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DNA Vaccination against Persistent Viral Infection

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This study shows that DNA vaccination can confer protection against a persistent viral infection by priming $CD8^+$ cytotoxic T lymphocytes (CTL). Adult BALB/c (*H-2^d*) mice were injected intramuscularly with a plasmid expressing the nucleoprotein (NP) gene of lymphocytic choriomeningitis virus (LCMV) under the control of the cytomegalovirus promoter. The LCMV NP contains the immunodominant CTL epitope (amino acids 118 to 126) recognized by mice of the *H-2^d* haplotype. After three injections with 200 μg of NP DNA, the vaccinated mice were challenged with LCMV variants (clones 13 and 28b) that establish persistent infection in naive adult mice. Fifty percent of the DNA-vaccinated mice were protected, as evidenced by decreased levels of infectious virus in the blood and tissues, eventual clearance of viral antigen from all organs tested, the presence of an enhanced LCMV-specific $CD8^+$ CTL response, and maintenance of memory CTL after clearance of virus infection. However, it should be noted that protection was seen in only half of the vaccinated mice, and we were unable to directly measure virus-specific immune responses in any of the DNA-vaccinated mice prior to LCMV challenge. Thus, at least in the system that we have used, gene immunization was a suboptimal method of inducing protective immunity and was several orders of magnitude less efficient than vaccination with live virus. In conclusion, our results show that DNA immunization works against a persistent viral infection but that efforts should be directed towards improving this novel method of vaccination.

Direct injection of DNA into animals is a novel and promising method for delivering specific antigens for immunization (7, 14, 51, 55, 61). This approach has been successfully used to generate protective immunity against influenza virus in mice and chickens, against bovine herpesvirus 1 in mice and cattle, and against rabies virus in mice (12, 17, 53, 64). In most cases, strong, yet highly variable, antibody and cytotoxic T-cell responses were associated with control of infection. Indeed, the potential to generate long-lasting memory cytotoxic T lymphocytes (CTL) without using a live vector makes this approach particularly attractive compared with those involving killed-virus vaccines and subunit vaccines.

We were interested in determining whether DNA vaccination could induce protective immunity against a disseminated persistent viral infection (6). To address this issue we have used the mouse model of infection with lymphocytic choriomeningitis virus (LCMV) (63). The immune response against LCMV has been extensively studied, and the importance of virus-specific $CD8^+$ CTL in viral clearance has been well established (2, 9, 10, 22, 26, 27, 35, 37, 54, 66, 67). Adult mice infected with the Armstrong strain of LCMV generate a potent $CD8^+$ CTL response and control the infection within 2 weeks. In contrast, adult mice infected with macrophage-tropic LCMV variants such as clone 13 and clone 28b become persistently infected and contain low levels of virus-specific CTL (3–5, 8, 25, 32, 36, 38). Immunity against clone 13 infection can be achieved by vaccination with the Armstrong strain, by adoptive transfer of spleen cells from Armstrong strain-immunized mice, or by using vaccinia virus recombinants expressing the LCMV CTL epitopes (25, 39). These studies show that the presence of memory CTL can confer protection against persistent infection by clone 13. It should be noted that several LCMV CTL epitopes have been identified and that none of them differ between the Armstrong strain and the clone 13 variant. In mice of the *H-2^d* haplotype, the majority of the LCMV-specific CTL response is directed against LCMV nucleoprotein (NP) amino acids 118 to 126, while in *H-2^b* mice, CTL recognize NP amino acids 396 to 404 and glycoprotein amino acids 33 to 41 and 278 to 286 (23, 24, 32, 40, 44–48, 57–59). The extensive knowledge about LCMV immunobiology that is available makes this model particularly instructive for studying DNA vaccination. Here we describe our initial studies concerning immunization of mice with purified DNA expressing the NP of LCMV under the control of the cytomegalovirus (CMV) promoter.

MATERIALS AND METHODS

Mice. For DNA immunization experiments, 6- to 8-week-old BALB/c ByJ (*H-2^d*) mice were purchased from Jackson Laboratory, Bar Harbor, Maine. The LCMV carrier BALB/c colony was bred and established at the University of California at Los Angeles as described previously (21). Six- to twelve-week-old LCMV carrier mice were used as donors of LCMV-infected spleen cells. LCMV-immune mice were made by injecting 6- to 12-week-old BALB/c ByJ mice intraperitoneally with 10^5 PFU of LCMV Armstrong. The mice were killed at 30 to 90 days after infection, and their spleen and lymph node cells were used as LCMV-specific immune cells.

Virus. The Armstrong CA 1371 strain of LCMV (Arm-7) and two variants derived from this virus (clones 13 and 28b) were used (3, 4, 32). Clones 13 and 28b were isolated from the spleens of an 8-week-old BALB/c LCMV carrier mouse and a 12-week-old BALB/c LCMV carrier mouse infected at birth with the Armstrong strain, respectively. Arm-7 was used to make LCMV-immune mice, whereas clone 13 and clone 28b were used to challenge DNA-immunized mice. Virus stocks were triple plaque purified on Vero cells and grown in BHK-21 cells. Stocks at the passage 2 level were used in all experiments. Vesicular stomatitis virus, Mudd-Summers strain of the Indiana serotype, was used for interferon (IFN) assays (a gift from John Holland, University of California at San Diego).

Plasmids. Plasmid pCMVNP was obtained by subcloning the complete genomic sequence of the LCMV NP from plasmid pArmNP-B (a gift from Lindsay Whitton, The Scripps Research Institute, La Jolla, Calif.) into the *Not*I site in the polylinker of plasmid pRcCMV from Invitrogen (San Diego, Calif.). This eukaryotic expression vector contains the CMV early promoter and polyadenylation and 3′ splicing signals from the bovine growth hormone. The inserted 1.7-kb *Not*I fragment from plasmid pArmNP-B comprises the LCMV cDNA sequences corresponding to the NP gene and part of the intergenic region of the small LCMV RNA segment (50). For large-scale preparations of plasmid DNA, transformed *Escherichia coli* TOF 10 bacteria were grown in TB medium

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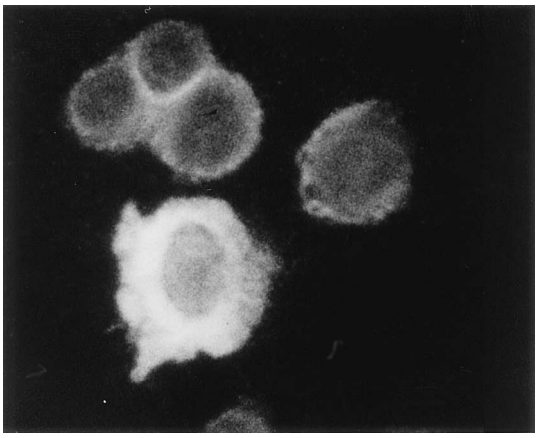

FIG. 1. Expression of LCMV NP in transiently transfected cells. L-929 cells were transfected with 20 μg of NP DNA by $CaCl_2$ precipitation. Forty-eight hours later the cells were fixed in acetone and NP expression was assessed by immunofluorescence staining with guinea pig anti-LCMV serum and a fluorescein isothiocyanate-conjugated secondary antibody. Note the cytoplasmic localization of NP.

(34) in the presence of ampicillin, and plasmids were extracted by the alkaline lysis method followed by two rounds of purification on cesium chloride density gradients (34). DNA concentrations were determined by measuring the optical density at 260 nm, and the integrity of the plasmids as well as the absence of contaminating *E. coli* DNA or RNA was checked by agarose gel electrophoresis. DNA was stored at −20°C in TE (10 mM Tris-HCl, 1 mM EDTA) buffer (pH 8.0). For injections, DNA was diluted in phosphate-buffered saline (PBS) to a final concentration of 2 μg/μl. DNA solutions in PBS were analyzed by agarose gel electrophoresis for degradation and DNA precipitation.

Cell transfection and plasmid expression. L-929 cells expressing the L^d H-2 antigen and NIH 3T3 cells were transfected with pCMV-NP DNA by $CaCl_2$ precipitation as described previously (11), except that the conditions of incubation of the DNA precipitate with the cells were changed to 18 h at 37°C under 5% CO_2. Briefly, 20 μg of plasmid DNA was precipitated with $CaCl_2$ in 2× BBS {50 mM BES [*N,N-bis*-(2-hydroxyethyl)-2-aminoethane sulfonic acid], 280 mM NaCl, 1.5 mM Na_2PO_4} at room temperature; after 20 min, the precipitate was slowly added to the cells and the mixture was incubated for 18 h at 37°C under 5% CO_2 in the presence of freshly changed Dulbecco modified Eagle medium supplemented with 5% fetal calf serum, L-glutamine, and antibiotics. At 48 and 72 h after transfection, cells were harvested by gentle trypsinization and washed in PBS, and 2×10^4 cells were collected into glass coverslips by cytocentrifugation at 500 rpm for 3 min (Cytospin 2, Shandon, Pa.). Expression of the NP gene was monitored by indirect immunofluorescent staining for LCMV antigen with a polyclonal guinea pig anti-LCMV serum (1:200) followed by fluorescein isothiocyanate-conjugated rabbit anti-guinea pig immunoglobulin G (1:1,000) as described previously (32). Plasmids pRcCMV and pCMVCAT (Invitrogen) were used as controls. For chloramphenicol acetyltransferase assays, transfected cells were harvested, washed in PBS, resuspended in Tris buffer, and lysed by 3 cycles of freezing and thawing. Chloramphenicol acetyltransferase assay reactions were performed on 1 μg of total cell lysate protein for 45 min at 37°C, and products were resolved by thin-layer chromatography as described previously (18).

Genetic immunization. Mice were injected three times with 200 μg of pCMVNP DNA or control DNA vector in the quadriceps muscle. At 0, 3, and 6 weeks (see Fig. 2), insulin syringes with 28½-gauge needles were used to inject 100 μl of the DNA solutions intramuscularly into the right legs of anesthetized mice. In one experiment, four mice received two intravenous injections of 200 μg of DNA and one intramuscular injection. Blood was collected from the retro-orbital plexus between DNA injections and at different times after the last DNA injection.

Detection of anti-LCMV antibody. LCMV-specific antibody was quantitated by a solid-phase enzyme-linked immunosorbent assay (ELISA) using plates coated with either purified LCMV virions or LCMV-infected BHK cell lysates (4). Briefly, LCMV antigen-coated 96-well plates were incubated with threefold serial dilutions of serum for 90 min, washed three times with PBS containing 0.05% Tween 20, and reincubated for a further 90 min with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. Plates were washed three times and incubated with 0.1 M sodium citrate buffer (pH 5) containing 2.2 mM *O*-phenylenediamine and 3 mM hydrogen peroxide. The LCMV-specific antibody titer is given as the reciprocal of the highest dilution whose optical density at 492 nm was 0.1 U greater than that of normal BALB/c ByJ mouse serum.

Virus challenge. At 2 to 8 weeks after the third DNA injection, mice were challenged intravenously with 2×10^6 PFU of LCMV clone 13 or 28b.

Virus titration. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (4).

Immunohistochemistry. Immunoperoxidase staining for LCMV antigen was performed as described previously (32). Briefly, 6-μm frozen sections were fixed in acetone and stained with polyclonal guinea pig anti-LCMV serum (1:300) followed by biotinylated goat anti-guinea pig immunoglobulin G (1:75) that had been previously adsorbed on mouse serum. Avidin-biotin-horseradish peroxidase complexes (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.) and the substrate 3-amino-9-ethylcarbazole (Sigma Laboratories, St. Louis, Mo.) were added according to the manufacturers' instructions, and tissues were then counterstained with hematoxylin.

Proliferation assays. For proliferative responses, 5×10^5 spleen cells from experimental mice were cocultured in 96-well plates with 10^5 spleen cells from either LCMV carrier mice or normal BALB/c mice. At different times, cells were pulsed with 1 μCi of [^{3}H]thymidine per well and incubated for a further 18 h. Cells were collected onto glass-wool filters by using a cell harvester (Titertek 530; Flow Laboratories), and the incorporated [^{3}H]thymidine was counted by liquid scintillation.

IFN-γ production assay. Single cell suspensions of spleen cells from experimental mice (8×10^6 cells) were incubated for 5 days with 2×10^6 spleen cells of either LCMV carrier mice or normal uninfected BALB/c ByJ mice in 24-well plates. Serial dilutions of culture supernatants collected at 24, 48, and 72 h were then incubated with L929 cells and tested for their ability to inhibit vesicular stomatitis virus infection as described previously (43). The end point was defined as the highest dilution able to inhibit 50% of the cytopathic effect of vesicular stomatitis virus. Recombinant IFN-γ (Genentech, San Francisco, Calif.) was used as a standard to calculate the units of IFN-γ in test samples.

CTL assays. Primary and secondary LCMV-specific CTL responses in spleens and lymph nodes were determined by a 6-h ^{51}Cr release assay as described previously (4, 5).

In vitro depletion of CD8 cells. Spleen cells of mice previously challenged with LCMV were treated with either rabbit complement alone (Cedarlane Laboratories, Hornby, Ontario, Canada) or an anti-Ly 2.2 (anti-CD8) monoclonal antibody plus complement. The anti-Ly 2.2 monoclonal antibody was purchased in the form of ascites fluid from Cedarlane Laboratories and used at the concentration specified by this supplier. The lymphocytes were then tested as effector cells in a cytotoxicity assay as described above. Specific depletion of the $CD8^+$ T-cell population was assessed by flow cytometry.

RESULTS

Analysis of the anti-LCMV immune response in DNA-vaccinated mice. In order to deliver the LCMV NP into mice by genetic immunization, the LCMV NP gene was subcloned into a mammalian expression vector containing the CMV promoter and the bovine growth hormone polyadenylation sequences. The plasmid obtained, pCMV-NP, expresses the LCMV NP

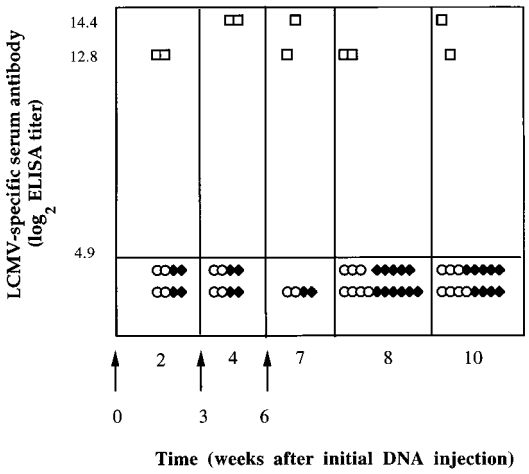

FIG. 2. Lack of seroconversion in DNA-vaccinated mice. BALB/c ByJ mice were injected three times with 200 μg of NP DNA or control DNA vector in the quadriceps muscle. DNA injections, indicated by vertical arrows, were given at 0, 3, and 6 weeks, and the presence of LCMV-specific antibody in the serum was assessed by ELISA at various times after DNA injection. The results from four independent experiments are summarized here. ○, control DNA-injected mice; ◆, NP DNA-injected mice; □, LCMV-immune mice.

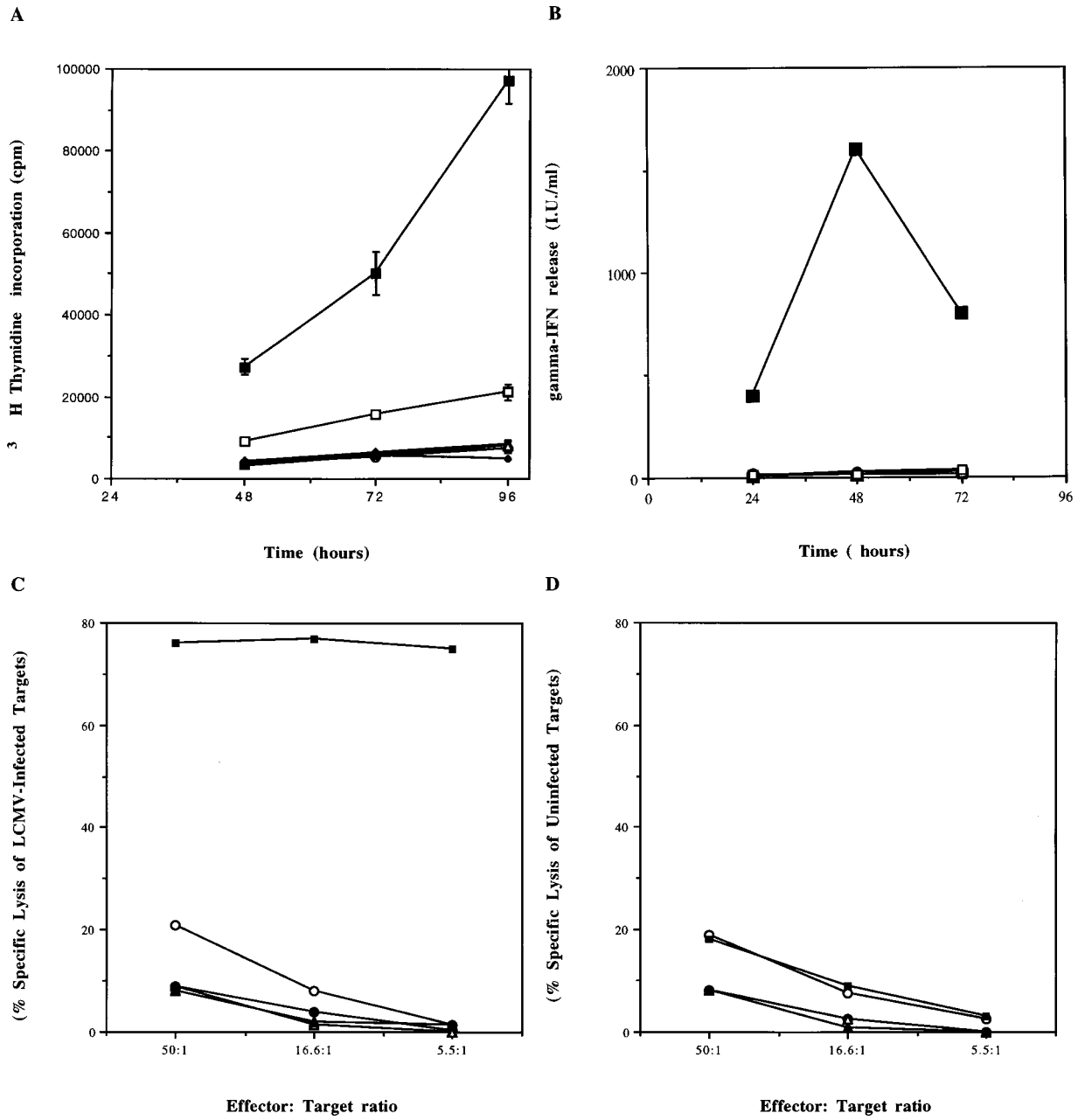

FIG. 3. LCMV-specific T-cell responses are below detection in DNA-vaccinated mice. BALB/c ByJ mice were injected three times with 200 μg of NP DNA or control DNA vector, and at 4 days after the last injection they were tested for LCMV-specific T-cell responses. LCMV-immune mice (~60 days after infection with 10^5 PFU of LCMV Armstrong) were included in all experiments as a positive control. (A) Proliferative response. Spleen cells (5×10^5) from experimental and control mice were stimulated in vitro with 10^5 LCMV carrier or normal spleen cells. Proliferative responses were determined by measuring [^{3}H]thymidine incorporation at 24, 48, and 72 h after stimulation. Cultures were pulsed with 1 μCi of [^{3}H]thymidine per well and harvested 18 h later. Results shown are the average counts per minute for triplicate wells ± standard errors. (B) IFN-γ production. Supernatants were collected at 24, 48, and 72 h after stimulation of spleen cells from experimental and control mice with LCMV carrier spleen cells or normal spleen cells. The supernatants were assayed for IFN activity by their relative abilities to inhibit vesicular stomatitis virus infection of L-929 fibroblast monolayers compared with a recombinant IFN-γ standard. In panels A and B, solid symbols indicate stimulation with LCMV carrier cells and open symbols indicate stimulation with normal cells. Triangles, individual NP DNA-vaccinated mice; circles, control DNA vaccinated-mice; squares, LCMV-immune mice. (C and D) Secondary CTL response. Spleen cells from DNA-vaccinated mice were stimulated in vitro with LCMV carrier cells. Five days later, CTL activity was measured by standard 6-h ^{51}Cr release assays using clone 13-infected BALB CL-7 cells (C) or uninfected BALB CL-7 cells (D) as targets. In panels C and D, individual NP DNA-vaccinated mice are indicated by triangles, control DNA-vaccinated mice are indicated by circles, and LCMV-immune mice are indicated by squares.

upon transient transfection of L-929 cells (Fig. 1) and NIH 3T3 cells (data not shown). The cytoplasmic expression of LCMV NP in transfected cells was intense and could be detected by immunofluorescence staining with either polyclonal anti-LCMV antibody (Fig. 1) or a monoclonal antibody against the LCMV NP (data not shown). On the basis of immunofluorescence staining the level of NP in transfected cells was comparable to that seen in virus-infected cells.

For in vivo studies, purified pCMV-NP (referred to as NP DNA) and control DNA (either pRcCMV, the backbone vec-

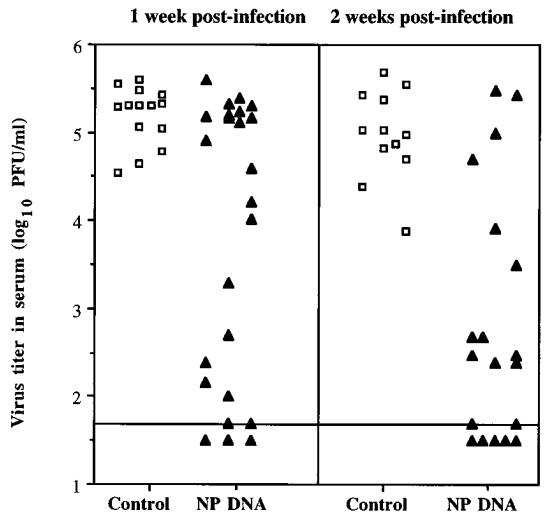

FIG. 4. Control of persistent LCMV infection in DNA-vaccinated mice: serum titers. BALB/c ByJ mice were injected at 0, 3, and 6 weeks with 200 μg of NP DNA (▲) or control DNA vector (□). Mice were challenged intravenously with 2×10^6 PFU of clone 13 or clone 28b at either 2 weeks (7 mice) or 8 weeks (40 mice) after the last DNA injection. Blood was collected from the retroorbital plexus at 1 and/or 2 weeks after virus infection, and the level of infectious LCMV in serum was quantitated by plaque assay on Vero cell monolayers. The horizontal line indicates the limit of detection of the plaque assay (<50 PFU/ml).

tor used for constructing pCMV-NP, or pCMVCAT) were injected intramuscularly into BALB/c ByJ mice. Mice were injected at 0, 3, and 6 weeks, and the presence of LCMV-specific antibody in the sera was checked by ELISA at 2, 4, 7, 8, and 10 weeks (Fig. 2). While antibody titers in the sera of LCMV-immune mice, i.e., mice inoculated intraperitoneally with 10^5 PFU of the Armstrong strain of LCMV, ranged between 7,930 and 21,870, antibody titers in the sera of NP DNA-injected mice could not be detected by this method (titers were <30). In addition, attempts to immunoprecipitate the LCMV NP present in radiolabeled extracts of LCMV-infected cells, by using pooled serum from five DNA-vaccinated mice, were unsuccessful (data not shown).

TABLE 1. Clearance of virus from tissues of DNA-immunized mice challenged with clone 13[a]

Vaccination	Mouse no.	LCMV titer (log_{10} PFU/ml or g of tissue) at 2 wk postinfection				
		Serum	Spleen	Liver	Lung	Brain
NP DNA	1	2.7	5.1	<2.3	5.7	<2.7
	2	2.5	5.8	2.7	4.1	2.7
	3	2.7	3.6	<2.0	3.4	<2.5
	4	<1.7	2.8	<2.2	<3.3	<2.4
	5	2.4	4.4	<2.4	<3.9	<2.6
	6	4.7	7.0	5.9	7.1	5.9
	7	5.4	7.4	6.3	7.8	6.2
Control DNA	1	4.4	6.9	6.4	7.6	5.6
	2	5.4	6.7	6.7	8.3	4.4
	3	5.0	7.0	6.6	8.4	NT[b]
	4	4.8	6.7	6.3	8.0	6.2
	5	4.9	6.6	6.6	7.2	5.9
	6	5.0	6.6	6.3	7.7	6.4

[a] At 8 weeks after the third DNA injection, BALB/c mice were challenged with 2×10^6 PFU of clone 13. Levels of infectious LCMV in the sera, spleens, livers, lungs, and brains were determined by plaque assay on Vero cell monolayers at 2 weeks after infection.
[b] NT, not tested.

TABLE 2. Clearance of virus and presence of memory CTL in DNA-vaccinated mice

Vaccination	Mouse no.	LCMV titer in serum (log_{10} PFU/ml) after virus challenge[a]			LCMV-specific CTL response (lytic units/10^6 cells)[b]
		Day 8	Day 15	Day 35	
Control DNA	1	5.5	5.6	3.7	
	2	5.3	5.7	4.8	2
	3	5.4	3.8	4.0	3
NP DNA	1	4.9	3.9	2.4	15
	2	4.2	<1.7	<1.7	55
	3	<1.7	<1.7	<1.7	80
	4	<1.7	<1.7	<1.7	118
	5	<1.7	<1.7	<1.7	105
LCMV	1	<1.7	<1.7	<1.7	117

[a] Mice were challenged with clone 13 after three DNA injections, and LCMV titers in the sera were determined by plaque assay at days 8, 15, and 35 after virus challenge.
[b] Single cell suspensions of spleen cells from mice sacrificed at day 35 were stimulated with spleen cells from LCMV carrier mice for 5 days, and their cytotoxic activity was determined in a 6-h ^{51}Cr release assay. Spleens from the control DNA-injected mice showed considerable atrophy due to the clone 13 infection. Since the numbers of spleen cells recovered from mice 1 and 2 were very small, the suspensions were pooled for the CTL analysis.

The T-cell response against LCMV was tested between DNA injections, and at different times after the last DNA booster, and compared with the response of LCMV-immune mice. Once again, the anti-LCMV responses in mice injected with NP DNA were below the level of detection and indistinguishable from the responses of mice immunized with control DNA. Figure 3A shows the lymphoproliferative response of spleen cells from NP DNA-injected mice, showing clearly that these cells were unable to proliferate in the presence of LCMV. In addition, while spleen cells from immune mice released IFN-γ after stimulation in vitro with LCMV, the presence of this cytokine was not detected in the supernatants of spleen cell cultures from DNA-injected mice (Fig. 3B). LCMV-specific cytotoxic activity was assessed in a standard 6-h ^{51}Cr release assay after 5 days of restimulation. All our attempts to demonstrate a secondary LCMV-specific CTL activity in spleens and draining lymph nodes from mice injected with NP DNA were unsuccessful. The results of one of these experiments are shown in Fig. 3C and D. Note the potent CTL response generated by virus-immunized mice.

Protection against a persistent infection by LCMV clones 13 and 28b. Although we were unable to directly measure an anti-LCMV immune response in the DNA-vaccinated mice, we next determined whether these mice had any degree of protection against viral challenge. For these experiments, DNA-vaccinated mice were challenged with LCMV variants that persistently infect naive adult mice. As the outcome of infection for the vaccinated mice should be indicative of the capacity of NP DNA to induce priming of LCMV-specific T cells, mice were challenged with 2×10^6 PFU of either LCMV clone 13 or LCMV clone 28b and tested for their ability to control viral infection and prevent the establishment of a persistent infection.

Figure 4 shows LCMV titers in the sera of mice challenged at 2 to 8 weeks after the last DNA injection. As expected, at 1 and 2 weeks after virus challenge, mice previously injected with control DNA were unable to clear the infection and had high

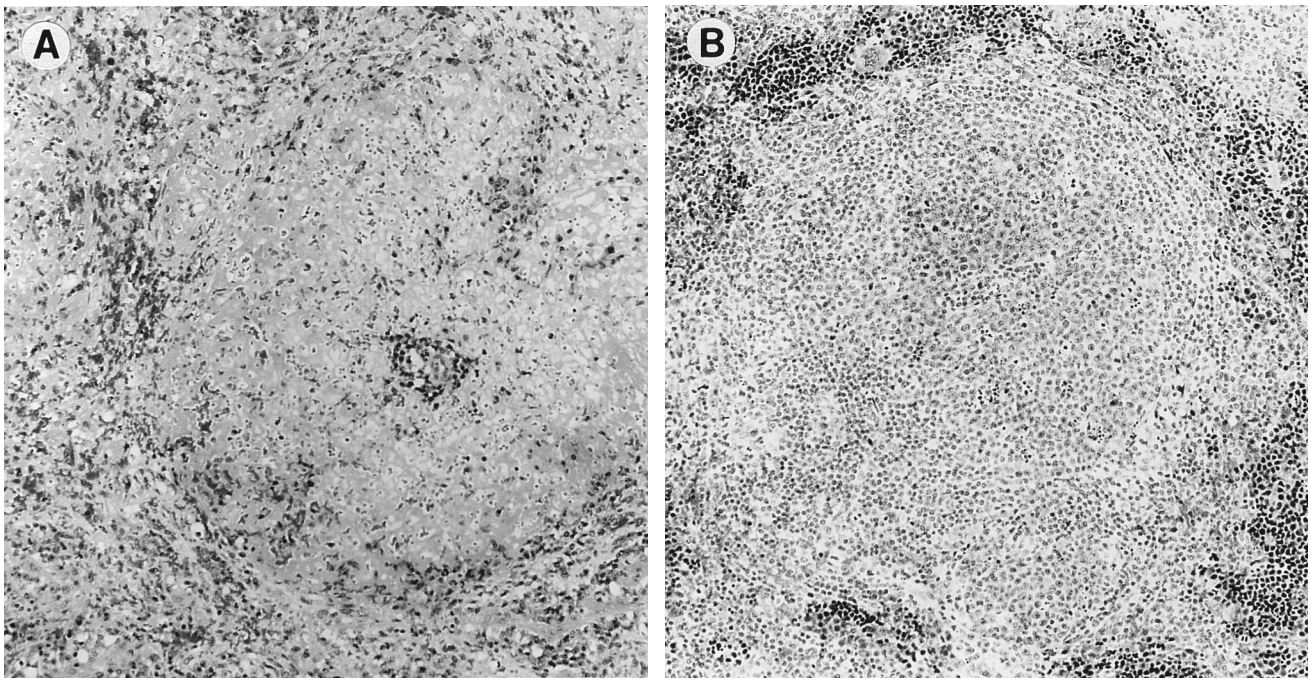

FIG. 5. DNA vaccination protects mice against damage to lymphoid tissue caused by immunosuppressive LCMV variants. Adult BALB/c ByJ mice were immunized with either control DNA or NP DNA and subsequently challenged with clone 13 or 28b. Spleens harvested at days 8 and 15 after viral challenge were fixed in paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. (A) Spleen section from a control DNA-vaccinated mouse at day 8 after clone 28b challenge, showing an empty follicle characteristic of an uncontrolled infection. Note the central artery in the center of the follicle. (B) Spleen section from a mouse protected by the NP DNA immunization at day 15 after clone 13 challenge. Splenic follicles are full and hyperplastic, whereas spleens from unprotected mice continued to show considerable damage. Magnification, ×225.

levels ($>10^4$ PFU/ml) of infectious virus in their sera. In contrast, mice injected with NP DNA showed a heterogeneous pattern of viremia: of 24 mice tested 1 week after challenge, 5 mice had virus levels at or below levels of detection (≤50 PFU/ml), 5 mice had 50- to 200-fold-lower levels of virus, and 2 mice had 10-fold-lower levels of virus than control DNA-vaccinated mice. Virus levels were significantly different between NP DNA- and control DNA-vaccinated mice ($P = 0.027$ by a two-tailed t test). At two weeks postchallenge, 7 of 19 mice had undetectable levels of infectious virus in their sera and 6 of 19 mice had virus titers up to 500-fold lower than virus levels in the sera of control mice. So, in addition to the mice that had low levels of infectious virus in the first week, additional NP DNA-injected mice controlled the infection by the second week. For the remaining mice (6 of 19), virus titers were not significantly different from those of the controls. These results show that DNA immunization induced various degrees of protection in the vaccinated mice. In some mice a sufficient threshold to confer complete protection was achieved, whereas in others the level of immunity generated was able to confer only partial or incomplete protection.

In order to confirm the protective effect of DNA vaccination, virus titers in several tissues were determined (Table 1). Infectious virus levels in the tissues of NP DNA-immunized mice that had controlled infection in their sera were consistently lower than virus levels present in the tissues of control DNA-injected mice. Infectious virus could not be detected in the brains and livers of protected mice, and these mice also showed substantially reduced levels of infectious virus in the lungs (100- to 10,000-fold lower) and spleens (100-fold lower). Virus titers in the tissues of viremic NP DNA-injected mice were comparable to virus levels in control DNA-injected mice (Table 1).

The protective effects of DNA vaccination were also evidenced by histologic evaluation of the spleens. One of the hallmarks of clone 13 and clone 28b infection of adult mice is splenic atrophy, characterized by empty follicles and disruption of normal splenic architecture (31, 52, 63). We examined the spleens of vaccinated mice for these characteristic changes at days 8 and 15 after viral challenge. At day 8 postinfection, the spleens of control DNA-vaccinated mice showed the characteristic drop-out of follicles and damage to splenic architecture (Fig. 5A). In sharp contrast, the spleens of mice protected by DNA immunization showed little damage. At day 15 postinfection, the spleens of control mice continued to show considerable damage, while the spleens of protected mice now had full hyperplastic follicles (Fig. 5B).

Although the DNA-vaccinated mice contained substantially lower levels of infectious virus in their sera and most tissues, high levels of infectious virus were still present in the spleens of these mice at day 15 postinfection (Table 1). In order to see if DNA immunization truly prevented the establishment of a persistent infection as opposed to delaying it, mice showing different levels of protection at days 8 and 15 postinfection were monitored for a longer period. By day 35, NP DNA-immunized mice, which previously showed a reduction of infectious virus in serum, continued to show declining titers in serum, and there was no evidence of enhanced viral replication in their tissues (Table 2 and data not shown). The persistence of viral antigen in the brain and liver was also investigated by staining frozen sections of these tissues with a guinea pig anti-LCMV antibody. LCMV antigen was not detected in the livers or brains of NP DNA-immunized mice, although it was readily detected in those of mice injected with control DNA (Fig. 6).

Control of persistent LCMV infection in DNA-immunized mice is associated with the presence of an LCMV-specific CTL

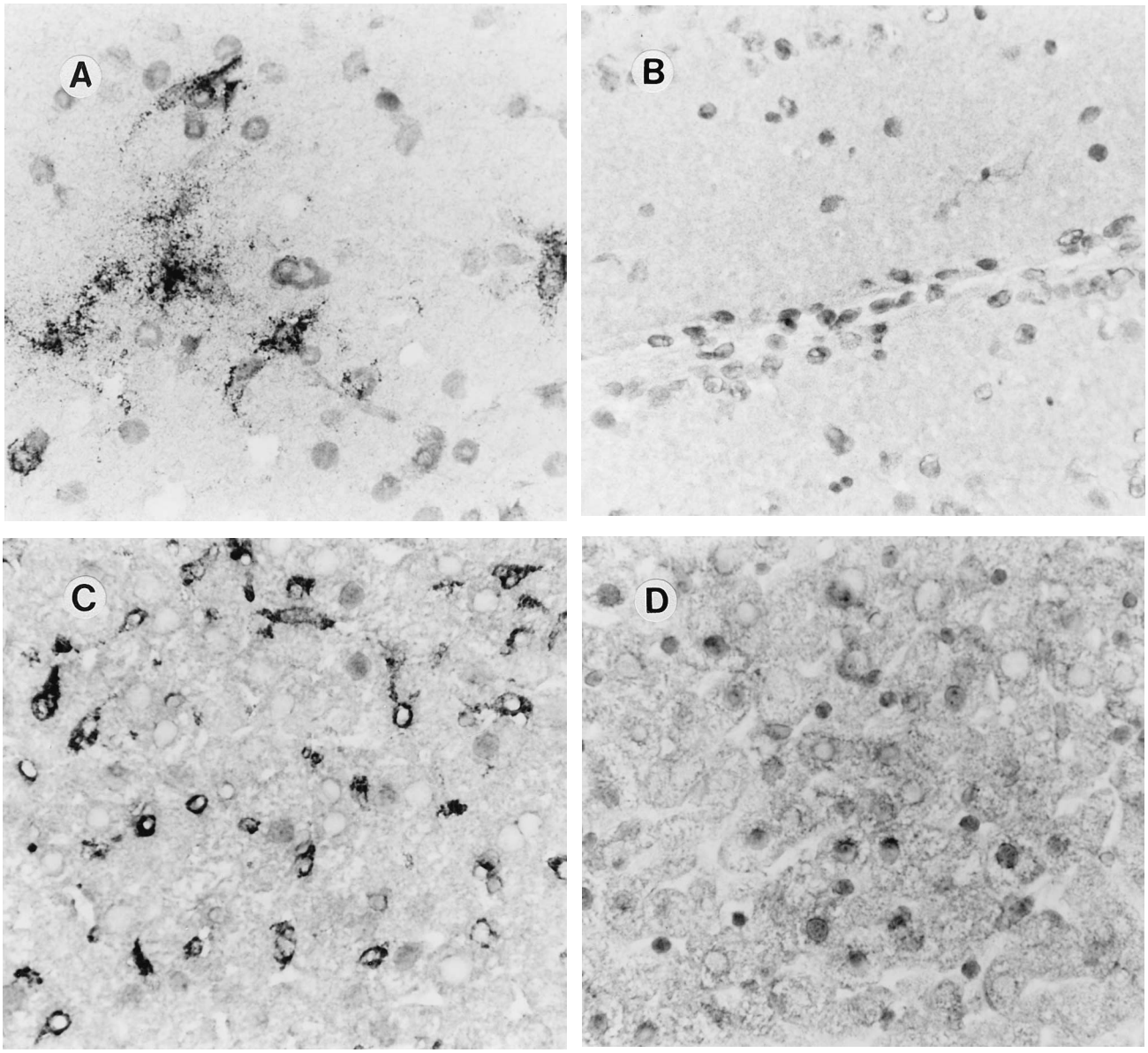

FIG. 6. Absence of LCMV antigen in the brains and livers of DNA-vaccinated mice challenged with clone 13. Immunostaining for LCMV antigen in brain and liver tissue of mice at 35 days after infection with LCMV clone 13. (A) Brain section from a control DNA-vaccinated mouse. Darkly staining cells indicate the presence of LCMV antigen within cells of the brain parenchyma. (B) Brain section from an NP DNA-vaccinated mouse that was protected from the clone 13 challenge, showing the absence of LCMV antigen within the brain. (C) Liver section from a control DNA-vaccinated mouse showing an abundance of LCMV antigen throughout the liver. (D) Liver section from an NP DNA-vaccinated mouse protected from the clone 13 challenge, showing the absence of LCMV antigen in the liver. Magnification, ×900.

response. Persistence of LCMV clone 13 and clone 28b infection in adult mice is associated with the abrogation of the LCMV-specific CTL response. Therefore, DNA-immunized mice challenged with clone 28b were tested for their ability to exhibit a CTL response at day 8 postinfection (Fig. 7). Spleen cells were checked for direct ex vivo CTL activity on syngeneic LCMV-infected targets. A strong LCMV-specific CTL response was detected only in those mice protected by the NP plasmid immunization. Mice injected with control DNA, or mice that were not protected despite the NP DNA injections, had only a weak CTL response against LCMV. The LCMV-specific CTL activity present in the protected mice was abrogated by in vitro depletion (anti-CD8 plus complement) of $CD8^+$ cells, showing that the effector cells were indeed $CD8^+$ T cells. These results documenting an LCMV-specific CTL response, associated with clearance of infection in NP DNA-injected mice, suggest that DNA immunization resulted in priming or activation of LCMV-specific $CD8^+$ T-cell precursors.

Another indication that NP DNA-injected mice controlled the LCMV infection because of previous priming of their $CD8^+$ T cells is the maintenance of a memory CTL response against LCMV (Table 2). At day 35 after infection with clone 13, spleen cells were collected and stimulated in vitro with LCMV for 5 days. The CTL response in DNA-vaccinated mice that had cleared the virus within 1 week was comparable to that observed in the spleens of mice immunized with live virus (80 to 118 and 117 lytic units/10^6 cells, respectively). A less

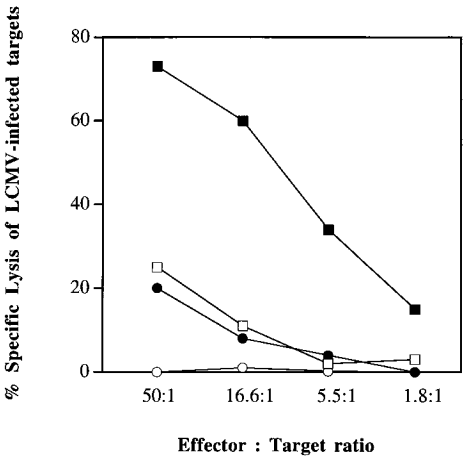

FIG. 7. Enhanced $CD8^+$ LCMV-specific CTL response in DNA-immunized mice after challenge with LCMV clone 28b. Adult BALB/c ByJ mice injected three times at 3-week intervals with either control DNA or NP DNA were challenged with clone 28b at 8 weeks after the last DNA booster. Eight days later spleen cells from the virus-challenged animals were checked for an LCMV-specific CTL response. To identify the effector cells, samples were treated with complement alone or with anti-CD8 plus complement to deplete $CD8^+$ T cells and then checked for CTL activity by a 6-h ^{51}Cr release assay against LCMV-infected or uninfected BALB CL-7 targets. □, NP DNA vaccinated, anti-CD8–complement treated; ■, NP DNA vaccinated, complement treated; ○, control DNA vaccinated, anti-CD8–complement treated; ●, control DNA vaccinated, complement treated. Lysis of uninfected BALB CL-7 targets is not shown (less than 3% at a 50:1 effector/target ratio).

potent secondary CTL response (15 and 55 lytic units/10^6 cells) could also be detected in the spleens of DNA-vaccinated mice that took longer to control the infection (Table 2).

DISCUSSION

This study shows that protection against a persistent viral infection can be achieved by DNA immunization. Despite the lack of a detectable immune response against LCMV after three DNA injections, 50% of the DNA-vaccinated mice were protected against challenge with LCMV variants that establish persistent infection in naive adult mice. In the DNA-vaccinated mice, protection was evidenced by decreased levels of infectious virus in the blood and tissues, undetectable levels of LCMV antigen in the brain and liver at day 35 postinfection, the presence of an enhanced $CD8^+$ CTL response, and maintenance of memory LCMV-specific CTL after clearance of viral infection.

It is most likely that protection in DNA-vaccinated mice was due to priming of LCMV-specific $CD8^+$ CTL precursors. Previous studies have shown that protection against persistent LCMV infection is mediated by virus-specific CTL (25, 39). The presence of memory CTL results in an accelerated response that effectively controls virus infection. Consistent with this was our finding that NP DNA-vaccinated mice that eventually cleared the LCMV clone 13 (and clone 28b) infection exhibited a potent $CD8^+$ CTL response whereas control DNA-vaccinated mice had minimal to undetectable CTL activity. It should be noted that memory CTL do not prevent infection but result in more effective clearance of the infection. The pattern of infection seen in DNA-vaccinated mice was in agreement with this; the "protected" mice also became initially infected, but they were able to eliminate the virus.

It should be emphasized that protection was seen in only ~50% of the mice and that we were unable to detect a virus-specific CTL response in any of the DNA-vaccinated mice prior to LCMV challenge. The inescapable conclusion from these results is that, at least as performed in our study, gene immunization was a suboptimal method for stimulating $CD8^+$ T cells. It was several orders of magnitude less efficient than vaccination with live LCMV. Also, 100% protection is seen in mice vaccinated with vaccinia virus recombinants or *Listeria monocytogenes* recombinants expressing LCMV NP, suggesting that these two delivery systems are, for now, better than purified DNA vaccination in stimulating $CD8^+$ CTL (19, 24, 25, 39, 47, 49). The pattern of immunization obtained here was similar to that obtained after injection of recombinant viral proteins (56).

Given the success of DNA immunization by the intramuscular route in other models of viral infection, particularly in the influenza virus system, it is somewhat surprising that the immune response seen in our DNA-vaccinated mice was so suboptimal (12, 14, 17, 33, 53, 64). It should be noted that the LCMV NP contains an immunodominant CTL epitope that induces a vigorous CTL response (47, 58, 59). Thus, it is unlikely that the suboptimal response seen after DNA vaccination is due to a lack of appropriate CTL epitopes. However, the inability to induce a strong immune response in mice immunized with NP DNA could be due to the expression plasmid that we used. In vitro, the plasmid utilized in our study strongly expresses the NP gene under the control of the CMV promoter. Although we have not determined the level of expression of NP after in vivo transfection, this same promoter was used in the influenza virus studies and in earlier studies using reporter genes, suggesting that this may not be a limiting factor (13, 14, 17, 33, 53, 61). However, in the absence of a direct comparison of our plasmid with the plasmids utilized in other systems, we cannot formally exclude this possibility. It should be noted that the plasmid utilized here does not contain intron A of the CMV enhancer/promoter, a transcriptional element related to increased expression of reporter genes in vivo (30, 33, 53).

A more likely explanation for the suboptimal immune responses that we have observed may be related to the inherent nature of this method of vaccination and the fact that we used a cytoplasmic protein that is not secreted. Despite its successful use in different viral systems, the mechanisms of genetic immunization are not well understood. Delivery of purified DNA by the intramuscular route results in efficient transfection of muscle cells and, perhaps, of limited numbers of antigen-presenting cells present in muscle tissue (13, 17, 33, 53, 61, 64). Several studies have shown that muscle is highly efficient in uptake and expression of injected DNA (1, 15, 30, 33, 60–62). However, muscle cells, as far as we know, do not express major histocompatibility complex class II molecules that are essential for presenting antigen to $CD4^+$ T cells, nor do they express costimulatory molecules such as B7-1/B7-2 that are necessary for optimal priming of $CD8^+$ CTL (16, 20, 28, 29). Thus, it is possible that most of the transfected DNA is essentially wasted in terms of stimulating T-cell responses. Only the small fraction of DNA that is taken up and expressed by antigen-presenting cells residing in muscle tissue may be relevant to the T-cell response. However, if the protein in question is efficiently secreted by the muscle cells, then this problem can be, at least partly, overcome. The secreted protein will be taken up by antigen-presenting cells, processed, and presented to $CD4^+$ T cells. There is some evidence that the influenza virus NP is, in fact, secreted (42). This may explain the better response seen by Montgomery et al. and Ulmer et al. in their experiments with influenza virus NP-vaccinated mice (33, 53). High levels of hepatitis B virus surface antigen (HBS) could be detected in the sera of mice injected with plasmid DNA ex-

pressing HBS; these mice also had high titers of anti-HBS antibody in their sera after DNA immunization (14). We are currently testing this hypothesis by adding a signal sequence to the LCMV NP so that it can be efficiently secreted and then using this modified DNA construct to vaccinate mice.

Finally, variation in the immune response obtained after DNA immunization is not a finding restricted to this work, and the relative inefficiency of in vivo transfection by the intramuscular route is leading to the development of novel methods of DNA immunization: from drugs and composites that enhance DNA uptake (e.g., bipuvocaine and liposomes) to the highly efficient biolistics approach (7, 17, 41, 51, 55, 65). Here we have shown that DNA vaccination can be used successfully against a disseminated persistent viral infection. Since protective immunity was seen in only 50% of the vaccinated mice, there is clearly room for improvement. Particular strengths and appeals of genetic immunization are its simplicity and the relative ease in manipulating the system. Appropriate modification of the plasmid, either by incorporating additional genes, such as those encoding costimulatory molecules, or by adding specific regulatory and signal sequences to the viral proteins, should lead to improved DNA vaccines.

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