

# Presentation by a Major Histocompatibility Complex Class I Molecule of Nucleoprotein Peptide Expressed in Two Different Genes of an Influenza Virus Transfectant

By Hideki Isobe,\* Thomas Moran,\* Shengqiang Li,\* Aideen Young,† Stanley Nathenson,† Peter Palese,\* and Constantin Bona\*

From the \*Department of Microbiology, Mount Sinai School of Medicine, New York 10029-6574; and the †Department of Microbiology and Immunology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

## Summary

Major histocompatibility (MHC) class I glycoproteins are specialized to present to CD8<sup>+</sup> T cells, peptides that originate from proteins synthesized within the cytoplasm. Conventional killed vaccines are unable to get into the cell cytoplasm and therefore fail to expand the CD8<sup>+</sup> T cell population. We have created a novel influenza transfectant virus, R10, which carries an immunogenic peptide from the nucleoprotein (NP) of PR8 influenza virus in its hemagglutinin (HA) and another similar peptide in its HK influenza virus NP. The two peptides are both presented by H-2D<sup>b</sup> and bind with approximately equal affinity. They can compete with one another for binding to H-2D<sup>b</sup>. Yet in cells infected with R10, both peptides are presented efficiently enough to expand the respective cytotoxic T lymphocyte (CTL) precursors *in vivo* and to serve as targets for CTL lysis *in vitro*. It has been proposed that proteins bearing signal sequences may be processed by a transporter-independent pathway. To investigate this, we infected the transporter-deficient cell line RMA-S with the R10 virus to see if the NP peptide expressed by the HA would be presented. The result shows that even the presence of a signal peptide in the HA does not overcome the lack of a transporter function, suggesting that the presentation of both peptides is dependent on functional transporter proteins. Our data also suggest the feasibility of creating by genetic engineering, recombinant vaccines expressing multiple epitopes that can effectively stimulate a cellular immune response.

CTL recognize short peptides derived from the processing of intracellular proteins that are presented at the cell surface by class I molecules of the major histocompatibility locus (1, 2). The isolation of naturally processed peptides from class I antigens has shown that for each allele the peptides exhibit a "motif" characterized by a few amino acids, and that these residues are crucial for binding to MHC antigens (3–7). This concept was strengthened by the resolution of the crystal structure of class I molecules showing that the consensus residues dock within pockets of the antigen-binding cleft (8–10).

Sensitization of cells with synthetic peptides showed that MHC molecules can bind and present specific peptides as well as analogues that differ in structure and length (10, 11). Whereas numerous studies have focused on the recognition of natural peptides and synthetic analogues by CD4 T cells (12–14), there is limited information on the effects of analogues on the presentation of endogenous viral peptides as-

sociated with class I antigens to CD8<sup>+</sup> T cells (15). These questions are particularly important for the development and the use of recombinant vaccines containing multiple epitopes.

Our study was aimed at investigating the presentation of two influenza virus nucleoprotein (NP)<sup>1</sup>-derived peptides that bind to the same allele (*i.e.*, D<sup>b</sup>). Conceptually, these two NP peptides may be considered to be analogues. To carry out this study, we took advantage of a new reverse genetics approach allowing the expression of foreign epitopes or antigens by influenza virus. This method is based on the rescue of cDNA-derived RNA into infectious viral particles (16). Using this technique it was possible to express in a hemagglutinin (HA) molecule the epitopes of HA of different virus

<sup>1</sup> Abbreviations used in this paper: ER, endoplasmic reticulum; HA, hemagglutinin; NP, nucleoprotein; SP, spleen cell; TAP, transporter-associated with antigen processing.

subtypes (17), an epitope derived from the V<sub>3</sub> loop of the HIV-1 glycoprotein (gp120) protein (18), and the epitope of the circumsporozoite protein of *Plasmodium yoelii* (19). For the present study, we created a chimeric influenza virus by inserting into antigenic site E of the HA of influenza A/WSN/33 virus, a nonamer peptide derived from the NP of influenza A/PR/8/34 virus (PR8). The chimeric HA gene was then transferred into a virus that derives the remaining seven RNA segments from influenza A/HK/8/68 virus (HK). Therefore, our chimeric virus (R10) expresses the PR8-NP peptide within the HA and the HK-NP peptide from its NP protein. PR8-NP and HK-NP peptides share the same anchoring motifs and differ by only two amino acid residues (20). This influenza virus transfectant expressing both peptides was used to study: (a) whether antigenic competition hinders the efficient presentation of two peptide analogues presented to CTLs by the same allele; and (b) how the transporter-associated with antigen processing (TAP) dependent transport system (21) affects the efficiency with which peptides are presented from proteins with and without signal sequences.

## Materials and Methods

**Mice.** C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

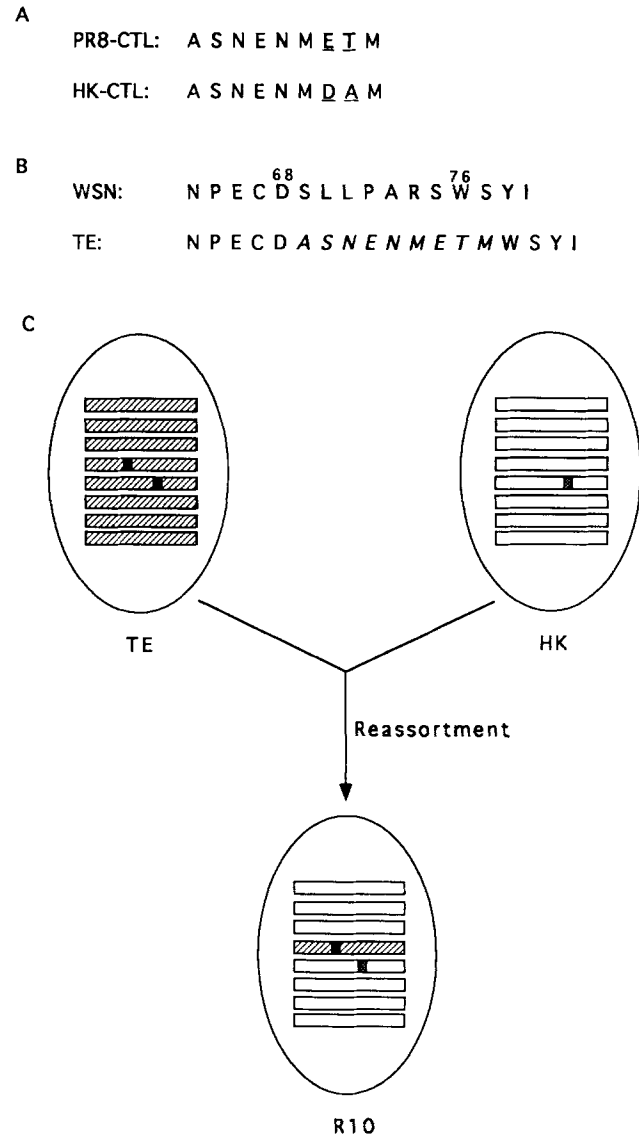
**Viruses.** Influenza A/PR/8/34 and A/HK/8/68 viruses were grown in embryonated eggs, whereas the chimeric influenza virus R10 was grown in Madin Darby canine kidney (MDCK) as previously described (16).

**Cell Lines.** EL-4, RMA, and RMA-S cells (kindly donated by Dr. J. Yewdell, National Institutes of Health, Bethesda, MD) were grown in DMEM medium supplemented with 10% FCS and were used as targets for the cytotoxic assays.

**Peptides.** Peptide ASNENMETM corresponding to amino acid residues 366-374 of the influenza virus A/PR/8/34 NP and ASNENMDAM corresponding to amino acid residues 366-374 of the influenza A/HK/8/68 NP were synthesized in the Department of Pharmacology, Mount Sinai Medical Center and were purified by HPLC. Two peptides corresponding to amino acid residues 229-237 (CKGVNKEYL) and 489-497 (QGINLDNL) of the SV40 T antigen were a gift from Dr. J. Yewdell.

**Construction of the Transfectant Virus TE Expressing the PR8-NP Epitope.** Plasmid pTE was constructed by replacing the BstEII-HindIII fragment of pT3/WSN-HA containing the influenza A/WSN/33 virus HA gene (19) with a PCR product, in which the nucleotide sequence encoding seven amino acid residues at the antigenic site E of the HA was replaced by the sequence encoding the PR8-NP peptide ASNENMETM. Rescue of infectious virus carrying the pTE plasmid-derived RNA was done as described (17). The nucleotide sequence encoding the PR8-NP peptide was confirmed by direct sequencing of the viral HA segment as previously described (17). All other genes of the virus are derived from influenza A/WSN/33 virus.

**Generation of the Reassortant R10 Virus.** The reassortment of viruses has been done as described (22). Briefly, Madin Darby canine kidney (MDCK) cells were infected with influenza HK virus and UV-irradiated TE virus. The supernatant of the infected cells was used for plaque assay in the presence of anti-HK antiserum. From 10 individually isolated plaques, R10 was genotyped and shown to contain its HA gene derived from the TE virus and its remaining genes from HK virus (Fig. 1).



**Figure 1.** Construction of the transfectant TE virus and the reassortant virus R10. (A) The amino acid sequences of the D<sup>b</sup>-restricted NP-specific CTL epitopes of influenza PR8 and HK viruses. Differences between the peptides are underlined. (B) The transfectant virus TE was designed to express the PR8 virus-specific CTL epitope in site E of the influenza A/WSN/33 virus HA. It was constructed by replacing the 7-amino acid peptide of the HA with the 9-amino acid CTL peptide (indicated in italics). (C) The reassortant virus R10 was generated by mixed infection of the transfectant TE virus and the influenza A/HK/8/68 virus. It derives the HA gene (RNA segment 4) from the TE virus and the remaining genes from HK virus. The CTL epitopes of the NP proteins of PR8 and HK viruses are indicated in black or gray boxes, respectively. It should be noted that the NP-specific PR8 CTL epitope is also present in the NP of the TE virus but not in reassortant virus R10 which contains HK virus NP segment.

**Induction of CTL Response.** C57BL/6 mice were immunized intraperitoneally with 0.2 ml of a viral suspension containing 10<sup>7</sup> PFU of PR8, HK, or R10 viruses. 7 d later, spleen cells (SC) were obtained and restimulated in vitro for 5 d with irradiated SC alone or coated with PR8-NP or HK-NP peptides or SC infected with PR8, HK, or R10 viruses as previously described (23). CTL clones

were obtained by limiting dilution (1 or 3 cells/well) and stimulated with SC coated with NP peptides.

**Cytolysis Assay.** EL-4, RMA, or RMA-S cells coated with PR-NP or HK-NP peptides or infected with PR8, HK, or R10 viruses were labeled with  $\text{Na}^{51}\text{CrO}_4$  ( $100 \mu\text{Ci}/10^6$  cells) for 1 h at  $37^\circ\text{C}$ . After two washings, the cells were transferred to V-bottom 96-well plates, the effector cells were added, and the mixture was incubated at  $37^\circ\text{C}$  in 7%  $\text{CO}_2$ . 4 h later, the supernatant was harvested and counted. The maximum chromium release was determined by incubating the cells with 1% NP-40. The percentage of specific lysis is calculated according to the following formula:  $100 \times [(\text{cpm samples} - \text{cpm spontaneous release}) / (\text{cpm maximum release} - \text{cpm spontaneous release})]$ .

**Immunostaining.** RMA-S cells were infected with  $10^7$  PFU of PR8 or R10 viruses. The presence of HA on the surface was detected by immunostaining as previously described (24). For immunostaining of cells, mAbs PY211 and 2G9 specific for the HA of PR8 virus and the HA of WSN virus, respectively, were used. As a negative control, mAb PY206 specific for the HA of X31 virus was used. Identical samples of infected cells were analyzed for hemadsorption.

## Results

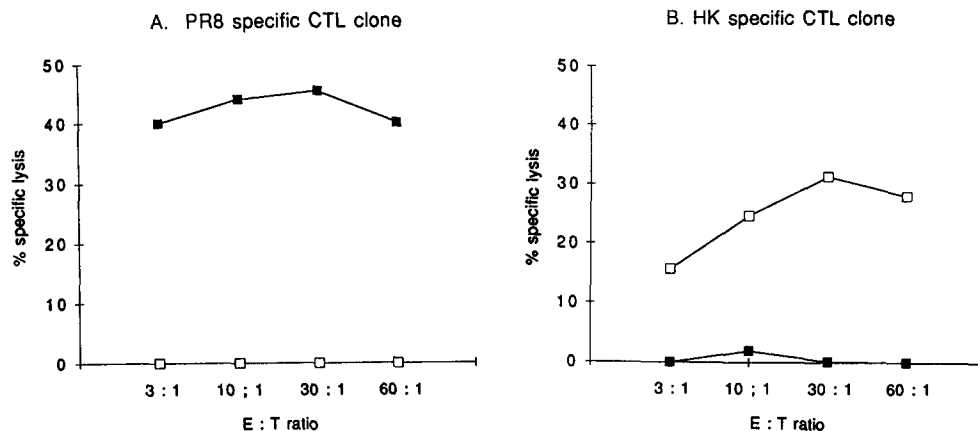
**Recognition of PR8 and HK-NP Peptides.** The two peptides which are derived from the NPs of influenza PR8 and HK viruses and bind to the  $\text{D}^b$  MHC glycoprotein, differ by two amino acids. The PR8-NP peptide has glutamic acid and threonine at positions 7 and 8 respectively, whereas the HK-NP peptide has aspartic acid and alanine in these positions (3). CTLs are able to discriminate between these subtle differences, as shown by our ability to generate NP peptide-specific CTL clones from C57BL/6 mice immunized with PR8 or HK viruses. Upon *in vitro* culture of splenic lymphocytes with PR8-NP or HK-NP peptides, the cells were cloned at 1–3 cells/well and expanded for several rounds. The data depicted in Fig. 2 show that PR8-NP-specific CTLs lysed EL-4 cells coated with PR8-NP but not with HK-NP peptide, and that HK-NP-specific CTLs lysed only EL-4 cells coated with HK-NP peptide. Whereas  $0.01 \mu\text{M}$  of PR8-NP and HK-NP peptides was required to sensitize EL-4 target cells for significant lysis by CTL clones,  $1 \mu\text{M}$  of peptide was necessary to reach the plateau of sensitization (data not

shown). Similar results were obtained with RMA and RMA-S cells (data not shown).

**Competition of Binding of PR8-NP and HK-NP Peptides to Surface  $\text{D}^b$  Glycoprotein.** A potential drawback to the use of virus transfectants, as vaccines expressing multiple peptides specific for the same allele, is that intramolecular competition may occur with one peptide being preferentially presented over others. The PR8-NP and HK-NP peptides differ by only two amino acid residues, and since the anchoring residues are identical, they may be considered to be analogues. Thus, if intramolecular competition occurs, it should be demonstrable using these peptides. To confirm that they had this capability, we studied the competition at the level of peptide presentation by simultaneously incubating constant amounts of PR8-NP peptide with variable amounts of HK-NP peptide and vice versa. When we incubated EL-4 cells with  $10 \mu\text{M}$  PR8-NP peptide and various amounts ( $10$ – $100 \mu\text{M}$ ) of HK-NP peptide, little inhibition of lysis of EL-4 cells by PR8-NP-specific CTL was observed. Only a weak inhibition was also observed when  $10 \mu\text{M}$  HK-NP peptide was incubated with various amounts of PR8-NP peptide and HK-NP specific CTLs (data not shown). In contrast, a strong inhibition was observed when only  $1 \mu\text{M}$  of PR8-NP peptide was simultaneously incubated with the HK-NP peptide or with peptides derived from SV40 virus which are recognized by  $\text{CD8}^+$  T cells in association with the  $\text{D}^b$  MHC class I glycoprotein (see Fig. 3 A). This was also true when EL-4 cells were simultaneously incubated with  $1 \mu\text{M}$  HK-NP peptide and various amounts of PR8-NP or the SV40 peptides (Fig. 3 B). These results suggest that PR8-NP and HK-NP peptides display a similar affinity for the  $\text{D}^b$  class I MHC glycoprotein and have the potential to compete with one another for occupancy in the  $\text{D}^b$  binding site.

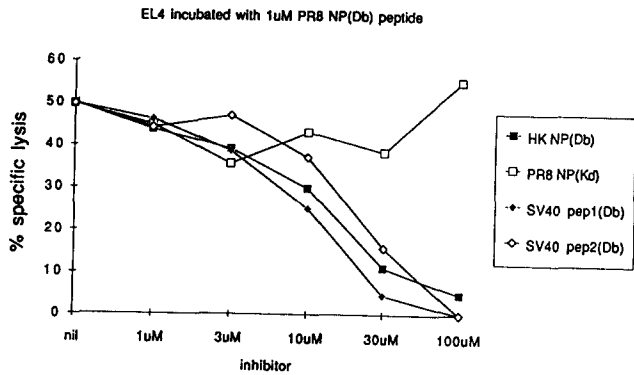
We incubated the target cells for 30 min with PR8-NP or HK-NP peptides, and then washed and incubated them for 2 h with various amounts of analogues. No inhibition of lysis of EL-4 cells by PR8-NP or HK-NP specific CTL was observed (data not shown). These results suggest that the two peptides have similar affinities for the MHC molecules.

**Presentation of the PR8-NP Peptide Expressed within the HA of Influenza Virus Transfectant R10.** In preliminary experi-

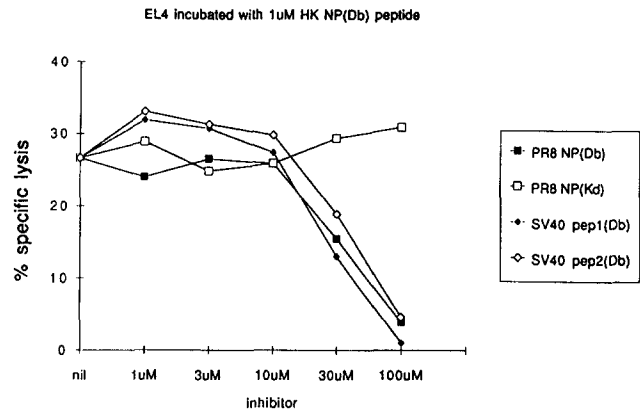


**Figure 2.** Specific lysis of EL-4 cells coated with PR8-NP or HK-NP peptides by PR8-NP or HK-NP peptide-specific CTL clones. (■) Coated with 366-374 PR8 NP ( $\text{D}^b$ ) peptide; (□) coated with 366-374 HK NP ( $\text{D}^b$ ) peptide.

### A. PR8 specific CTL clone



### B. HK specific CTL clone



**Figure 3.** Inhibition of CTL activity by peptide analogs. (A) Specific lysis by PR8-NP specific CTL clone of EL-4 cells simultaneously incubated with 1  $\mu$ M PR8-NP peptide and varying amounts of HK-NP and SV40 peptides. (B) Specific lysis by HK-NP-specific CTL clone of EL-4 cells simultaneously incubated with 1  $\mu$ M HK-NP peptide and varying amounts of PR8-NP and SV40 peptides. The experiments were carried out at a 40:1 E/T ratio.

ments we investigated the presentation of the PR8-NP peptide expressed by the HA gene of the transfectant virus R10 and that of PR8 virus which contains the peptide in the NP. C57BL/6 mice were immunized with PR8 virus and splenic lymphocytes were secondarily stimulated in vitro with SC coated with PR8-NP peptide. The CTL activity was determined using PR8-NP peptide-sensitized EL-4 target cells or cells infected with PR8 or R10 viruses. The data show that, as expected, cells from nonimmunized mice, whether or not secondarily stimulated with PR8-NP peptide-coated cells, did not lyse EL-4 cells (Fig. 4, A and B). Similarly, cells from PR8 virus-immunized mice which were not restimulated in vitro with PR8-NP peptide-coated cells did not lyse EL-4 cells (Fig. 4 C). In contrast, cells from PR8 virus-immunized mice after secondary in vitro stimulation with PR8-NP peptide-coated SC, lysed EL-4 target cells coated with homologous peptide or infected with PR8 or R10 viruses (Fig. 4 D). These results show that the PR8-NP peptide is generated from the PR8 virus NP as well as from the R10 virus HA and that it is efficiently presented to CTL. More importantly, the NP peptide generated from the R10 HA was immunogenic in vivo since upon in vitro incubation with PR8-NP peptide-coated SC, cells from mice immunized with R10 virus were able to lyse EL-4 target cells infected with R10 or PR8 viruses or sensitized with PR8-NP peptide (Fig. 4 F). These results demonstrate that the same core peptide expressed in genes coding for different viral proteins can prime the peptide-specific CTL precursors in vivo.

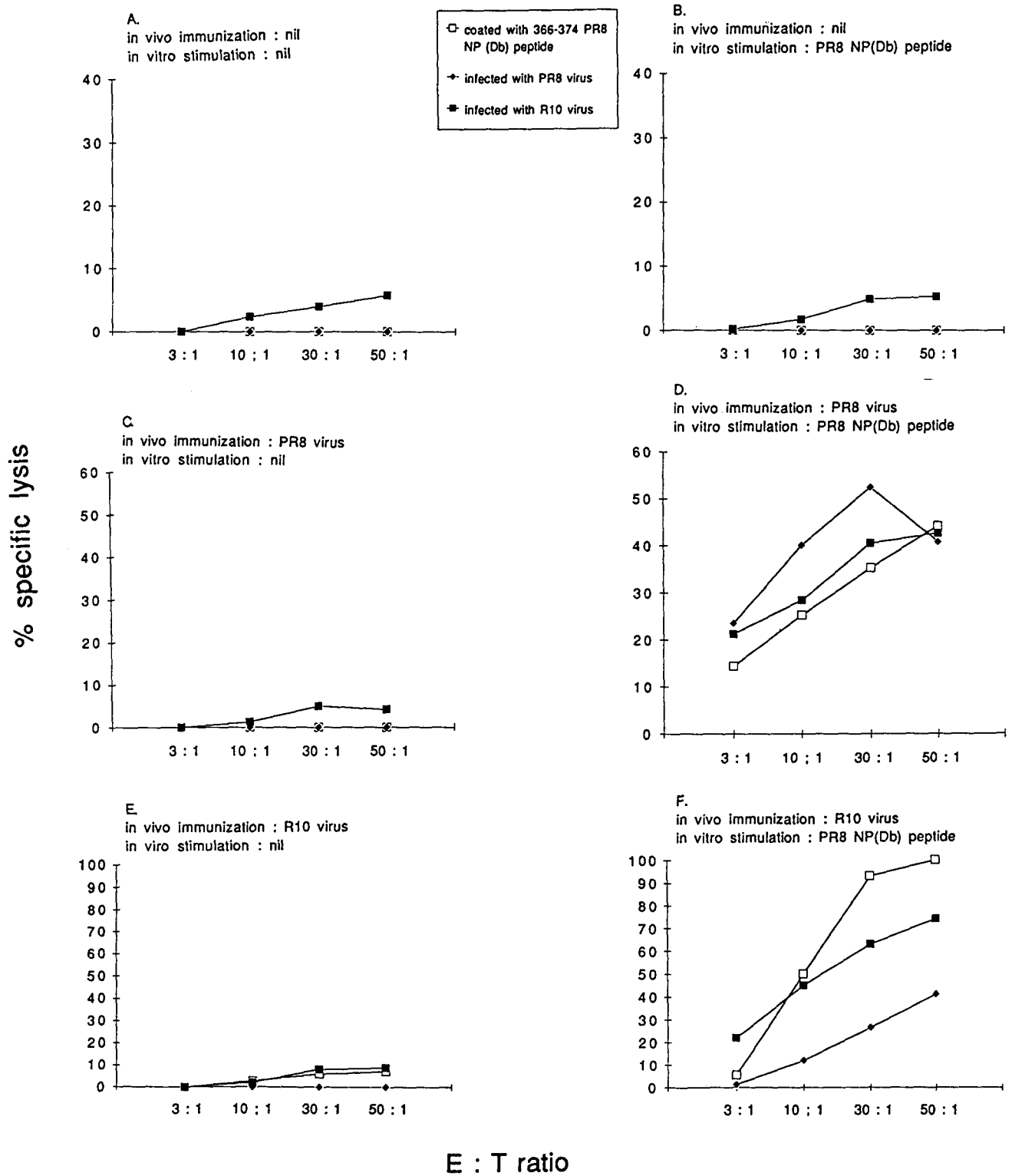
The results were confirmed by experiments in which splenic lymphocytes from mice immunized with PR8, HK or R10 viruses were secondarily stimulated in vitro with PR8-NP or HK-NP peptide coated SC, and then tested for CTL activity on PR8, HK, or R10 virus infected EL-4 cells. The data depicted in Table 1 show that as expected, cells from nonimmunized mice do not exhibit CTL activity upon in

vitro stimulation with PR8-NP or HK-NP peptide-coated SC. Cells from PR8 virus-immunized mice stimulated secondarily in vitro with PR8-NP-coated SC do not exhibit cytotoxic activity against HK virus-infected cells, however, they lyse target cells infected with PR8 or R10 viruses. In contrast, cells stimulated with HK-NP peptide-coated SC show a low but significant lytic capacity of EL-4 target cells infected with PR8 or R10 viruses. This activity can be attributed to cross-reactive CTL (25) that were primed in vivo and that are not eliminated by a single cycle of in vitro stimulation with peptides, the method used to generate CTL clones. Cells from mice immunized with HK virus show a significantly lower frequency of cross-reactive clones since upon in vitro stimulation with PR8-NP peptide-coated SC, low lytic activity is observed using target cells infected with PR8, HK, and R10 viruses. After in vitro stimulation with HK-NP peptide-coated SC, these cells showed significant lysis of EL-4 target cells infected with HK and R10 viruses.

It is important to note that whereas CTLs from PR8 virus-primed mice stimulated in vitro with HK-NP peptide were able to kill target cells infected with PR8 virus, those four animals primed with HK virus did not. Asymmetrical expansion of CTLs from PR8 virus-primed animals by HK-NP peptide suggest that the PR8 virus priming stimulated cross-reactive CTL or a discrete subset of CTLs exhibiting a promiscuous recognition. This may reflect the fact that PR8 virus replicates much more efficiently in mice than HK virus.

Table 1 also demonstrates that cells from animals immunized with R10 virus stimulated in vitro with PR8-NP or HK-NP peptide-coated SC display cytotoxic activity against PR8, HK, and R10 virus-infected EL-4 cells. Thus, the R10 virus can prime in vivo CTL for lysing target cells infected with both PR8 and HK virus.

These results clearly demonstrate that the R10 virus can efficiently prime both NP specific and cross-reactive CTLs



**Figure 4.** In vitro expansion of PR8-NP peptide-specific CTL obtained from mice that were immunized with PR8 virus or transfectant virus R10.

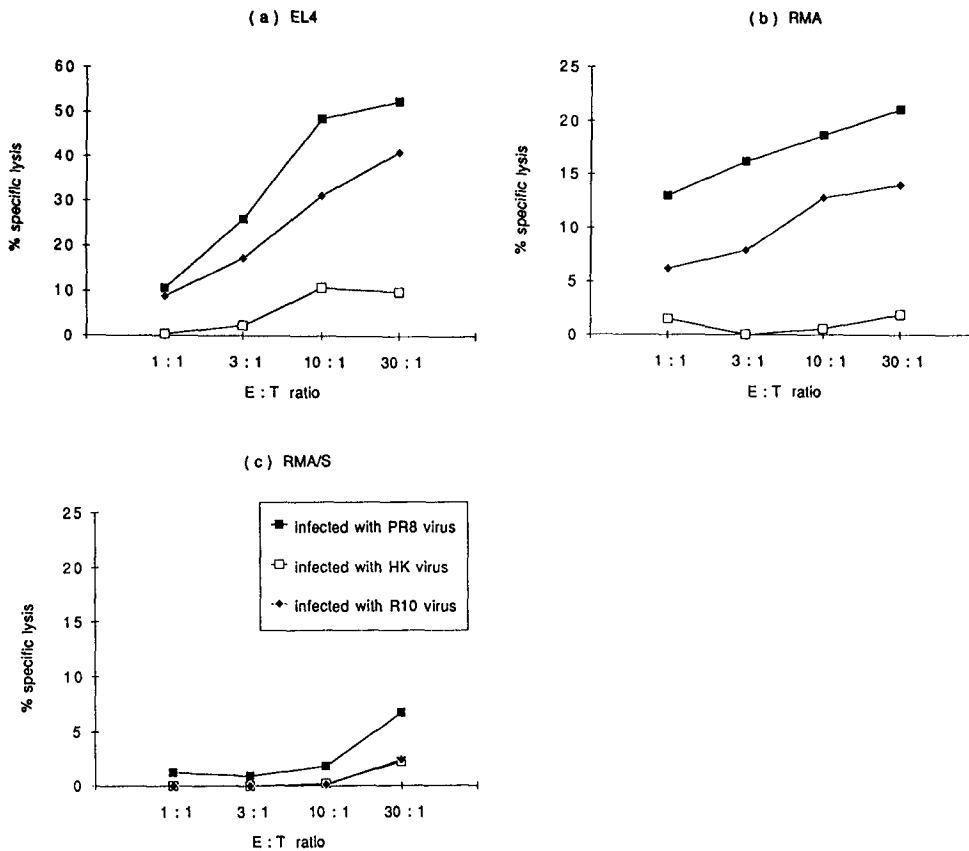
**Table 1.** *In Vivo Priming of CTL by R10 Influenza Virus Transfectant*

Mice immunized with	Number of mice started	In vitro stimulation with spleen cells coated with	EL-4 cells infected with		
			PR8 virus	HK virus	R10 virus
Saline	4	nil	1.2 ± 1.5*	1.3 ± 1.1	0
		PR8 NP	0.8 ± 1.2	0	0
		HK NP	0.8 ± 1.0	0	0
		nil	2.5 ± 1.8	0.3 ± 0.3	0
PR8 virus	6	PR8 NP	43.7 ± 21.5	2.2 ± 2.4	35.8 ± 10.9
		HK NP	28.6 ± 12.2	1.6 ± 2.7	37.4 ± 6.2
		nil	1.2 ± 1.0	1.0 ± 1.9	0.1 ± 0.4
NK virus	6	PR8 NP	11.2 ± 3.2	6.1 ± 7.3	14.1 ± 9.3
		HK NP	12.6 ± 4.6	37.7 ± 21.9	38.9 ± 8.0
		nil	3.7 ± 2.8	5.8 ± 5.2	0
R10 virus	6	PR8 NP	33.9 ± 16.8	43.5 ± 13.0	36.6 ± 16.0
		HK NP	18.2 ± 6.5	45.7 ± 24.0	37.2 ± 11.6

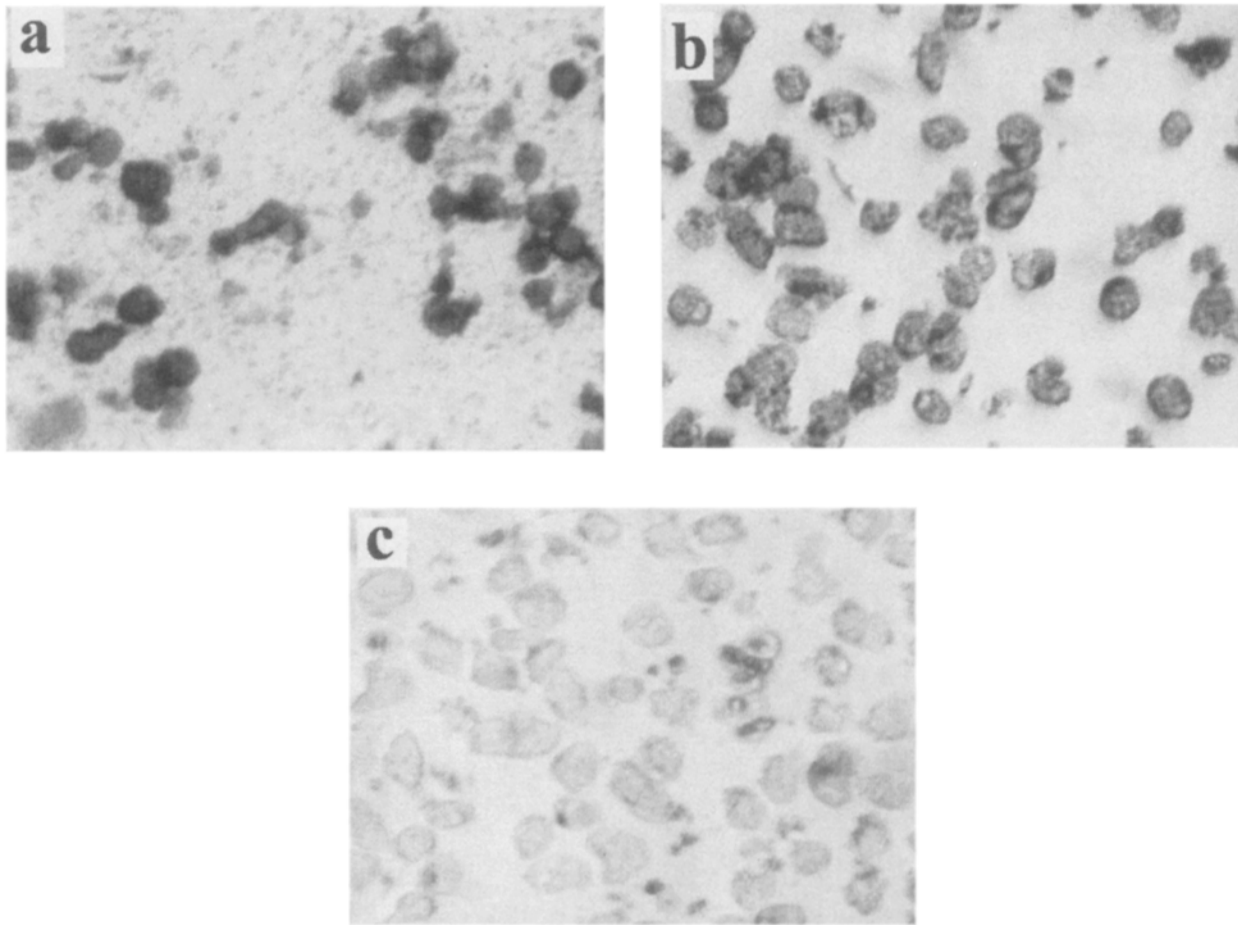
All the experiments were performed at a 30:1 E/T ratio.

NP, NP peptide

\* specific cytotoxicity—average ± SD.



**Figure 5.** Comparison of lysis of EL-4, RMA, and RMA-S cells infected with PR8, HK, or R10 viruses by PR8-NP peptide-specific CTL.



**Figure 6.** Expression of HA on the surface of RMA-S cell infected with PR8 and R10 viruses. RMA-S cells were incubated for 60 min with PR8 or R10 seed virus, washed, and cultured overnight.  $5 \times 10^4$  RMA-S-infected cells were fixed for 5 min with 1% paraformaldehyde, washed, and incubated for 60 min with mAb (10  $\mu$ g/ml). After washing, the cells were incubated for 45 min with peroxidase-labeled goat anti-mouse IgG (1:500 dilution), washed twice, and incubated in AEC substrate medium according to the manufacturer's instructions (Dako, Copenhagen, Denmark). (a) Immunostaining: virtually all cells infected with PR8 virus are stained after incubation with PY211 mAb-specific for PR8 virus HA (H1).  $\times 200$ . (b) Immunostaining: the majority of cells infected with R10 virus are stained after incubation with 2G9 mAb specific for WSN virus HA  $\times 200$ . (c) Immunostaining: lack of staining of cells infected with R10 virus after incubation with PY206 mAb-specific for X31 virus HA (H3).  $\times 200$ .

compared with PR8 or HK viruses. It is also possible that within APCs infected with R10 virus, both PR8 and HK-NP peptides are generated and therefore CTL precursors exhibiting specificities for these peptides were expanded. These results, taken together, indicate that both peptides are generated from R10-transfectant virus. Whether residing in the chimeric HA or the native NP molecule, they are efficiently presented by D<sup>b</sup> MHC and are able to expand precursor CTL in vivo and be recognized by CTL effectors in vitro.

*Presentation by RMA-S Cells of NP Peptides Expressed by the HA and the NP Transfectant Virus R10.* It has been reported that peptides derived from a signal sequence are processed by a transporter-independent pathway (26, 27). Since the PR8-NP peptide is harbored in the HA and has a signal sequence, whereas the HK-NP peptide is carried by the NP protein which is devoid of a signal sequence, it seemed possible that the HA peptide might be presented in the TAP-defective RMA-S cell line whereas the NP peptide would not. RMA-S cells have a defect in MHC class I assembly due to a defined muta-

tion in the *TAP-2* gene (21) that prevents expression of transporter molecules (28). These transporters are believed to be involved in the translocation of peptides to the endoplasmic reticulum (ER) where they encounter MHC class I glycoproteins (29, 30).

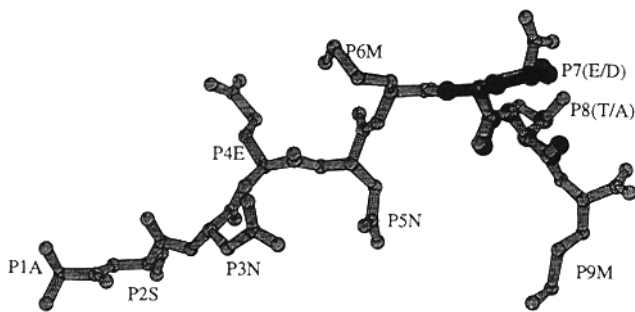
The control experiments show that both EL-4 (Fig. 5 a) and RMA (Fig. 5 b) cells infected with PR8 or R10 viruses (and not those infected with HK virus), are lysed by PR8 NP-specific CTL. The specific lysis of PR8 virus-infected cells was somewhat higher than that of cells infected with R10 virus. This difference reflects the reduced infectivity titer of the transfectant virus ( $10^8$  TCID<sub>50</sub>/ml) relative to that of PR8 virus ( $2.8 \times 10^9$  tissue culture infectious dose [TCID]<sub>50</sub>/ml). Although RMA-S cells sensitized with PR8-NP or HK-NP peptides were efficiently lysed by PR8-NP or HK-NP specific CTL (data not shown), lysis was not observed in the case of RMA-S cells infected with PR8 or R10 viruses (Fig. 5c). Lack of lysis of RMA-S target cells infected with PR8 or R10 viruses was not related to a defective production of the

HA trimer. The HAs are expressed on the surface of RMA-S cells as demonstrated by immunostaining with mAbs specific for the HA of PR8 or WSN viruses, respectively. (Fig. 6). The presence of HA of PR8 and WSN viruses on the surface of cells infected with R10 viruses was also visualized by hemadsorption (data not shown). Thus, the inability to lyse RMA-S-infected cells suggests that both peptides are processed by a transporter-dependent pathway.

## Discussion

Solution at 2.4 Å resolution of the structure of the H-2D<sup>b</sup> glycoprotein associated with the NP peptide (amino acids 366-374) of the PR8 virus has recently been reported (31). The structure reveals a hydrophobic ridge in the antigen-binding cleft of the glycoprotein, produced by the side chains of Trp73, Tyr156, and Trp147. This ridge was shown to be responsible for a bulge in the backbone of the bound peptide at P6, P7, and P8 that causes these peptide residues to be directed out of the cleft towards external solvent. Based on the high solvent-accessible surface areas of P6M, P7E, and P8T, Young et al. (31) suggested that these residues are available for contact by the TCR.

Our experimental data are in agreement with this prediction since CTL specific for the PR8-NP peptide (P7E and P8T) cannot lyse target cells coated with the HK-NP peptide (P7D and P8A) and HK-NP-specific CTL clones cannot lyse EL-4 cells coated with the PR8-NP peptide. Hence, since the identity of the side chains of the peptide residues at P7 and P8 are crucial to CTL recognition, these peptide residues must be involved in contact with the TCR. Fig. 7 shows the conformation of the PR8-NP peptide from the 2.4 Å resolution crystal structure. Superimposed on this are P7 and P8 from a model of the HK-NP peptide, obtained by replacing Glu and Thr at P7 and P8 of the PR8-NP peptide with Asp and Ala, respectively, and then performing 100 cycles of energy minimization using Insight (Biosym Technologies, CA). From this model, we predict that the differing CTL response of the two peptides is due to the fact that the HK-NP peptide has a smaller surface area available to the TCR, as a result of its having smaller side chains at P7 and P8 than the PR8-NP peptide.



**Figure 7.** The 2.4 Å resolution structure of the PR8-NP peptide (gray) and P7 and P8 of a model of the HK-NP peptide (black). Peptides are viewed from the side, looking from the  $\alpha_2$  helix toward the  $\alpha_1$  helix of the H-2D<sup>b</sup> molecule. Figure produced using Molscript (55).

Our results suggest that the two NP-specific peptides have similar affinity for the MHC molecule. When 1  $\mu$ M PR8-NP peptide was incubated simultaneously with varying amounts of HK-NP or of two SV40 peptides, similar levels of inhibition of target cell lysis by PR8 NP-specific CTL were observed. Comparable results were obtained when the cells were incubated with HK-NP peptide and varying amounts of PR8-NP or SV40 peptides. The conclusion that PR8-NP and HK-NP peptides have similar affinities for the D<sup>b</sup> MHC glycoprotein was strengthened by experiments in which EL-4 cells were first incubated with a peptide for 30 min, washed, and then incubated for 2 h with peptide analogues. In this case, lysis of EL-4 cells was not inhibited by the addition of peptide analogues. This result suggests that a peptide bound to surface class I molecules cannot be easily displaced by analogues.

We sought to determine, based on these findings, whether there is competition between the PR8-NP and the HK-NP peptides when they are expressed by the HA and NP proteins of the same virus. This is important if transfectant viruses such as R10 are to be used as vaccines, since peptides specific for a single allele may compete with one another for presentation.

Our results strongly suggest that in cells infected with the transfectant virus R10 both peptides are presented efficiently. Indeed, EL-4 cells infected with R10 virus were lysed by both PR8-NP and HK-NP peptide-specific CTLs. Furthermore, immunization of mice with R10 virus primed the precursors of both PR8-NP and HK-NP peptide-specific CTLs. These results are of twofold importance. First, they show that the PR8-NP peptide expressed by the HA of R10 virus is processed like the HK-NP peptide carried by the NP protein and therefore that it appears that the location of the epitope has little influence on its presentation in this system. Our data strengthen previous observations that a peptide recognized by T cells can be generated independent of flanking regions (23, 32, 33, 34). Actually, recent data showed that an influenza virus HA epitope recognized by CTL could be moved to a different site within the HA without precluding its generation from various locations and recognition by CTLs (35). Second, lack of competition between the peptides in our system suggests that live influenza viruses may be good vectors for presenting multiple CTL epitopes useful for the immunization of outbred species.

Whereas the CTL clones generated from acutely infected mice with PR8 or HK viruses and repeatedly stimulated in vitro with NP peptide exhibited high specificity for PR8 or HK-NP peptides, the polyclonal CTL obtained from animals infected with PR8 or R10 viruses exhibited cross-reactivity. Thus, CTLs from animals primed with PR8 virus and secondarily stimulated with HK-NP peptide lysed PR8-infected cells or secondarily stimulated with PR8-NP peptide lysed HK-infected cells.

There is little information on cross-reactivity in the recognition of unrelated viral antigens. Cross-reactive recognition was reported in the case of human CTL specific for influenza matrix protein and VP4 peptide of human rotavirus (36), as well as murine CTLs specific for influenza virus NP



and PB2 peptides (37), HA and NS1 (38), and lymphocytic choriomeningitis virus (LCM), Pichinde, and vaccinia viruses (39). Furthermore, cross-reactivity was also reported at a protective level (i.e., heterosubtypic immunity) against X31 virus in tracheal and lung CD8<sup>+</sup> T cells of animals immunized with PR8 virus (40).

Several hypotheses can be entertained to explain the presence of cross-reactive CTLs in a fraction of polyclonal population obtained from mice infected with PR8 or R10 viruses. The possibility of the presence of leader sequence in the chimeric HA molecule expressing PR8-NP epitope is in agreement with the observation reported by Bacik et al. (41) showing that leader sequence enhanced the degree of cross-recognition of vesicular stomatitis virus (VSV) peptide by Sendai virus NP-specific CD8<sup>+</sup> T cells. In addition, stimulation of memory cells that cross-react at a remote level with MHC-heterologous peptide complex may be due to enhanced expression of IL-2R or adhesion molecules that render the precursors more sensitive to low affinity peptides.

It is generally accepted that the peptides derived from proteins synthesized within the cytoplasm are generated by proteasome-associated enzymes and that they are then transported by TAP transporters to the ER where they bind to class I molecules (21, 42). The lack of competition is consistent with the observation that TAP contains a recognition peptide site with broad specificity (43) and that anchor residues important for the binding to class I molecules are not important for TAP recognition and translocation (44).

It has been shown that peptides derived from signal sequences themselves are not TAP dependent, suggesting that they are produced in ER by the action of signal peptidases (26, 27). However, the majority of peptides generated from endogenous proteins without (28, 29) or with (45) signal sequences are TAP dependent. Addition of leader sequences to NH<sub>2</sub>, but not COOH-termini of peptides which can be presented in a TAP-independent pathway, enhances the presentation in a TAP-dependent manner (41, 46). However, there are data indicating that signal sequences can bypass the requirement of TAP. Thus, Hammond et al. (47) showed that HIV-1 env protein is processed in infected cells after its cotranslation and translocation into ER via a TAP-independent pathway. Similarly, the addition of a signal sequence to an influenza virus matrix peptide can circumvent TAP deficiency in human mutant T2 cell lines. Mutant T2 cells expressing M57-68 peptide without signal sequence were not susceptible to lysis whereas those expressing the peptide with signal sequence were lysed by peptide-specific CTLs (48).

Thus, it was interesting to determine whether NP peptides expressed by the HA or NP proteins may use different processing pathways leading to their presentation on the MHC class I molecules. To this end, we have studied the lysis of R10 virus-infected RMA-S cells by PR8-NP-specific CTLs. RMA-S mutant cells have a defect in the assembly of MHC molecules (28) due to a defined mutation (21) preventing the expression of the TAP-2 gene product, a transporter involved in antigen presentation. Spies et al. (29) and later Attaya et

al. (49) have shown that CTL clones specific for the NP peptide were unable to kill influenza virus RMA-S-infected cells. In contrast, there are reports showing that RMA-S cells are leaky since they are able to present VSV, Sendai, and Rauscher virus-derived peptides (50–52). Our results showed that whereas both EL-4 and RMA cells infected with PR8 or R10 viruses are lysed by PR8-NP-specific CTLs, RMA-S cells infected with the same viruses are not. Our results are in agreement with the observation of Spies et al. (29) that RMA-S mutant cells do not present the peptides derived from the influenza virus NP. More importantly, we observed that RMA-S cells do not present the PR8-NP peptide expressed by the HA gene of the R10 virus.

Failure of RMA-S cells to present the NP peptide expressed in the HA can be interpreted in two ways. First, the PR8-NP peptide is generated by the processing of the HA in the cytosolic compartment. A portion of native polypeptides with signal sequences are processed within the cytosol before translocation to ER allowing for a transporter-dependent antigen presentation. This finding is in conflict with data demonstrating that an influenza virus NP peptide located COOH-terminal to an ER insertion sequence was efficiently presented by TAP-deficient cells (48, 53). A potential reason for this discrepancy is the location of the peptide versus the leader sequence. Whether in the above mentioned experiment the peptide was located at COOH termini of the insertion sequence, in R10 virus chimeric HA, the PR8-NP peptide was located in site E, distal to signal sequence. However, it is worth noting that it was found that signal peptides can function even when located far away from NH<sub>2</sub> terminus of the peptide (54). Another possible explanation in the case of peptide minigene encoding signal sequence is that the peptide is dumped in ER in a preprocessed form, whereas in the case of chimeric HA, the molecule must be further processed.

Alternatively, proteins with signal sequences are processed in the ER and transporter molecules are required within the ER for the capturing of peptides by class I molecules. The latter hypothesis is supported by recent findings (44) that TAP molecules are complexed with newly assembled class I molecules. The TAP disassociates from this complex upon the binding of peptide to class I (44).

In summary, our study indicates that transfectant viruses expressing multiple immunogenic peptides presented by the same allelic MHC product can be used to expand precursor CTL *in vivo* and serve as targets for CTL lysis *in vitro*. Thus, such viruses can be used as vehicles to introduce multiple immunogenic peptides into the endogenous processing pathway. It also suggests that the processing of proteins with signaling sequences occurs in the cytoplasm and that the influenza HA and NP viral proteins use the same transporter-dependent pathway for presentation to class I MHC antigens. Our results thus indicate the feasibility of genetically engineering recombinant vaccines that express multiple epitopes to stimulate cellular immunity and that play an important role in the host defense against obligatory intracellular parasites.

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Address correspondence to Dr. Constantin Bona, Department of Microbiology, Mount Sinai School of Medicine, One Gustave Levy Place, New York, NY 10029-6574.

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