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We have demonstrated that a conserved portion of the HA_2 subunit on the influenza virus hemagglutinin can induce a cytotoxic T lymphocyte response. This is a major development since it raises the possibility that this type of peptide could be used to provide protection that would be cross-reactive among influenza virus strains. The peptide we used was produced in E. coli using recombinant DNA techniques for the expression of segments of influenza viral genome. The molecule which stimulates this H-2 restricted cytotoxic T lymphocyte response is a fusion protein of the HA_2 subunit of H1 virus (A/PR/8/34 H1N1), and the induced lymphocytes kill target cells infected with strains of influenza A virus possessing the H1 hemagglutinin irregardless of the years isolated (e.g. 1934, 1978), the results indicate that the HA_2 subunit is a candidate for cross-reactive protection because there are substantial published data indicating that influenza virus induced cytotoxic T lymphocytes (Tc) are protective in challenged recipients

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Summary

Influenza A virus infection induces a major histocompatibility (MHC) antigen-restricted subtype-specific cytotoxic T cell (CTL) response and a cross-reactive response among the influenza A subtypes[1-4]. These CTL have been demonstrated to play a crucial role in the recovery from infection[5-8]. The definition of the viral determinants which are recognized by these CTL is not complete. At least five (haemagglutinin, neuraminidase, matrix protein, polymerase, and nucleoprotein) of the seven viral structural polypeptides have been reported to be responsible for the recognition by influenza virus-specific CTL or CTL clones[9-18]. We examined the abilities of several viral polypeptides prepared by gene cloning techniques to induce the secondary CTL response in vitro. These results show that a hybrid protein (c13 protein) of the first 81 amino acids of the viral NS_1 nonstructural protein and the HA_2 subunit of viral haemagglutinin (HA) stimulated H-2-restricted, subtype-specific secondary CTL in vitro. Furthermore, immunization of mice with c13 protein induced CTL in vivo. The precursor CTL frequencies of virus- and c13 protein-immune mice were estimated as $8,047^{-1}$ and $50,312^{-1}$, respectively, indicating that the c13 protein induces CTL in vivo but at a frequency below that observed in virus-immune mice.

Results

Twenty to 30 million immune spleen cells were cultured with various concentrations of polypeptides or A/PR/8/34 (H1N1) virus-infected syngeneic spleen cells. After incubation at 37°C for 5 days, cytotoxic activities of the stimulated cells were assayed on A/PR/8/34 virus-infected, $Na_2{}^{51}CrO_4$ labeled, P815 mouse mastocytoma cells. Table 1 shows that A/PR/8/34 virus-immune spleen cells stimulated with A/PR/8/34 virus-infected syngeneic cells were highly cytotoxic to A/PR/8/34 virus-infected P815 cells but not to uninfected P815 cells. Out of six peptides tested, only c13 protein, which is a hybrid protein between the first 81 amino acids of NS_1 and HA_2, stimulated the secondary CTL response in vitro, although the level of killing by c13-stimulated cells was lower than that obtained with effector cells induced by virus-infected stimulator cells. The induction of CTL by c13 protein was found to be dose-dependent and the killing of virus-infected target cells was H-2-restricted (data not shown). Interestingly, c36 and c7 proteins, which are HA_2 and the entire HA, respectively, did not induce any CTL responses.

Table 1. Induction of secondary CTL in vitro by E. coli-derived protein*

Exp.	Secondary stimulation	A/PR/8 30	A/PR/8 10	Uninfected 30	Uninfected 10
1	A/PR/8**	60.5	37.4	6.7	2.6
	NS1	-5.2	-8.7	4.3	0.0
	C13	15.0	-1.1	0.5	-0.5
	#13	-8.6	-10.1	-1.3	0.0
	#7	-3.2	-7.4	2.9	-0.9
	No	5.4	-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

*Influenza virus-specific polypeptides were produced in E. coli using the expression system described previously[19-21].

To determine the virus specificity of the secondary CTL induced by c13 protein, A/PR/8/34 virus- or A/Port Chalmers/1/73 (H3N2) virus-immune spleen cells were stimulated with c13 protein and tested for their ability to lyse P815 cells infected with various strains of influenza A viruses. As shown in Table 2, c13 protein can stimulate A/PR/8/34 (H1N1)-immune spleen cells but not A/Port Chalmers/1/73 (H3N2)-immune spleen cells. Although the effector cells stimulated by A/PR/8/34 virus- or A/Port Chalmers/1/73 virus-infected syngeneic cells could lyse all target cells, c13-induced effector cells lysed only target cells infected with A/PR/8/34 and A/Brazil/11/78 (H1N1) viruses, indicating that the c13 protein induced H1 subtype-specific CTL.

Table 2. Virus-specificity of c13-induced CTL

Stimulation		A/PR/8(H1N1)		A/BZ(H1N1)		A/SING(H2N2)		A/PC(H3N2)		Uninfected	
1°	2°	30	10	30	10	30	10	30	10	30	10
A/PR/8	A/PR/8	76.5	77.0	82.8	75.5	30.6	17.8	92.8	79.0	8.4	3.0
	c13	50.7	24.0	45.0	18.4	2.0	4.4	10.3	8.5	2.0	1.1
	No	1.3	-0.3	9.4	4.8	7.1	4.2	3.7	2.1	8.5	-0.6
A/PC	A/PR/8	96.0	72.9	nd*	nd	nd	nd	74.3	57.5	9.8	1.8
	c13	4.6	4.6	nd	nd	nd	nd	1.2	-3.7	2.4	-0.3
	No	-2.1	-2.1	nd	nd	nd	nd	-2.5	-3.5	1.2	-0.5

The following viruses were used in this experiment: A/PR/8/34 (A/PR/8), A/Brazil/11/78 (A/BZ), A/Singapore/1/57 (A/SING), and A/Port Chalmers/1/73 (A/PC). 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 (12 μg/ml) then assayed for cytotoxicity at E:T ratios of 30:1 and 10:1.

*Not done.

The observation that c13 protein was able to stimulate influenza virus-specific secondary CTL response in vitro led us to investigate whether this protein could stimulate CTL memory cells in vivo. Mice were immunized subcutaneously with 50 μg of the protein in Freund's complete adjuvant and boosted 3 weeks later by intraperitoneal inoculation with 50 μg of c13 protein. One week after the secondary injection, spleen cells were obtained and cultured with A/PR/8/34 virus-infected stimulator cells at 37°C for 5 days. As shown in Table 3, spleen cells taken from c13-immunized mice responded to the secondary stimulation, resulting in specific killing of A/PR/8/34 virus-infected P815 target cells. This table also shows that the cytotoxic cells induced by the stimulation of c13-immunized spleen cells with A/PR/8/34 virus-infected cells express influenza virus H1 subtype-specificity. These effector cells from H-2^d mice were also shown to be specific for the H-2^d haplotype and these c13 primed spleen cells were cytotoxic to H1N1 but not H3N2 virus infected targets (24.6% versus 2.9% specific lysis at E:T ratio of 10:1) after 3 weeks of incubation in the presence of 20% HuTCGF.

Table 3. Induction of memory CTL in mice immunized with c13 protein

Stimulation		A/PR/8		A/PC		Uninfected	
1°	2°	30	10	30	10	30	10
A/PR/8	A/PR/8	67.1	34.5	49.1	28.0	-0.8	-0.5
	No	2.0	-3.4	7.8	-6.0	-0.5	-0.2
c13	A/PR/8	13.7	1.0	-7.8	-8.1	0.2	-2.1
	No	-6.7	-8.3	-7.6	-9.6	-2.0	-1.9

BALB/c mice were immunized with 50 μg of c13 protein emulsified in an equal volume of Freund's complete adjuvant subcutaneously and boosted with 50 μg of c13 protein intraperitoneally without adjuvant 3 weeks later. One week after the booster injection, spleen cells were cultured with A/PR/8 virus-infected syngeneic spleen cells at 37°C for 5 days. 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.

The above results indicated that c13 protein has the ability to induce not only secondary CTL activity in vitro but also a memory CTL response in vivo. Therefore, we attempted to determine CTL precursor frequencies of c13-immune mice. One week 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 4. The frequencies of precursor CTL in spleen cells of A/PR/8/34 virus- and c13 protein-immunized mice were estimated to be $8,047^{-1}$ and $50,312^{-1}$, respectively. Spleen cells from non-immune mice did not contain detectable precursors. Although the precursor frequency of c13-immune spleen cells was lower than that of A/PR/8/34 virus-immune mice, these results showed that mice immunized with c13 protein had an

increased level of CTL precursors compared to non-immune spleen cells recognizing c13 protein. The precursor frequency of A/PR/8/34 virus-immune spleen cells reacting with c13 protein was estimated as $73,177^{-1}$, whereas that reacting with A/PR/8/34 virus-infected cells was $15,111^{-1}$.

Table 4. Comparison of precursor frequencies

Immunization with	Stimulation by	Precursor frequencies (1/n)	95% confidence range
A/PR/8	A/PR/8	8,047	5,710-11,341
c13	A/PR/8	50,312	37,140-68,154
No	A/PR/8	TLTC*	
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	No	TLTC*	

Precursor frequencies of A/PR/8/34- and c13-immunized mice were determined by the limiting dilution method[23]. Spleen cell suspensions diluted to desired concentrations were distributed into round-bottomed 96-well microplates (100 µl/well) and cultured with 1×10^6 x-irradiated (2,500 rad) syngeneic spleen cells infected with A/PR/8/34 virus in the presence of 20% human T cell growth factor (Meloy Lab. Inc.). After incubation at 37°C for 7 days, each well was assayed for cytotoxicity against A/PR/8/34 virus-infected P815 target cells (2,000 cells/well). Statistical analysis was performed according to the method described by Fazekas de St. Groth[24].
* Too low to count.

The observation that the entire HA, the HA_2 alone, and the NS_1 failed to induce the secondary CTL responses raises a question concerning which portion of the c13 protein, the NS_1 and/or the HA2, contains the determinant responsible for inducing the CTL response. In order to address this question we tested the ability of c13-induced effector cells to kill recombinant X-31 (H3N2)-infected target cells, because all of the genome coding for the internal viral proteins of X-31 virus was derived from the A/PR/8/34 (H1N1) parent virus but the surface glycoproteins were derived from the parent H3N2 virus[26]. C13-induced effector cells lysed A/PR/8/34 (H1N1)-infected target cells (38.4% specific lysis at E:T ratio of 10:1), but they did not lyse X-31 (H3N2)-infected targets (4.1% at E:T of 10:1). These results indicate that c13-induced H1-specific CTL recognize the antigenic difference expressed on external viral glycoproteins, indicating that the HA_2 portion of the c13 protein has a determinant which is recognized by CTL precursors. The inability to stimulate CTL generation by HA and HA_2 may be explained by the fact that these polypeptides produced in E. coli are not glycosylated and that peptides without sugars may have different conformation compared to that of native protein. A preparation of the A/WSN (H1N1) virus HA produced in E. coli also failed to stimulate CTL induction (A. Yamada, F.A. Ennis, and D.P. Nayak;

unpublished observation). Although we do not know the precise role of the first 81 amino acids of NS_1 which is coupled to the HA_2 in c13, this region may be important for the tertiary structure of the protein in order to present the immunodominant site to the responding cells. Since a derivative of c13 protein (#13 protein) lacking 153 amino acids of the carboxy terminal end of the HA_2 did not stimulate a CTL response, the antigenic site may be mapped to this portion. These observations are in agreement with those of Wabuke-Bunoti and Fan[27] who noted that a cyanogen bromide cleavage product of HA_2 (between residues 103 and 123) could induce a subtype-specific secondary CTL response.

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[9]. The nature of the antigenic site(s) of the virus recognized by both cross-reactive and subtype-specific CTL is still unclear. Recently, Braciale et al.[28] 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 cross-reactive CTL. Although they did not show which subunit of HA was responsible for the recognition, it is conceivable that the important determinant for the recognition could be located on the HA_2 subunit. They also showed that one of the cross-reactive CTL clones failes to lyse HA-expressing target cells, suggesting that viral product(s) other than HA might be recognized by cross-reactive CTL. The observation of Kees and Krammer[29] that most of their short term CTL clones have a specificity for internal viral components appears to support this idea. Furthermore, the isolation of a CTL clone that reacts with viral nucleoprotein[17] also seems to provide evidence that an internal protein is responsible for the recognition by cross-reactive CTL.

Our results show that the frequency of CTL precursors reacting with the subtype-specific determinant on HA_2 is about 10-20% of the total CTL precursor, suggesting that 80-90% of CTL precursors recognize viral determinant(s) other than that on HA_2. This is in accordance with the results of Kees and Krammer[29] who reported that about 90% of CTL precursors recognized internal proteins rather than external glycoproteins such as HA and neuraminidase. It therefore seems likely that the numbers of CTL precursors which recognize influenza A subtype-specific determinants may be around 10% of the total CTL precursors; however, the frequency may be variable depending on the viral strain or mouse strain as was pointed out by Vitiello and Sherman[30].

In conclusion, we have demonstrated in this communication that an influenza virus-specific hybrid protein between NS_1 and HA_2 prepared by recombinant DNA techniques can produce both an in vitro secondary CTL response and an in vivo generation of memory CTL in a subtype-specific manner. As subtype-specific CTL can protect mice from lethal infection with influenza virus[31], it will be interesting to see whether this protein induces any protective immunity to the recipient mice. Experiments investigating this issue are now in progress.

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