JOURNAL OF VIROLOGY, Apr. 1995, p. 2684–2688
0022-538X/95/$04.00+0

Vol. 69, No. 4

DNA Immunization Confers Protection against Lethal Lymphocytic Choriomeningitis Virus Infection†

MASAYUKI YOKOYAMA, JIE ZHANG, AND J. LINDSAY WHITTON*

Department of Neuropharmacology, Scripps Research Institute, La Jolla, California 92037

Received 23 December 1994/Accepted 12 January 1995

DNA vaccination has been evaluated with the lymphocytic choriomeningitis virus (LCMV) model system. Plasmid DNA encoding the LCMV nucleoprotein, when injected intramuscularly, induces both antiviral antibodies and cytotoxic T lymphocytes. Injection of DNA encoding the nucleoprotein or the viral glycoprotein confers protection against normally lethal LCMV challenge in a major histocompatibility complex-dependent manner. The protection conferred is incomplete, but it is most probably mediated by the induced cytotoxic T lymphocytes.

Although remarkably effective, live-virus vaccines have some disadvantages. They may be pathogenic to the normal or to the immunosuppressed recipient; they must be grown in tissue culture, with the associated risk of contamination by adventitious agents; and some are relatively unstable, requiring a "cold chain" to maintain viability during their distribution, and thus are poorly suited to use in underdeveloped countries. These and other problems drive the search for new approaches to vaccination. One recently developed approach is the administration of plasmid DNA by direct inoculation.

Plasmid DNAs injected intramuscularly have been shown to confer protection against orthomyxoviruses (8, 18) and rhabdoviruses (29). Although most studies have relied on intramuscular injection of DNA (4, 8, 10, 16, 27–30), other means of administration are possible, including administration in liposomes (15), intravenous injection in complexes with cationic lipids (31), and attachment to microscopic gold particles which are impelled transdermally into cells (6, 9, 20). Each delivery system has its advantages, but all share the potential benefits of DNA immunization, which mandate its careful and thorough evaluation. First, DNA can be made inexpensively, in large quantities, and at high levels of purity. Second, the vector is unlikely to be, or to become, pathogenic, in contrast to live-virus vaccines. Third, there is little or no immune response to the vector. Thus, there is no antigenic competition, increasing the chance that encoded sequences will be immunogenic. Furthermore, these vectors may be effective in expressing proteins, and inducing immune responses, even in the presence of preexisting host antibodies which would have recognized and possibly eradicated an equivalent live-virus vaccine. Thus, for example, a DNA vaccine encoding measles virus proteins may be useful in infants who carry maternal antimeasles antibodies, in whom traditional measles vaccine would be ineffective.

If DNA vaccines are to be useful, then they should induce effective and long-lasting antiviral immunity, thus mimicking the immune response to live-virus infection. Live-virus vaccines induce both antibody and T-cell responses, and both of these effector arms confer marked antiviral effects. Although the antibody responses induced by vaccination have long been considered the critical factor in antiviral protection, in recent years it has become clear that cytotoxic T-lymphocyte (CTL) responses also are important. Virus-specific T-cell responses comprise both major histocompatibility complex (MHC) class II-restricted $CD4^+$ T cells, most often of helper phenotype, and MHC class I-restricted $CD8^+$ CTLs, which kill infected cells, usually through the action of the protein perforin (12), which is contained in granules and released by these cells. We (13, 14, 17, 23, 24) and others (1, 5, 11, 19, 21) have shown that "CTL vaccines," designed to induce antiviral CTL in the absence of antiviral antibodies, can confer sufficient advantage upon the host to allow protection against diseases caused by both acute (13) and persistent (17) virus infections.

In these studies we used the model system of lymphocytic choriomeningitis virus (LCMV) infection of the mouse. LCMV is the prototype of the arenavirus family, and it has a bisegmented single-stranded ambisense RNA genome (2). The short genomic segment encodes two proteins, the nucleoprotein (NP) and the glycoprotein (GP); the latter undergoes posttranslational modifications and cleavage into its mature virus structural proteins GP1 and GP2 (3). In C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice the CTL response has been precisely mapped to regions of NP and GP. In $H\text{-}2^b$ mice three epitopes have been detected, one on each of the three structural proteins encoded by the S segment (25). In $H\text{-}2^d$ mice the CTL response is centered upon NP residues 119 to 127 (26), with a very minor response to a region of GP1. When inoculated intracranially, LCMV induces a massive lymphocytic response in the choriomeninges (which gives the agent its name) and death supervenes usually at 7 to 8 days postinfection. The disease is immunopathological, and immunosuppression or specific deletion of the CD8 component of the T-cell response can protect against death. Nevertheless, induction of CTLs can protect against this disease, which is itself CTL mediated.

In this report we show that (i) intramuscular injection of a plasmid construct leads to detectable protein expression, (ii) injection of plasmid constructs encoding LCMV NP generates an anti-LCMV antibody response, (iii) injection of such DNA leads also to priming of LCMV-specific CTLs, and finally, (iv) protection is conferred upon a proportion of vaccinated mice by injection of plasmids encoding the LCMV NP or GP; protection is host strain dependent, reflecting the capacity of the vaccinee's MHC molecules to present the administered LCMV sequences. Thus, our studies confirm the potential effective-

* Corresponding author. Mailing address: Department of Neuropharmacology, Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 554-7090. Fax: (619) 554-6466. Electronic mail address (Internet): lwhitton@riscsm.scripps.edu.

† Manuscript number 8385-NP from the Scripps Research Institute.

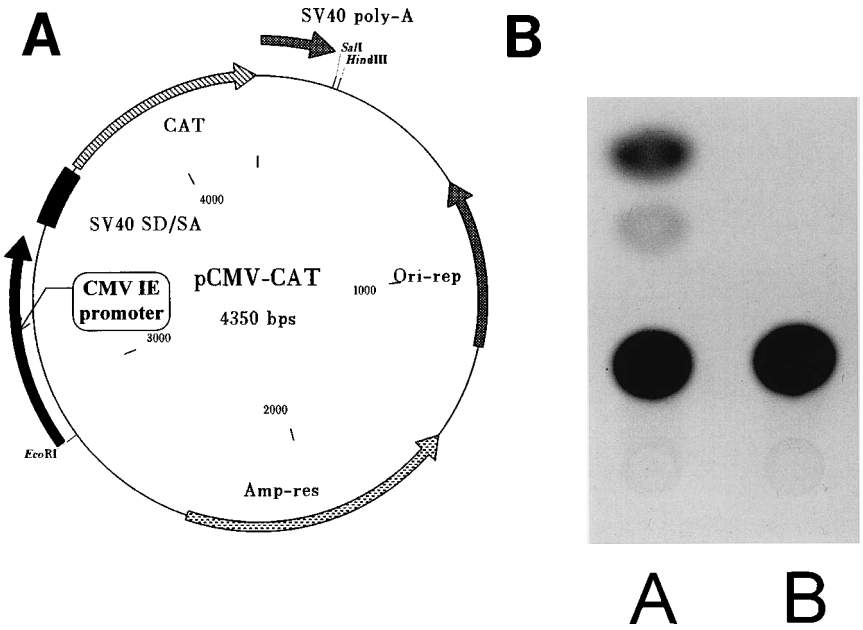

FIG. 1. Plasmids used in these studies and in vivo expression of a marker protein. (A) pCMV-CAT is shown. It is representative of all of the plasmids used; in the other three plasmids the CAT gene was replaced by either the LCMV NP gene or the LCMV GP gene. The simian virus 40 control sequences which flank the gene of interest are shown (SD/SA, splice donor-splice acceptor; poly-A, transcription termination and processing signals), as is the cytomegalovirus immediate-early (CMV IE) promoter. (B) pCMV-CAT was injected as described in the text. At 48 h postinjection, muscle was taken from the injection site (lane A) or the contralateral (noninjected) side (lane B) and processed as described in the text. An autoradiograph is shown, and the results are representative of findings for three mice.

ness of DNA plasmid immunization in antiviral protective vaccination.

Plasmid vector directs protein expression in vivo following intramuscular inoculation. Genes encoding LCMV NP and GP, as well as that encoding the chloramphenicol acetyltransferase (CAT) marker protein, were subcloned into the *Not*I site of plasmid pCMV (derived by excision of the β-galactosidase gene from pCMV-β [Clontech, Palo Alto, Calif.]). This plasmid contains the widely expressed (7) immediate-early promoter from human cytomegalovirus and a splice donor-acceptor site from simian virus 40, which precede the inserted sequences, and a transcription termination-polyadenylation site, which follows them (Fig. 1A). Expression from this plasmid of CAT, LCMV NP, and LCMV GP was confirmed in tissue culture by transient expression assays (data not shown). We used the CAT marker protein to confirm that pCMV could direct the expression of inserted genes when inoculated directly into murine muscle. Mice were injected intramuscularly with a single 100-μg dose of pCMV-CAT in saline, and 2 days later muscle was harvested and a cytoplasmic extract was prepared. Muscle from the injected limb and from the contralateral (noninjected) limb was tested for CAT activity. As shown in Fig. 1B, a high level of CAT activity was detectable in the injected muscle. Thus, our chosen plasmid was able to direct local protein expression, and our transcutaneous injection method was appropriate.

LCMV-specific antibody responses following administration of pCMV-NP. BALB/c mice were injected once, twice, or three times with 100 μg of pCMV-NP. The interval between injections was 14 days. At 14 days following each injection, blood was drawn and serum was prepared. Levels of anti-LCMV antibodies were assessed by an enzyme-linked immunosorbent assay (ELISA) using purified LCMV as the target antigen. In Fig. 2 serum dilutions from 1:10 through to 1:160 are shown. Mice injected twice or three times with pCMV-NP showed

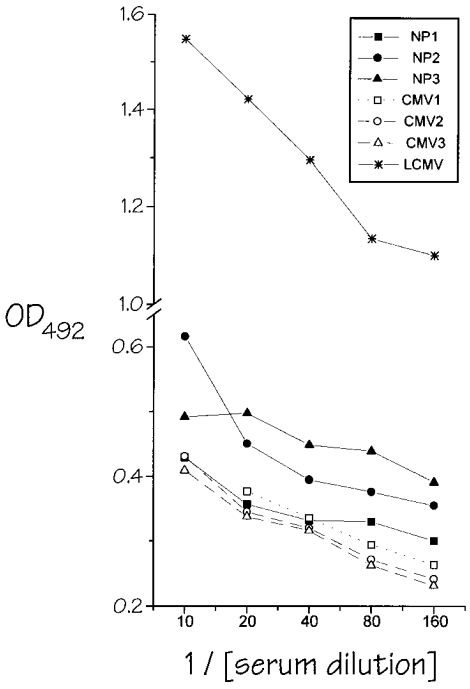

FIG. 2. LCMV-specific antibody levels induced by pCMV-NP injection. For each of two plasmid DNAs (pCMV alone [CMV] or pCMV encoding LCMV NP), groups of four BALB/c mice were injected once, twice, or three times (a total of 24 mice in six groups). Thus, for example, the notation NP2 refers to a group of four mice, each of which received two injections of pCMV-NP. Serum was drawn from all mice, and samples were analyzed individually. A control group of four mice received 2×10^5 PFU of LCMV intraperitoneally. Two weeks following the final injection, serum was drawn for ELISA. Within each group the standard deviation was <5%, and the results for each group therefore were averaged to clarify presentation. The optical density at 492 nm (OD_{492}) is shown on the ordinate, and the reciprocal serum dilution is shown on the abscissa.

elevated titers of antiviral antibody at all dilutions. Additionally, mice inoculated three times with pCMV-NP (NP3) showed higher titers than mice inoculated twice (NP2) or once (NP1), suggesting that modest boosting had occurred. Titers of virus-specific antibody induced by LCMV infection of a naive mouse were markedly higher than those induced by DNA inoculation. Thus, the levels of anti-NP antibody induced by pCMV-NP, while statistically significant ($P < 0.05$ at 1:80 and 1:160), were low compared with levels of antiviral antibodies induced by normal virus infection.

The low levels of antiviral antibody induced by this construct, even in mice receiving three doses, cannot be ascribed to low immunogenicity of NP in BALB/c mice, since LCMV itself and VV-NP induce strong anti-NP antibody responses in this mouse strain (14). Why might the response be so weak? First, LCMV is a cytoplasmic virus, and expression of its RNA sequences within a nuclear environment exposes them to unfamiliar transcriptional and posttranscriptional pathways, which may lead to low-level expression of the LCMV proteins. Second, the proteins encoded by the injected DNAs may not gain access to the appropriate antigen presentation pathways. During most virus infections, viral proteins are released into the extracellular space either through secretion from the infected cell or, very often, following lysis of the cell by the virus or by host antiviral effector mechanisms. These proteins are then available to B cells and to other specialized antigen-presenting cells, which potentially can present them via their MHC class II molecules, inducing helper T cells which may stimulate certain facets of the antibody response. In contrast, the introduction of plasmid DNA and the expression of proteins encoded thereupon may be noncytopathic, and the recipient cells may therefore retain the protein products, outside the reach of B-cell recognition and MHC class II presentation; only if the proteins are cell surface, secreted, or cytotoxic proteins, or if the cell itself dies or is otherwise scavenged by the host, will these plasmid-encoded proteins become available to the class II antigen-processing pathway. Thus, if the proteins are unavailable to the B cells or antigen-presenting cells it is possible that even with high-level intracellular expression of the encoded protein antibody responses would be minimal. To date, many of the proteins which have induced good antibody responses following DNA immunization have been cell surface or secreted proteins (8, 9, 18, 20, 29), although there are exceptions (21, 30). Our results do not allow us to discern the reason for the weak humoral response, and experiments to address these questions are under way.

Plasmid DNA immunization induces virus-specific CTLs. Control of LCMV infection is classically mediated by $CD8^+$ CTLs. We and others have shown that vaccine-induced protection against LCMV challenge also is conferred in large part by this cell type. Consequently it was of interest to determine whether or not the pCMV-NP construct could induce LCMV-specific CTLs. Injection of pCMV-NP plasmid DNA alone did not produce a CTL response detectable at the primary level (data not shown); that is, spleens harvested 7 days following injection with pCMV-NP and tested immediately, without restimulation, did not contain any detectable LCMV-specific CTL activity. Presumably, therefore, any priming of CTLs was at a level sufficiently low to be undetectable by our primary in vitro assay. Therefore, we carried out a secondary in vivo stimulation to determine whether or not mice inoculated with pCMV-NP had been primed for NP-specific CTLs.

CTL activity following LCMV infection of a previously nonimmune mouse peaked at 7 to 9 days postinfection and fell away thereafter. At day 4 postinfection CTL activity was difficult to detect in a previously unprimed animal, but it was readily detectable in an LCMV-immune animal, in which the presence of memory cells allowed an accelerated response to viral challenge. We took advantage of this phenomenon to determine whether intramuscular injection of pCMV-NP had induced CTLs in BALB/c mice. In Fig. 3 the in vivo responses at day 4 postinfection are shown individually for 14 BALB/c mice. Nine mice were immunized by intramuscular injection of pCMV-NP (five receiving a single injection of 100 μg of pCMV-NP and the other four receiving three identical injections, at 2-week intervals). Two mice were immunized with LCMV (2×10^5 PFU intraperitoneally), and three mice were nonimmune controls. At 2, 4, or 6 weeks later the mice were challenged with LCMV, and at 4 days postchallenge they were sacrificed and their spleens were analyzed. The three previously nonimmune mice showed uniformly low CTL activity, and both LCMV-immune mice showed elevated levels of activity (30 to 45% at a 50:1 effector/target ratio). Two of the five mice immunized once with pCMV-NP showed markedly enhanced activity; one mouse had marginally increased activity, and the remaining mice had levels similar to those of the previously nonimmune controls. Three of four triply immunized mice showed strong in vitro cytotoxicity. Thus, intramuscular injection of this plasmid DNA can induce levels of CTLs which, when assayed by this secondary in vivo stimulation, are found to be comparable to those induced by virus infection; but CTL induction does not occur in all recipients. This contrasts with the antibody findings in which equivalent responses were detected in all pCMV-NP-immunized mice. The reason for the sporadic failure of CTL induction, in the face of antibody induction, is not known.

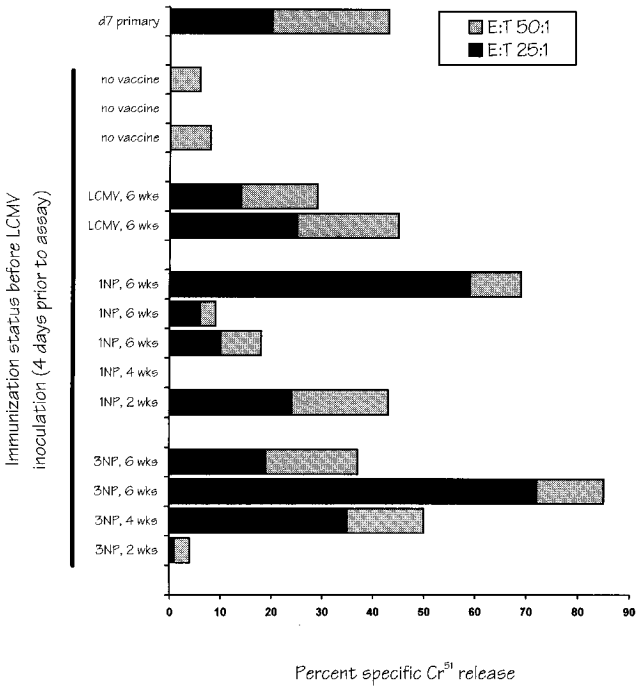

FIG. 3. Intramuscular DNA injection primes for LCMV-specific CTLs. In vitro cytotoxicity of splenocytes harvested 4 days following intraperitoneal injection of 2×10^5 PFU of LCMV. Primary splenocytes harvested from a previously nonimmune mouse 7 days following infection (top bar) served as the in vitro assay control. Positive and negative controls for the in vivo secondary stimulation were two LCMV-immune mice and three nonimmune (no vaccine) mice, respectively. The nine DNA-injected mice received pCMV-NP DNA (100 μg of DNA per intramuscular injection in saline) either as a single injection (1NP) or as three injections at 2-week intervals (3NP). E:T, effector-to-target ratio.

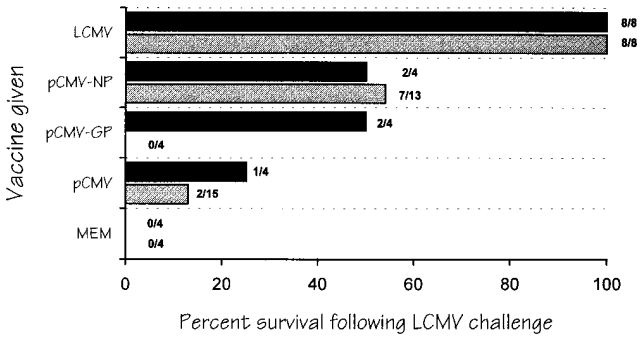

FIG. 4. Protection against normally lethal LCMV challenge conferred by DNA vaccination. BALB/c ($H\text{-}2^{dd}$) (▨) or C57BL/6 ($H\text{-}2^{bb}$) (■) mice were immunized with LCMV; with one, two, or three injections of plasmids encoding either the LCMV NP or the LCMV GP; with one, two, or three injections of a control plasmid, pCMV; or with medium alone (MEM). Six weeks later the mice were challenged with 20 50% lethal doses of LCMV intracranially. The percents surviving mice and the numbers in each group (survivors/total) are shown. There was no statistically significant difference in survival rates among mice receiving one, two, or three doses of plasmid DNA; for each plasmid DNA, therefore, results are combined.

Plasmid DNA inoculation protects against a normally lethal challenge dose of LCMV. BALB/c and C57BL/6 mice were inoculated with a variety of immunogens, and 6 weeks later they were subjected to intracranial challenge with a normally lethal dose of LCMV. As shown in Fig. 4, prior vaccination with intact LCMV conferred complete protection upon both mouse strains. pCMV-NP conferred protection upon approximately 50% of both BALB/c ($H\text{-}2^{d}$) and C57BL/6 ($H\text{-}2^{b}$) mice. LCMV NP contains major CTL epitopes for each of these MHC haplotypes (25). In contrast, a single injection of pCMV-GP conferred protection upon 50% of the C57BL/6 mice injected, but it conferred no protection upon BALB/c mice. LCMV-GP contains two major CTL epitopes for mice of the $H\text{-}2^{b}$ haplotype; it contains only a minor epitope for $H\text{-}2^{d}$ mice (22). Thus, the differing abilities of pCMV-NP and pCMV-GP to protect BALB ($H\text{-}2^{d}$) mice, in contrast to their similar protective efficacies in the case of C57BL/6 ($H\text{-}2^{b}$) mice, are consistent with the theory that protection is CTL mediated. The incomplete protection seen most probably reflects the sporadic failure of CTL induction shown in Fig. 3. The levels of protection afforded by DNA immunization (50%) were less than those seen when whole LCMV (100%; Fig. 4) or recombinant vaccinia viruses (80 to 90% [14, 22, 24]) were used as vaccines.

Our results confirm the theory that antiviral protection can result from this simple vaccine approach. We are currently evaluating various methods to enhance the antibody and CTL immunity and to overcome some of the other perceived defects of the DNA vaccine approach.

We acknowledge the skillful secretarial assistance of T. Calhoun. This work was supported by NIH grants AI-27028 and AI-37186.

REFERENCES

1. **Battegay, M., S. Oehen, M. Schulz, H. Hengartner, and R. M. Zinkernagel.** 1992. Vaccination with a synthetic peptide modulates lymphocytic choriomeningitis virus-mediated immunopathology. J. Virol. **66:**1199–1201.
2. **Bishop, D. H. L., and D. D. Auperin.** 1987. Arenavirus gene structure and organization, p. 5–17. *In* M. B. A. Oldstone (ed.), Current topics in microbiology and immunology, vol. 133. Arenaviruses: genes, proteins and expression. Springer-Verlag, New York.
3. **Buchmeier, M. J., and M. B. A. Oldstone.** 1979. Protein structure of lymphocytic choriomeningitis virus: evidence for a cell associated precursor of the virion glycopeptides. Virology **99:**111–120.
4. **Davis, H. L., M. L. Michel, and R. G. Whalen.** 1993. DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibody. Hum. Mol. Genet. **2:**1847–1851.
5. **del Val, M., H. J. Schlicht, H. Volkmer, M. Messerle, M. J. Reddehase, and U. H. Koszinowski.** 1991. Protection against lethal cytomegalovirus infection by a recombinant vaccine containing a single nonameric T-cell epitope. J. Virol. **65:**3641–3646.
6. **Eisenbraun, M. D., D. H. Fuller, and J. R. Haynes.** 1993. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. DNA Cell Biol. **12:**791–797.
7. **Furth, P. A., L. Hennighausen, C. Baker, B. Beatty, and R. Woychick.** 1991. The variability in activity of the universally expressed human cytomegalovirus immediate early gene 1 enhancer/promoter in transgenic mice. Nucleic Acids Res. **19:**6205–6208.
8. **Fynan, E. F., H. L. Robinson, and R. G. Webster.** 1993. Use of DNA encoding influenza hemagglutinin as an avian influenza vaccine. DNA Cell Biol. **12:**785–789.
9. **Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson.** 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. USA **90:** 11478–11482.
10. **Geissler, E. K., J. Wang, J. H. J. Fechner, W. J. Burlingham, and S. J. Knechtle.** 1994. Immunity to MHC class I antigen after direct DNA transfer into skeletal muscle. J. Immunol. **152:**413–421.
11. **Hany, M., S. Oehen, M. Schulz, H. Hengartner, M. Mackett, D. H. L. Bishop, H. Overton, and R. M. Zinkernagel.** 1989. Anti-viral protection and prevention of lymphocytic choriomeningitis or of the local footpad swelling reaction in mice by immunization with vaccinia-recombinant virus expressing LCMV-WE nucleoprotein or glycoprotein. Eur. J. Immunol. **19:**417–424.
12. **Kägi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner.** 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature (London) **369:**31–37.
13. **Klavinskis, L. S., M. B. A. Oldstone, and J. L. Whitton.** 1989. Designing vaccines to induce cytotoxic T lymphocytes: protection from lethal viral infection, p. 485–489. *In* F. Brown, R. Chanock, H. Ginsberg, and R. Lerner (ed.), Vaccines 89. Modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Klavinskis, L. S., J. L. Whitton, and M. B. A. Oldstone.** 1989. Molecularly engineered vaccine which expresses an immunodominant T-cell epitope induces cytotoxic T lymphocytes that confer protection from lethal virus infection. J. Virol. **63:**4311–4316.
15. **Leibiger, B., I. Leibiger, D. Sarrach, and H. Zuhlke.** 1991. Expression of exogenous DNA in rat liver cells after liposome-mediated transfection in vivo. Biochem. Biophys. Res. Commun. **174:**1223–1231.
16. **Lin, H., M. S. Parmacek, G. Morle, S. Bolling, and J. M. Leiden.** 1990. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation **82:**2217–2221.
17. **Oldstone, M. B. A., A. Tishon, M. Eddleston, J. C. de la Torre, T. A. McKee, and J. L. Whitton.** 1993. Vaccination to prevent persistent viral infection. J. Virol. **67:**4372–4378.
18. **Robinson, H. L., L. A. Hunt, and R. G. Webster.** 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine **11:**957–960.
19. **Schulz, M., R. M. Zinkernagel, and H. Hengartner.** 1991. Peptide-induced antiviral protection by cytotoxic T cells. Proc. Natl. Acad. Sci. USA **88:**991–993.
20. **Tang, D. C., M. DeVit, and S. A. Johnston.** 1992. Genetic immunization is a simple method for eliciting an immune response. Nature (London) **356:**152–154.
21. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martinez, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. A. Liu.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259:**1745–1749.
22. **Whitton, J. L.** 1990. Lymphocytic choriomeningitis virus CTL. Semin. Virol. **1:**257–262.
23. **Whitton, J. L.** 1994. The use of minigenes in antiviral vaccination, p. 429–438. *In* G. P. Talwar, K. V. S. Rao, and V. S. Chauhan (ed.), Recombinant and synthetic vaccines. Narosa Publishing House, New Delhi, India.
24. **Whitton, J. L., N. Sheng, M. B. A. Oldstone, and T. A. McKee.** 1993. A "string-of-beads" vaccine, comprising linked minigenes, confers protection from lethal-dose virus challenge. J. Virol. **67:**348–352.
25. **Whitton, J. L., P. J. Southern, and M. B. A. Oldstone.** 1988. Analyses of the cytotoxic T lymphocyte responses to glycoprotein and nucleoprotein components of lymphocytic choriomeningitis virus. Virology **162:**321–327.
26. **Whitton, J. L., A. Tishon, H. Lewicki, J. R. Gebhard, T. Cook, M. S. Salvato, E. Joly, and M. B. A. Oldstone.** 1989. Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity. J. Virol. **63:**4303–4310.

27. **Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner.** 1990. Direct gene transfer into mouse muscle in vivo. Science **247:**1465–1468.
28. **Wolff, J. A., P. Williams, G. Acsadi, S. Jiao, A. Jani, and W. Chong.** 1991. Conditions affecting direct gene transfer into rodent muscle in vivo. BioTechniques **11:**474–485.
29. **Xiang, Z. Q., S. Spitalnik, M. Tran, W. H. Wunner, J. Cheng, and H. C. Ertl.** 1994. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology **199:** 132–140.
30. **Yankauckas, M. A., J. E. Morrow, S. E. Parker, A. Abai, G. H. Rhodes, V. J. Dwarki, and S. H. Gromkowski.** 1993. Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmid DNA containing NP gene. DNA Cell Biol. **12:**771–776.
31. **Zhu, N., D. Liggitt, Y. Liu, and R. Debs.** 1993. Systemic gene expression after intravenous DNA delivery into adult mice. Science **261:**209–211.