JOURNAL OF VIROLOGY, Oct. 1997, p. 7881–7888 Vol. 71, No. 10
0022-538X/97/$04.00+0

Neonatal DNA Immunization with a Plasmid Encoding an Internal Viral Protein Is Effective in the Presence of Maternal Antibodies and Protects against Subsequent Viral Challenge†

DANIEL E. HASSETT, JIE ZHANG, AND J. LINDSAY WHITTON*

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

Received 9 May 1997/Accepted 10 July 1997

Conventional vaccines are remarkably effective in adults but are much less successful in the very young, who are less able to initiate a mature immune response and who may carry maternal antibodies which inactivate standard vaccines. We set out to determine whether DNA immunization might circumvent these problems. We have previously shown that intramuscular injection of plasmid DNA encoding the nucleoprotein (NP) gene of lymphocytic choriomeningitis virus (LCMV) is capable of inducing immune responses and protecting 50% of adult mice against lethal and sublethal challenge with LCMV. Here we demonstrate that mouse pups injected with the same plasmid hours or days after birth produce major histocompatibility complex-restricted, NP-specific cytotoxic T lymphocytes (CTL) that persist into adulthood; 48% of vaccinated pups responded to subsequent sublethal viral challenge by the accelerated production of anti-NP LCMV-specific CTL, indicating that these animals had been successfully immunized by the plasmid DNA. In addition, these mice showed a >95% reduction in splenic viral titers 4 days postinfection compared to control mice, demonstrating a more rapid control of infection in vivo. Furthermore, pups born of and suckled on LCMV-immune dams (and therefore containing passively acquired anti-LCMV antibodies at the time of DNA inoculation) responded to the DNA vaccine in a similar manner, showing that maternally derived anti-LCMV antibodies do not significantly inhibit the generation of protective immune responses following DNA vaccination. These findings suggest that, at least in this model system, DNA immunization circumvents many of the problems associated with neonatal immunization.

Antiviral vaccination has permitted the eradication of smallpox and the dramatic reduction, at least in the developed countries, of many other viral diseases, including measles and poliomyelitis. One challenge remaining is the successful vaccination of the very young. Neonates and infants are particularly susceptible to viral infection and consequent disease. In developing countries, measles remains a significant threat, being responsible, directly or indirectly, for 1 million to 2 million deaths annually. In addition, diarrheal diseases, many virus induced, are frequently lethal to young children in the absence of supportive therapy, leading to approximately 5 million deaths each year (5, 27, 29). Even in developed countries, infants remain a susceptible target for viruses; respiratory syncytial virus infection is a frequent cause of serious morbidity and sometimes mortality (2).

Several factors contribute to the vulnerability of neonates and infants to virus infection and disease, but arguably the most important, and perhaps the most approachable, is the relative ineffectiveness in this age group of protective antiviral immunization. Vaccination has been attempted but is fraught with difficulties, being hampered by at least two factors. First, the development and maintenance of a cytotoxic T-lymphocyte (CTL) response is critical for protection against many viral pathogens, but the neonatal immune system is functionally immature in this regard. Shortly after birth, the levels of mature T cells in the spleen are 1,000-fold lower than in adults (23), and large doses of antigen, such as those associated with virus infection or live virus vaccines, can overwhelm the developing immune system, leading to the induction of a T-helper type 2 (T_h2) immune response and a consequent inability of neonates to prime protective levels of $CD8^+$ CTL (12, 24).

A second critical factor in neonatal vaccine failure is the presence of maternal antiviral antibodies, transferred in utero or by suckling. Although these antibodies provide short-term passive immunity to the neonate, they can contribute to vaccine failure by inactivating certain live attenuated virus vaccines (28). Maternal antibodies are the primary cause of measles vaccine failure; in one study, successful vaccination occurred in only 38% of infants with preexisting maternal antibody, compared to 100% success in antibody-negative children of the same age (28).

Immunization of adults with plasmid DNA encoding a foreign protein is a recent approach to vaccination and results in the induction of both humoral and cellular immune responses against the encoded protein (reviewed in references 10, 13, and 14). In many cases, these host responses are capable of protecting the vaccinee against subsequent challenge with the relevant pathogen. The need for effective neonatal immunization strategies led us to examine the protective efficacy of plasmid-based immunization of very young animals. We considered that DNA immunization might redress some of the problems associated with neonatal vaccine failure, as intramuscular delivery of plasmid DNA appears to favor induction of CTL and T_h1 responses (11, 21, 22) and permits long-lived low-level expression of foreign proteins (33, 34), thus possibly overcoming the immaturity of the developing neonatal immune system. Furthermore, since the immunizing protein is not present in

* Corresponding author. Mailing address: Department of Neuropharmacology, CVN-9, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 784-7090. Fax: (619) 784-7380. E-mail: lwhitton@scripps.edu.

† Publication no. 10749 NP from the Department of Neuropharmacology of The Scripps Research Institute.

the vaccine preparation, plasmid DNA should not be susceptible to direct inactivation by maternal antibodies. However, a detrimental effect of maternal antibodies remains theoretically possible, since the immunogenicity of the expressed protein might still be diminished or abolished by maternal antibodies.

Our model system is the rodent pathogen lymphocytic choriomeningitis virus (LCMV), the prototypic member of the family *Arenaviridae*. Sublethal systemic infection of immunocompetent adult mice with wild-type LCMV leads to viral clearance and long-term immunity, while intracranial administration to a nonimmune immunocompetent adult mouse leads to a lethal choriomeningitis (8). Infection induces a variety of antiviral effector mechanisms, but protective immunity requires the presence of virus-specific major histocompatibility complex class I-restricted $CD8^+$ T lymphocytes (9, 15, 16, 31, 39, 40). Immunization with LCMV nucleoprotein (NP), expressed by recombinant vaccinia virus or plasmid DNA, can induce anti-LCMV CTL which confer protection on adult mice (31, 36, 37). Although antibody does not seem to play a vital role in virus clearance, maternally derived anti-LCMV antibodies can protect pups (3); however, this passive protection is transient and is greatly diminished by 5 weeks of age (2 weeks postweaning) (3). We have used the LCMV model to evaluate the immunity induced by neonatal DNA immunization in the absence and presence of maternal antibodies. In this report, we show that DNA immunization on the day of birth can prime brisk anti-LCMV CTL responses, which protect against virus challenge. Furthermore, even when immunization takes place in the presence of maternal antibodies, the neonates are primed for CTL and anti-LCMV antibody responses and are protected against viral challenge.

MATERIALS AND METHODS

Mice. All pups were derived from matings between 6- to 16-week-old female and male BALB/cByJ ($H\text{-}2^d$) mice. Immune female mice were generated by intraperitoneal (i.p.) infection with 2×10^5 PFU of LCMV (Armstrong strain). Such mice were not mated until at least 6 weeks later, to permit virus clearance and the development of good titers of antiviral antibodies (4). All animals were obtained from the Scripps Research Institute animal facility.

Plasmid DNA. Plasmids pCMV (derived by excision of the β-galactosidase gene from pCMVβ [Clontech, Palo Alto, Calif.]) and pCMV-NP have been previously described (36). Plasmid pCMV contains the human cytomegalovirus immediate-early promoter and splice donor/acceptor sites and a polyadenylation sequence derived from simian virus 40. Into this plasmid was inserted the full-length LCMV NP gene, to create pCMV-NP. Plasmid DNA was propagated in *Escherichia coli* according to standard methods and purified by using a Nucleobond affinity chromatography column (The Nest Group, Southboro, Mass.) according to the manufacturer's instructions. Endotoxin was removed directly by washing the column-bound DNA with 5% acetic acid in 40% (vol/vol) ethanol according to the column manufacturer's suggestions or following plasmid purification, by treatment of the plasmid DNA with an endotoxin removal system (Qiagen, Chatsworth, Calif.).

Plasmid immunizations and challenge. At the times indicated in the text, neonatal mice derived from and suckled on either nonimmune or immune dams were immunized with a single 25-μl injection containing 50 μg of plasmid DNA dissolved in saline. The DNA was injected into the back of the upper right thigh, using an 18-gauge needle affixed with a 0.2-cm Williams collar. The animals were challenged at 6 to 8 weeks of age with LCMV (Armstrong strain) (2×10^5 PFU i.p.).

Evaluation of NP-specific CTL priming by DNA immunization of young mice. Mice were immunized with DNA as pups and 6 to 8 weeks later were infected with LCMV (2×10^5 PFU i.p.). Spleens were removed 4 days postinfection (p.i.), and a portion was immediately frozen in liquid nitrogen for later virus titration (below). The remainder of each spleen was immediately assayed for cytolytic activity by using a standard chromium release assay (7). Briefly, a single spleen cell suspension was created by gentle Dounce homogenization followed by incubation with 0.83 M NH_4Cl to lyse erythrocytes. Spleen cells were washed twice in complete RPMI (RPMI plus 10% fetal calf serum [FBS], 20 mM L-glutamine, 50 U of penicillin G per liter, and 50 μg of streptomycin sulfate per liter) and resuspended in complete RPMI at a concentration of either 5×10^6 cells/ml (for an effector-to-target [E/T] ratio of 50:1) or 2.5×10^6 cells/ml (for an E/T ratio of 25:1). Target BALB/c17 ($H\text{-}2^d$) or MC57 ($H\text{-}2^b$) cells were incubated for 2 h at 37°C with ^{51}Cr (50 μCi/10^6 cells) and either an $H\text{-}2^d$- or $H\text{-}2^b$-restricted peptide representing an LCMV CTL epitope (sequence ERPQASGVYMGNLT or KAVYNFATCG, respectively). Effector and target cells were incubated together in triplicate wells at both E/T ratios for 5 h in a total volume of 200 μl per well, after which 100 μl of supernatant was harvested and assayed for released radioactivity. Specific chromium release was calculated for each well by using the formula [(sample release − spontaneous release) × 100]/(total release − spontaneous release), and the mean of each triplicate set was determined. Values for total and spontaneous release were obtained by incubation of target cells with and without 1% Nonidet P-40, respectively.

LCMV titrations. Virus titrations were done on Vero cells maintained in medium 199 (Gibco BRL) supplemented with 10% FBS, 20 mM L-glutamine, penicillin G (50 U/liter), and streptomycin sulfate (50 μg/liter). Spleen samples were weighed, thawed, and homogenized in 1 ml of Dulbecco's modified Eagle's medium containing 10% FBS, penicillin G (50 U/liter), and streptomycin sulfate (50 μg/liter) (DMEM). The homogenates were serially diluted in DMEM, and aliquots of each were used to infect Vero cells. After a 1-h absorption at 37°C, the inoculum was removed and the cells were overlaid with medium 199–5% FBS–0.5% agarose (FMC, Rockland, Maine) and incubated for 3 to 4 days at 37°C. The monolayers were fixed in 25% formaldehyde–1× phosphate-buffered saline (PBS; 1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl [pH 7.4]) and stained with 0.05% crystal violet in 1× PBS containing 20% ethanol.

Enzyme-linked immunosorbent assay (ELISA). Serum was obtained from immune and nonimmune dams following weaning of their pups. Ninety-six-well plates (Falcon 3912 Microtest III flexible assay plates) were coated with 100 μl of purified LCMV (200 ng of total protein/well) as antigen (a kind gift of Persephone Borrow). Following overnight incubation at room temperature, the unbound antigen was removed, and the wells were blocked for 1 h at room temperature with blocking buffer (3% bovine serum albumin [fraction V; Sigma, St. Louis, Mo.]–0.2% Tween 20 in PBS) and washed with wash buffer (0.2% Tween 20, 1× PBS). The serum samples were serially diluted in blocking buffer, and aliquots were added to the target plate (100 μl/well). Following a 1-h incubation at room temperature, the liquid was aspirated and the wells were washed three times with wash buffer. Bound immunoglobulin was detected with goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (Sigma A-9917; used at a 1:20,000 dilution, 100 μl/well). After a 1-h incubation at room temperature, the second antibody was removed and the wells were washed three times with wash buffer. Horseradish peroxidase activity was measured by incubation for 30 min with 100 μl of the substrate *o*-phenylenediamine dihydrochloride in 3% hydrogen peroxide (Sigmafast OPD tablets; Sigma) followed by the addition of 1 N HCl (100 μl/well). Absorbance at 492 nm was measured with a Multiscan Plus (Titertek, Flow Laboratories, McLean, Va.).

Statistical analyses. Student's *t* test, and calculation of means and standard errors, were carried out by using Sigmaplot for Windows, version 3.03 (Jandel Scientific, San Rafael, Calif.).

RESULTS

Neonatal immunization with pCMV-NP induces antiviral CTL. LCMV infection of naive adult mice induces a CTL response; virus-specific lytic activity is difficult to detect 4 days p.i. but is sufficiently developed to allow direct detection in splenocytes harvested 7 days p.i. CTL activity declines as virus is cleared, and several weeks later only memory CTL are present; these are not directly demonstrable, and their presence is revealed only following restimulation, which activates them and expands them to levels detectable in an in vitro assay. Such restimulation can be carried out in vitro or in vivo; in our studies, we take the latter course, infecting mice with LCMV and harvesting spleens 4 days later. As stated above, LCMV-specific CTL are undetectable at 4 days p.i. in previously nonimmune mice, but if the mice are immune to the antigen, and therefore contain memory CTL, the response is accelerated and lytic activity is readily detectable at 4 days postchallenge. Therefore, LCMV-specific cytolytic activity seen on day 4 p.i. is evidence of successful priming of an immune response by prior exposure to antigen. We use this approach to determine whether DNA immunization has successfully primed a vaccine for CTL. Thus, mice are immunized with DNA and, after 6 to 8 weeks, are infected with LCMV; 4 days later, splenocytes are harvested and tested for lytic activity.

To test whether DNA immunization shortly after birth could successfully prime an anti-LCMV T-cell response in neonatal mice, individual litters of BALB pups ($H\text{-}2^d$) received a single intramuscular injection of plasmid DNA (encoding either the LCMV nucleoprotein [pCMV-NP] or a vector control [pCMV])

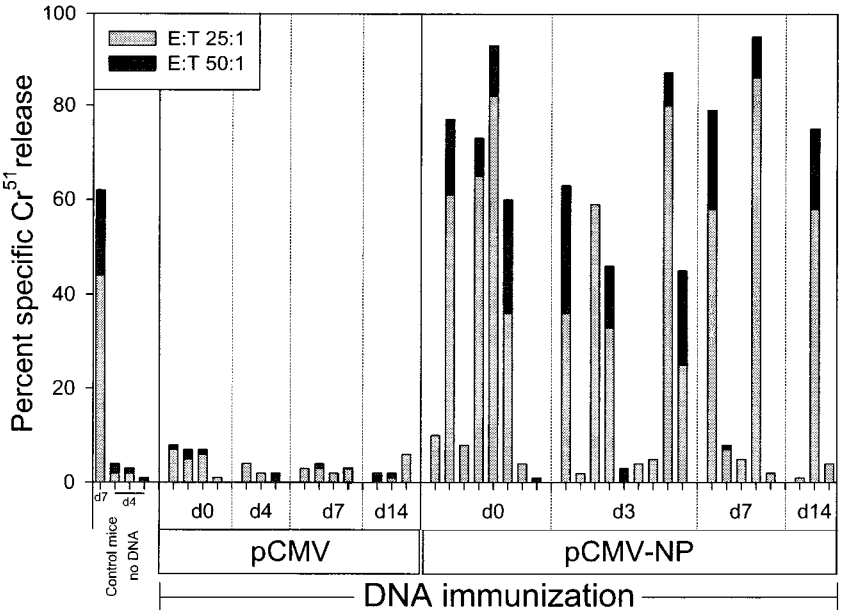

FIG. 1. Neonatal immunization with pCMV-NP induces antiviral CTL. Mice born of and suckled on nonimmune mothers were injected intramuscularly with 50 μg of either pCMV or pCMV-NP plasmid DNA on the indicated days. As additional controls, some nonimmune littermates received no plasmid injections. All mice were challenged with 2×10^5 PFU of LCMV by i.p. injection at 6 weeks of age, and spleens were taken 4 days p.i. (7 days p.i. for the control d7 sample). Splenocytes were tested for in vitro cytotoxicity as described in Materials and Methods, and results are plotted for individual mice.

on the day of birth (d0) or on day 3, 7, or 14 after birth (d3, d7, or d14). Six to eight weeks later, the mice were challenged with 2×10^5 PFU of LCMV i.p. Four days postchallenge, the spleens were removed and examined for CTL activity as described in Materials and Methods. Mice were considered positive for anti-NP CTL if the percent specific chromium release (at an E/T ratio of 50:1) was at least 20% above that seen from control target cells incubated in the absence of lymphocytes. The results are shown in Fig. 1. As expected, previously nonimmune animals did not develop detectable CTL 4 days postchallenge, but by 7 days, CTL responses were readily detectable. None of the 14 mice immunized as neonates with the control plasmid pCMV had detectable NP-specific CTL responses at 4 days p.i. In contrast, 48% (12 of 25) of mice immunized as pups with pCMV-NP showed evidence of NP-specific responses at this time point, indicating that a single injection of plasmid DNA in the first 2 weeks of life can prime a T-cell response. Note that the induced CTL recognize target cells coated with an epitope peptide, indicating that neonatal DNA immunization induces responses with the same epitope specificity as in adult mice. The fraction of pups responding to pCMV-NP did not appear to vary with age at immunization, being similar whether the DNA had been given on d0, d3, d7, or d14. Fifty percent (four of eight) of the pups immunized within hours of birth showed evidence of CTL priming when challenged as adults, demonstrating that DNA immunization in the first few hours of life can prime long-term immune responses against LCMV. Control experiments confirmed that lysis was NP specific and major histocompatibility complex restricted (data not shown). These results are almost identical to those obtained when adult mice were immunized with pCMV-NP; approximately 50% of pCMV-NP-immunized adult animals had significant CTL activity by day 4 postchallenge (36).

Neonatally immunized mice can limit LCMV replication following challenge as adults, and virus titers show an inverse correlation with CTL activity. In Fig. 1, we show that neonatal DNA immunization can prime for anti-LCMV CTL. To evaluate the biological relevance of this immunity, the virus titers in the spleens of the same mice were determined (Fig. 2). Four days p.i., the average titer in the nonimmune mice was 5.38×10^5 PFU/g. Compared to this, 11 of 25 mice immunized as pups with pCMV-NP showed reductions in virus titer of 95% or more, indicating that immunization of young mice induced protective immunity. There is a very strong correlation between the induction of CTL (as demonstrated in Fig. 1) and the ability to control virus replication (Fig. 2). All 11 mice showing >95% reduction in virus titers scored positive for NP-specific lytic activity, while none of the CTL-negative mice achieved this reduction. The average titer in mice inoculated with pCMV-NP but negative for CTL activity is 5.75×10^5 PFU/g (mean of titers in 13 mice), while the average titer in mice in which CTL were detected is reduced by >98% (8.88×10^3 PFU/g; mean of titers in 12 mice). The difference in virus titers between the CTL-positive and CTL-negative mice is statistically significant (t test, $P < 0.00004$).

DNA vaccination in the presence of antiviral maternal antibodies. In addition to the low number of functional T cells in neonates, another factor which often complicates neonatal antiviral immunization is the inactivation of live viruses and some recombinant protein vaccines by maternally derived antibodies (28). Nucleic acid vaccines should not be inactivated in the presence of maternally derived antibody, although the elicitation of an immune response by the expressed protein may be compromised in a manner similar to that seen with certain protein-based vaccines. To determine if anti-LCMV maternal antibodies adversely affect neonatal immunization with pCMV-NP, immune dams were generated by infection of BALB/c females with a nonlethal dose of LCMV at least 6 weeks before mating. Groups of BALB pups born of and suckled on these LCMV-immune dams were immunized with either pCMV or pCMV-NP at various times within the first 2 weeks of life, at which time they have levels of maternal antibody sufficiently high to confer protection against LCMV infection (3). Pups were weaned at 3 weeks of age and were challenged with live

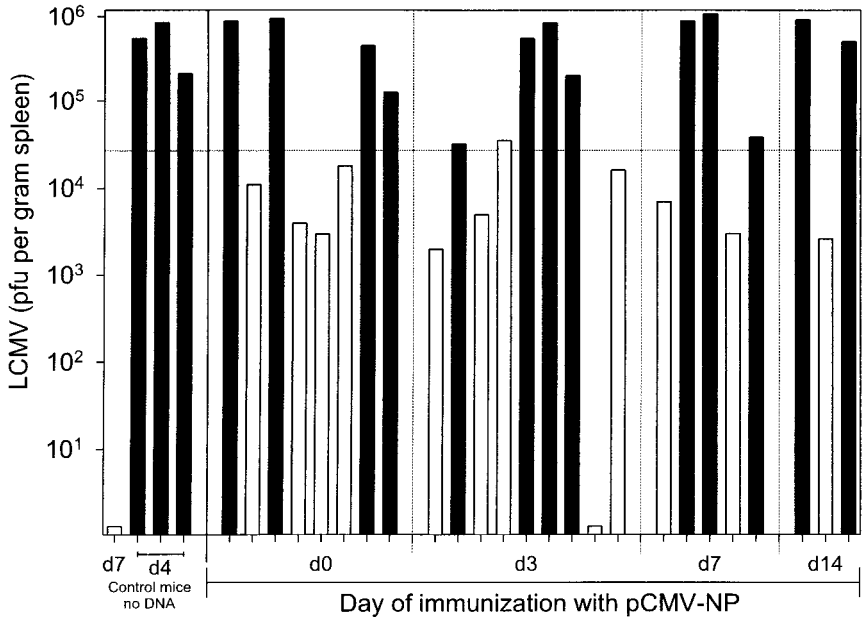

FIG. 2. Neonatally immunized mice can limit LCMV replication following challenge as adults, and virus titers show an inverse correlation with CTL activity. Spleen samples from the pCMV-NP-vaccinated mice represented in Fig. 1 were weighed and titrated on Vero cell monolayers. Results were normalized to PFU per gram of spleen. To facilitate data interpretation, black bars represent individual mice which were shown in Fig. 1 to be negative for CTL activity, while open bars represent CTL-positive mice. The horizontal line at 2.6×10^4 PFU/g represents a 95% reduction in virus titer compared to nonimmune mice.

LCMV 3 to 5 weeks later, by which time the protective effects of maternally derived antibodies had declined (3). High levels of anti-LCMV antibodies were present in the sera of immune dams postweaning (Fig. 3).

DNA immunization of neonatal mice can induce CTL even in the presence of maternal antibodies. CTL priming was evaluated as before, using in vivo restimulation. As shown in Fig. 4, all four of the pups born of and suckled on a nonimmune mother, and vaccinated with pCMV-NP on day 3, responded in the CTL assay, confirming the results shown in Fig. 1. One pup born of and suckled on an immune mother, but receiving no DNA, failed to respond in the CTL assay, indicating that maternal immunity in itself does not prime pups for accelerated anti-LCMV CTL responses. This conclusion was supported and extended by the finding that all of six pups born of and suckled on immune mothers and immunized multiple times with the vector alone (pCMV on d1, d3, d5, and d7) also failed to respond in the CTL assay. Of the 13 animals born of immune dams and immunized with pCMV-NP as neonates, 85% (11 of 13) showed NP-specific cytolytic responses following secondary in vivo stimulation. Thus, pups born of and suckled on immune dams develop active immunity in response to DNA immunization, since a single injection of pCMV-NP plasmid DNA between 1 and 14 days postpartum leads to the development of an accelerated NP-specific immune response upon exposure to the virus.

Enhanced anti-LCMV antibody responses in mice born to immune mothers and immunized as neonates with pCMV-NP. Antiviral antibody titers also were measured in the same mice sacrificed for evaluation of CTL priming (Fig. 4) and virus titers (see Fig. 6). For each mouse, blood was collected, serum was prepared, and the level of anti-LCMV antibody was determined by ELISA. Since these mice were 3 to 5 weeks postweaning, by which time maternal antibody levels have declined (3), any virus-specific antibody should reflect active immunity. To confirm that elevated titers did not result from residual maternal antibody, mice born of immune mothers, but receiving either no DNA or pCMV, were used as controls. As shown in Fig. 5, even previously nonimmune mice had low but detectable amounts of anti-LCMV antibody in their sera 4 days p.i. but mice immunized as neonates with pCMV-NP had increased antibody titers compared to the control groups, suggesting that they had been primed by the prior DNA immunization.

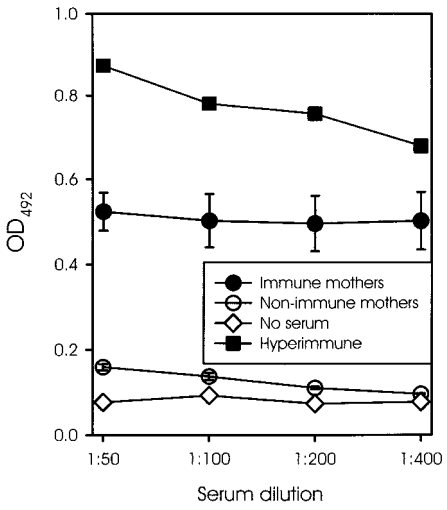

FIG. 3. Anti-LCMV antibody status of dams. Eight-week-old female BALB/c mice were inoculated with LCMV (2×10^5 PFU i.p.), and 6 weeks later, when virus had cleared, were mated with male BALB/c mice. The resulting pups were suckled on the mothers for 3 weeks; immediately postweaning, the dams were bled and their LCMV-specific antibody titers were determined by ELISA. Blood samples also were drawn postweaning from nonimmune dams. Standard error bars are shown. OD_{492}, optical density at 492 nm.

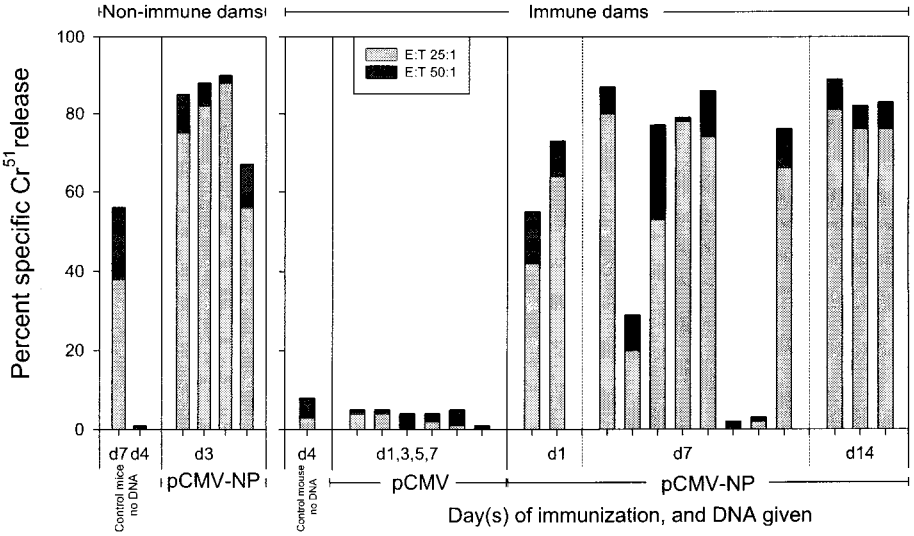

FIG. 4. DNA immunization primes for CTL activity even in pups born of immune dams. Mice born of and suckled on LCMV-immune dams either received no DNA, were immunized with pCMV on d1, d3, d5, and d7, or received a single injection of pCMV-NP on d1, d7, or d14. Mice born of and suckled on naive (nonimmune) dams received no DNA or were immunized with pCMV-NP on d3. Mice which received no DNA served as assay controls. All mice received 2×10^5 PFU of LCMV by i.p. injection at 6 to 8 weeks of age, and spleens were taken on day 4 (with the exception of the d7 mouse, whose spleen was harvested 7 days p.i. as a positive control for the CTL assay). Splenocytes were tested for in vitro cytotoxicity as described in Materials and Methods, and results are plotted for individual mice.

Viral clearance in neonatal mice vaccinated in the presence of antiviral maternal antibodies. Titers in spleens harvested for evaluation of CTL activity (Fig. 4) also were measured to determine the effects of immunization upon LCMV replication. The results are shown in Fig. 6. In total, 85% (11 of 13) of the pCMV-NP-vaccinated mice born of and suckled on immune mothers showed a >95% reduction in viral titers 4 days p.i. compared to the nonimmunized control mice. This result indicates that the pCMV-NP inoculation of neonatal mice, even in the presence of maternal antibodies, can confer protection against subsequent viral challenge. Of the 11 animals with detectable CTL (Fig. 4), 10 showed a reduction in virus titer of >95% (Fig. 6). One animal (represented by the gray bar in Fig. 6) had an in vitro cytolytic activity of 28% (effector/target ratio of 50:1) but did not show evidence of clearance by day 4 p.i. Although this particular animal had the lowest CTL response of any of the CTL-positive mice, it is surprising that the LCMV titer was not lower than that in nonimmune mice. Also, one mouse vaccinated with pCMV-NP on d7 had no demonstrable CTL and yet appeared to clear the infection from the spleen faster than the nonimmune controls.

Taken collectively, these data demonstrate that DNA immunization of neonates and young pups can successfully prime long-term protective antiviral immune responses in individuals immunized either in the presence or in the absence of maternal antibodies.

DISCUSSION

In the last half century, notable advances have been made in public health, reducing both the incidence of and the mortality associated with infectious diseases. None of these advances has been more significant, nor saved more lives, than the development of safe and effective vaccines. Common childhood viral diseases such as measles, poliomyelitis, and smallpox once killed or maimed millions annually. Widespread vaccination programs have reduced the incidence of measles and poliomyelitis in developed countries, while smallpox has been forever removed as a global health threat. However, the high production and distribution costs of many vaccines, the emergence of viruses such as hantavirus, Ebola virus, and human immunodeficiency virus, and the reemergence of bacterial diseases such as cholera and tuberculosis have made the development of new vaccines a public health imperative. Immunization with plasmid DNA offers several advantages over immunization with conventional live virus or protein-based vaccines (reviewed in references 10, 13, and 14).

Because a large percentage of the population at risk for infectious diseases are children, we began an examination of the efficacy of neonatal DNA vaccination. Others have hypothesized that the previous lack of success in neonatal vaccination regimens may have been due in part to large quantities of antigen delivered in the absence of adequate numbers of immune cells (23, 24); by the time the number of immune cells

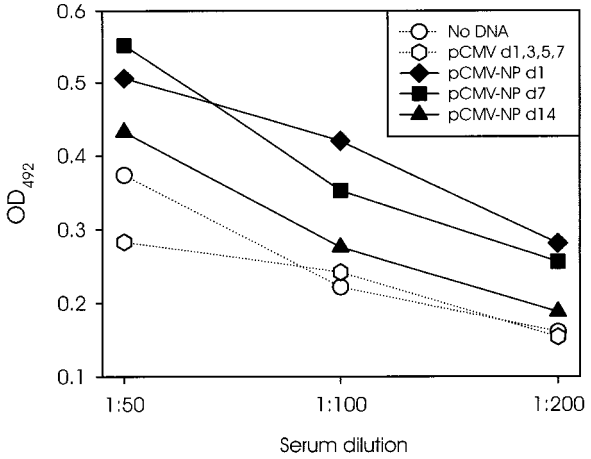

FIG. 5. DNA immunization primes for enhanced antibody responses in pups born of immune dams. Pups born of immune mothers, and immunized at various times as shown, were infected at 6 to 8 weeks of age with LCMV; 4 days later their blood was taken, serum was prepared, and anti-LCMV antibody titers were determined by ELISA. OD_{492}, optical density at 492 nm.

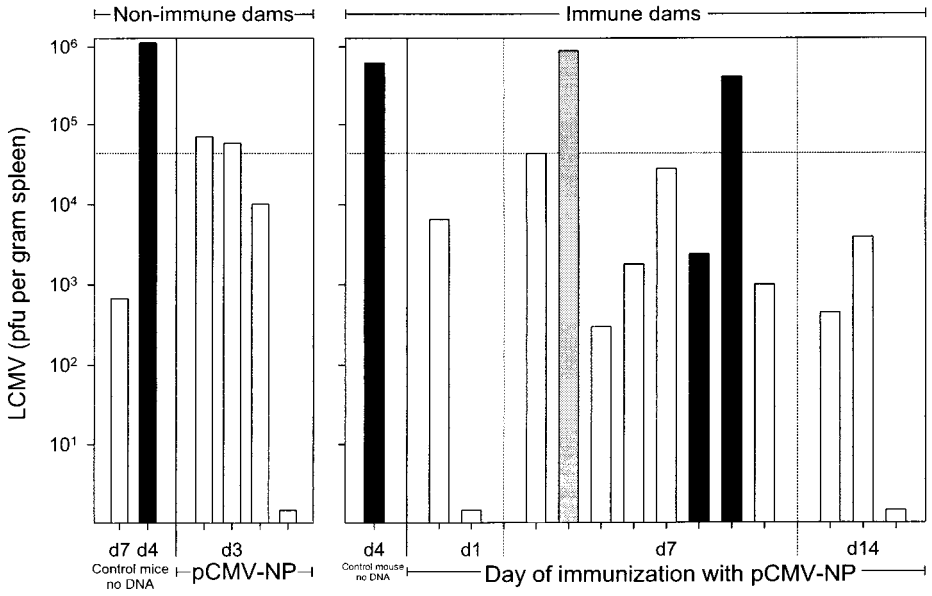

FIG. 6. Viral clearance in neonatal mice vaccinated in the presence of antiviral maternal antibodies. Virus titers were determined in spleen samples from the pCMV-NP-vaccinated mice represented in Fig. 4 and 5. All samples were weighed, and samples were titrated on Vero cell monolayers. Results were normalized to PFU per gram of spleen. To facilitate data interpretation, black bars represent individual mice which were shown in Fig. 4 to be negative for CTL activity, while open bars represent CTL-positive mice. The horizontal line at 4.3×10^4 PFU/g represents a 95% reduction in virus titer compared to nonimmune mice. The gray bar represents the single animal which was positive for in vitro cytotoxicity in Fig. 4 but failed to show reduced viral titer 4 days postchallenge.

had increased to potentially protective levels, the antigen either had already been cleared from the system or, in the case of self-replicating agents, had gained a crucial foothold allowing it to overwhelm the host, inducing tolerance and often resulting in viral persistence. Another factor contributing to neonatal vaccine failure is the presence of maternal antibodies, which can inactivate not only the pathogens but also the vaccines used to confer protective immunity. Thus, the timing of vaccination is crucial. If the vaccine is given too early in life, the immune system may be too immature to respond, and/or maternal antibodies may inactivate the vaccine; but delaying vaccination carries its own risks, since diminishing maternal antibody titers render the infant more susceptible to infection and disease. DNA immunization may circumvent these problems, since low levels of marker protein expression have been detected months after plasmid DNA inoculation (33). Thus, a single injection of plasmid administered shortly after birth should lead to long-term production of small amounts of antigen which, as the immune system matures, may permit induction of immunity against the plasmid-encoded antigen. In addition, plasmid DNA should not be inactivated by maternal antibodies.

The data presented here provide clear evidence that CTL responses can be primed by a single injection of plasmid DNA administered to neonatal mice and that the epitope specificity of the induced response appears the same as in adult mice. Even mice inoculated within hours of birth are successfully immunized, since accelerated antiviral CTL responses can be demonstrated several weeks later in 48% of pCMV-NP-immunized mice (Fig. 1). The immunity induced by neonatal DNA immunization was protective, as judged by decreases in virus titers in the spleens of 48% of the mice (Fig. 2). The correlation between detectable CTL activity and low virus titers was excellent, as might be expected given the critical role played by CTL in vaccine-induced protection against LCMV (15, 25, 26, 31, 32). These results clearly demonstrate that neonatal DNA immunization can induce protective CTL responses. However, the underlying mechanism remains unclear. For example, while injection of pCMV-NP leads to long-term antigen expression and to immunity, it is possible that the immunity is induced almost immediately following injection, despite the immaturity of the neonatal immune system, and that long-term antigen expression is unnecessary. It is also unclear why only 48% of the mice responded to the immunization, but these data are very similar to those obtained in previously published studies on adult DNA immunization against LCMV, in which around 50 to 75% of mice responded (20, 35–38). In light of the strong similarity in the rates of successful vaccination in adults and neonates, it appears unlikely that the vaccination failures in the present study reflect a specific defect in the ability to immunize young animals. The lack of a demonstrable protective immune response in half of the neonates (this study) or adults (other studies) immunized with pCMV-NP remains unexplained; a more detailed understanding of the mechanisms underlying DNA immunization is necessary before the technique can be rationally refined to achieve protection levels approaching 100% in the LCMV model system.

DNA immunization appears effective even when given to pups born of and suckled on immune dams. The mothers carry high levels of anti-LCMV antibodies which persist until after weaning (Fig. 3); these antibodies are transferred to the pups and can enhance recovery until approximately 2 weeks postweaning (3). However, the pups immunized with pCMV-NP show accelerated CTL (Fig. 4) and antibody (Fig. 5) responses and improved control of LCMV replication (Fig. 6). Our results with the LCMV nucleoprotein show that it is possible to design a DNA immunization approach that can successfully immunize infants in the presence of antiviral maternal antibodies. This is an important finding. For example, in humans, the optimal age for measles vaccination is debatable. Early studies suggested that immunization should be initiated only after 12 to 15 months (1, 28). However these recommendations were based on studies of babies born of mothers who had been naturally infected with measles virus and who therefore had

high levels of serum antibody; these high maternal levels were reflected in high levels of antibody passively transferred to the fetus or neonate. In general, children born of vaccinated mothers have, at a given age, lower levels of acquired antibody than children born of naturally infected mothers (17). In the United States, widespread measles vaccination began in 1963; therefore, most females currently of child-bearing age have been immunized by vaccination rather than by natural infection and have lower antibody levels than the older cohort of mothers; their children will therefore have lower levels of transferred antibody and will become susceptible to infection (and open to successful vaccination) sooner than children of older (naturally immune) mothers. The vaccine seroconversion rate in 9-month-old children was 93% when mothers had been born after 1963 and only 60% when the maternal birth date was 1957 or before (17). The earlier decline of passive immunity in children of vaccinated mothers has led to recommendations for earlier immunization, to avoid leaving a window of opportunity for measles virus. However, early administration of standard vaccines may result in vaccine failure for the reasons stated above. Our studies suggest that DNA immunization may overcome these problems by allowing vaccination of neonates, in the presence of maternal antibodies, thus permitting the development of protective immunity.

To our knowledge, there have been four published attempts to induce immunity in neonates by immunization with plasmid DNA. Wang and coworkers, using a plasmid expressing a rabies virus glycoprotein, examined the antibody and T_h-cell responses in mice inoculated within 24 h of birth (30). Plasmid DNA successfully primed B-cell and T_h-cell responses, and long-term antibody production, in vaccinated mice. CTL responses, and the effects of maternal antibodies, were not evaluated. A second study, using the influenza virus model system, showed clearly that CTL could be induced, but again the effect of maternal antibodies was not studied (6). These findings are consistent with our own. The two remaining studies, however, gave contrasting results. In the third study, an inhibitory effect of maternal antibodies was identified (18). Vaccination of neonatal swine born of nonimmune sows with a plasmid expressing pseudorabies virus (PRV) glycoprotein D induced low levels of anti-PRV antibodies. In contrast, when piglets from immune sows were inoculated with DNA, no antibody responses were detected, suggesting that colostral antibodies had inhibited DNA immunization; upon subsequent PRV challenge, the piglets (i) failed to show an accelerated antibody response, confirming the failure of DNA immunization to circumvent the effect of maternal antibodies, and (ii) succumbed, showing that protective immunity was not induced by the plasmid DNA. One possibly critical difference between this study and our own is the nature of the encoded protein; ours is internal, while the porcine study used a cell surface protein. In the fourth study, vaccination of neonatal mice used a plasmid expressing a malaria circumsporozoite protein previously shown to protect adult mice from challenge with malaria. Not only did the mice immunized at 2 to 5 days of age show no evidence of immunity; all vaccinated neonates instead showed evidence of tolerance typified by a lack of antibody, cytokine, or CTL responses when reinoculated with the plasmid DNA. Interestingly, soluble circumsporozoite protein induced antibody responses in these mice (19).

It is unclear why immunization with plasmid DNA could induce such widely disparate responses among neonatal vaccinees. Differences in antigenicity, susceptibility to tolerance induction, or inactivation by maternal antibodies between different antigens may account for the differential successes in inducing immunity. These results underscore the need for a systematic examination of the general mechanisms underlying DNA immunization in order to decipher why the induction of immunity is only sporadically successful.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI-37186 to J.L.W. and NIH training fellowship AI-07354 to D.E.H.

REFERENCES

1. **Albrecht, P., F. A. Ennis, E. J. Saltzman, and S. Krugman.** 1977. Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. J. Pediatr. **91:**715–718.
2. **Anderson, L. J., R. A. Parker, and R. L. Strikas.** 1990. Association between respiratory syncytial virus outbreaks and lower respiratory tract deaths of infants and young children. J. Infect. Dis. **161:**640–646.
3. **Baldridge, J. R., and M. J. Buchmeier.** 1992. Mechanisms of antibody-mediated protection against lymphocytic choriomeningitis virus infection: mother-to-baby transfer of humoral protection. J. Virol. **66:**4252–4257.
4. **Baldridge, J. R., T. S. McGraw, A. Paoletti, and M. J. Buchmeier.** 1997. Antibody prevents the establishment of persistent arenavirus infection in synergy with endogenous T cells. J. Virol. **71:**755–758.
5. **Bern, C., J. Martines, I. de Zoysa, and R. I. Glass.** 1992. The magnitude of the global problem of diarrhoeal disease: a ten-year update. Bull. W.H.O. **70:**705–714.
6. **Bot, A., S. Bot, A. Garcia-Sastre, and C. Bona.** 1996. DNA immunization of newborn mice with a plasmid-expressing nucleoprotein of influenza virus. Viral Immunol. **9:**207–210.
7. **Brunner, K. T., J. Mauel, J. C. Cerottini, and B. Chapuis.** 1968. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology **14:**181–196.
8. **Buchmeier, M. J., R. M. Welsh, F. J. Dutko, and M. B. A. Oldstone.** 1980. The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv. Immunol. **30:**275–331.
9. **Byrne, J. A., and M. B. A. Oldstone.** 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus in vivo. J. Virol. **51:**682–686.
10. **Donnelly, J. J., J. B. Ulmer, and M. A. Liu.** 1997. DNA vaccines. Life Sci. **60:**163–172.
11. **Feltquate, D. M., S. Heaney, R. G. Webster, and H. L. Robinson.** 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J. Immunol. **158:**2278–2284.
12. **Forsthuber, T., H. C. Yip, and P. V. Lehmann.** 1996. Induction of TH1 and TH2 immunity in neonatal mice. Science **271:**1728–1730.
13. **Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson.** 1995. DNA vaccines: a novel approach to immunization. Int. J. Immunopharmacol. **17:**79–83.
14. **Hassett, D. E., and J. L. Whitton.** 1996. DNA immunization. Trends Microbiol. **4:**307–312.
15. **Klavinskis, L. S., J. L. Whitton, E. Joly, and M. B. A. Oldstone.** 1990. Vaccination and protection from a lethal viral infection: identification, incorporation, and use of a cytotoxic T lymphocyte glycoprotein epitope. Virology **178:**393–400.
16. **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.
17. **Markowitz, L. E., P. Albrecht, P. Rhodes, R. Demonteverde, E. Swint, E. F. Maes, C. Powell, and P. A. Patriarca.** 1996. Changing levels of measles antibody titers in women and children in the United States: impact on response to vaccination. Pediatrics **97:**53–58.
18. **Monteil, M., M. F. Le Potier, J. Guillotin, R. Cariolet, C. Houdayer, and M. Eloit.** 1996. Genetic immunization of seronegative one-day-old piglets against pseudorabies induces neutralizing antibodies but not protection and is ineffective in piglets from immune dams. Vet. Res. **27:**443–452.
19. **Mor, G., G. Yamshchikov, M. Sedegah, M. Takeno, R. Wang, R. A. Houghten, S. Hoffman, and D. M. Klinman.** 1996. Induction of neonatal tolerance by plasmid DNA vaccination of mice. J. Clin. Invest. **98:**2700–2705.
20. **Pedroza Martins, L., L. L. Lau, M. S. Asano, and R. Ahmed.** 1995. DNA vaccination against persistent viral infection. J. Virol. **69:**2574–2582.
21. **Pertmer, T. M., T. R. Roberts, and J. R. Haynes.** 1996. Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. J. Virol. **70:**6119–6125.
22. **Raz, E., H. Tighe, Y. Sato, M. Corr, J. A. Dudler, M. Roman, S. L. Swain, H. L. Spiegelberg, and D. A. Carson.** 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc. Natl. Acad. Sci. USA **93:**5141–5145.
23. **Ridge, J. P., E. J. Fuchs, and P. Matzinger.** 1996. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science **271:**1723–1726.
24. **Sarzotti, M., D. S. Robbins, and P. M. Hoffman.** 1996. Induction of protec-

tive CTL responses in newborn mice by a murine retrovirus. Science **271:** 1726–1728.
25. **Schulz, M., P. Aichele, M. Vollenweider, F. W. Bobe, F. Cardinaux, H. Hengartner, and R. M. Zinkernagel.** 1989. Major histocompatibility complex-dependent T cell epitopes of lymphocytic choriomeningitis virus nucleoprotein and their protective capacity against viral disease. Eur. J. Immunol. **19:**1657–1668.
26. **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.
27. **Snyder, J. D., and M. H. Merson.** 1982. The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data. Bull. W.H.O. **60:**605–613.
28. **Stewien, K. E., V. Barbosa, O. S. de Lima, and K. Osiro.** 1978. The influence of maternally derived antibody on the efficacy of further attenuated measles vaccine. Infection **6:**207–210.
29. **Walsh, J. A., and K. S. Warren.** 1979. Selective primary health care: an interim strategy for disease control in developing countries. N. Engl. J. Med. **301:**967–974.
30. **Wang, Y., Z. Xiang, S. Pasquini, and H. C. Ertl.** 1997. Immune response to neonatal genetic immunization. Virology **228:**278–284.
31. **Whitton, J. L.** 1990. Lymphocytic choriomeningitis virus CTL. Semin. Virol. **1:**257–262.
32. **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.
33. **Wolff, J. A., J. J. Ludtke, G. Acsadi, P. Williams, and A. Jani.** 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Hum. Mol. Genet. **1:**363–369.
34. **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.
35. **Yokoyama, M., D. E. Hassett, J. Zhang, and J. L. Whitton.** 1997. DNA immunization can stimulate florid local inflammation, and the antiviral immunity induced varies depending on injection site. Vaccine **15:**553–560.
36. **Yokoyama, M., J. Zhang, and J. L. Whitton.** 1995. DNA immunization confers protection against lethal lymphocytic choriomeningitis virus infection. J. Virol. **69:**2684–2688.
37. **Yokoyama, M., J. Zhang, and J. L. Whitton.** 1996. DNA immunization: effects of vehicle and route of administration on the induction of protective antiviral immunity. FEMS Immunol. Med. Microbiol. **14:**221–230.
38. **Zarozinski, C. C., E. F. Fynan, L. K. Selin, H. L. Robinson, and R. M. Welsh.** 1995. Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle bombardment with DNA encoding an internal virion protein. J. Immunol. **154:**4010–4017.
39. **Zinkernagel, R. M., and P. C. Doherty.** 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature **248:**701–702.
40. **Zinkernagel, R. M., and P. C. Doherty.** 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. Adv. Immunol. **27:**51–177.