of a novel vaccine or approach to vaccination may not always be fully aware of the regulatory process by which a candidate vaccine becomes a licensed product. This chapter will provide an overview of the regulatory process and will discuss in more detail the quality and pre-clinical safety issues of plasmid DNA vaccines intended for human use. It is useful for research scientists to be aware of these processes as the development of a novel vaccine could be problematic due to the starting material often being developed in a research laboratory under ill-defined conditions.

2. Development of a Novel Vaccine

The initial nucleic acid vaccines produced for marketing are likely to be plasmid DNAs derived from bacterial cells. Future vaccines may consist of RNA instead of DNA or may be nucleic acid molecules complexed with other entities. In any case, the development of a novel vaccine from laboratory to licensed product is likely to take a considerable number of years and as the process advances, there will be a greater interaction between the vaccine manufacturer and the appropriate regulatory agencies. Major milestones in the development of a novel vaccine include:

- laboratory demonstration of "proof of concept"
- design and establishment of manufacturing
- demonstration of quality and pre-clinical safety
- approval for and conduct of clinical trials
- application for and attaining a product license

From: *Methods in Molecular Medicine, vol. 29, DNA Vaccines: Methods and Protocols*
Edited by: D. B. Lowrie and R. G. Whalen Humana Press Inc., Totowa, NJ

3. The Regulatory Process

Although the first official interaction between a vaccine manufacturer and a regulatory authority is likely to be when permission is sought to proceed to clinical trials, it is important and useful for industry and regulators to work together closely in the development of any novel vaccine. Informal contact during all stages of development is to be greatly encouraged. In order to proceed to clinical trials, the following information will be required,

- Sufficient laboratory-derived scientific data that indicates the potential of the candidate vaccine.
- Information on the quality of the candidate vaccine.
- Information on the pre-clinical safety of the candidate vaccine.

3.1. Quality

There are two major approaches to assuring the quality of a DNA vaccine:

1. The application of a variety of laboratory tests on the final purified vaccine, before and/or after formulation.
2. The application of "in-process" control.

In-process control is an approach that has proven very useful in the quality control of vaccines in general for many decades. It involves documenting the laboratory development of the vaccine, ensuring the quality of the starting materials, provision of a full description of the manufacturing process and the performance of appropriate tests at various stages of manufacture. Quality aspects are addressed in more detail in **Subheading 4.**

3.2. Pre-clinical Safety

Concerns about the safety of DNA vaccines are generally hypothetical in nature and due to a limited understanding of the complex biological systems involved. There are two principal concerns: (i) that a plasmid molecule may integrate into the host chromosome and disrupt the control of cell division, and (ii) that an unexpected and untoward immunological reaction may result from the use of a DNA vaccine. Pre-clinical safety concerns are dealt with in more detail later.

3.3. Clinical Trials

Permission to proceed with a clinical trial must be sought from an appropriate regulatory authority. Within the European Union, clinical trials are regulated by the licensing authorities within individual member states; in the USA, an IND (Investigational New Drug) is submitted to the U.S. Food and Drug

Administration (FDA). After documentation of the quality and pre-clinical safety of a new vaccine, clinical trials proceed through three progressive phases. Typically, in a phase 1 trial, which involves a small number of volunteers, short-term clinical tolerance and a gross assessment of immunogenicity of the vaccine are assessed. The phase 2 trial, involving a larger number of volunteers, is to investigate dosage and vaccination schedules. Phase 2 trials will also provide further information on safety and immunogenicity and will be pivotal in determining whether or not to proceed to a large-scale phase 3 trial in which the protective efficacy of the vaccine will be assessed with greater precision. A phase 3 trial will typically involve thousands of vaccinees and will also provide further data on safety. Clinical trials of a new vaccine generally take several years to complete.

3.4. Product License

Upon successful completion of clinical trials, the dossier submitted to the regulatory authorities for marketing authorisation should provide evidence of the following aspects of the candidate vaccine:

- Efficacy
- Safety
- Quality
- Consistency

The efficacy of the vaccine will be assessed from data generated during the phase 3 clinical trial. Evidence for safety will accrue from the clinical trials, such as the frequency and nature of any adverse reactions, and from pre-clinical safety (laboratory) tests generally performed prior to initiation of the clinical trials.

Much of the information on the quality of the vaccine will have been available at the time of initiation of the clinical trials. However, during the clinical trial period, it is likely that further development of the manufacturing process will take place and this in turn will result in further refinements to the quality control of the vaccine. By the time of submission for marketing authorisation, the final manufacturing procedure at the proposed production scale must be established and data supporting the consistency of the process will be an important part of the submission. Stability data on the vaccine should also now be available along with full toxicology data. In addition, it will be necessary for the manufacturing plant to be inspected to ensure that the vaccine is being prepared under good manufacturing practice, which involves manufacturing under highly defined, carefully controlled and reproducible conditions.

3.5. Post-licensing

In the case of vaccines, after a product license has been obtained, the regulatory work continues with phase 4, during which any adverse reactions continue to be reported, with the increasing numbers of recipients providing a more accurate estimation of the frequency and nature of adverse reactions, especially rare events. Thus, the full long-term efficacy and safety of a vaccine will only be properly established after many years of use and assessment of its performance. Further improvements or changes to the manufacturing procedure, such as production at a larger scale, or alterations to the final formulated vaccine, will require notification to the regulatory authorities and this is generally achieved by submission of a product license variation.

3.6. Guidelines

Specific guidelines for DNA vaccines to assist industry in submitting data for approval of a clinical trial or in support of an application for marketing authorisation have been developed by the World Health Organization (WHO) and the FDA. The WHO guideline, *Guidelines for Assuring the Quality of DNA Vaccines*, is available from the Chief, Biologicals Unit, WHO, CH-1211 Geneva 27, Switzerland. The FDA guideline, *Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications* is available from the Office of Communications, Center for Biologics Evaluation and Research, 1401 Rockville Pike, Rockville, MD 20852-1448. *A Guideline on the Production and Control of DNA Vaccines* is also under development by the European Union through the Biotechnology Working Party of the CPMP. This should be available during 1999. Additional useful guidelines are listed in **Subheadings 6.1.–6.3.**

4. Quality

The following information provided should be considered as generally applicable to all DNA vaccines. However, it should be remembered that individual vaccines may present particular quality control problems and any special features should be taken into account. Furthermore, the quality control of a particular vaccine should reflect its intended clinical use. Thus, different criteria may apply to a vaccine that is to be used prophylactically in healthy children universally, compared with one that is to be used therapeutically for a life-threatening condition.

Plasmid DNA vaccines should be considered along the same lines as traditional bacterial and viral vaccines, where adequate control of the starting materials and manufacturing process is just as important as that of the final product. Thus, “in-process” control is as important as comprehensive characterization

of the vaccine itself. Also, experience gained in the control of other types of biological products, for example those derived from genetically engineered *Escherichia coli*, will be invaluable in assessing the quality of a DNA vaccine. Fortunately, the manufacture of any plasmid DNA vaccine is a common process and industry already has considerable experience of large scale fermentation of *E. coli*. Large-scale purification of plasmid DNA is less well advanced.

Many of the general requirements for the quality control of biological products, such as tests for potency, endotoxin, stability, and sterility, also apply to DNA vaccines. The manufacture of a plasmid DNA vaccine should also abide by good manufacturing practice and relevant guidelines and points to consider documents should be applied during all stages of the development of a DNA vaccine.

4.1. Developmental Review

Assessment of quality includes the provision of a complete review of the development of the product. This could include such information as:

1. Description of the origin of the gene(s) encoding the protein against which an immune response is sought, such as the name of the micro-organism or cell from which the gene was derived, the origin of the micro-organism, its species, subtype, and passage history.
2. Description of cloning the gene into the vaccine plasmid, the sequence of the gene in the vaccine plasmid, a map, and the source of distinct regions within the plasmid and the choice of antibiotic selection marker. The development of a selection marker that avoided the use of antibiotics would be useful.
3. Description of transient expression in cell culture with assessment by immunofluorescence, Western blotting, or cell sorting.

4.2. Cell Banks/Starting Materials

Production will be based on a cell banking procedure. A cell banking system consists of a master cell bank (MCB) and a working cell bank (WCB) derived from the MCB. Cell banks consist of aliquots of a homogeneous lot of cells (typically these will be the bacterial cells containing the plasmid but may possibly be the plasmid by itself) kept under conditions (usually ultra-low temperature) such that each aliquot will provide a consistent amount of viable organisms for the manufacture of a batch of vaccine. The cells within the MCB and the establishment and maintenance of the MCB must be carefully validated with information provided on, e.g., the preparation of and viability of the cells in the cell bank, the sequence of the entire plasmid within the cells and phenotypic and genotypic characterisation of the cells within the bank. Less characterisation of the cells in the WCB is usually acceptable.

4.3. Production

The production process should be described in detail from the removal of an aliquot of cells from the WCB, through fermentation and purification, to the bulk purified plasmid. All materials used during fermentation and the parameters measured during growth should be described. At the end of a fermentation, tests will be performed to confirm the identity of the production cell, yield of cells or plasmid, and any other tests as appropriate to confirm the success and consistency of a fermentation batch.

A full description of harvesting, extracting and purifying the plasmid vaccine will be necessary. This will include validation of the purification system and of any additional materials used. Data will be required to demonstrate the reproducibility and consistency of the entire manufacturing process.

4.4. Bulk Purified Plasmid

A bulk purified plasmid will be produced from which the formulated vaccine will be prepared. The purified plasmid will be subjected to a number of tests to confirm its identity and to assess its purity. Some tests are likely to be performed on the purified bulk plasmid but others may be more appropriately applied to the formulated vaccine. Tests to establish the identity of a batch of plasmid might include sequencing the entire plasmid, analysis of its structural form (supercoils, denatured molecules, etc.), the extent of any modification, e.g., methylation, in vitro transfection experiments (immunofluorescence, Western blot) and possibly in vivo immunogenicity. Determination of the potency of each batch of vaccine will be crucial and careful consideration must go into the establishment of the appropriate assay for potency measurement and of potency units. It is not clear as to whether potency should be measured by an in vivo bio-assay, by an in vitro test or by physico-chemical analyses. To assist in potency assays, an in-house reference reagent or standard should be established.

A full description of the vaccine in its final form, and its preparation, will be required and limits on impurities such as undesirable plasmid molecular species, chromosomal DNA, RNA, *E. coli* protein, endotoxin and any materials used during manufacture must be established. Assays performed on the final purified vaccine should demonstrate the identity, purity and potency of the vaccine.

Plasmid DNA vaccines will be produced in batches and as with all other types of vaccines, DNA vaccines will be subject to "lot release testing," i.e., a sample of each batch of vaccine will have to be submitted to an appropriate national control laboratory for assessment prior to its release onto the market.

5. Safety Issues

There are several features about the use of a DNA vaccine which raise hypothetical concerns which, in the light of inexperience of their use, have to be addressed at the preclinical safety stage. These concerns are that:

- The plasmid DNA that is internalized by the cells of the vaccinee may integrate into the chromosomes of the vaccinee and disrupt the normal replicative state of that cell, causing uncontrolled cell division and tumorigenesis.
- The expression of a foreign antigen by such a novel mechanism and the duration of that expression may result in adverse immunopathology.
- The additional use of genes encoding cytokines or co-stimulatory molecules may themselves pose additional risks.
- Antibodies against the injected DNA itself may be formed and these may contribute toward undesired autoimmune reactions.
- The expressed antigen may itself have biological activity.

5.1. Integration

It is known that DNA taken up by mammalian cells can integrate into the cellular genetic material and be faithfully maintained during replication. This is the basis of the production of some recombinant therapeutic proteins. Theoretically, if the integration event resulted in the activation of a dormant oncogene or the deactivation of a suppresser gene, the control of normal cell division could be disrupted. Within an animal, this could result in tumour formation. Insertion of foreign DNA into a chromosome can occur in one of three ways, by random integration, by homologous recombination or by a retroviral mechanism. The most likely means in the present context would be by random integration. However, plasmids should be screened for sequences which might facilitate their integration.

After injection of DNA into an animal, only a small proportion of the DNA molecules enter cells. The probability of any DNA molecule integrating into the chromosome is also low and given that oncogenesis is a multi-factorial event, the risk of insertional mutagenesis must be exceedingly low. Nevertheless, given the high profile that this aspect of DNA vaccines is receiving, the limited data available to date and the potential consequences of such an event, it is important that this area is thoroughly investigated.

5.2. Adverse Immunopathology

The mechanism of the immune response to an antigen that is expressed from injected DNA is poorly understood, although it is by no means vital that this is fully understood in order to have an efficacious and safe vaccine. However, there are hypothetical concerns relating to the novelty of the manner in which expression is achieved and the unknown duration, level and site(s) of expres-

sion. Also, there is little information on the capacity for a plasmid DNA vaccine to induce adverse immunopathological conditions such as chronic inflammation or generalized immunosuppression.

5.3. Cytokine Genes

There is considerable interest in the co-administration of a gene encoding a cytokine, e.g., IFN-γ or IL-4, in order to direct and boost a specific immune response. However, there is evidence in mice, that excess of a cytokine, although it increases the response of certain T helper cells, can decrease or shut down the response of others, leading to generalized immunosuppression or chronic inflammation. This could have detrimental effects especially if the cytokine has been introduced on an expression plasmid whose expression cannot be terminated.

Bacterial DNA itself can also have a mitogenic or immunostimulatory effect and this property may be used to advantage in some DNA vaccines. As with the use of cytokines, the specific incorporation of immunostimulatory nucleic acid should proceed with care.

5.4. Anti-DNA Antibodies

Based on knowledge of the presence of specific anti-DNA antibodies in SLE-like autoimmune disorders, there is some concern that the inoculation of bacterial DNA may result in the production of high levels of anti-DNA antibodies. However, antibodies to DNA are present ubiquitously in man although they are of a different specificity and type to those found in SLE patients. It is difficult to induce antibodies against DNA and the consequence of immunising mice with bacterial DNA along with Freund's Complete Adjuvant and methylated-bovine serum albumin is the induction of antibodies to denatured DNA and not to dsDNA. Antibodies of the type found in SLE patients (anti-dsDNA Abs) have been induced only in mice prone to autoimmune disease (NZB or NZW mice) whose abnormal immunological background leads to the induction of the broadly specific SLE-like antibodies.

5.5. Biological Properties

An encoded antigen, e.g., a toxin, may exhibit undesirable biological activity and if this is the case appropriate steps may have to be taken, e.g., by deletion mutagenesis, to eliminate the activity while retaining the desired immune response.

6. Preclinical Safety Testing

The general aim of preclinical safety testing is to determine whether a novel vaccine candidate has the potential to cause unexpected and undesirable effects

in appropriate animal models. For plasmid DNA, as for many other biologicals, classical safety, toxicological or pharmacological testing, as recommended for chemical drugs, will only be of limited relevance. Thus, a flexible approach towards the preclinical evaluation of a plasmid DNA vaccine will be required taking into consideration the concerns regarding their safety.

Assays to assess the distribution, duration, and potential integration of a plasmid DNA vaccine in an experimental animal system will be expected. The duration of expression and the nature of the immune response should be investigated. Preclinical studies should also take into account any possibility of adverse immunopathological reactions arising from the use of the plasmid vaccine such as chronic inflammation, autoimmunity or immunosuppression. The possibility of inducing tolerance should be considered. There is much interest in the co-expression of a cytokine(s) to modulate the immune response and the possibility of this approach resulting in adverse immunopathology should also be considered carefully. Assays for anti-DNA antibodies should be established and the possibility that in vivo synthesized antigen may exhibit an adverse biological activity should be considered. The innate immunostimulatory properties of bacterial DNA are being investigated and this phenomenon should be borne in mind in designing preclinical studies and in assessing data derived from them.

The safety testing should involve a wide range of biological, molecular, biochemical, immunological, toxicological and histo-pathological investigative techniques, where appropriate, in the assessment of a plasmid's effect in an experimental animal, over an appropriate range of doses and during both acute and chronic exposure. Although preclinical safety and general toxicological and pharmacological testing will undoubtedly be required, the range of tests that need to be carried out will have to be decided on a case-by-case basis, in consultation with the regulatory authorities. Tumorgenicity studies would be appropriate if evidence of integration was uncovered although it is unlikely that a manufacturer would wish to continue with the development of the DNA vaccine if this were to occur. The future control of DNA vaccines will depend on our current state of knowledge of DNA vaccines and of the immune response to them.

Note: Any views expressed in this paper are those of the authors and do not necessarily represent the policy of the NIBSC or of WHO.

Appendix

6.1. WHO Documents

1. Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology, in *WHO Expert Committee on Biological Standardization*, Forty-first Report, Annex 3, Technical Report Series No. 814, World Health Organization, Geneva, 1991.

2. Good manufacturing practices for biological products, in *WHO Expert Committee on Biological Standardization*, Forty-second Report, Annex 1, Technical Report Series No. 822, World Health Organization, Geneva, 1992.
3. Guidelines for national authorities on quality assurance for biological products. In: WHO Expert Committee on Biological Standardization, Forty-second Report, Annex 2. Technical Report Series No.822. World Health Organization, Geneva, 1992.
4. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products, in *WHO Expert Committee on the Use of Essential Drugs*, Sixth Report, Technical Report Series No. 850, World Health Organization, Geneva, 1995.

6.2. FDA Documents

1. Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology (4/85).
2. Points to Consider in Human Somatic Cell Therapy and Gene Therapy (8/91).
3. Supplement to the Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology: Nucleic Acid Characterization and Genetic Stability (4/92).
4. Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (7/93).
5. Guideline on General Principles of Process Validation (5/87).
6. Guideline on the Preparation of Investigational New Drug Products (3/91).

6.3. EU Documents

1. Production and quality control of medicinal products derived by recombinant DNA technology (revised 1994). Note for Guidance, III/3477/92, European Commission.
2. Gene therapy products—quality aspects in the production of vectors and genetically modified somatic cells. Note for Guidance, III/5863/93, European Commission.