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Primary Pulmonary Cytotoxic T Lymphocytes Induced by Immunization with a Vaccinia Virus Recombinant Expressing Influenza A Virus Nucleoprotein Peptide Do Not Protect Mice against Challenge

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The nucleoprotein (NP) of influenza A virus is the dominant antigen recognized by influenza virus-specific cytotoxic T lymphocytes (CTLs), and adoptive transfer of NP-specific CTLs protects mice from influenza A virus infection. BALB/c mouse cells ($H\text{-}2^d$) recognize a single K^d-restricted CTL epitope of NP consisting of amino acids 147 to 155. In the present study, mice were immunized with various vaccinia virus recombinant viruses to examine the effect of the induction of primary pulmonary CTLs on resistance to challenge with influenza A/Puerto Rico/8/34 virus. The minigene ESNP(147-155)-VAC construct, composed of a signal sequence from the adenovirus E3/19K glycoprotein (designated ES) and expressing the 9-amino-acid NP natural determinant (amino acids 147 to 155) preceded by an alanine residue, a similar minigene NP(Met 147-155)-VAC lacking ES, and a full-length NP-VAC recombinant of influenza virus were analyzed. The two minigene NP-VAC recombinants induced a greater primary pulmonary CTL response than the full-length NP-VAC recombinant. However, NP-specific CTLs induced by immunization with ESNP(147-155)-VAC did not decrease peak virus titer or accelerate clearance of virus in the lungs of mice challenged intranasally with A/PR/8/34. Furthermore, NP-specific CTLs induced by immunization did not protect mice challenged intranasally with a lethal dose of A/PR/8/34. Sequence analysis of the NP CTL epitope of A/PR/8/34 challenge virus obtained from lungs after 8 days of replication in ESNP(147-155)-VAC-immunized mice showed identity with that of the input virus, demonstrating that an escape mutant had not emerged during replication in vivo. Thus, in contrast to adoptively transferred CTLs, pulmonary NP-specific CTLs induced by recombinant vaccinia virus immunization do not have protective in vivo antiviral activity against influenza virus infection.

Cytotoxic T lymphocytes (CTLs) appear to play an important role in host immune response, limiting replication and accelerating clearance of many different viruses. With respiratory viruses such as influenza A virus, adoptive transfer of murine primary or restimulated immune spleen cells causes a significant reduction of infectious virus levels in the lungs of mice and prevents death from intranasal (i.n.) challenge with a lethal dose of influenza A virus (1, 42). The specific effector-cell subset was found to be the $CD8^+$ major histocompatibility complex class I-restricted antiviral CTLs, and the nucleoprotein (NP) was identified as the dominant viral determinant recognized by these influenza virus-specific (38) and influenza virus subtype-cross-reactive CTLs (9, 43). Since NP is conserved among different strains of influenza A virus, it has been suggested that an NP-based vaccine may protect against different influenza A virus subtypes (13). Indeed, adoptive transfer of NP-specific CTL clones confers cross-protection from lethal challenge with heterologous influenza A viruses (36). CTLs specific for NS1 and hemagglutinin (HA) epitopes also have protective effects when adoptively transferred to mice (20, 21). In previous experiments in which protection was observed, polyclonal or clonal $CD8^+$-activated CTLs derived from spleens of immunized mice were expanded in vitro by restimulation with antigen and were then transferred intravenously to naive mice, often simultaneously with recombinant interleukin-2 (24, 25, 36, 41). A positive correlation of CTL cytolytic activity in vitro with the clearance rate of virus in the lungs of influenza virus-infected mice has been observed (26). However, studies in which immunization in vivo was used to induce CTL activity have generally resulted in poor protective immunity (5, 6, 14, 35) even after hyperimmunization of animals (2, 4, 13, 40). Therefore, CTLs naturally induced by infection in vivo, in contrast to results from adoptive transfer experiments, appear to be less effective in their antiviral function in vivo, and this was thought to be due to factors such as insufficient activation and poor recruitment of effector cells to the site of the virus infection.

$CD8^+$ T cells recognize a peptide in association with major histocompatibility complex class I molecules through the T-cell receptor (37), and such peptides may be derived from two different pathways of protein processing. In the major pathway, transporter proteins convey peptides produced in the cytosol into the endoplasmic reticulum (28). Alternatively, NH_2-terminal signal leader sequences adjacent to CTL epitopes can bypass transporter proteins, presumably by posttranslational transit through the protein-conducting channel, normally used for signal recognition particle-mediated export. Once inside the endoplasmic reticulum, signal peptidase presumably liberates the peptide from the leader (17). The recent availability of immunogenic signal sequence-CTL epitope constructs expressed by vaccinia virus vectors (3) provides an excellent

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opportunity to further examine the role of CTLs induced by immunization in the resolution of influenza A virus infection. Importantly, such studies can examine the contribution of $CD8^+$ CTLs in the absence of contributions of T-helper cells and antiviral antibodies specific for influenza A virus.

Several murine major histocompatibility complex class I-restricted CTL epitopes of NP from influenza A virus have been characterized, including a K^d-restricted epitope, amino acids (aa) 147 to 155 derived from A/Puerto Rico/8/34 virus. In the present study, we used a vaccinia virus recombinant, ESNP(147-155)-VAC, composed of a leader sequence from the adenovirus E3/19K glycoprotein (designated ES) and the NP minigene expressing the 9 aa 147 to 155 of NP preceded by an alanine and a similar construct lacking the ES signal sequence. We determined the ability of these viruses to induce primary pulmonary NP-specific CTLs in BALB/c mice, and we examined the role of such CTLs in the resolution of a respiratory influenza virus infection. We unexpectedly found that the primary pulmonary NP(147-155) peptide-specific CTLs present in the lungs of vaccinia virus recombinant-immunized mice lacked a biological effector function in the clearance of influenza A/PR/8/34 virus infection from the lungs and in the enhancement of survival of mice to a lethal challenge of influenza A/PR/8/34 virus in vivo.

MATERIALS AND METHODS

Mice. Female BALB/c ($H\text{-}2^d$) mice 6 to 10 weeks old were obtained from Jackson Laboratories, Bar Harbor, Maine, and Charles River Laboratories, Raleigh, N.C.

Viruses. The following vaccinia virus recombinants were used: HA-VAC, expressing the full-length HA sequence of A/PR/8/34 (H1N1); NP-VAC, expressing the full-length 495-residue NP of A/PR/8/34; minigene NP(147-155)-VAC (12), expressing a naturally processed peptide of NP (aa 147 to 155; MTYQRTRALV); minigene ESNP(147-155)-VAC (11), with the ES leader sequence and expressing the NP peptide (aa 147 to 155) preceded by an alanine (MRYMILGLLALAAVC SAATYQRTRALV); minigene ESN_{VSV}(52-59)-VAC, with the ES leader sequence and expressing the N peptide of vesicular stomatitis virus (aa 52 to 59). Recombinant vaccinia viruses were grown in BSC-1 cells, sonicated before freezing at −70°C, and titrated as described previously (10). Mouse-adapted influenza virus A/PR/8/34 (H1N1) was grown in 10-day-old embryonated hen's eggs and, after 2 days at 37°C, the infectious allantoic fluid was harvested and stored at −70°C. Influenza virus was titrated in MDCK cells, and the titers were expressed as 50% tissue culture infectious doses ($TCID_{50}$) per milliliter or per gram of lung tissue as described previously (33).

Virus immunization and challenge. Mice were infected simultaneously by the i.n and intraperitoneal (i.p) routes with 10^7 PFU in 100 μl (50 μl in each site) of vaccinia virus recombinant after anesthesia with methoxyflurane (Pitman-Moore Inc., Mundelein, N.J.). Anesthetized mice were challenged with either 10^2, $10^{4.3}$, or 10^5 $TCID_{50}$ of A/PR/8/34 virus by the i.n. route on day 6 postinfection (PI). Mice administered the vaccinia virus recombinants showed evidence of infection by the presence of serum antibodies (titers of approximately 1:4,000 at day 28 PI) to vaccinia virus antigens by enzyme-linked immunosorbent assay as previously described (15).

CTL assay. Target cells were prepared as described elsewhere (43). Briefly, P815 ($H\text{-}2^d$) cells, a DBA/2 mastocytoma cell line, were pulsed with NP peptide (Met aa 147 to 155; 25 μl of 1 mg/ml), were infected with A/PR/8/34 or vaccinia virus, or were left uninfected. Primary in vivo effector lymphocytes were prepared from pooled lungs or spleens of vaccinia virus recombinant-infected mice. Experiments used either lung exudate lymphocytes from i.n. and i.p. inoculated mice or splenic lymphocytes from intravenously (i.v.) inoculated mice. Cells were washed in calcium- and magnesium-free Iscove's medium and assayed the same day for cytotoxic activity in a standard ^{51}Cr-release assay. Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h, washed and resuspended at 10^4 cells per 100 μl. Effector cells were added in triplicate to targets at various ratios, starting at 100:1. After 8 h at 37°C, the supernatant was counted for ^{51}Cr release. The percent specific cell lysis was 100 × ([experimental counts per minute {cpm} − spontaneous cpm]/[total cpm − spontaneous cpm]).

Determination of virus titers in lungs. Virus titers are expressed as mean $\log_{10}$ $TCID_{50}$ ± standard error per gram of lung homogenate (10%, wt/vol) prepared 4 days postchallenge. Lung specimens from which virus was not recovered were assigned a titer of 1.5, since the lowest limit of detectable virus was 2.0 $TCID_{50}$/g.

Sequence analysis of NP CTL epitope. Influenza viral RNA was extracted from A/PR/8/34 virus present in allantoic fluids or lung homogenates of influenza virus-infected mice. Briefly, the virus suspension was incubated with proteinase K (Boehringer, Mannheim, West Germany) at 37°C for 10 min and phenol-chloroform extracted, and the RNA was precipitated with carrier yeast tRNA (Boehringer). Reverse transcription was carried out with the influenza A virus NP-specific primer, nucleotides 71 to 91 (5′-CTTACGAACAGATGGAGACTG -3′), and reverse transcriptase (AMV, Life Sciences Inc., St. Petersburg, Fla.). A 620-bp fragment of the synthesized NP cDNA was amplified by PCR using the above primer and a reverse NP primer, nucleotides 671 to 691 (5′-TTGTTTT TCGTCCATTCTCAC-3′). The PCR product was purified by using spun columns, and the sequence across the CTL epitope region was analyzed with the NP oligonucleotide, nucleotides 401 to 415 (5′-TCTGGCGCCAAGCTA-3′) by Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio) as described previously (22).

RESULTS

Induction of primary pulmonary CTLs by immunization of mice with vaccinia virus recombinants. Peak pulmonary CTL activity to vaccinia virus and to expressed proteins occurs at about day 6 PI (7). Mice infected with the influenza virus-vaccinia virus recombinants were therefore assessed for primary pulmonary CTL activity at this time point. BALB/c mice were inoculated with influenza virus A/PR/8/34-vaccinia virus recombinants simultaneously by the i.n. and i.p. routes since this immunization regimen induced $CD8^+$ CTL-mediated resistance to another respiratory virus, namely respiratory syncytial virus (RSV) (19). A high dose of ESNP(147-155)-VAC (10^7 PFU per mouse), but not 10-fold less (data not shown), induced primary CTL activity against NP peptide (147-155) and A/PR/8/34 virus as measured in the in vitro ^{51}Cr-release assay (Fig. 1). The ESNP(147-155)-VAC and NP(Met 147-155)-VAC induced a high level of primary pulmonary CTL activity to NP peptide-pulsed targets (Fig. 1). Primary CTL responses to A/PR/8/34, and to a lesser extent to NP peptide-pulsed targets, were detected in mice infected i.n. with A/PR/8/34 virus (Fig. 1, experiment 2). The other virus groups, NP-VAC, HA-VAC, and ESN_{VSV}-VAC, did not induce significant CTL activity against NP peptide or A/PR/8/34 virus. As expected, each group of mice given a vaccinia virus recombinant exhibited primary CTL activity against vaccinia virus antigens. These cytotoxic activities of lymphocytes obtained

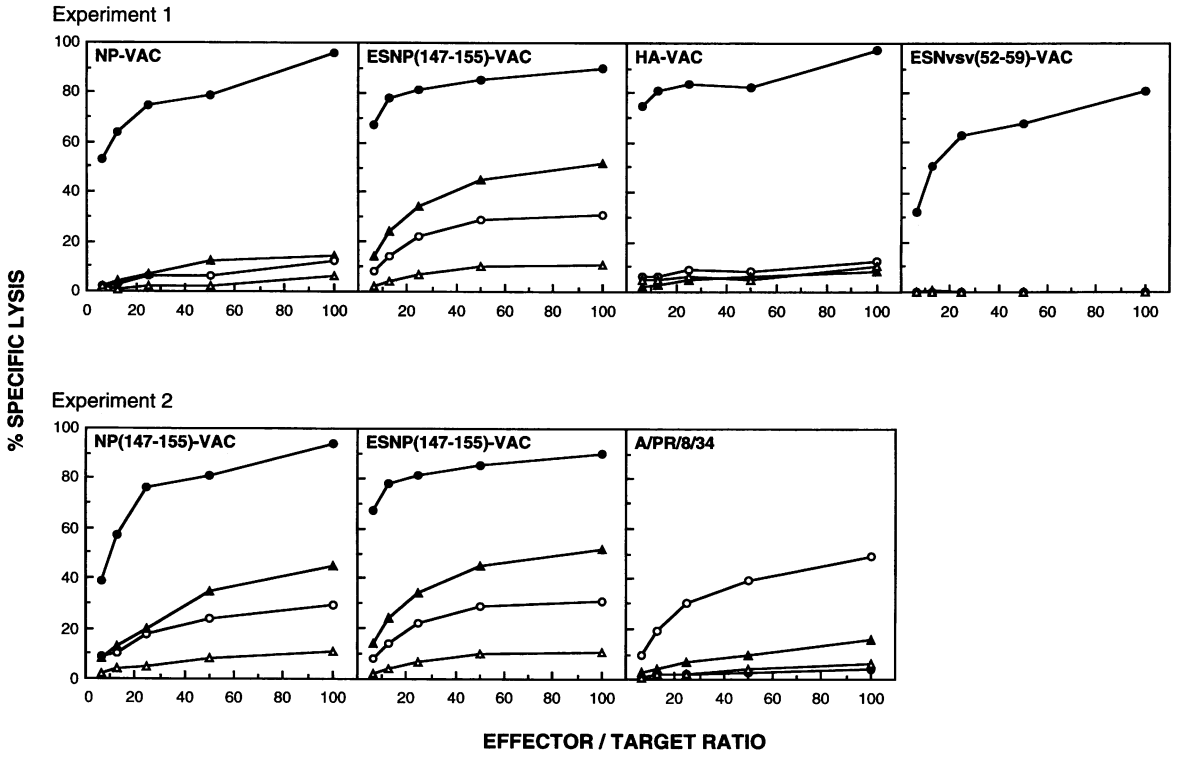

FIG. 1. Cytotoxic activity of primary pulmonary CTLs. BALB/c mice ($H\text{-}2^d$) were inoculated by the i.n. and i.p. routes with 10^7 PFU of NP-VAC, ESNP(147-155)-VAC, NP(147-155)-VAC, HA-VAC, or ESN_{VSV}(52-59)-VAC recombinant viruses or i.n. with $10^{3.7}$ $TCID_{50}$ of A/PR/8/34 virus. The titles above the graphs indicate viruses used to infect the mice. On day 6 PI, primary cytotoxic activity of pooled lung exudate lymphocytes from 5 mice was evaluated by using ^{51}Cr-labeled A/PR/8/34 virus-infected (○), vaccinia virus-infected (●), NP(Met 147-155) peptide-pulsed (▲), or uninfected (△) P815 ($H\text{-}2^d$) targets at the specific effector/target ratios. The top and bottom panels represent two separate experiments.

from the lung exudates of mice which were administered vaccinia virus recombinant viruses 6 days earlier were reproduced in four separate experiments (some data not shown) and confirm our findings of primary splenic CTL activity induced by standard i.v. immunization with ESNP(147-155)-VAC (Fig. 2). Thus, primary CTL responses to NP are induced by immunization of mice with the NP minigene constructs, ESNP(147-155)-VAC and NP(147-155)-VAC virus.

NP-specific CTLs induced by immunization do not reduce peak influenza virus challenge titers or accelerate clearance of virus in lungs of mice. Mice were inoculated i.n. and i.p. with vaccinia virus recombinants as described above and challenged i.n. with a low dose (10^2 $TCID_{50}$) of A/PR/8/34 virus 6 days later, and the amount of virus replication in the lungs was quantitated 4 days postchallenge (Table 1). This time of challenge was chosen since prior studies with the $M2_{RSV}$-VAC virus demonstrated CTL activity and resistance to RSV challenge to be highest at this time point (19). Vaccination with either the full-length NP-VAC, minigene ESNP(147-155)-VAC or minigene NP(147-155)-VAC did not induce resistance to A/PR/8/34 challenge virus replication in the lungs (Table 1). Nonetheless, HA-VAC, as previously found for C57BL/6 and other congenic mouse strains (14), induced resistance to replication of challenge virus (reduction of 10^5). As expected, the control ESN_{VSV}(52-59)-VAC virus did not mediate resistance to influenza A virus infection. We saw gross pulmonary pathology from all groups except the HA-VAC-immunized animals. These findings indicate that there is little to no reduction of influenza virus replication in vivo by minigene ESNP(147-155)-VAC or NP(147-155)-VAC vaccination of mice despite induction of CTL responses to NP.

We next studied whether immunization with minigene NP-vaccinia virus recombinant virus would accelerate the clearance of the influenza challenge virus from the lungs. BALB/c mice were immunized with the vaccinia virus recombinants and challenged i.n. with A/PR/8/34 on day 6 as described above, and the kinetics of virus replication in the lungs was determined from day 4 to day 10 postchallenge (Fig. 3). Virus was not detected in the lungs taken at days 4, 6, 8, and 10 postchallenge of HA-VAC-immunized mice. However, virus reached similar levels in the lungs of mice immunized with ESNP(147-155)-VAC or control ESN_{VSV}(52-59)-VAC at days 4 and 6 postchallenge. A difference in the clearance rate between these two groups was not observed, and virus was no longer detectable 10 days postchallenge. Therefore, NP-specific CTLs induced by immunization do not accelerate virus clearance from the lungs.

NP-specific CTLs induced by immunization do not enhance survival of mice challenged with a lethal dose of influenza virus. We next examined whether the minigene NP-VAC vaccines could protect BALB/c mice from a challenge with a lethal dose of A/PR/8/34. We compared the protective efficacies of immunization (i.n. and i.p.) with ESNP(147-155)-VAC, NP(147-155)-VAC, NP-VAC, HA-VAC, and ESN_{VSV}(52-59)-VAC (Fig. 4). Six days after immunization, A/PR/8/34 virus was given i.n. at a dose of $10^{4.3}$ $TCID_{50}$, (which represents approximately 1 50% lethal dose (Fig. 4A and B) or 10^5

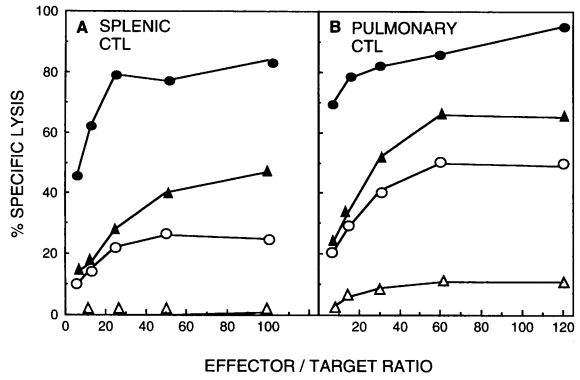

FIG. 2. Cytotoxic activity of primary CTLs from BALB/c mice primed with ESNP(147-155)-VAC. Mice were inoculated by the i.v. route with $10^{6.7}$ PFU (A) or by the i.n. and i.p. routes with $10^{7.0}$ PFU (B) of recombinant vaccinia virus. On day 6 PI, primary cytotoxic activities of pooled splenic (A) or lung exudate (B) lymphocytes were evaluated by using ^{51}Cr-labeled A/PR/8/34 virus-infected (○), vaccinia virus-infected (●), NP(Met 147-155) peptide-pulsed (▲), or uninfected (△) P815 targets at the specified effector/target ratios.

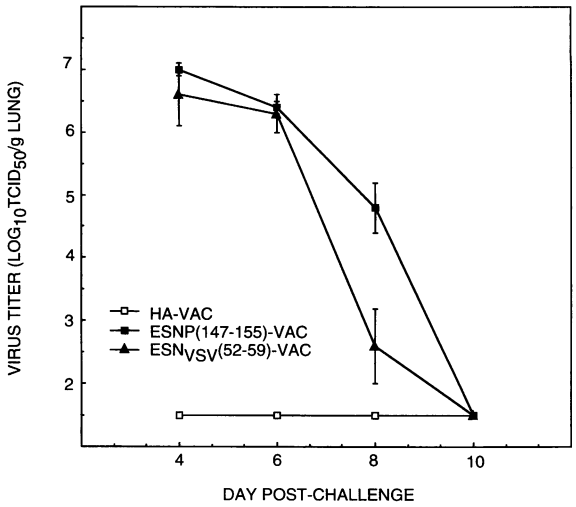

FIG. 3. Kinetics of virus clearance in lungs of immunized mice after respiratory challenge with influenza A/PR/8/34 virus. Groups of three to five BALB/c mice received 10^7 PFU of HA-VAC, ESNP(147-155)-VAC, and ESN_{VSV}(52-59)-VAC by the i.n. and i.p. routes and were challenged i.n. on day 6 PI with 10^2 $TCID_{50}$ of influenza A/PR/8/34 virus. Lungs were removed 4, 6, 8, and 10 days later for quantitation of virus. Mean $\log_{10}$ virus titers ($TCID_{50}$) ± standard error per gram of lung tissue are shown. The limit of detectable virus was 2.0 $TCID_{50}$/g of lung.

$TCID_{50}$ (Fig. 4C and D), and the mice were observed daily over the following 2 weeks for mortality. Immunization with HA-VAC completely protected mice from influenza virus, with 100% survival. In all experiments, the survival rate of mice immunized with NP-VAC or NP(147-155)-VAC was similar to that of the control group, ESN_{VSV}(52-59)-VAC. Surprisingly, mice immunized with ESNP (147-155)-VAC had a lower survival rate than the control virus group ESN_{VSV}(52-59)-VAC following challenge with the lower virus dose ($10^{4.3}$ $TCID_{50}$). High mortality rates were evident in the groups immunized with ESNP(147-155)-VAC, NP(147-155)-VAC, ESN_{VSV}(52-59)-VAC, or NP-VAC and challenged with 10^5 $TCID_{50}$ of A/PR/8/34 virus. These studies show that although ESNP(147-155)-VAC and NP(147-155)-VAC induce strong CTL responses to NP peptide and A/PR/8/34 as measured in vitro, they do not protect against a lethal course of respiratory disease in vivo and may possibly contribute to immunopathology.

Analysis of the NP epitope. To assess whether the K^d-restricted NP CTL epitope present in the A/PR/8/34 challenge virus had undergone mutation during in vivo replication in ESNP(147-155)-VAC-immunized animals, we sequenced the region of NP cDNA derived from viral RNA (encoding the NP peptide epitope) of influenza A virus obtained from lung homogenates of challenged mice. Mice were infected with ESNP(147-155)-VAC and challenged i.n. with A/PR/8/34 virus on day 6 PI. Virus present in the lungs harvested after 8 days of replication of challenge virus (day 14 after immunization) was selected for nucleotide sequence analysis. The nucleotide sequences of the NP CTL epitope region of parental and recovered virus were identical, nucleotides 484 to 513 (cDNA, 5′-ACTTATCAGAGGACAAGAGCTCTTGTT). Thus, failure of the effector function of CTL in vivo does not reflect the emergence of A/PR/8/34 escape mutants expressing an altered CTL epitope.

TABLE 1. Effect of vaccinia virus recombinant infection on resistance to replication of challenge influenza A/PR/8/34 virus in the lungs of BALB/c mice

Vaccinia recombinant virus[a]	No. of expts	No. of mice[b]	Lung virus titer[c] ($\log_{10}$ $TCID_{50}$/g)	$\log_{10}$-fold reduction[d]
HA-VAC	2	11	≤1.5 ± 0.0	5.3
NP-VAC	2	11	6.0 ± 0.2	0.8
NP(Met 147-155)-VAC	1	5	6.9 ± 0.1	0.0
ESNP(147-155)-VAC	3	25	6.8 ± 0.1	0.0
ESN_{VSV}(52-59)-VAC	3	15	6.8 ± 0.2	NA[e]

[a] BALB/c mice received 10^7 PFU of each vaccinia virus-influenza virus recombinant i.n. and i.p. on day 0. Mice were challenged i.n. on day 6 PI with 10^2 $TCID_{50}$ of influenza A/PR/8/34, and lungs were removed 4 days later for quantitation of virus.

[b] Total number of mice in group.

[c] Mean $\log_{10}$ virus titer ± standard error ($TCID_{50}$/g of lung tissue) of total number of animals for specified group. The lowest level of detectable virus was 2.0 $TCID_{50}$/g of lung tissue; undetectable virus was assigned a value of 1.5 $TCID_{50}$/g.

[d] Reduction in virus replication was determined by subtracting the mean $\log_{10}$ titer of influenza virus-vaccinia virus recombinant-primed animals from that of mice primed with the control ESN_{VSV}(52-59)-VAC virus.

[e] NA, not applicable.

DISCUSSION

In previous studies, we and others have found that one or two doses of NP-VAC did not significantly decrease the replication of A/PR/8/34 virus in the lungs of mice of several major histocompatibility complex haplotypes, including $H\text{-}2^b$, $H\text{-}2^k$, and $H\text{-}2^d$ (5, 6, 14, 35). In the present study, it was found that a single immunization of BALB/c ($H\text{-}2^d$) mice with full-length NP-VAC ($10^{5.3}$ or 10^7 PFU) did not induce primary pulmonary NP CTL activity, and this lack of induction of antiviral effector cells was thought to be largely responsible for the observed lack of protection of NP-VAC against influenza A virus infection. It was therefore exciting to find that minigene ESNP(147-155)-VAC and NP(Met 147-155)-VAC, vaccinia virus recombinants expressing the $H\text{-}2$ K^d-restricted NP CTL epitope (aa 147 to 155), induced primary pulmonary CTL activity against NP in BALB/c mice 6 days after immunization. However, mice with primary pulmonary NP-CTL activity were not resistant to influenza virus respiratory infection in vivo.

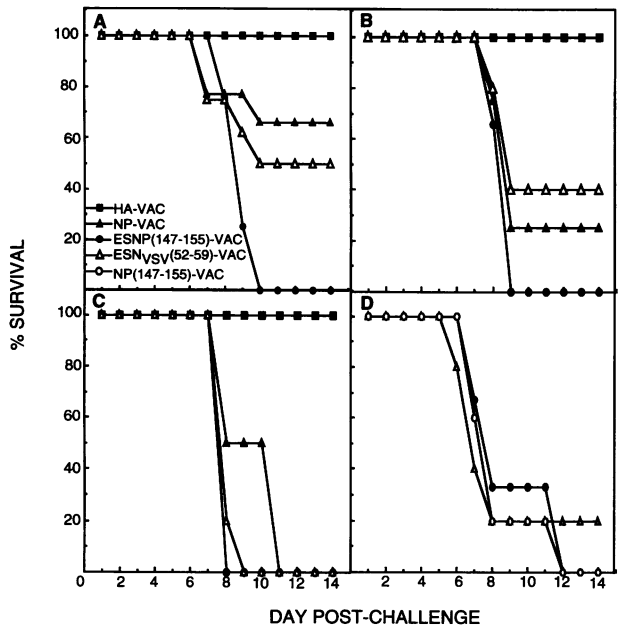

FIG. 4. Survival of vaccinated mice after respiratory challenge with influenza A/PR/8/34 virus. Groups of five to nine BALB/c mice were immunized with 10^7 PFU of HA-VAC, NP-VAC, NP(Met 147-155)-VAC, ESNP(147-155)-VAC, or ESN_{VSV}(52-59)-VAC, as specified, by the i.n. and i.p. routes and were challenged i.n. on day 6 PI with $10^{4.3}$ (A and B) and 10^5 (C and D) $TCID_{50}$ of A/PR/8/34 virus in four separate experiments. Mice were observed for mortality over 14 days postchallenge.

This finding was surprising to us, since primary pulmonary CTL effectors induced by immunization with $M2_{RSV}$-VAC readily decreased replication of RSV challenge virus administered 6 days after immunization (19). Although protection against RSV was short-lived, resistance correlated with the level of primary pulmonary CTL activity mediated by $CD8^+$ T cells (19). Differences in the virulence levels of these viruses could contribute to the observed differences in efficacy, since RSV infection is only semipermissive in mice, while influenza A/PR/8/34 virus infection can be lethal.

CTL activity induced by immunization with vaccinia virus recombinants can induce resistance to certain virus infections. The immediate-early nonstructural antigen, pp89, of murine cytomegalovirus expressed by vaccinia virus recombinant induces $CD8^+$ CTLs which protect mice from lethal murine cytomegalovirus disease (8, 18). A spectrum of CTL activities to NP of lymphocytic choriomeningitis virus (LCMV) and protective efficacies has been observed in NP_{LCMV}-VAC-infected mouse strains (16). NP-CTL activity was not detected in B10.BR ($H\text{-}2^k$) mice, a low and transient NP-CTL activity associated with partial resistance was seen in B10.D2 ($H\text{-}2^d$) mice, and a high and longer-lasting NP-CTL activity associated with strong resistance in C57BL/6 ($H\text{-}2^b$) mice was found. Importantly, minigenes of CTL epitopes of LCMV NP and glycoprotein GP coexpressed by vaccinia virus can induce a protective CTL response in certain mouse strains (31, 32). These findings with minigene-vaccinia virus recombinants suggest that CTLs, in the absence of other immunological effectors such as $CD4^+$ T cells or antibodies, by themselves are capable of mediating resistance to certain viruses. Thus, there are several examples of the effectiveness of vaccinia virus-based immunogens in which $CD8^+$ CTLs induced by immunization provide protection.

Recently, intramuscular injection of full-length naked cDNA for NP of influenza virus expressed by a mammalian plasmid vector has been reported to protect mice against lethal influenza virus infection (39). Following the inoculation of NP-cDNA into skeletal muscle cells of mice, both CTLs and antibodies to NP antigens are induced. Partial resistance to both homologous and heterosubtypic influenza A virus challenge was observed, but the mediators of the resistance were not defined. Clearly, both $CD4^+$ and $CD8^+$ T cells were induced, and are possible mediators of the observed protection. Perhaps, prolonged presence of NP antigen in transfected muscle cells induces an immune response qualitatively different from that induced by vaccinia recombinant virus-infected cells, and this could be an explanation for the differences in efficacy of NP-cDNA versus NP-VAC.

In contrast to the findings from our present study, adoptive transfer of murine influenza virus-specific CTLs has provided levels of protection ranging from 0.7 to 4.0 $\log_{10}$ reduction of virus titers in lungs determined at day 4 to 6 postchallenge associated with 50 to 100% survival from lethal influenza virus challenge (see the introduction). Experiments using a variety of inbred mouse strains (CBA, BALB/c, and CB6F1), virus strains (A/WSN, A/PR/8/34, A/Jap/57, A/X-31, A/USSR/90/77, and A/PC), and protocols for in vitro restimulation of CTLs were performed. In the vast majority of these studies, polyclonal or clonal CTLs derived from spleen or lungs of immunized mice have been used for adoptive transfer. Numbers of cells ranging from approximately 10^6 to 10^8 have been transferred by the i.v. route with or without simultaneous injection of recombinant interleukin-2. Memory $CD8^+$ T cells were found more efficient at virus clearance than memory $CD4^+$ T cells (23), and a positive correlation between the restimulation of such CTLs in vitro and the reduction of influenza virus in lungs of mice was observed.

Several immunological mechanisms could underlie the seemingly contradictive evidence of the protective effects of influenza virus-specific CTLs generated in vivo and of those restimulated in vitro. First, the number of NP-specific CTLs stimulated in vivo by immunization and present at the tissue site of challenge virus replication may not be sufficient to restrict virus replication, whereas larger numbers of adoptively transferred CTLs would traffic to the lung and accumulate in the lung parenchyma, bronchial mucosa, epithelium, and airway lumen (27). We have found a total of approximately 10^7 lymphocytes present in the lungs of ESNP(147-155)-VAC-infected BALB/c mice at day 6 PI, and of this population only a fraction will be CTLs specific for NP. In cell transfer experiments, larger numbers of NP-specific T cells are transferred (up to 10^8 cells). The minimum number of adoptively transferred primary immune T cells (5×10^7) was found to be higher than that for secondary polyclonal T cells (2×10^7) for a 100% survival rate of CBA mice to challenge influenza A/WSN virus infection (41). Second, the inherent in vivo antiviral activities of CTL populations may differ depending on whether the CTLs are restimulated in vitro. For example, it is possible that $CD8^+$ T cells with high densities and/or affinities of the T-cell receptors specific for the NP epitope might be selectively amplified during in vitro restimulation. Such CTLs may have stronger in vivo antiviral activities than CTLs induced by a single immunization of NP in vivo. Third, since the avidities of the T-cell receptors for NP can be affected by accessory and/or adhesion molecular interactions (34), it is possible that CTLs induced by in vitro restimulation have a lower energy threshold for activation than primary CTLs

induced by immunization and therefore might be active earlier during a virus infection in vivo. Fourth, it is possible that in vitro restimulation of CTLs optimizes the secretion of antiviral molecules such as gamma interferon or the degranulation of the CTL, resulting in the release of lethal perforins during interaction with virus-infected cells. Fifth, the presence of T-helper cells specific for influenza virus may augment resistance mediated by CTLs that are induced by immunization in vivo. Such T-helper cells would likely not be induced by NP-vaccinia virus minigene recombinants bearing only the CTL epitope. Exogenous helper factors present as lymphokine supplements in the populations of CTLs restimulated in vitro may alleviate the need for T-helper cells in the adoptive transfer experiments (30). Sixth, since CTLs clearly differ in their protective activities in vivo depending on the epitope recognized (16), it is possible that the NP epitope (aa 147 to 155) was one capable of only a very weak in vivo effect. Polyclonal nonrestimulated splenic-influenza-virus-immune T cells adoptively transferred to mice have been reported to induce resistance to challenge influenza virus infection (41, 42). However, the mediators of this protection are undefined and may include multiple effector T-cell ($CD8^+$ and/or $CD4^+$) clones. Exhaustion of antiviral CTLs possibly by anergy or apoptosis has been demonstrated for persistent viral infections such as LCMV (29). It is unknown whether the diminished NP-specific CTL protective immunity seen in our influenza virus-challenged mice is due to exhaustion of CTLs driven by NP antigen, but this remains a possibility. However, we think this is unlikely to be a major factor, since a low dose of challenge virus was used in the present study and exhaustion with LCMV required high doses of virus. Importantly, the present study in which the in vitro cytotoxic activity and in vivo efficacy of $CD8^+$ CTLs have been found to be dissociated provides a system in which the mediators of antiviral resistance in vivo can be further defined.

The potential usefulness of the inclusion of CTL epitopes in vaccines for respiratory virus infections such as influenza A virus has been examined in the present study. Our findings, in the context of those of others (40), emphasize the importance of a complete analysis of the immunogenicity and protective efficacy of a CTL epitope before its inclusion in a vaccine is validated. First, the proposed CTL epitope or set of CTL epitopes must be recognized by a high percentage of the target human population. Second, the immunogenicity of the CTL epitope in the vaccinees should be readily measurable by in vitro assays. Third, and most importantly, the CTL response induced by immunization with the virus vaccine must adequately protect the host from disease caused by the wild-type virus and this protection should be long lasting. Clearly, the present study demonstrates that the presence of in vitro cytotoxic activity in vaccinated animals is insufficient to predict in vivo efficacy of the vaccine for influenza virus and this underscores the need for the efficacy studies in vivo. Furthermore, the NP-specific CTL epitope examined in the present study, or another CTL epitope with comparable in vivo properties, would not appear to be a useful component of an influenza A virus vaccine.

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