

INFLUENZA VIRUS HEMAGGLUTININ-SPECIFIC
CYTOTOXIC T CELL RESPONSE INDUCED BY
POLYPEPTIDE PRODUCED IN *ESCHERICHIA COLI*

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During the course of influenza A virus infection, two different subsets of cytotoxic T lymphocytes (CTL)¹ are induced in mice and in humans. There are both subtype-specific CTL responses and responses that are crossreactive among the influenza A subtypes; both responses are restricted by class I major histocompatibility complex (MHC) antigens (1–4). These CTL have been shown to play a crucial role in the recovery from influenza virus infection (5–8).

The definition of the viral determinants that are recognized by these subsets of CTL is not complete (for review, see ref. 9). Preparations of hemagglutinin (HA) have been reported (10, 11) to stimulate a subtype-specific response. Wabuke-Bunoti and Fan (12) reported that the smaller subunit of HA (HA2), purified from bromelain-treated virions, induced a weak but significant secondary CTL response, which was subtype-specific. Becht et al. (13), however, failed to show the induction of the secondary CTL response using purified HA2 obtained by the selective digestion of virions with acid protease followed by treatment with mild detergent. On the other hand, several CTL clones apparently recognizing determinant(s) on the internal proteins, such as viral nucleoprotein (NP), have been isolated (14). Using a panel of recombinant viruses, Townsend and Skehel (14, 15) reported that the gene coding for NP determined the specificity of the effector lymphocytes, in that the target cells infected with the virus strain sharing only NP gene with the virus used for stimulation could be lysed by certain clones of CTL, or CTL generated in bulk cultures and stimulated in special ways. For the recognition of the target cells by crossreactive CTL, in addition to viral HA (16, 17), more conserved viral proteins, such as matrix protein and NP have been postulated to be responsible (9, 18, 19).

To elucidate which viral proteins are involved in the recognition by MHC-restricted CTL, we examined the abilities of several influenza viral polypeptides prepared by gene cloning techniques to induce the secondary CTL responses.

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¹ *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; E:T, effector/target; HA, hemagglutinin; IL-2, interleukin 2; MHC, major histocompatibility complex; NP, nucleoprotein; SNF, supernatant fluid.

Our results show that one of them (c13 protein), which is a hybrid protein of the first 81 amino acids of the viral NS1 nonstructural protein and the HA2 subunit of viral HA, stimulated H-2-restricted, influenza virus subtype-specific secondary CTL *in vitro*. Furthermore, immunization of mice with c13 protein induced the generation of memory CTL response *in vivo*.

Materials and Methods

Mice. Male BALB/c mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA), 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/Chile (A/Chile/1/83 [H1N1]), A/JAP (A/Japan/305/57 [H2N2]), A/BK (A/Bangkok/1/79 [H3N2]), and A/PC (A/Port Chalmers/1/73 [H3N2]), were propagated in 9-d-old embryonated chicken eggs. Infected allantoic fluids were harvested on day 3 after infection and clarified by low-speed centrifugation. The supernatant fluid was divided into small vials and stored at -80°C until use. A/X31 (H3N2), a recombinant strain obtained by cross between A/PR/8/34 and A/Hong Kong/1/68 (H3N2) was generously provided by Dr. E. D. Kilbourne of the Mount Sinai School of Medicine, New York.

Production of Influenza Virus-specific Proteins in *E. coli*. Influenza virus-specific polypeptides were produced in *E. coli* using the expression system described previously (20–22). Briefly, plasmids containing DNA fragments complementary to the viral RNA of A/PR/8 virus (23) were manipulated to achieve expression of the following products: (a) the NS1 nonstructural protein; (b) c13, the first 81 amino acids of NS1 fused to HA2; (c) $\Delta 13$, the first 81 amino acids of NS1 fused to the first 69 amino acids of HA2; (d) c7, the entire HA (minus the signal peptide); (e) $\Delta 7$, the HA1 and the first 69 amino acids of HA2; and (f) c36, the HA2 alone. The NS1 protein was purified as described previously (22). Unlike the NS1 protein, the HA-containing polypeptides were insoluble when produced in *E. coli*. Therefore, after lysis of the bacteria, two 0.1% deoxycholate extractions, and one 1% Triton X-100 extraction to remove contaminating *E. coli* proteins, the HA-specific proteins were solubilized with 4 M urea (4°C for 30 min). The urea was then removed by dialysis at 4°C . All proteins were stored in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA. The purity of these products was $>80\%$.

Immunization of Mice. Mice were immunized with $\sim 1,000$ plaque forming units of the virus intranasally without anesthesia. To immunize mice with a polypeptide, the solution containing 500 $\mu\text{g}/\text{ml}$ of c13 protein was mixed with an equal amount of complete Freund's adjuvant, and 0.2 ml of the mixture was inoculated into mice subcutaneously. 3 wk after the primary injection, mice were given an intraperitoneal booster inoculation of 50 μg without adjuvant.

Induction of Secondary CTL *In Vitro*. Spleens were aseptically removed from mice immunized with the virus at least 3 wk before, and were teased through a stainless steel mesh. After lysing red blood cells by treatment with ACK lysing buffer (155 mM NH_4Cl , 10 mM KHCO_3 , and 190 mM EDTA), cells were washed and resuspended in RPMI 1640 medium containing 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin supplemented with 10% heat-inactivated fetal bovine serum at a concentration of $2.5\text{--}3.0 \times 10^6$ cells/ml. Responder cells were cultured with virus-infected syngeneic spleen cells taken from normal mice at a ratio of 10:1, or with various concentrations of peptides at 37°C for 5 d in 5% CO_2 .

CTL Assay. P815 (H-2^d) cells were used as target cells for the standard ^{51}Cr -release assay. Single-cell suspensions of P815 cells were infected with the desired virus at an input multiplicity of >10 PFU/cell. Immediately after inoculation of the virus, 250 μCi of $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear, Boston, MA) was added. After incubation at 37°C for 90 min, the target cells were washed twice with RPMI and further incubated at 37°C for 6 h. 10^4 ^{51}Cr -labeled target cells were incubated with effector cells at various ratios in a 96-well, round-bottom microplate (Linbro, McLean, VA) at 37°C for 4 h. The supernatant fluid (SNF) of cultures was harvested by the aid of harvesting frames (Skatron Inc.,

Sterling, VA), and the radioactivities of SNF were counted in a gamma counter (Packard Instrument Co. Inc., Downers Grove, IL). Percent specific lysis was calculated as follows: percent specific lysis = (experimental release - minimum release) \times 100/(maximum release - minimum release). Maximum and minimum release were obtained by incubating target cells with 10% Renex 30 solution (Ruger Chem. Co., Irvington, NJ) and medium alone, respectively.

Maintenance of CTL in Presence of Interleukin 2 (IL-2). In vitro-stimulated spleen cell cultures were maintained for 3 wk in RPMI 1640 containing 10% FBS, 5×10^{-5} M 2-mercaptoethanol, and either 4% rat (Collaborative Research Inc., Lexington, MA) or 20% human (Meloy Laboratories Inc., Springfield, VA) IL-2.

Determination of Precursor Frequencies of CTL. Precursor frequencies of influenza virus-specific CTL were determined by a limiting-dilution method (24). Spleen cell suspensions diluted to desired concentrations were distributed into round-bottom, 96-well microplates (100 μ l/well), and cultured with 10^6 X-irradiated (2,500 rad) syngeneic spleen cells infected with A/PR/8 virus in the presence of exogenous IL-2. At least seven dilutions of the cell suspension were prepared, and 24 wells were seeded with each dilution. After incubation at 37°C for 7 d, each well was assayed for cytotoxicity against A/PR/8 virus-infected P815 target cells (2,000 cells/well) directly, or after splitting into two replicas. Estimates of the CTL precursor frequency were calculated by an iterative procedure based on maximum likelihood analysis described by Fazekas de St. Groth (25).

Results

Induction of Secondary CTL Response by Various Polypeptides. We examined the ability of various polypeptides to stimulate A/PR/8 virus-immune lymphocytes to generate a secondary CTL response in vitro. $2.5\text{--}3.0 \times 10^7$ immune cells were cultured with various concentrations of polypeptides of A/PR/8 virus-infected syngeneic lymphocytes at 37°C for 5 d. Table I shows that immune spleen cells stimulated with A/PR/8 virus-infected syngeneic cells were highly cytotoxic to A/PR/8 virus-infected P815 cells but not to uninfected P815 cells. c13 protein, which is a hybrid peptide between the first 81 amino acids of NS1 and HA2, stimulated the secondary response in vitro. Although the level of killing by c13

TABLE I
Induction of Secondary CTL In Vitro by E. coli-derived Protein

Exp.	Secondary stimulation	Percent specific lysis			
		A/PR/8		Uninfected	
		30	10	30	10
1	A/PR/8 [†]	62.5	40.5	6.7	2.6
	NS1	0.1	-3.2	4.3	0.0
	C13	19.3	4.0	0.5	-0.5
	Δ 13	-2.5	-4.6	-1.3	0.0
	Δ 7	2.1	-2.0	2.9	-0.9
	None	0.0	2.0	1.9	3.2
2	A/PR/8	91.9	89.2	16.4	11.5
	c13	28.6	11.3	-1.2	-1.0
	c36	6.2	-2.5	-2.1	-1.1
	c7	4.4	-3.7	-0.8	-0.8

Protein concentrations were 5 μ g/ml and 12 μ g/ml, in exp. 1 and 2, respectively.

E:T (effector/target) ratios were 30:1 and 10:1.

[†] A/PR/8 (H1N1)-infected syngeneic stimulator cells.

protein-stimulated cells was lower than that obtained with effector cells induced by virus-infected stimulator cells, the induction of CTL response by c13 protein was found to be dose-dependent, and the killing of virus-infected target cells was H-2-restricted (not shown). Interestingly, neither c36 protein, which is HA2 alone, nor c7 protein, which is the entire HA, induced any CTL responses.

Virus Specificity of Secondary CTL Induced by c13 Protein. To determine the virus specificity of the secondary CTL induced by c13 protein, A/PR/8 virus- or A/PC virus-immune spleen cells were stimulated with c13 protein and tested for their ability to lyse both A/PR/8 virus- and A/PC virus-infected P815 cells. As shown in Table II, the effector cells stimulated by A/PR/8 virus- and A/PC virus-infected cells lysed both A/PR/8 virus- and A/PC virus-infected target cells. c13 protein-induced effector cells, however, lysed only A/PR/8 virus-infected target cells. We then tested c13 protein-induced effector cells for their cytotoxicity on P815 cells infected with other H1 and H3 viruses. Although these effector cells lysed all H1 virus-infected target cells, regardless of the year of isolation, they did not lyse H3 virus-infected cells. Target cells infected with H2 viruses were not killed by c13 protein-induced effector cells (Fig. 1). These results indicate that c13 protein induced an H1 virus subtype-specific CTL response. To know whether internal proteins of A/PR/8 virus were involved in the recognition by c13 protein-induced CTL, their cytotoxicity against A/X31 (H3N2) virus-infected target cells, which contain the internal proteins derived from A/PR/8 virus and the external glycoproteins from H3N2 virus, was examined. A/X31 virus-infected cells were not lysed by c13 protein-induced CTL, whereas they were lysed by A/PR/8 virus-induced CTL (Fig. 1*b*). This finding indicates that c13 protein-induced CTL recognize viral external glycoproteins.

Induction of CTL Memory In Vivo by c13 Protein. The observation that c13 protein was able to stimulate influenza virus-specific secondary CTL response in vitro led us to investigate whether this protein has the potential to generate memory CTL response in vivo. Mice were immunized with 50 μ g of c13 protein

TABLE II
Virus Specificity of c13-induced CTL

Stimulation		Percent specific lysis					
Primary	Secondary	A/PR/8(H1N1)		A/PC(H3N2)		Uninfected	
		30	10	30	10	30	10
A/PR/8	A/PR/8	80.5	59.4	72.8	55.8	4.2	0.2
	A/PC	77.9	76.1	74.2	59.3	2.3	2.1
	c13	33.2	14.8	-0.4	-0.7	1.7	1.3
	None	1.7	-0.8	0.5	-1.9	-0.9	-1.3
A/PC	A/PR/8	96.0	72.9	74.3	57.5	9.8	1.8
	A/PC	92.3	75.6	85.1	80.0	10.2	3.8
	c13	6.7	1.0	4.3	-5.0	2.6	0.2
	None	-2.1	-1.1	-2.5	-3.5	1.2	-0.5

A/PR/8 virus and A/PC virus were used in this experiment. Spleen cells taken from A/PR/8 virus- and A/PC virus-immune mice were stimulated with A/PR/8 virus-infected syngeneic spleen cells or with c13 protein (24 μ g/ml), then assayed for cytotoxicity at E:T ratios of 30:1 and 10:1.

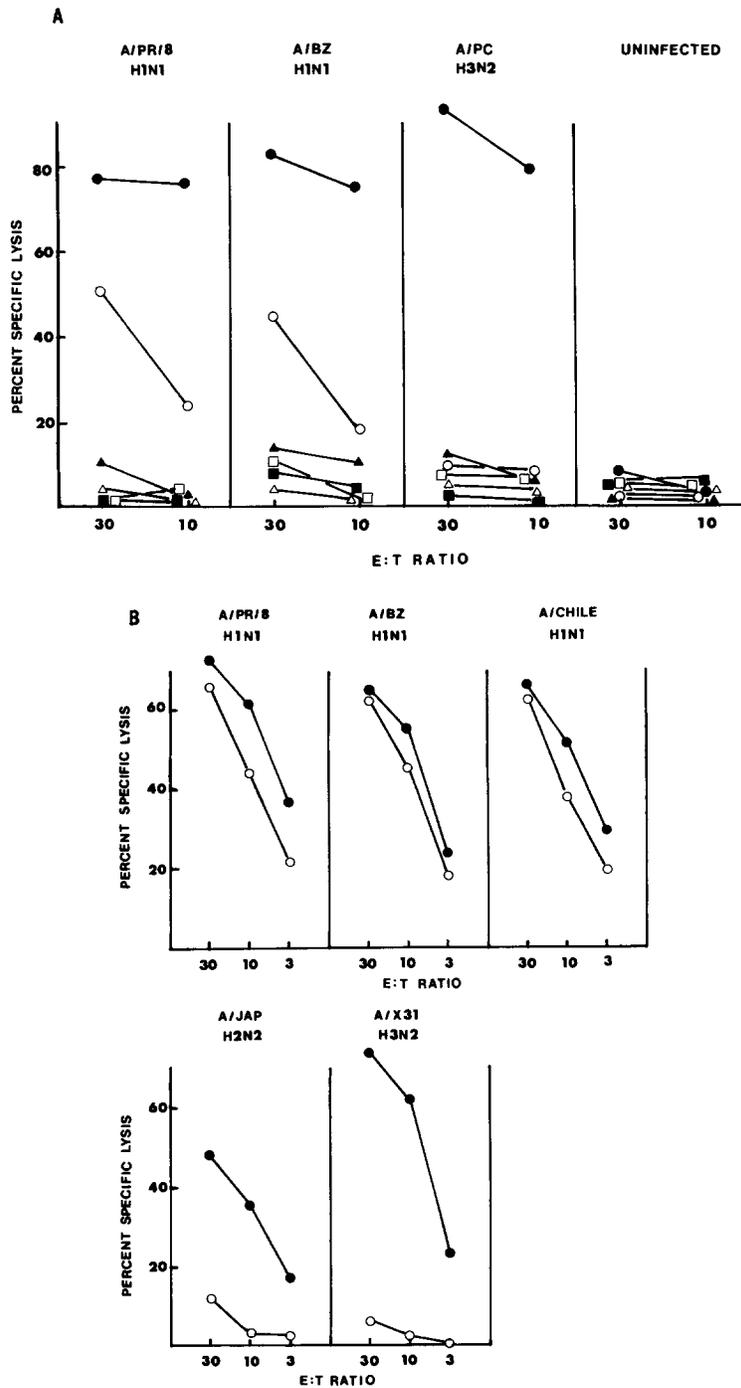


FIGURE 1. Virus specificity of c13 protein-induced effector cells. (a) Effector cells obtained by stimulation of A/PR/8 virus-immune spleen cells with A/PR/8 virus-infected cell (●) and those induced with c13 protein (○) were tested for their cytotoxicity on P815 cells infected with A/PR/8 (H1N1), A/BZ (H1N1), and A/PC (H3N2) viruses. Normal spleen cells stimulated with A/PR/8 virus-infected cells (▲), and those induced with c13 protein (△) were also tested along with immune (■) and normal (□) spleen cells cultured without stimulation. (b) A/PR/8 virus-immune spleen cells stimulated with A/PR/8 virus-infected spleen cells (●) and c13 protein (○) were tested on P815 cells infected with A/PR/8 (H1N1), A/BZ (H1N1), A/Chile (H1N1), A/JAP (H2N2), and A/X31 (H3N2) viruses.

in complete Freund's adjuvant, and were boosted 3 wk later by the intraperitoneal inoculation of 50 μ g of c13 protein. 1 wk after the secondary injection, spleen cells were obtained and cultured with A/PR/8 virus-infected stimulator cells at 37°C for 5 d. As shown in Table III, spleen cells taken from c13 protein-immunized mice responded to the secondary stimulation with A/PR/8 virus-infected stimulator cells, resulting in the specific killing of A/PR/8 virus-infected P815 target cells. This table also shows that these effector cells did not lyse A/PC virus-infected target cells, suggesting that the response was virus subtype-specific. These effector cells failed to lyse A/PR/8 virus-infected L929 (H-2^k) cells (data not shown).

Cytotoxicity of Cultured CTL Derived from In Vitro Stimulation with c13 Protein. We tried to maintain these effector cells in vitro in the presence of exogenous IL-2 after the secondary stimulation. The effector cells obtained by stimulation of A/PR/8 virus-immune cells with A/PR/8 virus-infected cells showed a high level of cytotoxicity to A/PR/8 and A/PC virus-infected target cells at lower E:T ratios after maintenance for 3 wk (Table IV). c13 protein-stimulated A/PR/8 virus-immune cells lysed A/PR/8 virus-infected target cells more efficiently than A/PC virus-infected target cells. c13 protein-immune spleen cells stimulated with A/PR/8 virus-infected cells killed A/PR/8 virus-infected target cells, but did not kill A/PC virus-infected target cells. These data indicate that c13 protein-induced cytotoxicity was maintained in vitro for a long period in the presence of IL-2 without losing virus-specificity.

Determination of Precursor Frequency of CTL. These results indicate that c13 protein has an ability to induce not only secondary CTL activity in vitro but also memory CTL response in vivo. Therefore, we attempted to determine the CTL precursor frequencies of c13 protein-immune mice. 1 wk after the booster inoculation with c13 protein, spleen cells were tested for their CTL precursor frequencies by limiting-dilution analysis. The results are contained in Table V. The frequencies of A/PR/8 virus- and c13 protein-immune spleen cells were

TABLE III
Induction of Memory CTL in Mice Immunized with c13 Protein

Stimulation		Percent specific lysis					
Primary	Secondary	A/PR/8		A/PC		Uninfected	
		30	10	30	10	30	10
A/PR/8	A/PR/8	68.7	37.7	55.7	30.8	-0.1	0.2
	None	6.8	1.7	4.5	-1.8	0.1	-0.6
c13	A/PR/8	18.0	5.9	-0.8	-3.9	0.8	-1.4
	None	-1.5	-3.0	0.1	-5.3	-1.3	-1.2
None	A/PR/8	3.3	2.1	-3.2	-3.5	-2.7	-2.2
	None	4.3	2.8	-2.1	-3.6	-2.0	-0.1

BALB/c mice were immunized subcutaneously with 50 μ g of c13 protein emulsified in an equal volume of complete Freund's adjuvant and boosted intraperitoneally 3 wk later with 50 μ g of c13 protein without adjuvant. 1 wk after the booster injection, spleen cells were cultured with A/PR/8 virus-infected syngeneic spleen cells at 37°C for 5 d. CTL activity was assayed on P815 cells infected with A/PR/8 virus and A/PC virus using E:T ratios of 30:1 and 10:1.

TABLE IV
Cytotoxicity of CTL after Maintenance In Vitro in Presence of IL-2

Stimulation		Percent specific lysis					
Primary	Secondary	A/PR/8		A/PC		Uninfected	
		10	3	10	3	10	3
A/PR/8	A/PR/8	73.3	57.5	63.3	50.2	2.4	3.7
	c13	47.6	24.6	17.5	2.9	2.0	1.4
c13	A/PR/8	26.6	7.2	6.4	-1.5	1.6	-0.6
None	A/PR/8	-3.4	-4.8	-0.3	0.7	2.0	-0.4
	c13	0.8	-3.0	4.0	-2.1	0.1	-0.8

Immunization of mice with the virus and c13 protein is given in the Materials and Methods. Protein concentration used for in vitro stimulation was 12.5 $\mu\text{g/ml}$. Cytotoxicity was assayed after 3 wk of in vitro culture, using E:T ratios of 10:1 and 3:1.

TABLE V
Comparison of Precursor Frequencies

Immunization with:	Stimulation by:	Precursor frequencies (1/f)	95% confidence limit
A/PR/8	A/PR/8	8,047	5,710-11,341
c13	A/PR/8	50,312	37,140-68,154
None	A/PR/9	Nil	
A/PR/8	A/PR/8	15,111	11,004-20,752
A/PR/8	c13	73,177	49,482-108,219
A/PR/8	None	Nil	—

Precursor frequencies of A/PR/8 virus- and c13 protein-immunized mice were determined by the limiting-dilution method (24). The estimates were calculated by solution of maximum likelihood equation, as described by Fazekas de St. Groth (25).

estimated as one in 8,047 and 50,312, respectively. Spleen cells from nonimmune mice did not contain detectable precursors. Although the level of precursor frequency of c13 protein-immune spleen cells was lower than that of A/PR/8 virus-immune mice, these results showed that mice immunized with c13 protein had increased amounts of CTL precursors compared to nonimmune mice. We also determined the frequency of CTL precursors in A/PR/8 virus-immune spleen cells recognizing c13 protein. The frequency of A/PR/8 virus-immune spleen cells reacting with c13 protein was estimated as 1/73,177, whereas those reacting with A/PR/8 virus-infected cells were 1/15,111. To further define the virus specificity of c13 protein-induced effector cells at the clonal level, we made two replicas from each plate of limiting-dilution analysis and tested them against target cells infected with H1 and H3 virus strains. As shown in Table VI, the precursor frequencies of CTL reacting with A/PR/8 virus-infected stimulator cells were very close when we tested them against H1 and H3 virus-infected target cells. However, the precursor frequency of CTL induced by c13 protein was strikingly lower against H3 virus-infected targets than against H1 virus-infected targets, indicating that virus specificity observed in bulk cultures (Table

TABLE VI
Virus Specificity of c13 Protein-induced CTL at the Clonal Level

Exp.	Stimulation	Precursor frequency (1/f)	
		H1N1	H3N2
1	PR8	19,791 (14,477–27,057)*	20,813 (14,904–29,065)
	c13	23,620 (17,679–31,560)	233,575 (146,644–372,040)
2	PR8	21,256 (15,840–28,524)	25,813 (18,318–36,375)
	c13	85,699 (59,164–124,228)	256,684 (137,692–478,506)
3	c13	63,047 (44,818–88,689)	290,846 (145,539–581,231)
4	c13	114,501 (81,137–161,585)	Nil

A/PR/8 virus-immune spleen cells were cultured in limiting-dilution conditions with A/PR/8 virus-infected, syngeneic, X-irradiated spleen cells, or with c13 protein (12.5 µg/ml) in the presence of X-irradiated, normal syngeneic spleen cells. After 7 d of incubation, the contents of each well were split into two aliquots, and their CTL activity was tested against both H1 and H3 virus-infected target cells. The estimates were calculated as mentioned in the Materials and Methods. A/PR/8 (H1N1) and A/PC (H3N2) viruses were used to infect target cells in exp. 1 and 2. In exp. 3, A/BZ (H1N1) and A/BK (H3N2) viruses were used. In exp. 4, A/BZ and A/X31 virus were used as H1N1 and H3N2, respectively.

* 95% confidence limit.

II and Fig. 1) reflected the recognition pattern of CTL precursors at a clonal level.

Discussion

In this paper, we have shown that an influenza virus-specific hybrid protein, made of the first 81 amino acids of NS1 and the HA2 subunit of viral HA, and produced in *E. coli* by recombinant DNA techniques, induced a secondary CTL response in vitro, and a memory CTL response in vivo.

The observation that the entire HA, the HA2 alone, and the NS1 failed to induce the secondary CTL responses raised a question concerning which portion of the c13 protein, the NS1 and/or the HA2, contains the determinant responsible for inducing the CTL response. To address this question, we tested the ability of c13 protein-induced effector cells to kill recombinant A/X31 (H3N2) virus-infected target cells, because all of the genome coding for the internal viral proteins of this virus was derived from the A/PR/8 parent virus, but the surface glycoproteins were derived from the parent H3N2 virus (26). The observation that c13 protein-induced effector cells were cytotoxic to A/PR/8 virus-infected cells but not to A/X31 virus-infected target cells (Fig. 1*b*) indicates that c13 protein-induced H1 subtype-specific CTL recognize the antigenic difference expressed on the external glycoproteins. These results indicate that the HA2 portion of c13 protein has a determinant that is recognized by CTL precursors.

It has been reported that type A influenza virus-specific CTL generated in bulk culture show a broad specificity among type A viruses (2), while subtype-specific CTL have been also described (3, 10). The nature of the antigenic site(s) of the virus recognized by both subtype-specific and crossreactive CTL is still unclear. Recently, Braciale et al. (27) reported that influenza virus HA expressed on murine cells, using DNA-mediated gene transfer, was recognized by both subtype-specific CTL and a subset of crossreactive CTL. Although they did not show which subunit of HA was responsible for the recognition, it is conceivable that the important determinant for the subtype-specific recognition could be located on the HA2 subunit. They also showed that some crossreactive CTL clones failed to lyse HA-expressing target cells, suggesting that viral product(s) other than HA might be recognized by crossreactive CTL. Kees and Krammer (28) reported that most of their short term CTL clones were specific for internal viral components, which appears to support this idea, even though the pattern of the crossreactivity was different from that of crossreactive CTL in bulk cultures, because those clones crossreacted among the viruses sharing genes coding for internal proteins. Furthermore, the isolation of CTL clones that react with internal proteins (14), and the observation that mouse L cells transfected with viral NP gene can serve as target cells to crossreactive CTL (29) also seem to provide evidence that the internal protein(s) are involved in the recognition by influenza virus-specific crossreactive CTL.

The frequency of CTL precursors reacting with the subtype-specific determinant on HA2 is generally lower than that obtained by stimulation with virus-infected cells, although the CTL precursor frequencies varied between experiments. These results suggest that the majority of CTL precursors recognize viral determinant(s) other than those on HA2.

The data presented in Table VI show that A/PR/8 virus-stimulated clones lysed both A/PR/8 virus- and A/PC virus-infected target cells. These observations are different from those of Kees and Krammer (28), who reported that most CTL clones lysed only target cells which were infected with a virus that shared internal proteins with the virus used for elicitation of the response. The reason for this discrepancy is not clear; however, since they used spleen cells taken from mice 1 wk after the primary immunization, when the primary response to infection was beginning (1, 3), it is conceivable that the specificity pattern is different from that of spleen cells obtained from animals at least 3 wk after immunization. On the contrary, our results are in accordance with those of Owen et al. (30), who reported that ~80% of A/PR/8 virus-stimulated CTL clones lysed both A/PR/8 (H1N1) virus- and A/Northern Territory/60/68 (H3N2) virus-infected cells. As the majority of CTL clones reactive with c13 protein lysed only target cells infected with H1 subtype viruses, it seems unlikely that fully crossreactive CTL precursors recognize conserved antigenic portions of the HA2 subunit. These results support the concept that highly conserved internal proteins are recognized by these crossreactive CTL.

Although we do not know the precise role of the first 81 amino acids of NS1, which is coupled to the HA2 in c13 protein, this region may be important for maintaining the tertiary structure of the protein, in order to present the immunodominant site to the responding cells, or for serving as an adjuvant. Since a

derivative of c13 protein (Δ 13 protein) lacking 153 amino acids of the carboxy terminal end of the HA2 did not stimulate a CTL response, the antigenic site may be mapped to this portion; however, we could not rule out the possibility that the failure of Δ 13 protein to induce CTL response may be due to the change of configuration because of the removal of this portion from c13 protein. In this respect, it seems worth pointing out that a CNBr cleavage product of HA2 (between residues 103 and 123) has been reported (12) to induce a subtype-specific secondary CTL response. The reason neither the entire HA nor HA2 alone induced a CTL response is not clear. The lack of glycosylation of these proteins may be an explanation, although it is also possible that special factor(s) may be required for the generation of CTL response by these proteins, as described by Wabuke-Bunoti et al. (31).

In conclusion, we have shown here that an influenza virus-specific hybrid protein including the first 81 amino acids of NS1 and the HA2 subunit of viral HA, prepared by recombinant DNA techniques, can produce both an in vitro secondary CTL response and in vivo generation of memory CTL in a subtype-specific manner. Since it has been reported (32) that a subtype-specific CTL clone can protect mice from lethal infection with influenza A virus, it will be interesting to see whether this protein induces protective immunity to the recipient mice. Experiments investigating this issue are now in progress.

Summary

We have tested the abilities of various polypeptides of A/PR/8/34 (H1N1) virus, constructed by recombinant DNA techniques, to induce influenza virus-specific secondary cytotoxic T lymphocyte (CTL) responses. A hybrid protein (c13 protein), consisting of the first 81 amino acids of viral nonstructural protein (NS1) and the HA2 subunit of viral hemagglutinin (HA), induced H-2-restricted, influenza virus subtype-specific secondary CTL in vitro, although other peptides did not. Using a recombinant virus, the viral determinant responsible for recognition was mapped to the HA2 portion of c13 protein. Immunization of mice with c13 protein induced the generation of memory CTL in vivo. The CTL precursor frequencies of A/PR/8/34 virus- and c13 protein-immune mice were estimated as one in 8,047 and 50,312, respectively. These results indicate that c13 protein primed recipient mice, even though the level of precursor frequency was below that observed in virus-immune mice.

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References

1. Yap, K. L., and G. L. Ada. 1977. Cytotoxic T cells specific for influenza virus infected target cells. *Immunology*. 32:151.
2. Zweerink, H. J., S. A. Courtneidge, J. J. Skehel, and B. A. Askonas. 1977. Cytotoxic T cells kill influenza virus infected cells but not distinguish between serologically distinct type A viruses. *Nature (Lond.)*. 267:354.
3. Ennis, F. A., W. J. Martin, and M. W. Verbonitz. 1977. Hemagglutinin-specific cytotoxic T cell response during influenza infection. *J. Exp. Med.* 146:893.

4. McMichael, A. J., A. Ting, H. J. Zweerink, and B. A. Askonas. 1977. HLA restriction of cell-mediated lysis of influenza virus infected human cells. *Nature (Lond.)* 270:524.
5. Yap, K. L., G. L. Ada, and I. F. C. McKenzie. 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature (Lond.)* 273:238.
6. McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. S. Beare. 1983. Cytotoxic T-cell immunity to influenza. *New Engl. J. Med.* 309:13.
7. Wells, M. A., P. Albrecht, and F. A. Ennis. 1981. Recovery from a viral respiratory infection. I. Influenza pneumonia in normal and T-deficient mice. *J. Immunol.* 126:1036.
8. Wells, M. A., F. A. Ennis, and P. Albrecht. 1981. Recovery from a viral respiratory infection. II. Passive transfer of immune spleen cells to mice with influenza pneumonia. *J. Immunol.* 126:1042.
9. Sherman, L. A., A. Vitiello, and N. R. Klinman. 1983. T-cell and B-cell responses to viral antigens at the clonal level. *Ann. Rev. Immunol.* 1:63.
10. Zweerink, H. J., B. A. Askonas, D. Millican, S. A. Courtneidge, and J. J. Skehel. 1977. Cytotoxic T cells to type A influenza virus. Viral hemagglutinin induces A-strain specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol.* 7:630.
11. Braciale, T. J. 1979. Specificity of cytotoxic T cells directed to influenza virus hemagglutinin. *J. Exp. Med.* 149:856.
12. Wabuke-Bunoti, M. A. N., and D. P. Fan. 1983. Isolation and Characterization of a CNBr cleavage peptide of influenza viral hemagglutinin stimulatory for mouse cytolytic T lymphocytes. *J. Immunol.* 130:2386.
13. Becht, H., R. T. C. Huang, B. Fleischer, C. B. Boschek, and R. Rott. 1984. Immunologic properties of the small chain HA2 of the haemagglutinin of influenza viruses. *J. Gen. Virol.* 65:173.
14. Townsend, A. R. M., and J. J. Skehel. 1982. Influenza A specific cytotoxic T-cell clones that do not recognize viral glycoproteins. *Nature (Lond.)* 300:655.
15. Townsend, A. R. M., and Skehel, J. J. 1984. The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells. *J. Exp. Med.* 160:552.
16. Koszinowski, U. H., H. Allen, M.-J. Gething, M. D. Waterfield, and H.-D. Klenk. 1980. Recognition of viral glycoproteins by influenza A-specific cross-reactive cytolytic lymphocytes. *J. Exp. Med.* 151:945.
17. Webster, R. G., and B. A. Askonas. 1980. Monoclonal antibodies to hemagglutinin and to H-2 inhibit the cross-reactive cytotoxic T cell populations induced by influenza. *Eur. J. Immunol.* 10:151.
18. Reiss, C. S., and J. L. Schulman. 1980. Influenza type A virus M protein expression on infected cells is responsible for cross-reactive recognition by cytotoxic thymus-derived lymphocytes. *Infect. Immun.* 29:719.
19. Braciale, T. J. 1977. Immunological recognition of influenza virus-infected cells. II. Expression of influenza A matrix protein on the infected cell surface and its role in recognition by cross-reactive cytotoxic T cells. *J. Exp. Med.* 146:690.
20. Rosenberg, M., Y.-S. Ho, and A. Shatzman. 1983. The use of pKC30 and its derivatives for controlled expression of genes. *Methods Enzymol.* 101:123.
21. Shatzman, A., Y.-S. Ho, and M. Rosenberg. 1983. Use of phage lambda regulatory signal to obtain efficient expression of genes in *Escherichia coli*. In *Experimental manipulation of gene expression*. M. Inoue, editor. Academic Press, New York. 1-14.
22. Young, J. F., U. Desselberger, P. Palese, B. Ferguson, A. R. Shatzman, and M.

- Rosenberg. 1983. Efficient expression of influenza virus NS1 nonstructural proteins in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. 80:6105.
23. Young, J. F., U. Desselberger, P. Graves, P. Palese, A. Shatzman, and M. Rosenberg. 1983. Cloning and expression of influenza virus genes. *In* The origin of pandemic influenza viruses. W. G. Laver, editor. Elsevier North/Holland, Amsterdam. 129–138.
 24. Kees, U., G. Kynast, E. Weber, and P. H. Krammer. 1984. A method for testing the specificity of influenza A virus-reactive memory cytotoxic T lymphocyte (CTL) clones in limiting dilution cultures. *J. Immunol. Methods*. 69:215.
 25. Fazekas de St. Groth, S. 1982. The evaluation of limiting dilution assays. *J. Immunol. Methods*. 49:R11.
 26. Baez, M., P. Palese, and E. D. Kilbourne. 1980. Gene composition of high-yielding influenza vaccine strains obtained by recombination. *J. Infect. Dis.* 141:362.
 27. Braciale, T. J., V. L. Braciale, T. J. Henkel, J. Sambrook, and M.-J. Gething. 1984. Cytotoxic T lymphocyte recognition of the influenza hemagglutinin gene product expressed by DNA-mediated gene transfer. *J. Exp. Med.* 159:341.
 28. Kees, U., and P. Krammer. 1984. Most influenza A virus-specific memory cytotoxic T lymphocytes react with antigenic epitopes associated with internal virus determinants. *J. Exp. Med.* 159:365.
 29. Townsend, A. R. M., A. J. McMichael, N. P. Carter, J. A. Huddlestone, and G. G. Brownlee. 1984. Cytotoxic T cell recognition of the influenza nucleoprotein and hemagglutinin expressed in transfected mouse L cells. *Cell*. 39:13.
 30. Owen, J. A., M. Allouche, and P. C. Doherty. 1982. Limiting dilution analysis of the specificity of influenza-immune cytotoxic T cells. *Cell. Immunol.* 67:49.
 31. Wabuke-Bunoti, M. A. N., A. Taku, R. Garman, and D. P. Fan. 1984. Stimulation of anti-influenza cytotoxic T lymphocytes by a synthetic peptide of the influenza hemagglutinin can be modulated by at least three independent helper factors. *J. Immunol.* 133:2186.
 32. Lukacher, A. E., V. Braciale, and T. J. Braciale. 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* 160:814.