INFLUENZA VIRUS SUBTYPE–SPECIFIC CYTOTOXIC T LYMPHOCYTES LYSE TARGET CELLS COATED WITH A PROTEIN PRODUCED IN *E. COLI*

BY AKIO YAMADA,* JAMES F. YOUNG,[†] AND FRANCIS A. ENNIS*

*From the *Division of Infectious Disease, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605; and the Molecular Genetics Division, Smith, Kline, and French Laboratories, Philadelphia, Pennsylvania 19101*

Virus-specific cytotoxic T cells (CTL) recognize viral antigen in conjunction with products of genes of the major histocompatibility complex (MHC) (1); however, the nature of the viral antigen(s) recognized by specific CTL is not clear. Identification of viral determinant(s) recognized by CTL is an important issue; however, it has been difficult to demonstrate CTL recognition of target cells exposed to viral antigens. Previous studies (2) have indicated that the surface glycoproteins of Sendai virus were recognized by CTL, providing the fusion (F) protein was cleaved. Isolated viral surface glycoproteins were recognized on cell surfaces by CTL after incorporation into the cell membrane by liposomal carriers (3, 4), and by DNA-mediated gene transfer (5). CTL responses to internal influenza viral proteins have also been reported (6, 7). Extensive studies have been conducted (8) using influenza A virus infection as a model system, and two different subpopulations of CTL, crossreactive and subtype-specific, are induced.

We previously reported (9) that an influenza virus–specific polypeptide (designated c13 protein) produced in *E. coli* induced H1 influenza subtype–specific memory and secondary H-2-restricted CTL responses in mice. The c13 protein is a hybrid protein of the first 81 amino acids of the NS1 viral nonstructural protein and the HA2 subunit of viral hemagglutinin (HA) derived using genes from an H1N1 virus. It is, therefore, conceivable that the c13 protein interacts with cellular membrane to be presented to CTL precursors along with MHC products. In this study, we examined the ability of c13 protein to render target cells susceptible to lytic activity of influenza virus–specific CTL. The results showed that target cells exposed to c13 protein were lysed by CTL in a subtype-specific and H-2-restricted manner, suggesting that this protein interacts with target cell membrane with its hydrophobic region, and is presented on the cell membrane in proper conformation with H-2 antigens for recognition by the influenza virus subtype–specific CTL.

Materials and Methods

Mice. Male BALB/cAnH (H-2^d) and C3H/HeN (H-2^k) mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA), and used at the age of 4–5 wk.

Virus. Influenza A viruses, PR8 (A/Puerto Rico/8/34 [H1N1]), A/BZ (A/Brazil/11/78

[H1N1]), A/PC (A/Port Chalmers/1/73 [H3N2]), and a recombinant virus A/X31 (H3N2), were propagated in 9-d-old embryonated chicken eggs, as described previously (9).

Production of cl3 Protein.   cl3 protein was produced in *E. coli* as described before (9). Briefly, plasmids containing DNA fragments complementary to the viral RNA of PR8 virus were manipulated to achieve expression of cl3 protein, which is a hybrid of the first 81 amino acids of NS1 and HA2. Following 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 cl3 protein was solubilized with 4 M urea (4°C for 30 min). The urea was then removed by dialysis at 4°C and stored in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Induction of Secondary CTL In Vitro.   Secondary CTL were prepared as described previously (9). Briefly, spleen cells from mice immunized with virus were cultured with syngeneic normal spleen cells infected with the virus or exposed to cl3 protein (12.5 μg/ml) at 37°C for 5 d. After several washings, they were used as effector cells.

CTL Assay.   P815 cells (H-2^d) and BW5147 cells (H-2^k) were used as target cells. In preliminary experiments, we observed that P815 and BW5147 cells exposed to cl3 protein (12 μg/ml) expressed membrane antigen(s), detected by indirect immunofluorescence with a specific antiserum to cl3 protein, but P815 and BW5147 cells not treated with cl3 did not. In the CTL assay, target cells infected with virus, or exposed to cl3 protein diluted in RPMI medium, were labeled with Na$_2$^{51}CrO$_4$, and incubated with effector cells at various effector/target (E/T) ratios in a 96-well round-bottom microplate at 37°C for 4 h in a standard ^{51}Cr-release assay, as described earlier (9).

Cold-target Inhibition Test.   P815 cells infected with various viruses or treated with cl3 protein (12.5 μg/ml) at 37°C for 90 min were added to the CTL assay at various concentrations. The CTL assay was performed as described below.

Blocking of Cytotoxicity by Anti-H-2^d Monoclonal Antibody (mAb).   Various target cells were incubated with anti-H-2^d mAb (Litton Bionetics, Charleston, SC) at 37°C for 30 min, and then were incubated with appropriate effector cells at 37°C for 4 h. Antibody was used at 1:20 dilution (final), based on the results of a preliminary experiment. Cytotoxicity was determined as described above.

Results

Influenza Virus–specific Lysis of Target Cells Exposed to cl3.   We examined the ability of cl3 protein to render P815 cells susceptible to influenza virus–specific CTL. Effector cells were generated by stimulation of PR8 virus–immune spleen cells with PR8 virus–infected syngeneic spleen cells, and by cl3 protein. As shown in Fig. 1, cl3 protein–coated P815 cells were lysed by PR8 virus– and cl3 protein–induced CTL, although the level of lysis was below that of virus–infected target cells. The level of lysis of cl3 protein–coated target cells was dose-dependent up to a concentration of 3 μg/ml (data not shown). These results, (Fig. 1) suggested that target cells exposed to cl3 protein displayed an H1 virus subtype–specific determinant, since cl3 protein has been shown (9) to induce H1 subtype–specific CTL. We therefore prepared two different effector cells, PR8 virus–stimulated PR8 virus–immune spleen cells, and A/PC virus–stimulated A/PC virus–immune spleen cells. Both PR8-immune effectors and PC-immune effectors specifically lysed PR8 virus–infected target cells; (e.g., 63.8% and 66.2%, respectively, at E/T ratios of 10:1); however, cl3 protein–coated target cells showed little lysis by A/PC virus–induced A/PC virus–immune spleen cells, (2.8% at E/T 10:1), whereas they were definitely susceptible to PR8 virus–stimulated PR8 virus–immune spleen cells (34.0% at E/T 10:1). These findings suggest that cl3 protein–coated target cells were lysed by PR8 virus–specific CTL, but not by either crossreactive or A/PC virus–specific CTL.
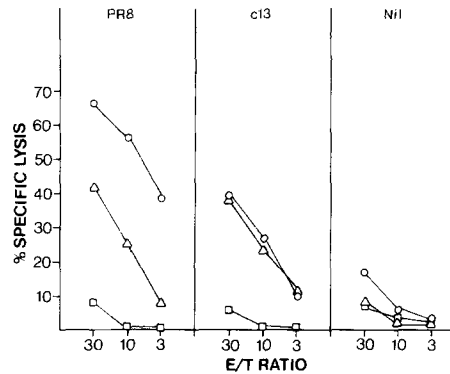
FIGURE 1.   Lysis of P815 target cells infected with PR8 virus, exposed to c13 protein, or untreated target cells (*Nil*). These target cells were incubated with PR8 virus–immune spleen cells stimulated with virus (O) or c13 protein (△). Unstimulated PR8 virus–immune spleen cells (□) were also used as effectors.

Cold-target Inhibition Study.   To further examine the antigen specificity of effector cells recognizing target cells coated with c13 protein, we tried to inhibit the killing activity of effector cells by cold target cells. Lysis of c13 protein–coated ^{51}Cr-labeled target cells was inhibited by both PR8 virus– and A/BZ virus–infected cold target cells, as well as by c13 protein–coated target cells (Fig. 2). A/X31 virus–infected ^{51}Cr-labeled target cells were not lysed by PR8 virus–induced effector cells in the presence of all cold target cells infected with type A viruses, but were lysed in the presence of c13 protein–coated or uninfected cold target cells. These findings indicate that c13 protein–coated target cells are recognized and lysed by H1 virus subtype–specific CTL.

H-2-restricted Killing of c13 Protein–coated Target Cells.   We attempted to determine whether there was H-2 restriction of killing of c13 protein–coated target cells. Effector cells were obtained from BALB/c (H-2^d) and C3H (H-2^k) mice immunized with PR8 virus at least 3 wk before. After stimulation with virus–infected syngeneic spleen cells, cytotoxicity was examined on virus-infected and c13 protein–coated P815 (H-2^d) and BW5147 (H-2^k) cells. Table I shows that the lysis of virus–infected target cells was definitely restricted by the H-2 complex. The lysis of P815 cells coated with c13 protein was manifested by effector cells from BALB/c mice, but not by those from C3H mice. To further examine a possible association of c13 protein with the H-2^d molecule, we attempted to block the killing of c13 protein–coated P815 cells by H-2^d effector cells using mAb directed to H-2K^d and -D^d. As shown in Table II, the lysis of P815 cells infected with PR8 virus by BALB/c-derived effector cells was reduced in the presence of anti-H-2^d mAb. The anti-H-2^d mAb inhibited BALB/c effector–mediated lysis of c13 protein–coated P815 cells, but did not affect the killing of PR8 virus–infected BW5147 cells by C3H-derived effectors. These results strongly suggest that c13 protein is recognized by influenza virus–specific CTL in conjunction with H-2K^d or -D^d molecules. BW5147 cells coated with c13 protein were not sensitive to the lytic activity of C3H-derived effector cells, even though they could lyse PR8 virus–infected BW5147 cells (Table I).

Discussion

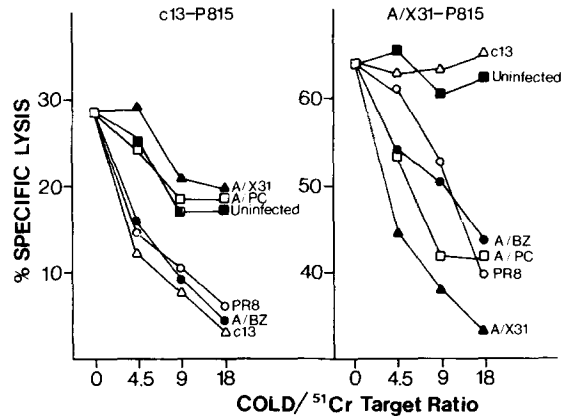We have shown that P815 cells coated with c13 protein, which is a hybrid

FIGURE 2. Cold-target inhibition test. Lysis of c13 protein–coated P815 cells by PR8 virus–stimulated PR8 virus–immune spleen cells was examined in the presence of various cold target cells. ^{51}Cr-labeled P815 cells infected with A/X31 virus were also included in this experiment.

TABLE I

H-2-restricted Lysis of c13 Protein–coated P815 Cells

Target	E/T ratio	Percent specific lysis by effectors*	
		BALB/c (H-2^d)	C3H (H-2^k)
PR8-P815	30	57.2	−1.6
	10	55.3	0.0
	3	36.1	0.0
c13-P815	30	34.6	1.8
	10	21.0	1.2
	3	11.8	−2.2
PR8-BW5147	30	2.6	38.7
	10	−1.6	31.8
	3	1.2	17.9
c13-BW5147	30	0.5	4.6
	10	−1.7	0.0
	3	−1.3	−1.2

* Obtained by subtraction of lysis of untreated target cells by each group of effector cells.

protein of the first 81 amino acids of NS1 and HA2 of PR8 virus, are lysed by influenza virus–specific CTL. Lysis of c13 protein–coated target cells was accomplished by CTL induced by virus strains of the H1 but not the H3 subtype. Cold-target inhibition analysis clearly demonstrated that c13 protein–coated target cells were recognized and killed by an H1 subtype–specific population of CTL, confirming our previous finding (9) that c13 protein has a determinant recognized by H1 subtype–specific CTL.

Morein et al. (3) showed that liposomes containing the surface glycoproteins of Semliki Forest virus could sensitize target cells to CTL in the presence of inactivated Sendai virus. Sendai virus, which has a potent protein, UV-inactivated virions (2), and the hemagglutinin-neuraminidase (HN) and F proteins, have been shown (4) to make target cells sensitive to CTL killing when introduced into target cells in liposome vehicles. Koszinowsky et al. (10) reported killing mediated by influenza virus–specific crossreactive CTL if target cells were fused

TABLE II

Effect of Anti-H-2^d mAb on Lysis of c13 Protein–coated Target Cells

Effector	Target	E/T ratio	Percent specific lysis in the presence of:*	
			SP2/0	Anti-H-2^d
BALB/c	PR8-P815	10	57.4	48.9 (14.8)
		3	41.4	36.2 (12.6)
	c13-P815	30	43.3	26.8 (38.1)
		10	22.2	10.6 (52.3)
		3	9.3	6.9 (25.8)
C3H	PR8-BW5147	10	48.0	45.5 (5.2)
		3	22.3	23.0 (−3.1)

* PR8 virus–infected and c-13 coated target cells were incubated with a 1:20 dilution of anti-H-2^d mAb, or with the supernatant fluid obtained from culture of parental SP2/0 cells at 37°C for 30 min, then incubated with effector cells. Percent inhibition of killing is indicated in the parentheses.

to liposomes containing influenza virus surface glycoproteins, HA and neuraminidase (NA).

As far as we know, there have been no successful reports of making target cells sensitive to CTL specific for viral proteins without using liposomes. In this regard, it was somewhat unexpected to observe killing of c13 protein–coated P815 cells. The HA2 portion of c13 protein has a long hydrophobic tail on its carboxy-terminal end (11); therefore, it is conceivable that this hydrophobic end can be embedded into the plasma membrane, although we do not know the precise mechanism of the interaction of c13 protein with the cellular membrane. The observation (12) that the tryptic peptide of hen's egg lysozyme, which has a hydrophobic portion, could be presented by fixed macrophages to T cell clones seems to support this concept.

The observation that C3H (H-2^k) mouse effector cells with the ability to lyse PR8 virus–infected BW5147 (H-2^k) cells did not lyse c13 protein–coated P815 (H-2^d) cells suggests that lysis of c13 protein–coated target cells was H-2 restricted. The association of c13 protein with an H-2 molecule was also suggested by a blocking experiment using mAb against H-2K^d and -D^d molecules. On the other hand, it is of interest that c13 protein–coated BW5147 cells were resistant to CTL derived from both H-2-compatible and -incompatible mice. Since c13 protein could be detected on the surface of BW5147 cells by immunofluorescent study using specific antibody to c13 protein (our unpublished observation), it is conceivable that influenza virus–specific, H-2^k-restricted CTL cannot recognize c13 protein in conjunction with H-2^k molecules. Selected use of certain H-2 molecules as restriction elements by CTL has been reported (13).

In conclusion, we have demonstrated that a particular protein produced in E. coli renders target cells susceptible to influenza virus H1 subtype–specific CTL. These approaches should provide us with more details concerning the antigenic specificity of virus–specific CTL and the interaction between viral antigen(s) and MHC product(s).

Summary

We have tested the ability of the c13 protein, which is a hybrid protein of the first 81 amino acids of the viral nonstructural protein (NS1) and the HA2 subunit of viral hemagglutination produced in E. coli, to render target cells susceptible to the lytic activity of influenza virus–specific cytotoxic T lymphocytes (CTL). The results showed that P815 cells coated with c13 protein were lysed by PR8

virus–induced secondary CTL derived from BALB/c mice. Cold-target inhibition tests clearly demonstrated that c13 protein–coated P815 cells were recognized by an H1 subtype–specific CTL population. Furthermore, PR8 virus–induced CTL derived from C3H mice did not lyse c13 protein–coated P815 cells, suggesting that c13 protein was recognized by CTL in conjunction with H-2^d products. These findings suggest that this protein interacts with the cellular plasma membrane and makes target cells recognizable by H-2-restricted, influenza virus subtype–specific CTL.

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