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Intramuscular injection of BALB/c mice with a DNA plasmid encoding nucleoprotein (NP) from influenza virus A/PR/8/34 (H1N1) provides cross-strain protection against lethal challenge with influenza virus A/HK/68 (H3N2). CTL specific for the H-2Kd-restricted epitope NP147–155 are present in these mice and are thought to play a role in the protection. To assess the effectiveness of NP DNA immunization in comparison with influenza virus infection in the induction of CTL responses, we monitored the frequency of CTL precursors (CTLp) in mice following i.m. injection with NP DNA or intranasal infection with influenza virus and showed that the CTLp frequency in NP DNA-immunized mice can reach levels found in mice that had been infected with influenza virus. We also measured the CTLp frequency, anti-NP Ab titers, and T cell proliferative responses in mice that were injected with titrated dosages of NP DNA and documented a correlation of the CTLp frequency and the Ab titers, but not proliferative responses, with the injection dose. Furthermore, we observed a positive correlation between the frequency of NP147–155 epitope-specific CTLp and the extent of protective immunity against cross-strain influenza challenge induced by NP DNA injection. Collectively, these results and our early observations from adoptive transfer experiments of in vitro activated lymphocytes from NP DNA-immunized mice suggest a protective function of NP-specific CTLp in mice against cross-strain influenza virus challenge. The Journal of Immunology, 1999, 162: 4163–4170.

The ability of CD8+ CTL to recognize viral Ags in the form of 8- to 11-amino acid peptides presented by MHC class I molecules on the cell surface (1–3) has suggested that they play a role in the clearance of intracellular virus during a viral infection (4–6). The role of CD8+ T cell responses in immunity against influenza virus in mice was established by early studies of adoptive lymphocyte transfer (7–10). It also has been demonstrated that CD4+ T cell responses can be induced against influenza virus infection, and in many cases these CD4+ T lymphocytes are protective when adoptively transferred into naive mice (11, 12). In some instances, CD4+ MHC class II-restricted CTL have been observed (6, 12). Therefore, although the specific in vivo effector functions of CD8+ or CD4+ T cells are still topics for debate, cell-mediated immunity, especially the cytotoxic T cell response, apparently plays a role in the clearance of influenza virus infection in mice (6, 13).

DNA vaccination represents a novel method for induction of both humoral and cellular immune responses (14). Intramuscular injection of naked plasmid DNA results in efficient expression of Ag in muscle cells (15). The initiation of immune responses, especially CTL responses, requires the participation of bone marrow-derived professional APCs (16–18) and may include an Ag transfer process from muscle cells to professional APCs, similar to cross-priming (17). Other studies from our laboratory demonstrated that i.m. injection of a plasmid DNA encoding influenza virus nucleoprotein (NP), derived from A/PR/8/34 (H1N1), can induce a robust CTL response specific for an H-2Kd-restricted NP147–155 epitope in BALB/c mice (19). In these studies immunization with NP DNA conferred protection against a lethal dose challenge with the influenza virus A/HK/68 (H3N2), a virus strain that conserves the sequence encoding NP including the H-2Kd-restricted CTL epitope 147–155, but contains significant changes in the viral surface glycoproteins (hemagglutinin (HA) and neuraminidase) compared with A/PR/8/34 (H1N1).

Although DNA immunization is being explored as a vaccine strategy in both preclinical animal studies and clinical trials, the immunogenicity of DNA vaccines for the induction of CTL responses has not been directly compared with natural viral infection. Moreover, different aspects of immune responses, especially CTL precursor (CTLp) frequency, as a function of the dose of DNA vaccine administered have not been fully characterized. Furthermore, the relationship between the frequency of DNA vaccine-induced Ag-specific CTLp and protective efficacy has not been addressed in the influenza virus challenge model. The primary objectives of this study are 1) to compare the immunogenicity of the NP DNA vaccine with that of influenza virus infection and to document the kinetics of NP147–155-specific CTLp frequency changes following DNA immunization vs influenza virus infection; 2) to characterize the dose response of the NP DNA vaccine in inducing CTLp, T cell proliferation, and Ab responses in mice; and 3) to determine whether a relationship exists between the NP147–155-specific CTLp frequency and the NP DNA-induced protective immunity observed in our cross-strain virus challenge.

Abbreviations used in this paper: NP, nucleoprotein; CTLp, cytotoxic T lymphocyte precursor; HA, hemagglutinin; LDA, limiting dilution analysis.

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model. In this paper we directly compare the immunogenicity of NP DNA vaccine with that of influenza virus infection in inducing NP\textsubscript{A}7\textsubscript{7}--A\textsubscript{155}--specific CTLp in BALB/c mice, demonstrate a dose-dependent relationship between the amount of NP DNA administered and the frequency of NP\textsubscript{A}7\textsubscript{7}--A\textsubscript{155}--specific CTLp elicited in mice, and present a correlation between the CTLp frequency and the protective efficacy in mice against cross-strain influenza virus challenge. This correlation provides a minimal estimate of the NP\textsubscript{A}7\textsubscript{7}--A\textsubscript{155}--specific reciprocal CTLp frequency of 100,000, namely, one CTLp in every 100,000 spleen cells, that is associated with protection from death or significant morbidity after cross-strain influenza challenge in the presence of an intact immune system that also is capable of CD\textsuperscript{+} T cell responses to NP.

**Materials and Methods**

**DNA constructs**

The expression vector used in the study, V1J, has been described previously (20). Briefly, it contains the human CMV immediate early gene enhancer and promoter, the intron A sequence, multiple restriction sites for development, 0.1 M citrate buffer (pH 4.5) containing hydrogen peroxide for immunization was purified from 

**Immunization, challenge, and adoptive transfer protocol**

For immunization, mice under anesthesia induced by the administration of ketamine-xylazine were injected with plasmid DNA dissolved in 100 μl of sterile saline three times at 3-wk intervals. Fifty microliters of DNA was injected bilaterally into the quadriceps muscles each time. To prime mice, 100 μl of complete RPMI 1640 medium in quadruplicate. Each well also contained purified recombinant NP at 10 μg/ml. The cultures were incubated for 3 days at 37°C. The target cells were then washed thoroughly and mixed with CTL at designated E:T ratios in 96-well plates and incubated at 37°C for 4 h in the presence of 5% CO\textsubscript{2}. A 20-μl sample of supernatant from each cell mixture was counted to determine the amount of 31Cr released from target cells. The percentage of specific lysis was calculated using the formula: percent specific lysis = ($E - S$/M - S) x 100, where $E$ represents the average counts per minute released from target cells in the presence of effector cells, $S$ is the spontaneous counts per minute released in the presence of medium only, and $M$ is the maximum counts per minute released in the presence of 2% Triton X-100.

**Limiting dilution analysis (LDA) for CTL**

The LDA protocol was modified from a previously published method (24). Briefly, splenocytes were cultured in twofold serial dilutions (1,000–128,000 cells/well) in 24 replicates in U-bottom 96-well plates in a total of 200 μl of complete RPMI 1640 medium supplemented with 10% Rat T-stim conditioned media (Collaborative Biomedical, Bedford, MA), 0.05 M methyl-α-D-mannopyranoside, and 5 μM IL-2 (Cellular Products, Buffalo, NY). Each well also contained 1 x 10\textsuperscript{6} gamma-irradiated (25) using an application program provided by R. H. Boon, Pennsylvania State University (Hershey, PA).

**Statistical methods**

Analysis of covariance (26) was utilized on data from experiments showing the relationship of reciprocal CTLp frequency with dosage on injected NP DNA to establish the similarity of the dose-response relationship among experiments and to estimate the overall effect. Analysis of covariance (26) was calculated to represent the association between CTLp frequency and survival rate in BALB/c mice. A repeated measures analysis of variance (28) was performed on percent of initial weight in

**Lymphocyte proliferation**

Spleen cells were plated in round-bottom 96-well plates at 1 x 10\textsuperscript{5} cells/ well in 200 μl of complete RPMI 1640 medium in quadruplicate. Each well also contained purified recombinant NP at 10 μg/ml. The cultures were incubated for 3 days at 37°C. The target cells were then washed thoroughly and mixed with CTL at designated E:T ratios in 96-well plates and incubated at 37°C for 4 h in the presence of 5% CO\textsubscript{2}. A 20-μl sample of supernatant from each cell mixture was counted to determine the amount of 31Cr released from target cells. The percentage of specific lysis was calculated using the formula: percent specific lysis = ($E - S$/M - S) x 100, where $E$ represents the average counts per minute released from target cells in the presence of effector cells, $S$ is the spontaneous counts per minute released in the presence of medium only, and $M$ is the maximum counts per minute released in the presence of 2% Triton X-100.
BALB/c mice to determine a difference in this measurement among groups of animals injected with HA DNA, Vector DNA, and NP DNA.

Results

Comparison of CTLp frequency after influenza virus infection and NP DNA immunization

Previous studies from our laboratory demonstrated that i.m. injection of plasmid DNA encoding NP derived from influenza virus A/PR/8/34 in BALB/c mice can induce a vigorous CTL response against an H-2Kd-restricted epitope, NP147–155, as measured by the percentage of specific lysis by the bulk CTL culture in a cytotoxicity assay (19, 23). However, we found that 51Cr release assays with bulk cultured splenocytes from NP DNA-immunized mice can rarely be used to distinguish the efficacy or document the dose relationships of DNA vaccines. Immunization of mice with 1 μg of NP DNA can easily yield a splenocyte CTL culture rendering similar percentages of specific lysis of target cells as those for mice receiving a 100-μg NP DNA inoculation or mice recovered from influenza virus infection (results not shown). Furthermore, a recent paper by Rodriguez et al. (29) has suggested that CTLp frequency, rather than the presence of lytic activity in bulk CTL culture upon restimulation, is a better correlation for protective immunity. Thus, we decided to quantitatively measure the functionality of CTL in immunized mice by performing LDA of CTLp frequency.

The efficacy as well as the kinetics of the CTL response following i.m. injection of NP DNA are shown longitudinally in Fig. 1 for BALB/c mice that had been either intranasally infected with influenza virus A/HK/68 or injected i.m. with NP DNA. Spleen cells obtained from the mice at different time points after virus infection or NP DNA injection were restimulated in limiting dilution cultures with syngeneic spleen cells pulsed with NP147–155 epitope peptide for 7 days and then tested against P815 cells pulsed with NP147–155 peptide in the standard cytotoxicity assay. The cultures from individual wells were considered positive if the specific lysis of the target cells was >20% (see Materials and Methods for details). As shown in Fig. 1, mice infected with influenza virus rapidly mounted a CTL response against the NP147–155 epitope, marked as a decline in the reciprocal CTLp frequency, i.e., a rise in total numbers of CTLp specific for NP147–155 peptide (Fig. 1, upper panel). By 2 wk postinfection, the reciprocal CTLp frequency had reached 40,000–50,000, namely, one CTLp in every 40,000–50,000 spleen cells, compared with fewer than one CTLp in 200,000 spleen cells before infection in these mice. The reciprocal CTLp frequency in these infected mice remained at the lower levels until 10 wk postinfection, with the lowest reciprocal frequency being close to 20,000 (one CTLp in every 20,000 splenocytes). A reciprocal CTLp frequency of 20,000–60,000 can be detected in these mice even months after infection (results not shown). The NP DNA-immunized mice, on the other hand, displayed a slow rise in the number of CTLp, reflected in a gradual decline in the reciprocal CTLp frequency after injection (Fig. 1, lower panel). Boosting injections at 3 and 6 wk improved the CTL immune response, and the reciprocal CTLp frequency reached levels similar to those induced by viral infection by 8–10 wk. These results indicated that three i.m. injections of 30 μg of NP DNA can induce comparable numbers of CTLp against the NP147–155 epitope as those induced by the viral infection in BALB/c mice, but after multiple exposures and with slower kinetics.

Dose dependence of the immune responses induced by NP DNA

To further assess the immunogenicity of the NP DNA vaccine and define the dose dependence of the immune response to DNA vaccines, we titrated the injection dose of NP DNA in mice and measured different parameters of NP-specific immune responses. First, we tested the NP147–155-specific CTLp frequency in mice immunized three times with different doses of NP DNA. Repeated injections were conducted to minimize any injection variation that might occur. The reciprocal CTLp frequency in correlation with the dose of injected NP DNA is depicted in Fig. 2A. The reciprocal frequency of the CTLp against the NP147–155 peptide was inversely correlated with the dose of NP DNA injected, with the higher injection dose of NP DNA corresponding to the lower reciprocal frequency of the NP147–155-specific CTLp in mice, i.e., injecting a higher dosage of NP DNA elicited more CTLp in mice. Injection of 1 μg or more of NP DNA consistently yielded a reciprocal CTLp frequency <100,000 (i.e., more than one CTLp in every 100,000 splenocytes), whereas injection of 0.03 μg yielded a reciprocal CTLp frequency close to that detected in naive mice (generally with a reciprocal CTLp frequency >200,000, namely, less than one CTLp in every 200,000 splenocytes; see Fig. 1). The results from three experiments demonstrated very similar inverse relationships. An analysis of variance was performed on the log of the reciprocal CTLp frequency vs the log of the dose of injected NP DNA. There is no evidence of a difference in this relationship among the replicate experiments (p = 0.33); therefore, the data were pooled to determine the rate of decrease in CTL with dose. The slope of the relationship is −0.27 (95% confidence interval, −0.19 to −0.35), which corresponds to a 32% decrease in reciprocal CTLp frequency per 10-fold increase in dose (95% confidence interval, 21–43%). This represents a statistically significant dose-response relationship due to the fact that the confidence interval on the rate of decrease excludes zero (p < 0.05).

Second, we measured the anti-NP Ab responses in an end-point titration ELISA (Fig. 2B). The immunogenicity of NP DNA for humoral responses also was found to be generally correlated with
the injection dose. Increasing the injection dose of NP DNA resulted in higher ELISA end-point titers in mice, and an injection dose of 0.03 μg yielded a titer very close to that detected in naive mice (100).

Last, we measured the T cell proliferative responses in each dose group. The T cell proliferative responses were measured as [3 H]thymidine incorporation by spleen cells in culture upon stimulation with recombinant NP protein. The proliferative responses of T cells from these mice, however, were found not to exhibit the linear dose dependence seen with Ab and CTLp responses (Fig. 2C). Rather, a threshold effect was observed, in which only the group injected with a 30-μg dose showed a significant level of proliferation in response to NP protein stimulation in culture. Much weaker positive responses were seen at immunization doses from 0.1 to 10 μg. Spleen cells from all groups demonstrated significant proliferative responses when stimulated with Con A (results not shown). These results are consistent with our previous observations that proliferative responses and cytokine secretion by CD4+ lymphocyte cultures were most vigorous when a large quantity of NP DNA (50–200 μg/i.m. injection) was inoculated (30).

Restimulated NP DNA-primed lymphocytes can confer protection in naive mice

We previously showed that NP DNA-primed spleen cells, upon in vitro restimulation with influenza virus A/PR/8/34, can confer protection against lethal cross-strain challenge with influenza virus A/HK/68 when adoptively transferred into naive mice (22). To more precisely assess the role of NP147–155 peptide-specific lymphocytes in the protection, we restimulated spleen cells from mice immunized with NP DNA or infected with influenza virus with syngeneic spleen cells pulsed with NP147–155 synthetic peptide for 7 days. This condition hypothetically would only expand the NP147–155 peptide-specific CTL in culture, since the peptide consists of amino acids of a minimum H-2Kd-restricted CTL epitope (31, 32), and the recognition of this epitope by NP-specific CTL requires the presentation by H-2Kd class I molecules (33). It is also possible that restimulation of NP147–155-specific CD8+ T cells may result in the production of cytokines that may, in turn, activate other antiviral effector cells during the in vitro restimulation period. As shown in Fig. 3, naive mice that received these NP147–155 peptide-restimulated splenocytes from the NP DNA-immunized or A/PR/8/34 virus-primed mice were fully protected, whereas the naive mice that received no cells succumbed to the challenge. These results indicated the ability of the NP147–155-specific T cells
induced by NP DNA immunization to contribute to protection from cross-strain influenza challenge.

To estimate the number of NP DNA-primed and in vitro fully activated lymphocytes needed for complete protection in naive mice, we titrated the cultured spleen cells in an adoptive transfer experiment. These cells had been activated by restimulation with influenza virus in vitro and were cytolytic against NP147–155 peptide-pulsed target cells at the time of the transfer (results not shown). Three groups of mice that had been challenged with influenza virus A/HK/68 received 5 × 10^7, 1.7 × 10^7, or 5 × 10^6 cells/mouse, respectively. As shown in Fig. 4A, only the group that received 5 × 10^7 cells was completely protected (100% survival), whereas the group that received 1.7 × 10^7 showed an 80% survival rate, and the group that received 5 × 10^6 showed almost no protection. The control group of naive mice was not protected (this challenge was performed at the same time as that shown in Fig. 3; therefore, the results for control group of naive mice in this experiment can be found in Fig. 3).

**FIGURE 4.** Number of NP DNA-induced fully activated CTL needed to achieve optimal protection in naive mice. Spleen cells from groups of 20 mice injected with NP DNA (3 × 100 μg/mouse) were in vitro restimulated with syngeneic spleen cells infected with A/PR/8/34 for 7 days and adoptively transferred into groups of 10 naive mice at the indicated cell number per mouse. The recipient mice were infected with a lethal dose of A/HK/68 before cell transfer, and survival (A) and mass loss (B) were monitored for up to 4 wk. This challenge was performed at the same time as the challenge shown in Fig. 3; therefore, the results for control group of naive mice in this experiment can be found in Fig. 3.

prevent the establishment of viral infection or eliminate the virus from the host before the onset of morbidity. This experiment, however, was not designed to offer an accurate estimate of how many NP147–155-specific CTLp are needed for optimal protection, since all the NP-specific lymphocytes transferred would have been fully activated by in vitro restimulation with infectious virus.

**NP147–155-specific CTLp frequency as a correlate of protection**

To study the association of NP147–155 CTLp frequency and cross-strain protection by NP DNA immunization, we challenged the groups of mice that had been injected with different doses of NP DNA with a lethal dose of influenza virus A/HK/68 and monitored for weight loss and survival up to 4 wk (Fig. 5A). The survival rates from these experiments were plotted against their corresponding CTLp frequency, shown in Fig. 2A (B). The correlation coefficient (r) was determined for all the correlates in three experiments.
with the reciprocal frequency of the NP<sub>147–155</sub>-specific CTLp (Fig. 5B). The Spearman correlation coefficient between CTLp frequency and survival rate was −0.81 (p < 0.05). Thus, there is evidence of a significant association between CTLp frequency and survival rate. The group of mice with a reciprocal CTLp frequency <100,000, i.e., having more than one CTLp in every 100,000 splenocytes, generally survived the lethal cross-strain challenge with influenza virus A/HK/68 (>80% survival), whereas the group with a higher reciprocal CTLp frequency (>1/120,000, namely, having less than one CTLp in every 120,000 splenocytes) exhibited suboptimal or no protection in the challenge experiments. These results indicated that the minimal reciprocal CTLp frequency associated with optimal cross-strain protection is 100,000, namely, one NP<sub>147–155</sub> epitope-specific CTLp in every 100,000 splenocytes. This does not mean that the function of CTLp is directly correlated with the protection we documented in the challenge experiment, but only indicates that the NP<sub>147–155</sub>-specific CTLp frequency in NP DNA-immunized mice can serve as a surrogate marker for an individual’s chance to survive the lethal challenge.

**Protection mediated by immune responses against HA**

To illustrate the difference between protective immunities directed against the variable surface protein HA and those against the conserved internal protein NP, we challenged two groups of mice that had been injected three times with 100 μg of A/PR/8/34 HA DNA or 100 μg of blank vector DNA, respectively, with a lethal dose of influenza virus A/PR/8/34 (H1N1). The group that received A/PR/8/34 HA DNA achieved a 100% survival rate against the homologous strain virus challenge, while none of the mice in the group that received blank vector DNA survived (Fig. 6A). Noticeably, the immunized group suffered no significant weight loss at any time point monitored. However, the mice immunized with A/PR/8/34 HA DNA were not protected when challenged with a heterologous strain of influenza virus of a different subtype, A/HK/68 (H3N2). As shown in Fig. 6B, the group that received the A/PR/8/34 HA DNA showed similar levels of weight loss as the group that received blank vector DNA, whereas the group immunized with NP DNA clearly demonstrated improved recovery from weight loss resulting from influenza virus infection. The group weight change between the groups illustrated the morbidity suffered by the mice, although in this particular experiment the majority of the mice survived the challenge. A repeated measures analysis of variance was performed on percentage of initial weight. Missing values for the animals that had died were imputed from the information retained from the surviving animals. There is a statistically significant difference among treatment groups in the average percentage of initial weight through 20 days (p < 0.001), where this outcome is the result of the difference between groups receiving HA DNA and vector DNA relative to the group receiving NP DNA. A graph of the average percentage of initial weight for the three groups is presented in Fig. 6A. Error bars represent pairwise comparisons of the groups at each time point. A significant difference between groups at a time point is signified in error bars that do not overlap (p < 0.05). Significant differences in the percentage of initial weight were observed between groups receiving HA DNA or vector DNA relative to that in the group receiving NP DNA from 9 days postinfection on. These data indicated that the predominantly strain-specific Ab-based protective immunity can prevent both mortality and morbidity inflicted by influenza virus infection with the homologous strain virus (A/PR/8/34), but offers no protection against infection with heterologous strain virus A/HK/68 (H3N2), whereas cellular immunity induced by NP DNA immunization can provide cross-strain protection against mortality and morbidity caused by influenza virus infection.

**Discussion**

The present study was designed to use influenza virus NP as a model Ag to quantitatively assess the immunogenicity of DNA vaccines. The i.m. injection of a plasmid DNA encoding NP from influenza virus A/PR/8/34 (H1N1) can induce a robust CTL response against the H-2K<sup>b</sup>-restricted epitope 147–155, and the immune response induced by NP DNA immunization can provide protection in mice against a cross-strain challenge with influenza virus A/HK/68 (H3N2) (19, 23). Here we initially recorded the kinetics of the CTLp response following i.m. injection of NP DNA compared with those of natural influenza virus infection. Second, we documented the dose dependency of NP<sub>147–155</sub>-specific CTLp frequency following i.m. injection of titrated doses of NP DNA in mice. Third, we demonstrated an association between NP<sub>147–155</sub>-specific CTLp frequency and survival rate in mice and assessed the minimal CTLp frequency associated with protective immunity against cross-strain influenza virus challenge. Last, we illustrated the difference in cross-strain immunities between immunization with NP DNA and A/PR/8/34 HA DNA. Although HA DNA provided sterilizing immunity in mice against challenge with the homologous (H1N1) strain of influenza virus, it offered no protection against the challenge with a heterologous (H3N2) strain of influenza virus even when the challenge was capable of causing only...
Immunizing mice with titrated doses of NP DNA provided a means to quantitatively measure the different aspects of NP-specific immune responses. A reproducible correlation with the injection dose was documented for NP<sub>147–155</sub>-specific CTLp frequency by LDA as well as anti-NP Ab responses by ELISA. The dose dependence of the Ab response induced by DNA immunization has also been demonstrated with other influenza virus gene constructs, such as HA DNA vaccine (J.B.U., et al., unpublished observations). Surprisingly, no such obvious correlation with the immunizing doses of NP DNA could be demonstrated for T cell proliferative responses. Yet, it is likely that CD4<sup>+</sup> T cells were activated even at low doses of DNA immunization, since IgG Ab responses were detected, and IgM to IgG isotype switching in mice generally requires the involvement of Th cells. This may indicate that there is a threshold dose of NP DNA vaccine required for the positive detection of proliferative responses in mice. This is supported by our recent observations that T cell proliferation and cytokine secretion by CD4<sup>+</sup> lymphocyte cultures were most vigorous when a high immunization dose of NP DNA of 50–200 μg was applied (30). This may also be a function of the adjuvant effects of bacterial DNA described by Krieg (34) and others (35). In addition, the NP-specific CD4<sup>+</sup> T cell responses may not be as monospecific as the CTL responses in BALB/c mice. Potentially, a greater diversity of MHC class II-restricted epitopes, in contrast to the single dominant CD8<sup>+</sup> CTL epitope 147–155 (23), might also limit our ability to detect a linear dose-dependent relationship, in contrast to the threshold effect that was seen (36).

In contrast, comparison of NP<sub>147–155</sub>-specific reciprocal CTLp frequency with survival rate strongly indicated that a reciprocal CTLp frequency of 100,000 or lower, i.e., one or more CTLp in every 100,000 splenocytes, was associated with the highest survival rates after cross-strain influenza virus challenge (Fig. 5B). Thus, these experiments indicate that NP<sub>147–155</sub>-specific CTLp frequency can serve as a surrogate marker to assess the cross-strain resistance to lethal influenza virus challenge in BALB/c mice. The ability of a single epitope response to exhibit such a relationship may be surprising given that the potential for recognition of multiple epitopes exists. However, immune dominance appears to limit the diversity of the effector CTL response, at least in BALB/c mice. We and others have described an immunorecessive CTL epitope within NP, residues 218–226. However, the CTL response against this epitope could only be detected when we deleted the immunodominant CTL epitope, NP<sub>147–155</sub>, in NP DNA construct (22), and the CTLp frequency for this epitope appears low even in multiply immunized mice (T.-M.F., unpublished observations). Furthermore, when we challenged naive mice that had received activated lymphocytes from the mice immunized with NP DNA in which the 147–155 epitope had been modified (lymphocytes were in vitro restimulated with influenza virus), they were only partially protected (40–50% vs 90–100%) (T.-M.F., unpublished observations).

The CTLp frequency estimated in this study is lower than that reported in previous studies (29, 37–39). There are three points to be considered regarding these data: 1) our estimation for the CTLp frequency is a conservative one, since our arbitrary cut-off value used to score a positive microcultures in the standard cytotoxicity assay is highly stringent (20% of specific lysis compared with others that generally apply spontaneous lysis counts + 3 SD as cut-offs to score a positive culture well; see Materials and Methods for details); 2) the LDA method for estimating CTLp frequency is far more conservative, as the in vitro culture conditions may fail to expand every in vivo functional CTLp, thus presenting a lower estimate of precursor frequency compared with those assessed by other methods, such as the enzyme-linked immunospot assay and the tetramer-staining assay (40–44); and 3) the effector cells estimated in this study were splenic CTL precursors induced by DNA immunization and thus were probably predominantly or exclusively memory T cells and were different from the lymphocytes detected in mice recovering from an acute viral infection or suffering a persistent viral infection. They were also different from the lymphocytes used in the adoptive transfer experiments, which were fully activated by in vitro restimulation and were highly cytolytic in vitro (i.e., functional effector cells). Although the conclusions from adoptive transfer experiments are generally definitive about the roles of CD4<sup>+</sup> or CD8<sup>+</sup> T cells in protective immunity (7, 8, 12), the infusion of large quantities of active cytolytic cells in recipients may not necessarily be reflective of the natural protective responses induced by immunization. Therefore, the approach taken in the present study not only provides a quantitative estimate of host-generated cytotoxic T cell immune responses and protective efficacy by NP DNA immunization, but also offers a physiological assessment of the levels of CTLp associated with cross-strain protection when other immune factors associated with host anti-viral immunity are present.

In vivo depletion studies have shown that CD4<sup>+</sup> T cells contribute to cross-strain protection in mice immunized with NP DNA, and T cell subset adoptive transfer experiments have shown that activated CD4<sup>+</sup> T cells can provide cross-strain protective immunity in mice (30). The roles of CD4<sup>+</sup> T cells in antiviral immunity are complex and may include production of antiviral cytokines, lysis of virus-infected host cells, amplification of CD8<sup>+</sup> T cell responses, and helper function for Ab production (6, 13).

Our results, showing little if any correlation of spleen cell proliferative responses with injection doses of NP DNA (Fig. 2C), should not be considered evidence against the role of CD4<sup>+</sup> T cells in the protective immunity induced by NP DNA immunization. Rather, our results indicate that bulk spleen cell proliferative responses are probably not an appropriate surrogate marker to assess the antiviral immunity in this system. T cell proliferative responses in bulk cultures, like the percentage of specific lysis recorded with bulk CTL culture, may serve primarily as a semi-quantitative measure for CD4<sup>+</sup> lymphocyte responses. A more quantitative method, such as the enzyme-linked immunospot assay, should be considered in the future to assess CD4<sup>+</sup> T cell responses.

NP DNA-induced cellular immunity can accelerate the viral clearance and improve the host’s chance for survival. The virus titers recovered from the lung were much lower in NP DNA-immunized mice than in the mice injected with blank vector DNA (19). However, NP DNA-induced cellular immunity is by no means a sterilizing immunity, and the mice generally suffered about 10–20% weight loss before recovering from viral infection (Figs. 4B and 5A). In contrast, the mice injected with A/PR/8/34 HA DNA, in which the HA type-specific Ab responses were elicited (results not shown), were completely protected against lethal challenge with the homologous strain of influenza virus in terms of both mortality and morbidity, but were provided no protection against even a relatively mild cross-strain challenge with the heterosubtypic influenza virus A/HK/68 (Fig. 6). Thus, an optimal prophylactic vaccine against influenza virus should include means to induce both Ab responses for type-specific protection and cytotoxic T cell responses for broad cross-strain protection (13).

References


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