

ACTIVE IMMUNIZATION AGAINST VIRUS INFECTIONS  
DUE TO ANTIGENIC DRIFT BY INDUCTION OF  
CROSSREACTIVE CYTOTOXIC T LYMPHOCYTES

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Current inactivated whole or subunit influenza vaccines induce antibodies to the hemagglutinin that are specific for the virus in the vaccine or for closely related virus strains; however, frequent antigenic variation of sites on the HA1 subunit of influenza virus hemagglutinin (HA)<sup>1</sup> (1, 2) has been postulated to explain low levels of vaccine efficacy (3, 4). A search for more crossreactive epitopes that may serve as protective immunogens appears reasonable in an effort to provide better vaccine efficacy against virus strains that vary antigenically in their antibody binding sites.

Several laboratories have reported that influenza virus-specific CTL recognize antigenic sites that are more conserved than the sites on the HA that bind antibodies (5-8). Furthermore, it has been demonstrated that adoptive transfer of influenza-specific CTL can limit virus replication in the respiratory tract of mice infected with influenza virus (9-11). Recently, influenza nucleoprotein (NP)-specific cytotoxic T cell clones have been shown to reduce the replication of virus and protect mice from lethal influenza infection (12), and Townsend et al. (13) defined a peptide region of NP that was recognized by this protective CTL clone. NP-enriched preparations have been reported to protect mice against challenge with virus that contained the same NP (14).

We have demonstrated that c13 protein, a hybrid protein of the first 81 amino acids (aa) of the viral NS1 nonstructural protein and the HA2 subunit of A/PR/8 (H1N1) HA, induced H1 subtype-specific CTL after secondary stimulation in vitro (15, 16). In addition, we have observed that passive transfer of CTL clones established by stimulation with a derivative of c13 protein, having 65 aa deleted from the NH<sub>2</sub>-terminal end of the HA2 subunit, significantly reduced virus titers in lungs of mice infected with virus strains of H1 or H2 subtypes (17). We recently demonstrated that the epitope recognized by the protective CTL clone maps to an 11-aa residue on the transmembrane region of the HA2 subunit (unpublished work). It

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<sup>1</sup> *Abbreviations used in this paper:* aa, amino acid; HA, hemagglutinin; NP, nucleoprotein; PFU, plaque-forming units.

is important to determine whether peptides or fusion proteins, which contain sites that induce CTL and are better conserved than the antibody binding sites on the HA1 subunit, might actively induce crossreactive protection against influenza A viruses that undergo frequent antigenic mutations. We now report the results of virus challenge experiments performed in mice immunized with the HA2 subunit containing fusion protein. The results demonstrate that active immunization with a fusion protein that contains a crossreactive CTL site confers significant protection against infection with viruses that have undergone marked antigenic drift and antigenic shift in the antibody binding sites on the HA.

### Materials and Methods

*Mice.* Male BALB/c mice were purchased from Charles River Breeding Laboratories, Inc. (Stone Ridge, NY) and used at the age of 4–5 wk.

*Virus.* Influenza A viruses, A/PR/8 (A/Puerto Rico/8/34 [H1N1]), A/BZ (A/Brazil/11/78 [H1N1]), A/TW (A/Taiwan/1/86 [H1N1]), A/JAP (A/Japan/305/57 [H2N2]), A/PC (A/Port Chalmers/1/73 [H3N2]), and A/X31 (H3N2), a recombinant strain obtained by reassortment of genes between A/PR/8 and A/Hong Kong/1/68 (H3N2), were propagated in 10-d-old embryonated chicken eggs. Infected allantoic fluids were harvested 2 d after infection, aliquoted, and stored at  $-80^{\circ}\text{C}$  until use.

*Production of Influenza Virus-specific Protein in Escherichia coli.* c13 and D proteins were produced in *E. coli* as described previously (15, 16). Briefly, plasmids containing DNA fragments complementary to the viral RNA of A/PR/8 virus were manipulated to achieve expression of c13 and D protein, which are hybrids of the first 81 aa of NS1 fused to the entire HA2 subunit (aa 222), or to the 157 aa from the COOH-terminal end of HA2, respectively. D protein has a linker sequence (glutamine-isoleucine-proline) inserted between aa 81 of NS1 and aa65 of HA2. After lysis of the bacteria, two 0.1% deoxycholate extractions and one 1% Triton X-100 extraction were performed to remove contaminating *E. coli* proteins, and the c13 and D protein were solubilized with 4 M urea at  $4^{\circ}\text{C}$  for 30 min. The urea was then removed by dialysis at  $4^{\circ}\text{C}$ . The prepared proteins were stored in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA.

*Immunization of Mice.* Mice were immunized with 200  $\mu\text{g}$  or 300  $\mu\text{g}$  of c13 protein intraperitoneally without adjuvants or with 100 plaque forming units (PFU) of the A/PR/8 virus intranasally under ether anesthesia.

*CTL Assay.* 1 wk after the last immunization, spleen cells of mice were taken for in vitro stimulation with virus. CTL assays were carried out as described previously (15, 16). Briefly,  $3 \times 10^7$  spleen cells from immunized or control mice were cultured with  $3 \times 10^6$  syngeneic normal spleen cells infected with A/PR/8 or A/PC virus at a multiplicity of 10 PFU per cell. After 5 d of culture these cells were used as effector cells. For target cells,  $2 \times 10^6$  P815 (mastocytoma: H-2<sup>d</sup>) cells were incubated with A/PR/8, A/JAP, or A/PC virus at a multiplicity of 10 PFU per cell in the presence of 250  $\mu\text{Ci}$   $^{51}\text{Cr}$ , and  $10^4$   $^{51}\text{Cr}$ -labeled target cells were incubated with effector cells at the indicated ratios in a 96-well round-bottomed microplate for 4 h. The supernatant fluids were harvested and  $^{51}\text{Cr}$  was measured in a gamma counter. Percent specific lysis was determined as follows: percent specific lysis =  $100 \times (\text{experimental release} - \text{minimum release}) / (\text{maximum release} - \text{minimum release})$  where spontaneous release was determined by incubating P815 cells in medium, and maximum release by incubating P815 cells in 10% Renex 30 solution (Ruger Chemical Co., Irvington, NJ). E/T ratios varied from 3:1 to 200:1 as listed, and quadruplicate samples were tested at each E/T ratio.

*Virus Challenge.* 1 wk after the last immunization, mice were challenged intranasally under ether anesthesia with  $5 \times 10^4$  PFU of A/PR/8, A/TW, A/JAP or A/PC viruses. 4 d later lungs were harvested for measurement of pulmonary virus titers.

*Titration of Pulmonary Virus.* Harvested lungs were manually homogenized in 1.5 ml of PBS followed by centrifugation (2,000 g for 15 min at  $4^{\circ}\text{C}$ ). Supernatants were frozen until they were titrated for virus. Madin Darby canine kidney (MDCK) cells were maintained in MEM containing 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 292  $\mu\text{g}/\text{ml}$  L-glutamine

supplemented with 10% heat-inactivated FCS, and were seeded ( $25 \times 10^4$  cells in 1 ml MEM) in 24-well tissue culture plates. Lung supernatants were thawed and serially diluted in PBS containing 0.1% BSA. After aspiration of medium from the wells, 100  $\mu$ l of the diluted virus solution was added to each well and incubated at 37°C for 1 h with occasional agitation. Each well then received 1 ml of agar medium containing MEM, 0.1% D-glucose, 0.01% DEAE-Dextran, 1% vitamins, 10  $\mu$ g/ml trypsin, and 1% agar. After 2 d incubation in 5% CO<sub>2</sub> at 37°C, 1 ml of 10% neutral red in PBS was overlaid on agar in each well. Plaques were counted after 10 h incubation. The results were expressed as the mean log<sub>10</sub> PFU/ml of duplicate samples.

**CTL Clones.** CTL clones were established as described previously (17, 18). Briefly, A/PR/8 immune spleen cells were cultured with normal syngeneic spleen cells infected with A/PR/8 virus or exposed to D protein. CTL responder cells were stimulated with A/PR/8 or D protein-pulsed normal syngeneic  $\gamma$ -irradiated spleen cells in the presence of 10% rat IL-2 for 5–8 wk. A limiting dilution was carried out to develop CTL clones. Viable responder cells (0.5, 1, 2, 4, 8, and 16 cells/well) were cultured in wells of 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) with 10<sup>6</sup> syngeneic, A/PR/8, or D protein-pulsed,  $\gamma$ -irradiated BALB/c spleen cell in 0.2 ml of medium. Stimulator cells were added every 7 d. B-7 or A-11 clones described in this paper grew from a well in which four or two responder cells had been seeded, respectively, as described (17).

**Cold Target Inhibition Test.** P815 cells infected with various viruses or coated with D protein (50  $\mu$ g/ml) for 90 min at 37°C were added to the CTL assay, in which B-7 CTL clone was incubated with <sup>51</sup>Cr labeled P815 target cells infected with A/PR/8, at the indicated ratios.

**Titration of Neutralizing Antibody.** Neutralizing antibody against A/PR/8 virus was determined by a plaque assay in MDCK cells. Serially diluted pooled sera were preincubated with 20 PFU of virus at 37°C for 1 h and were titrated for virus as described above. The 50% plaque-neutralizing antibody titers were calculated. Pooled sera of mice that had been infected intranasally 6 wk earlier with 100 PFU of A/PR/8 virus were included as a positive control.

## Results

**Virus Specificity of c13 Immune Spleen Cells Stimulated by Viruses.** We immunized BALB/c mice with c13 protein to determine whether immunization would induce protection against influenza infection via induction of CTL. Preliminary experiments had indicated that CFA did not significantly enhance the level of c13 protein induced CTL activity. Table I shows that the spleen cells of c13 immunized mice that were stimulated by A/PR/8 virus-infected normal syngeneic spleen cells in vitro were able to lyse A/PR/8 infected target cells, although to a lower degree than A/PR/8-immune effector cells stimulated by A/PR/8 infected normal spleen cells, and they did not lyse A/PC (H3N2) virus-infected target cells or uninfected target cells. We found no detectable CTL activity in c13 protein-immune spleen cells after stimulation by A/PC (H3N2) virus. Spleen cells of nonimmune mice also failed to show CTL activity on any target cells after stimulation with virus in vitro.

**Pulmonary Virus Titers of c13 Immune Mice Infected with A/PR/8 (H1N1) or A/PC (H3N2) Viruses.** We next examined the pulmonary virus titers of c13 protein-immunized mice and nonimmune mice after challenge with A/PR/8 (H1N1) or A/PC (H3N2) viruses. The c13-immunized mice had significantly lower pulmonary virus titers of A/PR/8 virus compared with the lung virus titers of nonimmunized mice. There was no difference in the pulmonary virus titers between c13-immunized mice and nonimmune mice after challenge with the heterotypic virus, A/PC (H3N2) (Table II).

**Survival of c13-immune Mice after A/PR/8 Challenge.** After a lethal challenge with  $5 \times 10^4$  PFU of A/PR/8 virus, seven of eight c13-immunized mice survived beyond day 60 (last day of observation), but all of nonimmune mice were dead by day 7

TABLE I  
*Virus Specificity of c13-immune Spleen Cells Stimulated by A/PR/8 (H1N1) or A/PC (H3N2) Viruses*

Stimulation		Percent specific lysis of target cells								
In vivo	In vitro	A/PR/8 (H1N1)			A/PC (H3N2)			Uninfected		
1°-4°	5°	200	60	20	200	60	20	200	60	20
c13	A/PR/8	30	13	5	1	0	2	-3	-3	-3
	A/PC	-1	3	3	0	-1	0	1	0	3
	None	2	0	-1	0	-1	-2	-4	-3	-3
None	A/PR/8	0	-2	-1	-1	-1	-1	-4	-4	-3
	A/PC	-1	-1	0	1	1	0	-1	-3	-1
	None	0	0	2	-1	0	2	-4	-4	-2
In vivo	In vitro	A/PR/8 (H1N1)			A/PC (H3N2)			Uninfected		
1°	2°	25	8	3	25	8	3	25	8	3
A/PR/8	A/PR/8	65	32	11	59	31	20	4	6	0
	A/PC	32	10	8	34	16	8	-3	-4	-1
	None	-1	-2	-3	0	-1	-1	-3	-4	-3

Virus-infected (A/PR/8, A/PC) and uninfected P815 target cells were tested at the indicated E/T cell ratios.

\* 4-wk-old mice were given 200 µg of c13 protein intraperitoneally and repeat doses 3, 4, and 5 wk later.

(Fig. 1). This protection induced by c13 protein reflects the specificity of lysis by the virus stimulated c13 immune spleen cells of A/PR/8-infected target cells in vitro, and the specific limitation of A/PR/8 virus replication in the lungs of mice that had been immunized by c13 protein.

*HA Epitope Specific for CTL Is Conserved.* We wanted to determine if immunization would afford protection against variant H1 subtype virus strains across the H1 subtype. First, we performed a CTL assay and a cold target inhibition assay using influenza H1 subtype viruses A/PR/8, A/BZ, and A/TW, which were isolated in 1934, 1978, and 1986, respectively. We used as effector cells a CTL clone designated B-7, which had been stimulated with D protein, a derivative of c13 protein (17). As shown in Table III, the B-7 clone lysed target cells infected with each of the H1 subtype virus strains, but failed to lyse A/PC (H3N2)-infected target cells. As a positive control, the A-11 CTL clone stimulated with live A/PR/8 virus killed target cells

TABLE II  
*Pulmonary Virus Titers of c13-immunized Mice*

Immunization	Recipients*	
	Virus challenge	Virus titer
c13	A/PR/8 (H1N1)	3.8 ± 0.9†
None	A/PR/8	5.4 ± 0.2†
c13	A/PC (H3N2)	5.4 ± 0.2
None	A/PC	5.0 ± 0.3

\* Each group consists of six mice.

†  $p < 0.005$ , determined by Student's *t* test.

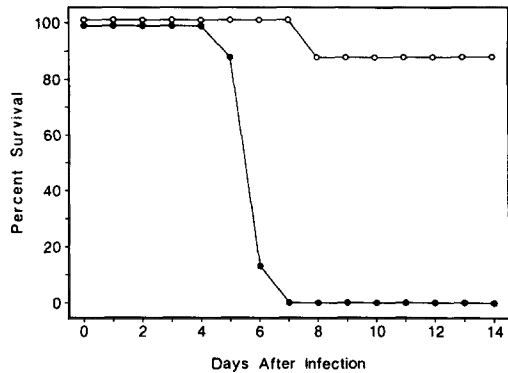


FIGURE 1. Survival of mice after lethal A/PR virus infection. Symbols are as follows: (O) c13 protein immunized mice; (●) nonimmunized mice. 1 wk after the fourth immunization (Table I), mice were intranasally challenged with A/PR/8 virus at a dose of  $5 \times 10^4$  PFU under ether anesthesia and were observed for survival. The difference in survival between the two groups was significant ( $p < 0.001$ ), as determined by Fisher's exact probability test.

infected with virus strains of the H1 and H3 subtypes. Cold target inhibition experiments revealed that lysis of A/PR/8-infected cells was inhibited by both A/PR/8 and A/BZ virus-infected cold targets and by D protein-coated cold target cells. Neither A/X31 (H3N2) infected cold target cells nor uninfected cold target cells competed with the  $^{51}\text{Cr}$ -labeled target cells (Fig. 2). These results indicate that an epitope on the HA2 subunit of influenza H1 subtype virus strains is highly conserved, and is recognized by the D protein-stimulated CTL clone.

*Protection of Mice Infected with A/TW by c13 Immunization.* We examined whether c13 protein-immune mice would also be protected against a recently isolated H1 subtype virus strain, A/Taiwan/1/86 (H1N1). The results shown in Table IV demonstrate that the c13 protein-immunized mice had significantly lower lung virus titers of A/Taiwan/1/86 after challenge than did nonimmune mice; however, neither the c13 protein-immunized nor control nonimmune mice had serum neutralizing antibodies against A/PR/8 virus, which was the source of the genes for NS1 and the HA2 subunit used to make this fusion protein c13. This was expected because the HA2 subunit does not contain sites that induce neutralizing antibodies. In contrast, A/PR/8 virus-primed mice, sampled as positive controls, showed high levels of neu-

TABLE III  
*Recognition of Target Cells Infected with H1 Subtype Virus Strains by an HA2 Subunit-specific CTL Clone*

CTL clone	E/T ratio	Percent specific lysis				Uninfected
		A/PR/8 (H1N1)	A/BZ (H1N1)	A/TW (H1N1)	A/PC (H3N2)	
B-7*	1.0	77	83	58	0	0
	0.5	61	68	47	0	0
A-11†	1.0	66	74	43	48	0
	0.5	45	49	31	29	0

\* Clone B-7, which expresses 97% of Thy-1.2, 95% of Lyt-2, and 0% of L3T4 surface antigens, was stimulated with D protein.

† Clone A-11, which expresses 99% of Thy-1.2, 94% of Lyt-2, and 2% of L3T4 surface antigens, was stimulated with A/PR/8 virus.

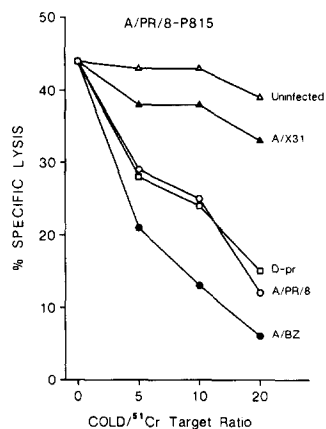


FIGURE 2. Cold-target inhibition test. Cytotoxicity of clone B-7 on A/PR/8-infected P815 cells was measured in the presence of various unlabeled target cells at the indicated ratios.

tralizing antibody titers. These results suggest that immunization with c13 protein induced protection against virus strains of the H1 subtype isolated in 1934 and 1986 by the induction of CTL responses, and not by the induction of neutralizing antibody.

*Induction of Crossreactive-specific CTL by c13-immune Spleen Cells.* We then examined whether c13-immune mice would be protected against challenge with a virus of the H2 subtype. Table V shows that c13-immune spleen cells stimulated by A/PR/8 virus lyse the A/PR/8 (H1) virus or A/JAP (H2) virus-infected target cells, but not A/PC (H3) virus-infected target cells. The level of A/JAP-infected target cells lysis by c13-immune spleen cells stimulated with A/PR/8 virus was about half of that of A/PR/8-infected target cells lysed by the effector cells and was significant. On the contrary, A/PC (H3) virus-stimulated c13-immune spleen cells did not lyse any of virus-infected target cells. As a positive control, A/PR/8 virus-immune spleen cells stimulated by A/PR/8 virus or A/PC virus showed significant lysis of all of the virus-infected target cells. These results suggest that c13-immune spleen cells contain a population of H1-H2 crossreactive CTL precursors, since c13-immune spleen cells stimulated by A/PR/8 virus showed crossreactive lysis of target cells infected with A/PR/8 (H1) or A/JAP (H2) viruses. In addition, we have observed that the lysis of A/PR/8 virus infected target cells by clone B-7 specific for HA was inhibited by

TABLE IV  
*Pulmonary Virus Titer of c13-immunized Mice*

Immunization	Recipients		
	Virus challenge	Virus titer	Neutralizing antibody titer
c13	A/TW (H1N1)	2.5 ± 0.9*	<4
None	A/TW (H1N1)	4.1 ± 0.3*	<4
A/PR/8	ND	ND	256

\*  $p < 0.005$ , as determined by Student's  $t$  test. An additional 200- $\mu$ g dose of c13 protein was given to mice (five mice each group) 3 wk after the fourth immunization (Table I). Before challenge, serum was obtained from c13 protein-immunized mice for titration of neutralizing antibody to A/PR/8.

TABLE V  
Crossreactive Specificity of c13-immune Spleen Cells Stimulated by A/PR/8 Virus

Stimulation*		Percent specific lysis of target cells											
In vivo 1°-6°	In vitro 7°	A/PR/8 (H1N1)			A/JAP (H2N2)			A/PC (H3N2)			Uninfected		
		200	60	20	200	60	20	200	60	20	200	60	20
c13	A/PR/8	73	50	24	38	18	10	10	4	0	11	4	1
	A/PC	1	0	0	-1	0	2	0	0	0	0	0	0
	None	6	1	1	5	1	4	3	1	1	3	0	1
In vivo 1°	In vitro 2°	A/PR/8 (H1N1)			A/JAP (H2N2)			A/PC (H3N2)			Uninfected		
		30	10	3	30	10	3	30	10	3	30	10	3
A/PR/8	A/PR/8	54	35	20	50	27	12	51	27	13	3	1	1
	A/PC	39	19	7	37	21	9	48	20	8	1	0	0
	None	2	1	0	3	1	2	4	2	0	2	1	1

Virus-infected (A/PR/8, A/JAP, A/PC) or uninfected P815 target cells were tested at the indicated E/T ratios.  
\* 4-wk-old mice were given 300 µg of c13 protein intraperitoneally and repeat doses 3, 4, 5, 6, and 7 wk later.

A/JAP virus-infected target cells (data not shown), which supports the existence of H1-H2 crossreactive CTL population in the c13-immunized mice.

*Reduction of Pulmonary Virus Titer of c13-immune Mice Infected with A/JAP (H2N2) Virus.* We assessed the ability of c13 immunized mice against challenge with virus of the H2 subtype. We injected mice that had been immunized with six doses of c13 protein as shown in Table V, with A/JAP (H2N2) virus, A/PR/8 (H1N1), or A/PC (H3N2) viruses. The sera from these c13-immunized mice were tested before the mice were challenged with virus. The sera did not contain any detectable plaque neutralizing antibodies (<1:4) against A/PR/8, A/JAP, or A/PC viruses, as expected from the results of our earlier experiment shown in Table IV. Table VI shows that c13 immune mice had significantly lower lung virus titers than control mice after challenge with the A/PR/8 (H1) or A/JAP (H2) viruses, but not with A/PC (H3) virus. These results appear to reflect the in vitro CTL activity in Table V, where the induction of H1-H2 crossreactive CTL was shown.

TABLE VI  
Reduction of Pulmonary A/JAP Virus Titer of c13-immunized Mice

Immunization	Recipients*	
	Virus challenge <sup>†</sup>	Virus titer
c13	A/PR/8 (H1N1)	4.7 ± 0.4 <sup>§</sup>
None	A/PR/8	5.8 ± 0.8 <sup>§</sup>
c13	A/JAP (H2N2)	4.0 ± 0.3 <sup>  </sup>
None	A/JAP	4.8 ± 0.4 <sup>  </sup>
c13	A/PC (H3N2)	4.4 ± 0.3
None	A/PC	4.2 ± 0.2

\* Each group consists of four mice.

<sup>†</sup> Influenza viruses were challenged one week after the sixth immunization as shown on Table V.

<sup>§</sup> *p* < 0.02, determined by Student's *t* test.

<sup>||</sup> *p* < 0.02, determined by Student's *t* test.

### Discussion

The composition of influenza vaccines is regularly revised due to the frequent antigenic variation of the antibody binding sites on the HA1 subunit of the HA. Despite these efforts, vaccine efficacy is less than desired. We hypothesized that the induction of CTL responses to conserved epitopes would protect mice against infection with influenza virus strains with altered antibody binding sites on the HA. Mice immunized without adjuvants with a hybrid protein that contains the HA2 subunit of A/PR/8/34 (H1N1) HA developed specific CTL responses, and were protected against challenge with A/PR/8/34 virus and the recently isolated A/Taiwan/1/86 virus strain, without the induction of neutralizing antibodies. In addition to this cross-reactive protection within the H1 subtype, immunized mice were also protected against challenge with virus of the H2 subtype, reflecting the *in vitro* specificity of the CTL epitope on the HA2 subunit. These results demonstrate that immunization with a fusion protein that contains the HA2 subunit of influenza A (A/PR/8) virus induced protection against influenza A H1 subtype virus strains isolated over 50 yr apart that have great diversity in hemagglutinating-antibody specificities, and also provides crossreactive protection against challenge with an H2 subtype virus. Although we have not tested it directly, c13 protein might induce protection against all of H1 and H2 subtype virus strains. This seems reasonable because we have previously demonstrated that CTL clones stimulated with a derivative of c13 protein exhibit crossreactive lysis of target cells infected with H1 and H2 subtype virus strains, and that passive transfer of such a clone restricts virus replication in mice infected with virus strains of the H1 or H2 subtypes (17). Further, we now report reduced lung virus titers after challenge with H1 and H2 subtype viruses. We assayed the sera of c13 immunized mice for neutralizing antibodies to rule out the possibility that such antibodies might contribute to this protection and found none. This was expected because the antibody binding sites for virus neutralization are on the HA1 subunit of HA (19, 20). Thus, such a fusion protein that contains a conserved CTL epitope offers promise for the potential development of influenza vaccines by inducing protective crossreactive CTL responses. We have recently observed that the crossreactive CTL epitope on the HA2 maps to an 11-aa residual in the transmembrane region of the HA2. We have been able to stimulate the proliferation of the protective B-7 CTL clone, induced by the fusion protein, using this 11-aa peptide, and this peptide renders target cells susceptible to lysis by this CTL clone. We have also succeeded in inducing a CTL line from A/PR/8 immune spleen cells using this synthetic peptide (data not presented). We will attempt to prime mice *in vivo* with this peptide in future experiments.

Crossreactive memory CTL responses to influenza viruses have been detected in humans (21-23), and these may contribute to recovery from infection (24) with virus strains that are antigenically different at HA1 antibody-combining sites, thus bypassing neutralizing antibodies. It is not clear why these crossreactive memory CTL responses, which result from natural infection, are not more protective to humans after viral challenge with variant strains. Studies in mice (25) and humans (26) suggest that the ability of current influenza vaccines to induce CTL responses is limited, and the reasons for this are not known. Perhaps immunization with crossreactive CTL epitopes can be better accomplished by use of small fusion proteins or peptides. It



is, however, reasonable to expect that such small peptides or fusion proteins may demonstrate HLA haplotype preferences. Gotch et al. (27) have noted haplotype preference with peptides of NP and matrix protein, and we have reported haplotype preference in mice (16), and have also noted it with human PBL lymphocytes (data not presented) with this fusion protein. These questions must be addressed in future studies, but the design of more crossreactive influenza vaccines based on conserved sites that are active in CTL induction appears worthwhile as an approach for future vaccine development.

Despite their promise, synthetic peptides have not been successfully developed as vaccines, because of their failure in general to induce protective immunity (28). The limited protection that has been noted usually required carriers and adjuvants (29), but an exception was reported with a hybrid synthetic peptide (141-158 + 200-213 aa) of the virus coat protein (VP1) of foot and mouth disease virus, which induced neutralizing antibody (30). The ability of this influenza-derived fusion protein to actively immunize against influenza A virus strains, which vary markedly in their antibody binding sites on the HA1, may be due to the presence of as yet undefined T helper determinants on the fusion protein that assist the CTL responses to the conserved cytotoxic T cell epitope on the HA2 subunit. Perhaps the failure of peptides to protect in other systems (28) may be due to the absence of such T helper and CTL sites. The results reported here suggest a model for more crossreactive vaccines against antigenically variant viruses, such as influenza A, or HIV-1.

### Summary

We have examined whether active immunization with c13 protein, a hybrid protein of the first 81 amino acids of the viral NS1 nonstructural protein and the HA2 subunit of A/PR/8 (H1N1) hemagglutinin, could protect BALB/c mice from challenge with A/PR/8 H1 subtype virus. Mice immunized with the c13 protein had a significant reduction of pulmonary virus titers with A/PR/8 (H1) virus, but failed to limit the replication of A/PC (H3) virus, which reflects the *in vitro* CTL activity of c13 immune spleen cells. We observed that the epitope recognized by HA2 specific CTL, which are induced by a derivative of c13 protein, is highly conserved among H1 and H2 subtype virus strains. This led us to test whether active immunization with c13 protein would also limit pulmonary virus replication in mice infected with the A/TW virus, a virus of the H1 subtype, which was isolated in 1986, and with a virus of the H2 subtype, A/Japan/305/57. Immunized mice had significantly lower lung virus titers than did control mice, and did not possess any neutralizing antibodies to the challenger viruses. These results indicate that active immunization with a fusion protein containing the cross-reactive CTL epitope protects mice from influenza infection by inducing CTL against influenza A H1 and H2 subtype virus strains, which markedly vary in their antibody binding sites on the HA1. The ability to induce active cross-reactive immunization with a fusion protein which contains a highly conserved CTL epitope offers a model for vaccine approaches against viruses which undergo significant variations in their antibody binding sites.

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