Gene Vaccination with Naked Plasmid DNA: Mechanism of CTL Priming
By Maripat Corr, Delphine J. Lee, Dennis A. Carson, and Helen Tighe

Summary
The injection of naked plasmid DNA directly into the muscle cells of mice has been shown to induce potent humoral and cellular immune responses. The generation of a cytotoxic T lymphocyte (CTL) response after plasmid DNA injection may involve the presentation of the expressed antigen in the context of the injected myocytes' endogenous major histocompatibility (MHC)-encoded class I molecules or may use the MHC molecules of bone marrow-derived antigen presenting cells (APC) which are capable of providing co-stimulation as well. To resolve which cell type provides the specific restricting element for this method of vaccination we generated parent→F1 bone marrow chimeras in which H-2bxa recipient mice received bone marrow that expressed only H-2bc or H-2dh MHC molecules. These mice were injected intramuscularly with naked plasmid DNA that encoded the nucleoprotein from the A/PR/8/34 influenza strain, which as a single antigen has epitopes for both H-2Db and H-2Kd. The resulting CTL responses were restricted to the MHC haplotype of the bone marrow alone and not to the second haplotype expressed by the recipient's myocytes. The role of somatic tissues that express protein from injected plasmids may be to serve as a reservoir for that antigen which is then transferred to the APC. Consequently, our data show that the mechanism of priming in this novel method for vaccination uses the MHC from bone marrow-derived APC, which are efficient at providing all of the necessary signals for priming the T cell.

Direct injection of naked plasmid DNA either intramuscularly or intradermally induces strong, long-lived immune responses to the antigen encoded by the gene vaccine. While the intradermal route of administration appears to be the most efficient, there is evidence that either route leads to production of antibody and the activation of both major histocompatibility complex (MHC) class I-restricted, antigen-specific cytotoxic T lymphocytes (CTL) and MHC class II-restricted CD4 T cells secreting Th1-type cytokines (1–9). Plasmid DNA immunization has potential advantages compared to traditional protein vaccination due to the strong CTL and Th1 responses induced, the prolonged antigen expression, and the resistance of the antigen source to antibody mediated clearance. As a consequence, gene vaccination has potential applications in the fields of infectious diseases, allergy and cancer.

The question of the mechanism by which DNA vaccines activate the immune system has been raised by a number of investigators (6, 10). The majority of CD4 T cells recognize peptides derived from exogenous proteins endocytosed by antigen-presenting cells (APC), degraded to peptide fragments and loaded onto MHC class II molecules (11). In contrast, CD8 T cells generally recognize peptides derived from endogenous proteins presented in the context of MHC class I molecules. Peptides derived from proteo-
latory molecules is more likely to tolerate than stimulate T cells (16–19). This poses the following question: which cell type presents gene encoded antigen to prime the immune system after intramuscular gene vaccination?

Although not answering this question directly, a number of experiments have provided a theoretical basis by which such priming could occur. Non-professional APC such as transfected fibroblasts are able to induce an antigen-specific MHC class I–restricted response if they are physically relocated to secondary lymphoid tissue (20). This implies that antigen presentation and co-stimulation do not need to be provided by the same cell but must be in the same local environment. Thus it is possible that CTL could receive a first signal from peptide/MHC class I complexes expressed by the muscle tissue and a second signal from hemopoietic cells recruited by a local inflammatory response to the site of injection.

In addition, it has been shown that under certain circumstances exogenous antigens can be presented in the context of MHC class I (21–30). Consequently, presentation may occur by protein transfer from transfected muscle to a professional APC. This potential mechanism is supported by the original cross–priming experiments in which bystander cells were shown to present MHC class I–restricted minor histocompatibility antigens in vivo (31) and by more recent experiments in which a test antigen expressed by tumors of one haplotype can cross-prime CTL restricted to another MHC haplotype when transferred to F1 recipient mice (32).

The primary aim of the experiments reported here was to determine whether somatic cells at the site of plasmid DNA injection can prime a specific CTL response by presenting antigen in the context of their endogenous MHC complexes or whether presentation is restricted to professional APC of hemopoietic origin.

Materials and Methods

Parental → F1 Bone Marrow Chimeras. Female BALB/c, C57Bl/6, and CB6 F1 mice (C57Bl/6 × BALB/c) were purchased from Jackson Laboratories (Bar Harbor, Maine). CB6 mice, aged 10–12 wk, were used as bone marrow recipients. To minimize the risk of bacterial infection before reconstitution, recipient mice were fed autoclaved food and housed in micro-isolator cages with autoclaved bedding. Antibiotics were given (sulfamethoxazole/trimethoprim) in their drinking water for 5 d pre-irradiation, and for 3 wk post-irradiation. This regimen was supplemented with intramuscular injection of antibiotics (gentamicin) during the first week post irradiation. Recipient female CB6 F1 mice were given 1,200 rads and then injected intravenously with 5 × 10⁶ T-depleted bone marrow cells from 5-wk-old donor female BALB/c, C57Bl/6, or CB6 F1 mice in a total volume of 100 µl serum-free RPMI 1640 (BioWhittaker, Walkersville, MD). Chimerism was confirmed at time of death by cytofluorographic analysis of harvested splenocytes.

T Cell Depletion of Bone Marrow. Bone marrow was expressed from the tibia and femurs of 5-wk-old donor mice using sterile serum-free RPMI 1640, a 3-ml syringe and 22-gauge needle. Bone marrow preparations were depleted of T cells by treatment with a cocktail of anti-Thy-1 (YTS 154), anti-CD4 (RL172), and anti-CD8 (3.155) antibodies at 4°C, followed by batch selected guinea pig complement (PelFrez Biologicals, Rogers, AK) and 10 µg/ml DNAase I (Sigma Chem. Co., St. Louis, MO) at 37°C. Cells were then washed and viable cells counted. The efficiency of depletion was then tested by antibody staining of cells and analysis on the cytofluorograph.

Cytofluorographic Analysis. T-depleted bone marrow was stained using antibodies against CD4 and CD8 coupled to phycoerythrin (Caltag, San Francisco, CA). Staining antibodies were selected which bound to alternative epitopes of CD4 and CD8 to the depleting antibodies to avoid false readings due to competition. Splenocytes from bone marrow chimeric mice were analyzed at the time of death. Cells were stained with either anti-Thy-1-FITC (Caltag) to determine number of T cells, anti-B20-PE (PharMingen, La Jolla, CA) to determine B cell levels and antibodies against H-2ª-PE and H-2ª-FITC (PharMingen) alone and together to determine whether all the leukocytes were of donor type or whether residual recipient (H-2ª and H-2ª) leukocytes still remained. Cells were then analyzed on the FACScan® flow cytometer (Becton-Dickinson, San Jose, CA) using the Lysys II analysis programs. All mice used in these experiments were shown to contain less than 4% of residual recipient leukocytes.

Preparation of Plasmid DNA. The nCMV-int and nCMV-NP vectors have been described previously (1). DNA was prepared using Qiagen maxi prep kits (Qiagen, Chatsworth, CA), with the modification of adding one-tenth volume 10% Triton X-114 (Sigma) to the clarified bacterial lysate before applying it to the column in the kit. Before injection the residual endotoxin level was quantified using a limulus extract clot assay (Associates of Cape Cod, Woods Hole, MA). Plasmid DNA with a level of <5 ng endotoxin/mg DNA was resuspended in sterile isotonic saline solution before injection.

Immunization of Mice. Unmanipulated adult mice (>12-wk-old, BALB/c, C57Bl/6, and CB6 F1 mice) were injected intramuscularly in the rear quadriceps with 100 µg of either nCMV-NP encoding the influenza virus nucleoprotein (four mice per group) or nCMV-int as control (three mice per group) in a total volume of 50 µl saline using a 25-gauge needle. Injections were given twice, separated by a two-week interval. Four weeks after the final injection, mice were killed and spleens removed for in vitro restimulation of CTL. Bone marrow chimeric mice (four mice per group) were injected as above, beginning six and eight weeks post bone marrow transplant. Six weeks after the final DNA immunization, mice were killed by cervical dislocation and spleens removed for in vitro restimulation of CTL.

Assay for CTL. Mice were killed by cervical dislocation, their spleens removed and teased apart in RPMI media supplemented with 2% fetal bovine serum (FBS). In 24-well plates (Costar, Cambridge, MA) 7 × 10⁴ responder splenocytes were incubated with 6 × 10⁵ stimulator splenocytes (derived from C57Bl/6 mice or BALB/c mice) in the presence of 50 IU/ml recombinant IL2. The culture media was RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 50 mM 2-mercaptoethanol, and 1% penicillin and streptomycin. The stimulator cells were irradiated (2,000 rads) syngeneic splenocytes pulsed with 4 µg/ml synthetic peptide (Molecular Research Laboratories, Durham, NC). Synthetic NP peptides used were TYQTRALV (restricted to H-2ª) and ASNENMETM (restricted to H-2ª) (33). After 5 d the restimulated cells were harvested and separated from dead cells on a Lympholyte M (Accurate Chemicals, Westbury, NY).
gradient. In 96-well round-bottom plates, target cells were incubated in 200-μl volumes with restimulated T cells at graded effector to target ratios for 4 h. Assay medium used was phenol red-bated in 200-bd volumes with restimulated T cells at graded gradient. In 96-well round-bottom plated and lysed by measuring lactate dehydrogenase release using the Cytotox 96 assay kit (Promega Corp., Madison, WI). Controls were included on each plate for spontaneous LDH release from target and effector cells. Percent lysis was calculated according to the manufacturer’s instructions by a formula that approximates to:

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\frac{(\text{test release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100.
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**Results and Discussion**

**Intramuscular Injection of Plasmid DNA Encoding the Influenza Virus Nucleoprotein (NP) is a Highly Effective Method of Inducing NP-specific CTL.** Intramuscular gene immunization of unmanipulated C57Bl/6, BALB/c or F1 (C57Bl/6 × BALB/c) mice with plasmid DNA encoding the gene for influenza virus nucleoprotein (NP) of A/PR/8/34 influenza strain was highly effective at inducing strong NP-specific CTL (Fig. 1). Groups of four mice of each strain were immunized intramuscularly with either 100 μg of nCMV-NP or control vector, nCMV-int, at weeks 0 and 2. Mice were killed at week 6 and after in vitro restimulation their spleens were assayed for their ability to lyse H-2b target cells pulsed with the H-2Dk-restricted peptide from NP residues 366-374 or H-2d target cells pulsed with the H-2Kd-restricted peptide from NP residues 147-155. A single plasmid encoding NP was able to induce the generation of specific H-2b-restricted CTL in both C57Bl/6 and F1 mice and H-2d-restricted CTL in both BALB/c and F1 mice. The F1 mice were capable of processing the NP antigen such that CTL were generated to both the H-2b- and H-2d-restricted epitopes in the same animal (Fig. 1, A and B). The primary signal for this priming may have been either from the MHC/peptide complexes on the myocytes or from bone marrow-derived APC.

**Intramuscular Plasmid DNA Injection of Parent→F1 Bone Marrow Chimeras Yields CTL Restricted to the Parental (Bone Marrow-derived) Haplotype.** Muscle cells injected with plasmid DNA can produce large amounts of the gene encoded protein (15). The efficacy of intramuscular plasmid DNA injection for inducing antigen-specific CTL combined with the relative paucity of professional APC at the site of injection and an apparent tendency of non-professional APC to induce tolerance rather than immunity, leads to the question of whether muscle cells themselves can act as antigen-presenting cells or whether protein (or DNA) is transferred to hemopoietic cells which then stimulate antigen responsive T cells.

To determine the relative roles of bone marrow-derived APC and muscle cells in the induction of the CTL response to intramuscular immunization with DNA encoding influenza virus nucleoprotein we generated parent→F1 chimeras. F1 hybrid mice were given lethal irradiation to obliterate their immune system, and then reconstituted with T-depleted bone marrow of either parental haplotype (or T-depleted bone marrow from CB6 F1 mice as control). This generated chimeric mice whose muscle cells were of both H-2b and H-2d haplotypes, and whose immune system had the potential to recognize antigens restricted by either haplotype due to the presence of an F1 thymus (34), but whose bone marrow-derived APC could only present antigen in the context of one parental haplotype.

Mice were immunized intramuscularly as described above and six weeks after the final injection were killed, their spleens removed and splenocytes restimulated in vitro before assay for CTL. At the time of death, chimerism was confirmed by cytofluorographic analysis of the splenocytes which showed that all mice had <4% residual recipient’s leukocytes. As shown in Fig. 2, immunization of parent→F1 chimeras with nCMV-NP induced NP-specific CTL that were restricted only to the haplotype found on the parental bone marrow-derived APC and not the alternative haplotype also expressed by somatic cells. This finding was true regardless of whether the bone marrow donor was of H-2b (A) or H-2d (C) haplotype. Mice reconstituted with CB6 bone marrow produced NP-specific CTL restricted to either H-2b or H-2d (B). Control mice injected
In summary, intramuscular injection of plasmid DNA encoding the influenza virus nucleoprotein results in the induction of NP-specific CTL restricted to the MHC class I molecules expressed by bone marrow-derived APC.

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Address correspondence to Helen Tighe, Department of Medicine, University of California, San Diego, Clinical Sciences Building, Room 126, 9501 Gilman Drive, La Jolla, CA 92093-0663.

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