

Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes

(vaccinia virus vector/influenza vaccine/internal viral protein/cell surface expression/cellular defense mechanism)

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ABSTRACT Influenza A virus-specific cytotoxic T lymphocytes (CTL) capable of lysing cells infected with any influenza A virus ("cross-reactive CTL") constitute a major portion of the host CTL response to influenza. The viral nucleoprotein (NP), a major internal virion structural protein, has been implicated as a possible target antigen for cross-reactive CTL. To directly examine CTL recognition of NP, a vaccinia virus recombinant containing a DNA copy of an influenza A virus NP gene was constructed. We found that murine cells infected with this virus were efficiently lysed in a major histocompatibility complex-restricted manner by cross-reactive CTL populations obtained by immunization with a variety of influenza A virus subtypes. In addition, the recombinant vaccinia virus containing the PR8 NP gene was able to both stimulate and prime for a vigorous secondary cross-reactive CTL response. Significantly, splenocytes from mice primed by inoculation with the recombinant vaccinia virus containing the PR8 NP gene could be stimulated by influenza A viruses of all three major human subtypes. Finally, unlabeled target competition experiments suggest that NP is a major, but not the sole, viral target antigen recognized by cross-reactive CTL.

Major histocompatibility complex-restricted, virus-specific cytotoxic T lymphocytes (CTL) are a prominent feature of host immune responses to many viruses, where they appear to play a role in limiting viral replication and dissemination (1-3). The most intensively studied anti-viral CTL are those specific for influenza A virus-infected cells. Even so, the viral antigens recognized by anti-influenza virus CTL remain largely undefined.

Studies of murine anti-influenza virus CTL identified two general CTL populations, one specific for the immunizing strain and closely related strains within the same subtype ("strain-specific") and the other able to lyse cells infected with any influenza A (but not B) virus ("cross-reactive") (4-6). In humans, only cross-reactive CTL have been identified (7, 8). The two viral glycoproteins, hemagglutinin and neuraminidase, are expressed on infected cell surfaces in large quantities (9). For this reason, it was thought that they served as the principal target antigens for strain-specific and even cross-reactive CTL, despite the fact that these glycoproteins derived from different subtypes are not serologically cross-reactive (10). Present evidence suggests, however, that the glycoproteins may represent target antigens for only a minority of both CTL populations (11, 12). This would mean that CTL predominantly recognize one or a number of the eight nonglycosylated viral structural and nonstructural proteins. The amino acid sequences of these proteins are highly conserved among influenza A viruses, providing a possible explanation for cross-reactive recognition. Although none of them are classical integral membrane pro-

teins, at least the nucleoprotein (NP) (13, 14), and perhaps several other internal virion proteins (14-19), are present on infected cell surfaces, where they could serve as CTL target structures.

To directly examine recognition of influenza A virus proteins by CTL, we have constructed live recombinant vaccinia (Vac) viruses containing DNA copies of influenza virus genes. In a previous report, we showed that a recombinant Vac virus containing an HA gene derived from influenza virus strain Jap [A/Japan/305/57 (H2N2)] (20, 21) is able to prime or stimulate (or both) a secondary anti-Jap CTL response in mice and that cells infected with this recombinant virus are lysed by anti-Jap CTL. It was also found that inoculation of mice with the recombinant virus primed largely for a strain-specific CTL response. In this report, we continue these studies using a recombinant Vac virus containing the NP gene of influenza virus strain PR8 [A/Puerto Rico/8/34 (H1N1)]. We demonstrate (i) that cells infected with the recombinant virus express the NP gene and are efficiently lysed by cross-reactive CTL and (ii) that this virus is able to both prime for and stimulate a vigorous secondary cross-reactive CTL response.

MATERIALS AND METHODS

Influenza Viruses. The influenza virus strains PR8, Jap, HK [A/Hong Kong/107/68 (H3N2)], NT60 [A/Northern Territories/60/68 (H3N2)], and B Lee (B/Lee/40) were grown in the allantoic cavities of 10-day, embryonated hens' eggs. After 3 days of incubation at 37°C, the infectious allantoic fluid was harvested, aliquoted, and stored at -70°C.

Vac Viruses. A DNA copy of the NP gene derived from PR8 (generously provided by P. Palese, Mount Sinai School of Medicine, New York) was inserted into Vac virus under control of a Vac virus promoter by using protocols described for expression of foreign genes in Vac virus (22, 23). Details of the construction of this recombinant Vac virus containing the PR8 NP gene (NP-Vac) and other recombinant viruses expressing genes from PR8 will be presented elsewhere. Viruses were grown in HeLa cells at 37°C for 3 days. Infected cells were pelleted by centrifugation, resuspended in Eagle's minimal essential medium containing 2.5% fetal bovine serum, trypsinized, sonicated, diluted, aliquoted, and stored at -70°C.

Cells. P815 cells [a DBA/2 (*H-2^d*) mastocytoma cell line] were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (RPMI medium) in a humidified air/CO₂ atmosphere at 37°C.

Mice. Six- to 8-week-old BALB/c (*H-2^d*) and C3H (*H-2^k*)

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Abbreviations: CTL, cytotoxic T lymphocyte(s); NP, nucleoprotein; PR8, A/Puerto Rico/8/34 (H1N1); Jap, A/Japan/305/57 (H2N2); HK, A/Hong Kong/107/68 (H3N2); B Lee, B/Lee/40; Vac, vaccinia; NP-Vac, recombinant Vac virus containing the PR8 NP gene; NT60, A/Northern Territories/60/68 (H3N2).

mice were obtained from Dominion Laboratories (Dublin, VA).

Preparation of Target Cells. P815 cells were pelleted and washed twice with Dulbecco's modified phosphate-buffered saline (pH 7.2) before being resuspended at 10^7 cells per ml in Eagle's minimal essential medium supplemented with 20 mM Hepes/0.1% bovine serum albumin. Cells were then incubated for 1–2 hr at 37°C with 10–100 egg infectious doses or plaque-forming units per cell for influenza and Vac viruses, respectively, pelleted, and resuspended in RPMI medium. Following a 6-hr incubation at 37°C, cells were pelleted, resuspended in 100–200 μ l of RPMI medium supplemented with 250–500 μ Ci (1 Ci = 37 GBq) $\text{Na}^{51}\text{CrO}_4$, and incubated for 1 hr at 37°C. Cells were then pelleted, washed, and suspended at a concentration of 10^5 cells per ml for use in microcytotoxicity assays.

Preparation of Effector Cells. Splenocyte suspensions were prepared from mice primed 3–8 wk earlier by intraperitoneal inoculation with 1 ml of phosphate-buffered saline containing 10^8 egg infectious doses of influenza virus or by intravenous injection with 0.2 ml of phosphate-buffered saline containing 10^7 plaque-forming units of Vac virus. Approximately 6×10^7 splenocytes were co-cultured for 6 days at 37°C with 3×10^7 autologous splenocytes infected with influenza or Vac viruses in 40 ml of Iscove's modified Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Microcytotoxicity Assays. *In vitro*-stimulated splenocytes in 100 μ l of Iscove's modified Dulbecco's modified Eagle's medium were added at ratios of 20:1, 7:1, 3:1, and 1:1 to 10^4 ^{51}Cr -labeled target cells in 100 μ l of Iscove's modified Dulbecco's modified Eagle's medium, in round-bottom, 96-well polystyrene plates. Following a 4-hr incubation at 37°C, 100 μ l of the supernatant was removed and the concentration of released ^{51}Cr was determined with an LKB Rackgamma γ -counter. Data are expressed as % specific release defined as [experimental cpm – spontaneous (no CTL) cpm]/[total (detergent) cpm – spontaneous cpm]. Spontaneous release values were always <20% of total cpm. All assays were performed in triplicate or quadruplicate. Unlabeled target inhibition assays were performed by adding unlabeled target cells to ^{51}Cr -labeled targets at ratios of 50:1, 25:1, 12:1, and 6:1 using an effector-to-labeled target ratio of 3:1.

RESULTS

Characterization of NP Produced by Recombinant Virus-Infected Cells. A recombinant Vac virus containing a DNA copy of the PR8 NP gene under the control of an early Vac virus promoter (referred to as NP-Vac) was constructed by using methods described previously (22, 23). Infection of monkey kidney cells with this virus resulted in the production of a polypeptide that was immunoprecipitable by rabbit anti-PR8 sera and that co-migrated in sodium dodecyl sulfate/polyacrylamide gels with NP produced during PR8 infection of the same cells. Detergent extracts from NP-Vac-infected monkey kidney cells adsorbed to polyvinyl 96-well plates bound to all 48 murine anti-NP monoclonal antibodies tested in an indirect enzyme immunoassay. The specificity of binding was demonstrated by the failure of these antibodies to bind similarly prepared extracts from cells infected with a Vac virus recombinant containing the PR8 HA gene. These findings demonstrate that NP produced during NP-Vac infection is very similar, if not identical, to NP produced during PR8 infection.

Recognition of NP-Vac-Infected Cells by Cross-Reactive CTL. Preliminary experiments established that NP-Vac-infected P815 cells expressed roughly similar amounts of cell surface NP as PR8-infected cells, as determined by indirect

immunofluorescence using monoclonal anti-NP antibodies. NP-Vac-infected cells were then tested for recognition by cross-reactive anti-influenza A virus CTL. Splenocytes derived from NT60-primed BALB/c mice and stimulated *in vitro* with Jap-infected autologous splenocytes lysed NP-Vac-infected cells to a similar extent as PR8-infected cells but did not lyse wild-type Vac- or B Lee-infected cells (Fig. 1A). The failure of similarly stimulated CTL derived from CBA mice ($H-2^k$) to lyse NP-Vac-infected P815 cells above control values (Fig. 1B) demonstrates the major histocompatibility complex-restricted nature of NP recognition [the same CBA CTL were able to lyse L cells ($H-2^k$) infected with PR8, Jap, or HK]. A further experiment demonstrated that a number of cross-reactive CTL populations induced by priming and stimulation with a variety of influenza A virus subtypes specifically lysed NP-Vac-infected cells (Table 1).

To roughly assess the proportion of NP-specific CTL present in cross-reactive CTL populations, "unlabeled target" competition studies were performed. The lysis of ^{51}Cr -labeled NP-Vac-infected cells by a cross-reactive CTL population induced by Jap priming and NT60 *in vitro* stimulation was almost completely inhibited by unlabeled NP-Vac- or

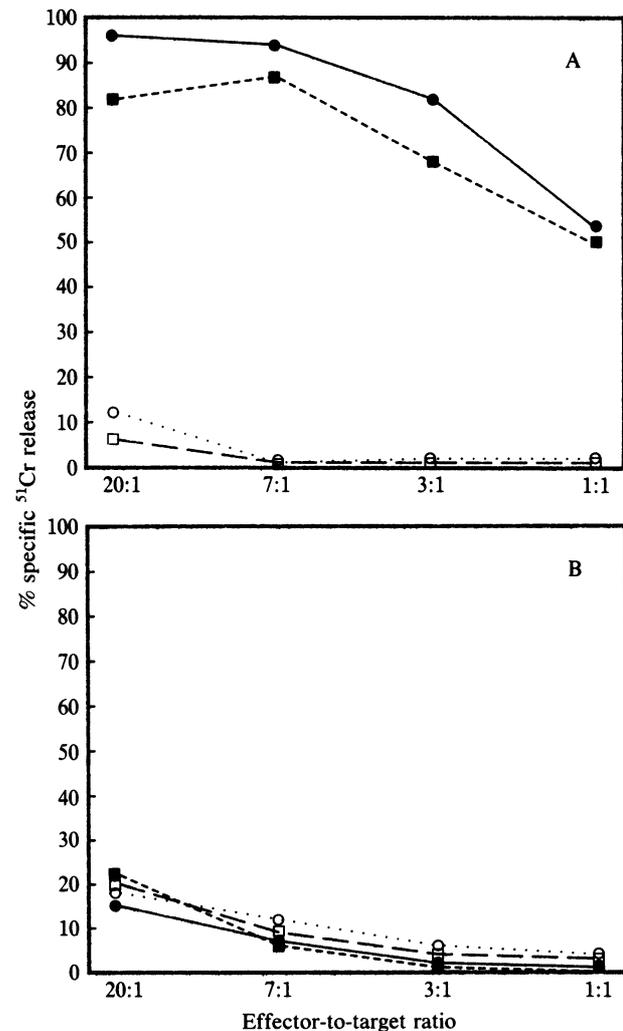


FIG. 1. Recognition of NP-Vac-infected cells by cross-reactive CTL. Cytotoxicity assays were performed by adding ^{51}Cr -labeled influenza or Vac virus-infected P815 ($H-2^d$) to cross-reactive anti-influenza A virus-specific CTL at various ratios. ●, PR8 targets; ○, B Lee targets; ■, NP-Vac targets; □, Vac targets. (A) Jap (H2N2) *in vitro*-stimulated splenocytes derived from NT60 (H3N2)-primed BALB/c ($H-2^d$) mice. (B) NT60 *in vitro*-stimulated splenocytes derived from Jap-primed CBA ($H-2^k$) mice.

Table 1. Recognition of NP-Vac-infected cells by a number of cross-reactive CTL populations

Splenocytes		% specific release of infected P815 cells			
		NP-Vac		Vac	
Primary	Secondary	3:1	1:1	3:1	1:1
PR8 (H1N1)	Jap (H2N2)	78	51	10	5
PR8	NT60 (H3N2)	67	34	11	3
Jap	PR8	45	19	14	6
Jap	NT60	54	25	7	5
NT60	PR8	76	37	11	4
NT60	Jap	65	30	2	0

Cytotoxicity assays were performed by using BALB/c splenocytes and ⁵¹Cr-labeled virus-infected P815 cells at the effector-to-target ratios indicated.

PR8-infected cells (Fig. 2A). The specificity of this inhibition is demonstrated by the failure of Vac- or B Lee-infected cells to significantly inhibit lysis. When ⁵¹Cr-labeled PR8-infected cells were used as targets for the same CTL, inhibition by NP-Vac-infected cells plateaued at roughly 50% of the killing

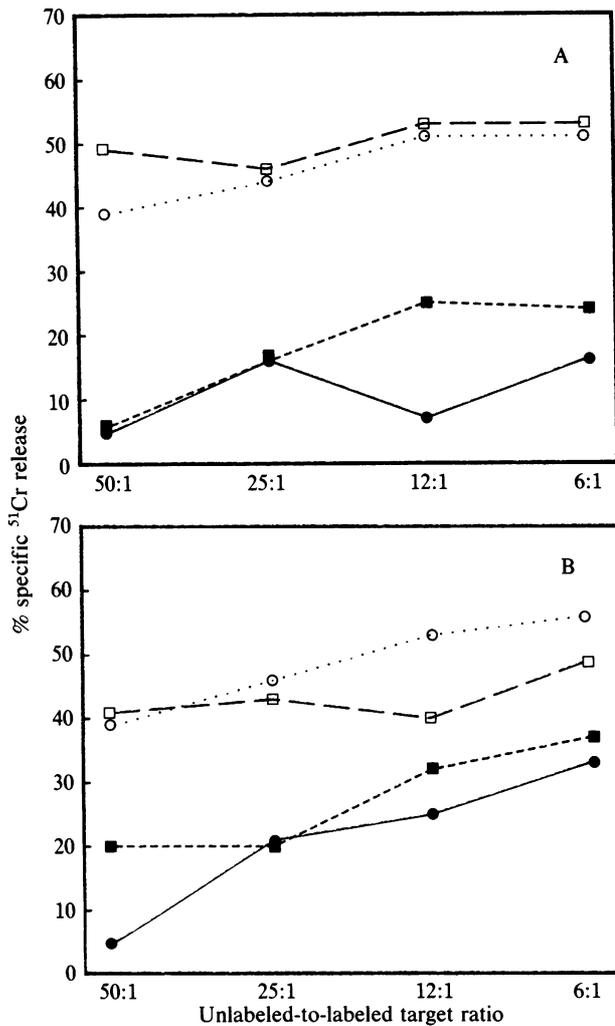


FIG. 2. Inhibition of cross-reactive CTL by unlabeled NP-Vac-infected cells. Unlabeled target inhibition assays were performed by using unlabeled influenza- or virus-infected P815 cells to inhibit lysis of ⁵¹Cr-labeled P815 cells by NT60 *in vitro*-stimulated splenocytes derived from Jap-primed BALB/c mice. ●, PR8 unlabeled competitors; ○, B Lee unlabeled competitors; ■, NP-Vac unlabeled competitors; □, Vac unlabeled competitors. (A) ⁵¹Cr-labeled NP-Vac-infected P815 cells. (B) ⁵¹Cr-labeled PR8-infected cells.

observed with the control unlabeled target cell inhibitors. In contrast, unlabeled PR8-infected cells were able to almost completely inhibit lysis (Fig. 2B). The inability of NP-Vac-infected cells to inhibit cross-reactive CTL to a similar extent as cells infected with PR8 or other influenza A viruses was found using other cross-reactive CTL populations as well. These data suggest that a significant portion of cross-reactive CTL recognize NP but that other viral antigens may be recognized by cross-reactive CTL as well.

Priming and Stimulation of Cross-Reactive CTL by NP-Vac. To examine the ability of the NP to stimulate cross-reactive CTL, splenocytes derived from BALB/c mice previously immunized with NT60 were stimulated *in vitro* with autologous NP-Vac-infected splenocytes and tested for their ability to lyse P815 cells infected with various influenza viruses. As seen in Fig. 3A, CTL stimulated by NP-Vac were able to efficiently lyse cells infected with influenza A viruses from each of the major human subtypes but failed to lyse cells infected with an influenza B virus. The specificity of NP-Vac stimulation is demonstrated by the failure of wild-type Vac virus to stimulate anti-influenza virus-specific CTL (Fig. 3B).

NP-Vac also primed for a cross-reactive CTL response. Incubation of splenocytes derived from mice primed with

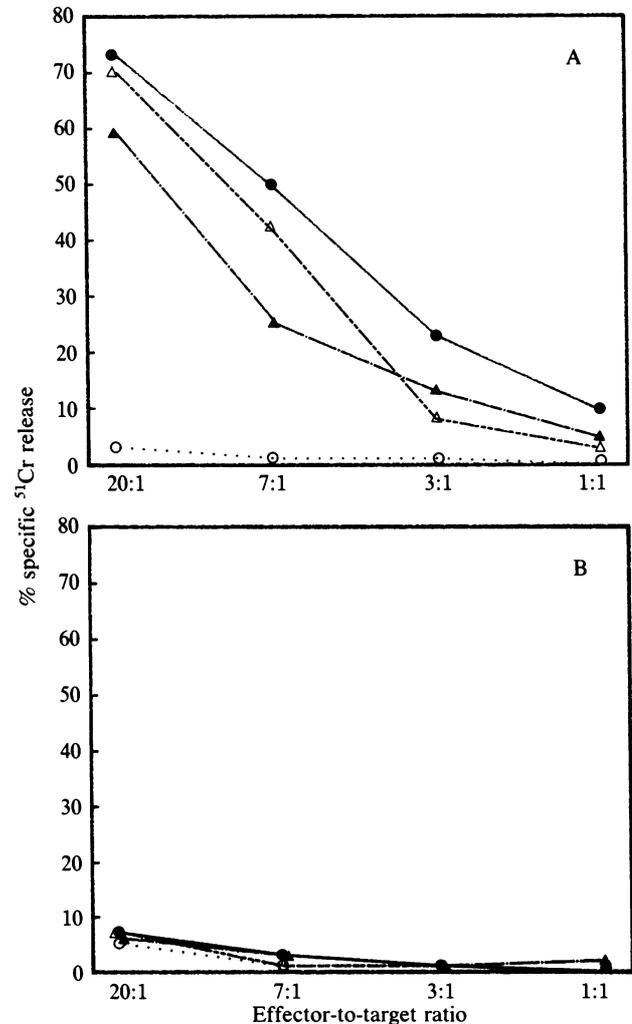


FIG. 3. NP-Vac stimulation of splenocytes derived from influenza A-primed BALB/c mice. Cytotoxicity assays were performed by using ⁵¹Cr-labeled influenza virus-infected P815 cells and *in vitro*-stimulated CTL derived from NT60-primed BALB/c mice. ●, PR8 targets; ○, B Lee targets; ▲, Jap targets; △, HK targets. (A) NP-Vac-stimulated splenocytes. (B) Vac-stimulated splenocytes.

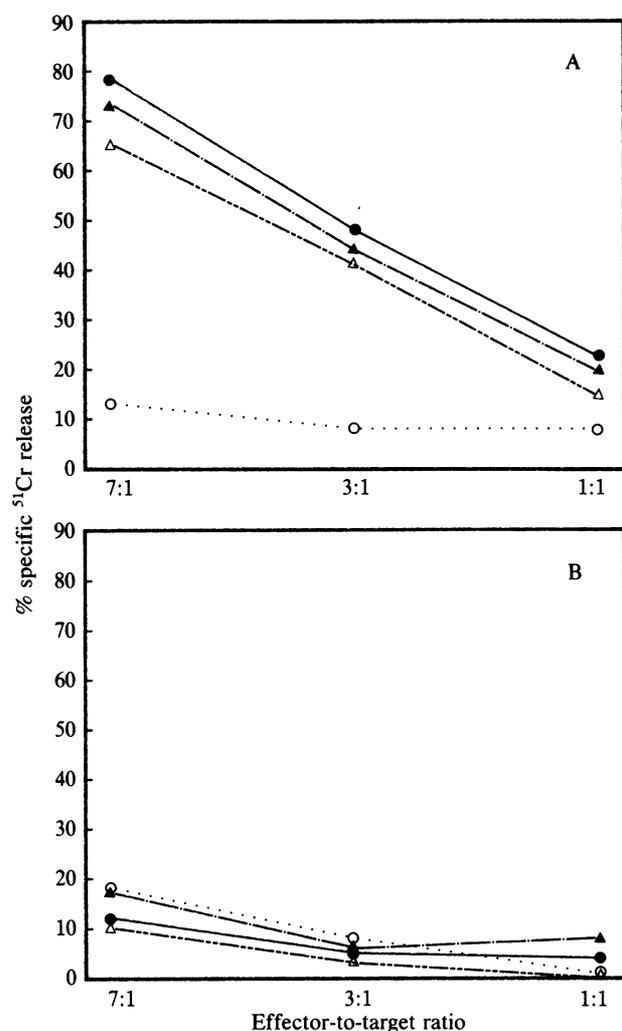


FIG. 4. NP-Vac priming for a cross-reactive anti-influenza A virus CTL response. Cytotoxicity assays were performed by using ⁵¹Cr-labeled influenza virus-infected P815 cells and NT60 *in vitro*-stimulated splenocytes derived from BALB/c mice. ●, PR8 targets; ○, B Lee targets; ▲, Jap targets; △, HK targets. (A) NP-Vac-primed mice. (B) Vac-primed mice.

NP-Vac with autologous NT60-infected splenocytes resulted in the generation of CTL that lysed cells infected with all influenza A viruses tested but not with an influenza B virus (Fig. 4A). In contrast, similarly stimulated splenocytes from Vac-primed mice produced only background lysis (Fig. 4B). An additional experiment revealed that inoculation with NP-Vac resulted in priming for a cross-reactive CTL response upon stimulation with autologous cells infected with any human influenza A subtype virus (Table 2). The specificity of

Table 2. NP-Vac primes for a cross-reactive secondary CTL response

Splenocytes		% specific release of infected P815 cells							
		PR8 (H1N1)		Jap (H2N2)		HK (H3N2)		B Lee	
Primary	Secondary	3:1	1:1	3:1	1:1	3:1	1:1	3:1	1:1
NP-Vac	PR8	47	23	32	15	37	14	12	5
NP-Vac	Jap	80	57	66	33	72	44	8	5
NP-Vac	NT60	58	30	41	19	50	25	10	4
B Lee	B Lee	19	8	11	5	11	5	45	17

Cytotoxicity assays were performed by using BALB/c splenocytes and ⁵¹Cr-labeled virus-infected P815 cells at the effector-to-target ratios indicated.

this recognition is demonstrated by the low levels of specific release given by these CTL on B Lee-infected targets and by the similar low levels given by B Lee-stimulated CTL on influenza A virus-infected targets. These data demonstrate that NP can both prime for and stimulate a vigorous cross-reactive CTL response and, along with the data presented above, indicate that NP is a major target antigen for cross-reactive CTL.

DISCUSSION

In a previous communication, we demonstrated the potential of recombinant Vac viruses for studying the specificity of murine anti-influenza virus CTL (21). We also suggested that, used as live vaccines, these viruses would have the advantage over currently used inactivated or subunit vaccines in priming for secondary CTL responses. In the present study, we have extended this work by placing an influenza virus NP gene into Vac virus and have shown that NP is a major target structure for cross-reactive CTL. This provides direct confirmation of recent reports in which indirect evidence for CTL recognition of NP was presented (24, 25). It is important to recognize that the present findings do not directly identify the target structure(s) recognized by anti-NP CTL. Thus, it is possible that CTL recognize either native NP, alternative forms of NP produced by cellular processing of the native molecule, or NP-induced alterations in cellular components. At present, the first possibility seems marginally more likely since we have found that anti-NP monoclonal antibodies that lose reactivity upon denaturation of NP bind well to NP expressed on infected cell surfaces (unpublished results). This would suggest that at least some cell surface NP is present in its native form and available as a CTL target structure.

Experiments gauging the effects of NP-Vac inoculation on subsequent murine pulmonary infection with influenza viruses from different subtypes have begun; preliminary results indicate that intravenous injection of NP-Vac primes for a secondary cross-reactive CTL response in lungs of mice subsequently challenged by aerosol infection with HK. Based on present knowledge of CTL function, the priming effect of NP-Vac on a secondary cross-reactive CTL response would be expected to result in reduced viral replication in vaccinated animals (1, 2). Since the priming effect of influenza virus infection appears to be relatively short-lived (lasting perhaps a few years) (26), such vaccines may prove useful for certain individuals previously infected with influenza A virus as well as immunologically naive individuals.

This report provides additional evidence for the expression of NP on infected cell surfaces and indicates that its cell surface expression does not require the presence of other influenza virus gene products. The mechanism responsible for NP surface expression is unknown, but previous data suggest it is not due to readsorption of NP leaked or secreted from infected cells (14). NP is located internally in virions, where it is the major viral protein in the ribonucleoprotein complex. Following infection, it first appears in large quantities in the nucleus and at later times in the cytoplasm as well (9). In addition to its role as a major viral structural protein, studies with temperature-sensitive mutants suggest it functions in viral RNA transcription and replication (27). None of these known functions and characteristics suggest a role for cell surface NP in the infectious cycle.

If the expression of NP on cell surfaces is of no functional significance to a productive viral infection, there are two explanations for its occurrence. First, NP may be inadvertently (from the standpoint of both cell and virus) transported to the cell surface, due perhaps to a chance modification of the internal form of the protein. Alternatively, NP may be transported to the cell surface by a deliberate cellular process that

functions normally to transport internal cellular proteins to the cell surface. Although the primary purpose of this process may be the renewal and maintenance of cellular machinery, it could also be of value to the organism as a whole in allowing for immune surveillance of internal cellular alterations. A possible example of the functioning of this process is the surface expression of simian virus 40 T antigen, a protein with no known surface function that elicits a vigorous CTL response (28–30). In the case of internal oncogene products such as this, or lytic infections with viruses for which expression of viral antigens on the cell surface is not an integral part of the infectious cycle, the existence of such a mechanism would be of obvious benefit. Even in the case of infection with influenza viruses and other viruses whose replication depends on the incorporation of viral proteins into the plasma membrane, the surface expression of internal viral components made early in the infectious cycle could speed immune cytolysis and help limit the amount of infectious progeny produced.

The basis for the cell surface expression of NP must be investigated to determine whether surface NP represents a modified form of the internal protein. Recombinant Vac viruses containing other influenza virus genes can also be used to determine which other nonglycosylated viral proteins are expressed on infected cell surfaces. It is hoped that these studies will help determine the basis for the expression of internal viral proteins on infected cell surfaces.

Note Added in Proof. Since the original submission of this manuscript, similar findings of cross-reactive CTL recognition of a cloned NP gene product have been reported by Townsend *et al.* (31).

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