SPECIFICITY OF CYTOTOXICITY T CELLS DIRECTED TO INFLUENZA VIRUS HEMAGGLUTININ

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Because the initial realization that T cells comprised a discrete lymphocyte class, there has been a great deal of experimental work directed toward an analysis of the nature of the antigen receptor on T cells and, concomitantly, toward an assessment of the comparative specificity of T and B cells. Perhaps a major advance in understanding the molecular nature of the antigen receptor on T cells has come from studies demonstrating the expression, on the surface of specific T cells, of idiotypic determinants (immunoglobulin heavy chain variable region gene products [V_H])[1] which are shared with the corresponding antibody (1–3). Although from such observations it might be anticipated that T cells and antibody would have comparable degrees of specificity for antigen, analyses of T-cell specificity have not necessarily fulfilled this expectation (reviewed in reference 4).

A major limitation in many analyses of T-cell specificity has been the need to rely on ancillary T-cell activities, i.e., the capacity of T cells to help or suppress humoral responses or exhibit delayed-type hypersensitivity manifestations in order to assess T-cell recognition of antigen. This limitation would appear to be less severe in the case of cytotoxic T lymphocyte (CTL) responses as antigen recognition by CTLs is directly measured by their capacity to lyse target cells expressing the appropriate antigen (5). Thus an analysis of the fine specificity of CTL recognition could provide useful information on the comparative specificities of T and B cells.

Studies on the CTL response to conventional (i.e., non major histocompatibility complex [MHC] encoded) antigen have already yielded some information with regard to CTL specificity. For example, virus-specific CTLs readily distinguish target cells infected with unrelated viruses (6). Furthermore, more recent evidence along these lines suggests that distinct viral antigens are specifically recognized by CTLs (7–11). Perhaps more detailed information on CTL specificity has come from the analysis of the CTL response to hapten-modified cells (12–15). Overall, these studies have demonstrated a high degree of specificity in CTL recognition of hapten-modified cell surfaces; however, it is not clear from these studies whether hapten per se is exclusively

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[1] *Abbreviations used in this paper*: CTL, cytotoxic T cells; EID_{50}, egg infectious dose yielding 50% positive response; H, influenza virus hemagglutinin antigen; HAU, hemagglutination units of virus; MHC, major histocompatibility complex; N, influenza virus neuraminidase antigen; PBS, phosphate-buffered saline; V_H, immunoglobulin heavy chain variable region.

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recognized (12, 15) or whether the structures to which the hapten is coupled influence the specificity of CTL recognition (13, 14).

An alternative approach to an analysis of CTL specificity has come from the recent observation of Zweerink et al. (10) that purified influenza H stimulates a cytotoxic cell response from primed precursors that is directed to target cells expressing the immunizing hemagglutinin. Because influenza virus strains that possess hemagglutinins with varying degrees of serologic cross-reactivity are available, the comparative specificities of CTLs directed to influenza H and anti-hemagglutinin antibody can be assessed. In the present report, we have examined the specificity of the CTL response of primed mouse spleen cell precursors after in vitro stimulation with purified type A influenza hemagglutinin. This analysis demonstrates that influenza hemagglutinin stimulates an H-2-restricted CTL response which is highly specific for the immunizing hemagglutinin. These CTLs readily distinguish target cells expressing serologically unrelated hemagglutinins from those expressing hemagglutinin serologically related to the stimulated antigen. Analysis of the fine specificity of CTL recognition by using hemagglutinins with varying degrees of serologic cross-reactivity revealed, however, a hierarchy of cross-reactivity that was the converse of the serologic pattern. Implications of these findings with respect to the specificity repertoire of T and B cells and the requirements for CTL induction are discussed.

Materials and Methods

General. Male BALB/c (*H-2D*) and CBA/H (*H-2K*) mice were bred at the John Curtin School and used at 8–12 wk of age. P815 (*H-2D*) mastocytoma and L929 (*H-2K*) fibroblast cell lines, maintained in tissue culture, served as target cells in cytotoxicity assays (16). Eagle's minimal essential medium (No. 410–1500, Grand Island Biological Co., Grand Island, N. Y.) supplemented with antibiotics and 10% heat-inactivated fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia) was used as medium in all cytotoxicity assays.

Viruses. Influenza virus strains A/WSN [A/WSN(H_0N_1)], A/JAP/57 [A/JAPAN/305/57 (H_2N_2)], A/JAP/62 [A/JAPAN/170/62 (H_2N_2)], A/AA/67 [A/Ann Arbor/7/67 (H_2N_2)], A/JAP/BEL [A/JAP/305/57 × A/BEL/42 (H_2N_1)], A/X-7F_1 [A/NWS/43 × A/RI/5/57 (H_0N_2)] and B/LEE were grown in the allantoic cavity of 10-d-old embryonated eggs and stored as infectious allantoic fluid as described previously (16). Virus strain A/JAP/BEL is a recombinant strain that possess the hemagglutinin of the prototype A/JAP/57 virus (H_2) and the neuraminidase of A/BEL/42 (N_1) which is unrelated serologically to the A/JAP/57 neuraminidase (N_2). Similarly, A/X-7F_1 is a recombinant strain with the prototype neuraminidase (N_2) and a serologically unrelated hemagglutinin (H_0).

Hemagglutinin Preparation. Purified A/JAP/57 hemagglutinin was prepared from the recombinant virus A/JAP/BEL. The virus was purified from infectious allantoic fluid by adsorption-elution from fowl erythrocytes followed by velocity sedimentation over a preformed linear sucrose gradient (17). The virus band was diluted, pelleted, and resuspended in phosphate-buffered saline (PBS) to a final concentration of approximately 10^6 hemagglutinating units (HAU)/ml. Viral hemagglutinin was prepared from the purified virus preparation by disruption of the purified virus with 10% (wt/vol) sodium dodecyl sulfate at 20°C followed by cellulose acetate strip electrophoresis according to the method of Laver (18). Viral hemagglutinin, eluted from the strips, was freed of detergent by precipitation with cold (−20°C) ethanol. The precipitated hemagglutinin was resuspended in cold (4°C) PBS and freed of residual detergent and ethanol by dialysis against PBS. Viral hemagglutinin prepared in this fashion, when examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate under reducing conditions, was resolved into two bands corresponding to the 45,000 and 25,000 dalton polypeptide subunits of the hemagglutinin (19). Hemagglutinin protein concentration was determined by the method of Lowry (20) with bovine serum albumin standards. Purified

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hemagglutinin in PBS was stored at 4°C in the presence of penicillin (200 U/ml) and streptomycin (200 μg/ml).

Immunization. Mice were inoculated by the intravenous route. Donors of primed cytotoxic cell precursors were inoculated with 100–200 HAU (1.5–3.0 × 10^7 EID_{50} U [egg infectious dose yielding 50% positive response]) of infectious virus. Spleen cells from these donors were used as responder cells for in vitro responses 3–8 wk after immunization. Immune sera for hemagglutination inhibition assays were pooled from four BALB/c donors. These mice had received two intravenous inoculations of 100 HAU of infectious virus at monthly intervals. Immune serum was collected 7 d after the second inoculation. Hyperimmune rabbit antiserum directed to the A/JAP/305/57 hemagglutinin (H_2) (a generous gift of Dr. W. G. Laver, Australian National University) was prepared as described (21).

In Vitro Secondary Responses. CTLs were generated in vitro essentially as described (16, 22). Usually, 40 × 10^6 spleen cells from previously primed donors were cultured with stimulator cells in 25 cm^2 Falcon tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) containing 15 ml of medium (22). The responder to stimulator cell ratio was 10:1. In some experiments, 100 × 10^6 responder spleen cells were cultured under the above conditions with 75 cm^2 Falcon flasks and 40 ml of culture medium. Stimulator cells consisted of normal syngeneic spleen cells. These cells were either infected with 5 U EID_{50} of infectious virus per nucleated cell or incubated with purified viral hemagglutinin. In preliminary experiments, stimulator cells were incubated with viral hemagglutinin at 37°C for 30 min and either washed extensively before culturing with responder cells or cultured directly. Under both conditions CTL responses could be stimulated. However, uniformly higher cytotoxic activity was achieved when unbound hemagglutinin was not removed by washing of the stimulator cells. In all experiments reported here, unwashed stimulator cells were used. Viable cells were tested for cytotoxic activity after 5 d of culture. Recovery of viable cells was 40–50% when infected stimulators were used and 10–30% when stimulation was carried out with hemagglutinin-treated stimulator cells.

Assay for Cell-Mediated Cytotoxicity. The ^{51}Cr release cytotoxicity assay was carried out as described (16). Briefly, ^{51}Cr-labeled P815 or L929 cells were infected in suspension with 10 U EID_{50} of infectious virus per cell and incubated at 37°C for 30 min. After extensive washing to remove unbound virus, 2 × 10^4 target cells in 0.1-ml vol of medium were added to individual wells of 96-well, flat-bottomed microtiter culture plates. Various numbers of effector cells in 0.1-ml vol were added to the wells, and the plates incubated at 37°C for 6.5 h as described (16). Spontaneous ^{51}Cr release from target cells incubated with medium only usually ranged from 10 to 15% and was always <20%. Percent specific ^{51}Cr release was obtained from the formula:

$$\frac{\text{test counts} - \text{spontaneous release}}{\text{water lysis counts} - \text{spontaneous release}} \times 100.$$

All values represent the mean percent specific ^{51}Cr release of four replicate wells.

Inhibition of Cytotoxicity by Unlabeled Targets. Cytotoxicity assays with unlabeled infected competitor cells (cold targets) were carried out as described (16). Competitor cells were mixed with cytotoxic effectors, and the appropriate effector-competitor mixtures in 0.1-ml vol of medium were added to wells containing 2 × 10^4 ^{51}Cr-labeled target cells in 0.1 ml of medium. The competitor cells were infected with the indicated influenza strain 4–5 h before infection of ^{51}Cr-labeled targets and maintained at 37°C for that period to ensure viral antigen expression.

Assay for Serologic Cross-Reactivity among Virus Strains. Viruses were assessed for serologic cross-reactivity by the microtitration hemagglutination-inhibition test (22). Four HAU of a given virus in a volume of 0.025 ml PBS were added to serial twofold dilutions of each sera in a final volume of 0.025 ml in PBS. After 35 min of incubation at room temperature, 0.025 ml of a 1% (vol/vol) suspension of fowl erythrocytes was added to each well. After 30 min of incubation at room temperature, the hemagglutination-inhibition end point was determined. All sera were treated with *Vibrio cholera* receptor-destroying enzyme (Center for Disease Control, Atlanta, Ga.) and heated to 56°C for 30 min before testing.

Results

Characteristics of the In Vitro Secondary CTL Response to Influenza Hemagglutinin. Stimulator cells infected with influenza A/JAP/57 virus or treated with purified

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TABLE I

*Induction of an In Vitro Secondary Cell-Mediated Cytotoxic Response by Influenza Hemagglutinin**

In vitro stimulation	Effector to target cell ratio‡	Percent specific ^{51}Cr release from target cells§			
		Uninfected	A/WSN (H_0N_1)	A/JAP/57 (H_2N_2)	B/LEE
A/JAP/57	1:1	0.4	4.5 ± 0.2	27.7 ± 1.3	1.1 ± 0.1
hemagglutinin	2.5:1	0.6	13.0 ± 0.3	58.6 ± 1.4	2.4 ± 0.1
A/JAP/57-	1:1	1.7 ± 0.1	58.1 ± 1.2	71.3 ± 0.8	6.0 ± 0.4
infected cells	2.5:1	4.7 ± 0.2	81.7 ± 2.1	92.2 ± 1.5	6.4 ± 0.4

* BALB/c mice were inoculated intravenously with 1.5×10^7 U EID_{50} of infectious A/JAP/57 virus. 3 or more wk later spleen cells from pools of three donor spleens were cultured in vitro either with 15 μg A/JAP/57 hemagglutinin or with A/JAP/57-infected stimulator spleen cells as described (Materials and Methods). After 5 d of culture, the cytotoxic cell activity of the respective responder cell populations was examined.

‡ 2×10^4 ^{51}Cr-labeled, infected (or uninfected) P815 target cells/well.

§ Values are the means ± SE of the mean from four replicate wells with spontaneous release subtracted.

TABLE II

*Specificity of the Cell-Mediated Cytotoxic Response to Influenza Hemagglutinin**

In vitro stimulation	Effector to target cell ratio‡	Percent specific ^{51}Cr release from target cells§					
		Uninfected	A/WSN (H_0N_1)	A/JAP/57 (H_2N_2)	A/JAP/BEL (H_2N_1)	A/X-7F_1 (H_0N_2)	B/LEE
A/JAP/57	1:1	0.6	2.0 ± 0.1	13.4 ± 0.3	11.0 ± 0.4	1.7 ± 0.1	0.2
hemagglutinin	2.5:1	2.4 ± 0.1	7.6 ± 0.7	34.2 ± 1.7	30.0 ± 0.9	5.2 ± 0.3	2.9 ± 0.1
	5:1	5.5 ± 0.3	17.6 ± 1.9	53.1 ± 1.1	49.0 ± 2.3	13.6 ± 0.5	7.9 ± 0.4
A/JAP/57-	1:1	3.8 ± 0.2	62.5 ± 1.9	66.9 ± 1.2	62.3 ± 1.5	52.3 ± 0.4	4.6 ± 0.2
infected cells	2.5:1	9.3 ± 0.2	90.7 ± 1.5	88.7 ± 2.3	78.6 ± 1.9	72.8 ± 1.9	12.8 ± 0.4

* As in Table I.

‡ As in Table I.

§ As in Table I.

influenza A/JAP/57 hemagglutinin induced a CTL response in vitro from spleen cells previously primed to A/JAP/57 virus (Table I). As has been demonstrated (16, 23, 24), the CTL response to stimulator cells infected with a given type A influenza virus is characterized by a high degree of cross-reactivity for target cells infected with type A virus of any subtype. This response is mediated by a discrete subpopulation of cross-reactive CTLs (16, 24). In contrast, the cytotoxic cell response to purified hemagglutinin was directed almost exclusively to target cells infected with A/JAP/57 virus. There was also a low degree of cytotoxic activity for A/WSN-infected target cells that was above the background activity observed on B/LEE-infected targets. The possible significance of the "cross-reactivity" is discussed below (see Discussion).

The above results suggested that the bulk of the cell-mediated cytotoxic activity stimulated by purified A/JAP/57 hemagglutinin was specifically directed to the viral hemagglutinin expressed on the infected target cell surface. This point was further substantiated in experiments with target cells infected with recombinant virus strains (Table II). Cytotoxic cells generated in response to A/JAP/57 hemagglutinin efficiently lysed target cells infected with either A/JAP/57 or A/JAP/BEL virus. The latter virus is a recombinant virus that possesses the A/JAP/57 hemagglutinin and a serologically unrelated neuraminidase. In contrast, targets infected with A/X-7F_1 virus (which possesses the neuraminidase of the A/JAP/57 virus and a serologically unrelated hemagglutinin) were lysed to a minimal extent by these effector cells. The magnitude of the lysis on A/X-7F_1-infected targets was comparable to that observed

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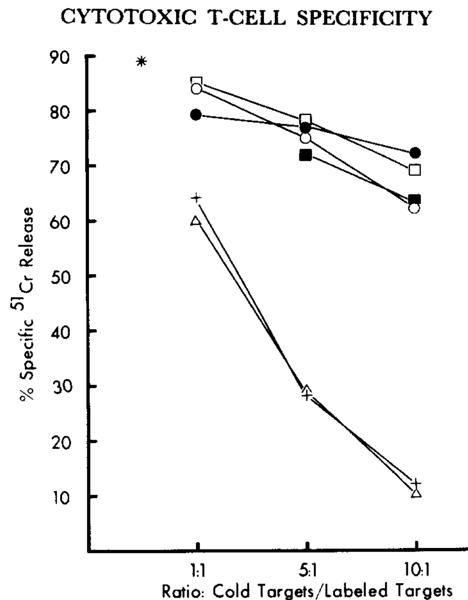

FIG. 1. Inhibition of cell-mediated cytotoxicity by unlabeled "cold" competitors. Spleen cells from BALB/c mice previously immunized with A/JAP/57 virus were stimulated in vitro with 15 μg of A/JAP/57 hemagglutinin as described (Materials and Methods). After 5 d in culture, the cytotoxic activity of the spleen cells was examined on ^{51}Cr-labeled, A/JAP/57-infected P815 target cells in the presence of increasing numbers of unlabeled cold competitors. The effector to target cell ratio is 10:1. The ratio of unlabeled competitors to labeled targets is as indicated. Unlabeled competitors are P815 cells infected with influenza A/WSN (□), A/JAP/57 (△), A/JAP/BEL (+), A/X-7 F_1 (■), B/LEE (●), or uninfected (○). Values are the means of four replicate wells. SE >3% in all cases are omitted. Asterisk (*) indicates percent specific ^{51}Cr release in the absence of competitor cells.

on target cells infected with A/WSN virus (whose hemagglutinin and neuraminidase is serologically unrelated to those of A/JAP/57). Target cells infected with any of the type A influenza viruses were readily susceptible to lysis by CTLs generated in response to A/JAP/57-infected stimulator cells.

Results similar to those observed in Table II were obtained when the specificity of cytotoxic cell recognition was examined by "cold target" competition (Fig. 1). Lysis of ^{51}Cr-labeled A/JAP/57-infected target cells by cytotoxic cells generated in response to A/JAP/57 hemagglutinin was inhibited to a similar extent either by unlabeled A/JAP/57-infected or A/JAP/BEL-infected target cells. On the other hand, the degree of lysis inhibition observed with A/X-7F_1- or A/WSN-infected cold targets was comparable to that obtained with B/LEE or uninfected cold targets. Thus, only target cells that expressed the A/JAP/57 hemagglutinin efficiently acted as competitors.

The magnitude of the cell-mediated cytotoxic response to viral hemagglutinin was directly proportional to the stimulating dose of hemagglutinin (Table III). Furthermore, the lytic activity of the cytotoxic cells so generated was restricted to infected target cells that were compatible at the *H-2* locus with the effector cells (Table IV). Thus the cytotoxic cell response to purified influenza hemagglutinin exhibits the typical pattern of *H-2* restriction observed in a variety of cytotoxic T-cell responses.

Recognition of Serologically Cross-Reactive Hemagglutinins. The above results indicated that influenza A/JAP/57 hemagglutinin stimulated an in vitro CTL response directed

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TABLE III
Antigen Dose Dependence of the Cell-Mediated Cytotoxic Response to Influenza Hemagglutinin

Hemagglutinin concentration*	Effector to target cell ratio‡	Percent specific ^{51}Cr release from target cells§		
		Uninfected	A/JAP/57	B/LEE
μg				
0.15	1:1	0	1.2 ± 0.1	1.7 ± 0.1
	2.5:1	1.0 ± 0.1	3.2 ± 0.1	2.5 ± 0.1
	5:1	1.2 ± 0.1	7.5 ± 0.2	5.0 ± 0.1
1.5	1:1	0	2.2 ± 0.1	0.8
	2.5:1	0.8	7.9 ± 0.1	1.8 ± 0.1
	5:1	1.7 ± 0.1	17.5 ± 0.5	3.6 ± 0.1
15	1:1	0	7.5 ± 0.1	0.9
	2.5:1	0.1	19.7 ± 0.5	1.9 ± 0.1
	5:1	1.6 ± 0.1	33.1 ± 1.3	3.9 ± 0.1

* Spleen cells from BALB/c mice previously immunized with A/JAP/57 virus were stimulated in vitro with indicated dose of A/JAP/57 hemagglutinin and tested for cytotoxicity 5 d later as described (Materials and Methods).
‡ As in Table I.
§ As in Table I.

TABLE IV
*H-2 Restriction of the Cell-Mediated Cytotoxic Response to Influenza Hemagglutinin**

Immune cell donor (mouse strain)	In vitro stimulation	Percent specific ^{51}Cr release from target cells‡§			
		P815 (*H-2D*)		L929 (*H-2K*)	
		Uninfected	A/JAP/57	Uninfected	A/JAP/57
BALB/c (*H-2D*)	A/JAP/57 hemagglutinin	4.4 ± 0.1	51.1 ± 0.5	3.3 ± 0.1	4.6 ± 0.1
BALB/c (*H-2D*)	A/JAP/57-infected cells	14.9 ± 0.3	86.3 ± 1.1	8.5 ± 0.1	11.5 ± 0.2
CBA/H (*H-2K*)	A/JAP/57-infected cells	—‖	—	6.3 ± 0.1	60.1 ± 1.4

* Spleen cells from BALB/c or CBA/H donor mice, previously immunized with influenza A/JAP/57, were stimulated in vitro either with syngeneic-infected stimulator cells or in the case of BALB/c responder cells with 15 μg of A/JAP/57 hemagglutinin. Cytotoxic activity was examined after 5 d of culture.
‡ The effector to target cell ratio was 5:1. 2 × 10^4 ^{51}Cr-labeled P815 or L929 target cells were added per test well.
§ As in Table I.
‖ Not tested.

preferentially to target cells bearing the A/JAP/57 hemagglutinin. Target cells expressing serologically unrelated hemagglutinins, i.e., A/WSN- and A/X-7F_1-infected cells, were only minimally susceptible to lysis by these cytotoxic T cells and were inefficient as cold competitors. Given the apparent specificity of these effector cells for the A/JAP/57 hemagglutinin, it was of interest to examine their capacity to lyse target cells expressing hemagglutinins that were serologically related to the A/JAP/57 hemagglutinin. Two type A influenza virus strains of the H_2N_2 subtype—A/JAP/62 and A/AA/67—were chosen for this analysis. The former strain (A/JAP/62) exhibits a high degree of cross-reactivity with antibody directed to the A/JAP/57 hemagglutinin (25), whereas the latter strain (A/AA/67) demonstrates only a weak reaction with antibody directed to the A/JAP/57 hemagglutinin (25). The cytotoxic activity of A/JAP/57 hemagglutinin-specific CTLs for target cells infected with

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TABLE V

*Reactivity of Hemagglutinin-Specific Cytotoxic Cells for Serologically-Related Hemagglutinins**

In vitro stimulation	Effector to target cell ratio‡	Percent specific ^{51}Cr release from target cells§				
		Uninfected	A/JAP/57 (H_2N_2)	A/JAP/62 (H_2N_2)	A/AA/67 (H_2N_2)	B/LEE
A/JAP/57	1:1	0	21.1 ± 0.8	6.5 ± 0.2	9.8 ± 0.8	2.7 ± 0.1
hemagglutinin	2.5:1	0	42.9 ± 1.0	14.2 ± 0.9	13.8 ± 1.2	3.3 ± 0.1
	5:1	0	66.2 ± 3.1	21.1 ± 1.0	35.4 ± 3.3	4.9 ± 0.2
	10:1	0	89.0 ± 1.4	34.2 ± 1.9	56.3 ± 2.2	11.6 ± 0.3
A/JAP/57-infected cells	1:1	0	81.4 ± 0.5	73.4 ± 4.0	69.4 ± 4.1	3.3 ± 0.1
	2.5:1	4.1 ± 0.1	94.5 ± 1.7	95.1 ± 3.2	99.3 ± 3.6	9.7 ± 0.5

* As in Table I.
‡ As in Table I.
§ As in Table I.

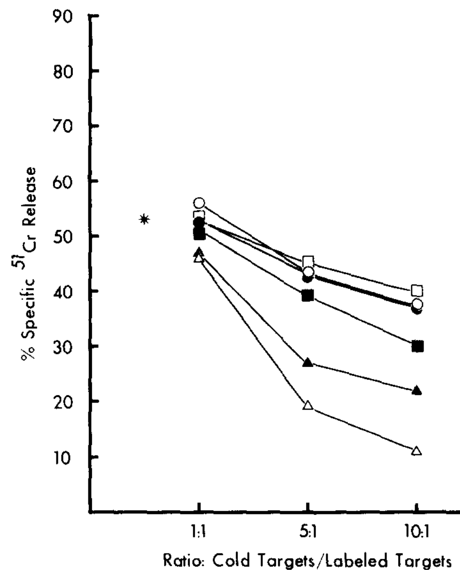

FIG. 2. Inhibition of cell-mediated cytotoxicity by unlabeled "cold" competitors expressing serologically cross-reactive hemagglutinins. The effector to target cell ratio is 5:1. Unlabeled competitors are P815 cells infected with influenza A/WSN (□), A/JAP/57 (△), A/JAP/62 (■), A/AA/67 (▲), B/LEE (●), or uninfected (○). Other information as in Fig. 1.

homologous (A/JAP/57) virus and the two serologically cross-reactive viruses is shown in Table V. Target cells infected with A/JAP/57 virus were most efficiently lysed by effector cells directed to the A/JAP/57 hemagglutinin. On the other hand, at most of the effector to target ratios tested, target cells expressing the weakly cross-reactive A/AA/67 hemagglutinin were more efficiently lysed than target cells expressing the highly cross-reactive A/JAP/62 hemagglutinin. Indeed, a similar hierarchy was obtained by cold-target competition analysis (Fig. 2). Lysis of ^{51}Cr-labeled A/JAP/57-infected target cells by A/JAP/57 hemagglutinin-specific CTLs was most efficiently inhibited by A/JAP/57 cold targets. A/AA/67 cold targets were less efficient inhibitors than A/JAP/57 cold targets but were more efficient than A/JAP/62 cold targets. Cold targets expressing the serologically unrelated A/WSN hemagglutinin-inhibited lysis to the same extent as uninfected and B/LEE-infected cold targets.

Inasmuch as these results indicated a possible disparity in specificity between humoral and the effector activity of CTLs, it was of interest to test the specificity of

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TABLE VI

*Stimulation by A/JAP/57 Hemagglutinin of an In Vitro Cytotoxic Cell Response from Precursor Cells Primed to Serologically-Related Hemagglutinins**

Primed precursor source	In vitro stimulation	Effector to target cell ratio‡	Percent specific ^{51}Cr release from target cells§				
			Uninfected	A/JAP/57 (H_2N_2)	A/JAP/62 (H_2N_2)	A/AA/67 (H_2N_2)	B/LEE
A/JAP/62-immune	A/JAP/57 hemagglutinin	1:1	0.7	6.1 ± 0.4	5.5 ± 0.2	5.6 ± 0.2	1.1 ± 0.1
		2.5:1	0.9	15.6 ± 0.4	17.2 ± 0.6	13.7 ± 0.5	2.6 ± 0.1
		5:1	4.2 ± 0.2	28.2 ± 1.0	34.7 ± 1.2	26.5 ± 0.3	6.5 ± 0.3
A/AA/67-immune	A/JAP/57 hemagglutinin	1:1	0.3	46.9 ± 1.4	17.2 ± 0.8	40.5 ± 2.0	1.0 ± 0.1
		2.5:1	1.9 ± 0.1	68.4 ± 1.7	43.7 ± 1.2	67.4 ± 2.6	5.9 ± 0.1
		5:1	6.2 ± 0.4	83.5 ± 0.5	64.8 ± 1.1	81.3 ± 2.1	10.3 ± 0.5
B/LEE-immune	A/JAP/57 hemagglutinin	1:1	1.7 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	3.8 ± 0.1	2.3 ± 0.1
		2.5:1	3.1 ± 0.1	5.4 ± 0.2	6.0 ± 0.2	6.6 ± 0.5	4.2 ± 0.1
		5:1	7.7 ± 0.3	16.7 ± 0.5	13.3 ± 0.3	13.7 ± 0.7	12.0 ± 0.7
A/JAP/62-immune	A/JAP/57-infected cells	1:1	1.1 ± 0.1	83.4 ± 2.2	79.7 ± 3.3	84.7 ± 1.5	4.7 ± 0.2
		2.5:1	5.3 ± 0.3	94.6 ± 1.6	90.8 ± 1.3	93.6 ± 1.3	9.3 ± 0.5
		5:1	9.7 ± 0.1	98.9 ± 1.5	92.3 ± 0.7	93.4 ± 1.3	13.7 ± 0.3
A/AA/67-immune	A/JAP/57-infected cells	1:1	1.4 ± 0.1	46.7 ± 2.3	49.5 ± 0.9	58.6 ± 2.5	2.5 ± 0.1
		2.5:1	2.5 ± 0.1	81.3 ± 1.2	83.9 ± 1.1	81.1 ± 1.9	4.2 ± 0.1
		5:1	7.2 ± 0.3	94.4 ± 2.4	88.9 ± 1.3	89.4 ± 2.5	8.7 ± 0.4
B/LEE-immune	B/LEE-infected cells	1:1	2.6 ± 0.1	3.8 ± 0.4	3.4 ± 0.1	1.9 ± 0.1	56.1 ± 2.5
		2.5:1	6.5 ± 0.2	9.6 ± 0.3	8.0 ± 0.1	5.9 ± 0.2	73.5 ± 4.2

* BALB/c mice were inoculated intravenously with 1.5–3.0 × 10^7 U EID_{50} of infectious A/JAP/62, A/Ann Arbor/67, or B/LEE virus. 3 or more wk later spleen cells from pools of three donor spleens were cultured either with 15 μg of A/JAP/57 hemagglutinin or with stimulator cells infected with A/JAP/57 or B/LEE virus. After 5 d of culture, the cytotoxic activity of the respective responder populations was examined.

‡ As in Table I.

§ As in Table I.

hemagglutinin recognition at the level of CTL induction. It was not possible to examine the stimulation of A/JAP/57-primed precursors by purified A/JAP/62 and A/AA/67 hemagglutinin as the appropriate recombinant virus strains needed for hemagglutinin isolation were not available. It was, however, possible to test the capacity of A/JAP/57 hemagglutinin to stimulate a response from CTL precursors primed to A/JAP/62 and A/AA/67 viruses (Table VI). A/JAP/57 hemagglutinin could stimulate a detectable CTL response from both responder populations. However, the magnitude of the response from A/AA/67-primed spleen cells was greater than that of A/JAP/62-primed spleen cells. Furthermore, as both precursor populations could be efficiently stimulated by A/JAP/57-infected stimulator cells, the difference in the magnitudes of the responses to purified hemagglutinin appeared not to be attributable to a difference in the efficacy of primary immunization. It is also worthwhile to note that precursors primed to B/LEE virus were not stimulated by A/JAP/57 hemagglutinin but did specifically respond to B/LEE-infected stimulator cells.

Serologic Cross-Reactivity among Type A Influenza Viruses of the H_2N_2 Subtype. The antigenic hierarchy among the three serologically cross-reactive hemagglutinins examined herein was established with chicken antisera (25). In light of the above results on cross-reactivity among these hemagglutinins at the level of CTLs, it was necessary to establish the serologic relationship of these hemagglutinins in the mouse. This analysis is shown in Table VII. Consistent with previous observations (25), murine antiserum to A/JAP/57 virus exhibits a high degree of cross-reactivity for A/JAP/62 virus in the hemagglutination-inhibition test. Similarly, rabbit antibody directed to purified A/JAP/57 hemagglutinin showed even greater reactivity with A/JAP/62 virus than with homologous virus. Both antisera demonstrated minimal reactivity

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TABLE VII

Serologic Cross-Reactivity between Type A Influenza Viruses of the H_2N_2 Subtype in the Hemagglutinin-Inhibition Test

Antiserum*	Hemagglutination-inhibition titer virus‡		
	A/JAP/57	A/JAP/62	A/AA/67
A/JAP/57 hemagglutinin (rabbit)	5,120§	8,610	160
A/JAP/57‖	1,280	760	<10
A/JAP/62‖	20	1,810	20
A/AA/67‖	<10	226	1,280

* Hyperimmune rabbit antiserum to the purified hemagglutinin of influenza A/JAP/305/57 was prepared as described (Materials and Methods). The remaining three antisera were produced in BALB/c mice after secondary immunization with intact influenza virus of the indicated strain.

‡ Titer of hemagglutination-inhibiting antibody as measured in the microtitration hemagglutination-inhibition test.

§ Values are the reciprocals of the average of the highest serum dilution, giving complete inhibition of hemagglutination from duplicate samples.

‖ Mouse antisera were pooled from four immune donors.

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with A/AA/67 virus. Murine antibody to the A/AA/67 virus showed the converse pattern of reactivity with little cross-reactivity demonstrable for A/JAP/57 virus. Thus the overall pattern of cross-reactivity with mouse antibodies was comparable to published patterns. A quite different hemagglutination-inhibition pattern was observed with antiserum to A/JAP/62 virus. In this case, the antibody reacted almost exclusively with A/JAP/62 virus. This pattern of "asymmetric cross-reactivity" is not totally unexpected because type A influenza hemagglutinins possess specific determinants as well as common determinants which are shared by hemagglutinins of the same subtype (26, 27). The possible relevance of this observation to the issue of CTL specificity is discussed below.

Discussion

The results presented here demonstrate that purified influenza hemagglutinin stimulates in vitro an H-2-restricted CTL response from primed precursors which is highly specific for the stimulating hemagglutinin. The specificity of this response is comparable to the specificity of the virus strain-specific subpopulation of CTLs generated in response to infectious influenza virus in vivo (16, 23) or to virus-infected stimulator cells in vitro (16). The present findings are therefore consistent with the concept that influenza hemagglutinin glycoprotein is a target antigen for the strain-specific CTL subpopulation stimulated by infectious influenza virus. Also consistent with this concept is our previous observation that nascent viral glycoprotein synthesis is required to render target cells susceptible to lysis by strain-specific CTLs (11). It is also evident from the present results showing that the high degree of cross-reactivity for target cells infected with type A influenza virus of unrelated subtypes, which is generated in response to infectious influenza virus (16, 23, 24) and is likewise mediated by a discrete subpopulation of cross-reactive CTLs (10, 16, 23) cannot be readily explained on the basis of reactivity to the influenza hemagglutinin. Although CTLs directed to the influenza N have not as yet been directly demonstrated, it is reasonable to assume that such CTLs would have specificity characteristics comparable to those reported here for CTLs directed to influenza H. Thus, at present, the most likely

candidate for the target antigen of the cross-reactive cytotoxic subpopulation is the internal virion antigen influenza matrix protein which is serologically cross-reactive among all type A influenza viruses and expressed on the surface of influenza-infected cells (11, 28, 29).

In undertaking this analysis, the primary objective was to determine whether CTL directed to influenza hemagglutinin exhibited a degree of specificity comparable to that of the corresponding antihemagglutinin antibody. Cytotoxic T cells directed to the prototype hemagglutinin readily distinguished target cells expressing serologically unrelated hemagglutinins (Tables I and II; Fig. 1). Similarly, these CTLs specifically recognized target cells expressing serologically cross-reactive hemagglutinins (Table V; Fig. 2). On one hand then, CTL response to influenza hemagglutinin was indeed as specific as the humoral response. On the other hand, when the fine specificity of CTL recognition was examined with target cells expressing serologically cross-reactive hemagglutinins, the hierarchy of cross-reactivity was the converse of the serologic hierarchy (Table V; Fig. 2), i.e., target cells expressing the serologically poorly cross-reactive hemagglutinin were more susceptible to lysis (and more efficient cold competitors) than cells expressing the highly cross-reactive hemagglutinin.

Although these two sets of observations are in apparent disagreement and pose a potential paradox with regard to the issue of CTL specificity, they should be considered in light of two considerations. First, at least two sets of antigenic determinants are demonstrable on the type A influenza hemagglutinin molecule—specific determinants which are unique to a particular hemagglutinin and common determinants which are shared to varying degrees with hemagglutinins of the same subtype (26, 27). That such sets of determinants are distinguishable is illustrated here (Table VII) where the antihemagglutinin response of BALB/c mice to A/JAP/62 virus is directed almost exclusively to that virus in spite of the high degree of cross-reactivity between this virus and the prototype A/JAP/57 virus demonstrated by other antisera. Second, analysis of the monoclonal humoral response to influenza virus has revealed the presence of a large variety of B-cell clones whose homogeneous antibody products exhibit varying degrees of serologic cross-reactivity with a series of cross-reactive hemagglutinins (30, 31).

Based on these considerations, the apparent difference in fine specificity between CTLs and antihemagglutinin antibody could solely reflect a quantitative difference in the frequency of particular precursors. Thus, within the subset of CTL precursors directed to "common" (shared) determinants on the A/JAP/57 hemagglutinin, those that cross-react with the serologically distantly related A/AA/67 hemagglutinin could be present in a higher frequency than precursors directed to determinants shared with the highly cross-reactive A/JAP/62 hemagglutinin. Alternatively, the repertoire of CTL specificities directed to a given hemagglutinin could be restricted as compared with the B-cell repertoire. In the present instance, our failure to detect a high degree of cross-reactivity between A/JAP/57 and A/JAP/62 hemagglutinin at the level of the CTL response would represent a relative lack of precursors (specificities) directed to determinants shared by these two hemagglutinins. Finally, as the data presented here on asymmetric serologic cross-reactivity imply, it is possible that those determinants on the A/JAP/57 hemagglutinin that are shared with A/AA/67 hemagglutinin readily stimulate a response from the appropriate CTL precursors, whereas the determinants shared with the A/JAP/62 hemagglutinin are poorly stimulatory. These

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alternative explanations may be resolved by an analysis of the CTL response to influenza hemagglutinin at the level of individual CTL precursors.

The fact that purified influenza hemagglutinin stimulates an *H-2* restricted CTL response raises a variety of questions regarding the requirements for CTL induction. Perhaps the most immediate question involves antigen presentation during CTL induction. There is now a good deal of evidence that is consistent with the concept that the target antigen of *H-2*-restricted CTLs must be displayed on the target cell as an integral cytoplasmic membrane component in order to render the target cell susceptible to lysis (15, 22, 32–34). If such a proviso holds as well at the level of CTL induction, then exogenous hemagglutinin must be integrated into the cytoplasmic membrane of the relevant stimulator cell for induction to occur. Alternatively, hemagglutinin either adsorbed to the stimulator cell surface or free in solution could interact directly with the relevant recognition structure on the CTL precursor and stimulate a response; however, as demonstrated herein, the response is still *H-2* restricted. Resolution of this point would appear to be crucial to an understanding of the process of CTL recognition. Experiments are now in progress addressing these alternatives.

It has recently been reported that influenza A viruses of the H_2N_2 subtype act as T- and B-cell mitogens and that this mitogenic activity appears to be associated with the H_2 hemagglutinin glycoprotein (35). Although in the present report a hemagglutinin of H_2 subtype was used for in vitro stimulation, it is unlikely that the capacity of primed precursor to respond to this hemagglutinin is due to nonspecific mitogenic properties of this molecule. As discussed above, the CTL response of primed precursors to this antigen was highly specific in spite of the presence in the primed spleen cell population of cross-reactive CTL precursors which were readily stimulated by virus-infected stimulator cells. Further CTL precursors primed to influenza B/LEE did not exhibit significant cytotoxic activity on any of several different target cells after co-culture with A/JAP/57 hemagglutinin (Table VI). It is possible that the low level of cytotoxic activity for target cells expressing serologically unrelated hemagglutinins that CTLs directed to A/JAP/57 hemagglutinin exhibit could be due to nonspecific stimulation of cross-reactive CTL precursors. Analysis of the CTL response to hemagglutinins of other subtypes should resolve this issue.

In conclusion, the findings reported here indicate that the CTL response to influenza hemagglutinin is highly specific and exhibits a degree of fine specificity of recognition that we consider to be comparable to that of anti-hemagglutinin antibody. These findings support, from a functional standpoint, the concept that the antigen recognition structure on cytotoxic T cells could consist, in part at least, of idiotypes (immunogobulin V_H region gene products) shared with the B-cell pool. Also the present observations are easily accommodated by a two-receptor model for cytotoxic T-cell recognition where determinants on the influenza H are recognized by a distinct receptor combining site that is independent of the combining site for *H-2 K/D* gene products. Indeed, recent evidence suggesting that the capacity of cytotoxic T-cell precursors to recognize self-MHC gene products and foreign antigens is independently acquired (36, 37) more forcefully supports the two-receptor model. It is also evident that the present results could be incorporated into a single receptor model for cytotoxic T-cell recognition where a single receptor combining site simultaneously encompasses determinants presented by both the MHC gene product and the foreign antigen.

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Based on the present observations and previous results on the CTL response of mice-bearing mutations in the *H-2K* portion of the murine MHC (6, 38), this combining site must exhibit a high degree of specificity for both sets of determinants. Detailed biochemical analysis of the antigen recognition structure(s) on the cytotoxic T cell will ultimately settle this issue.

Summary

Purified type A influenza viral hemagglutinin stimulates an in vitro cell-mediated cytotoxic cell response that exhibits a high degree of specificity for the immunizing hemagglutinin. The response magnitude is proportional to the hemagglutinin dose used for stimulation. The lytic activity of the effector cells is H-2 restricted. Analysis of the specificity of the response indicated that these cytotoxic T cells readily distinguish target cells expressing serologically unrelated hemagglutinin from target cells bearing hemagglutinins serologically related to the stimulating hemagglutinin. Further analysis of the fine specificity of cytotoxic T-cell recognition with serologically cross-reactive type A influenza hemagglutinins revealed a hierarchy of cross-reactivity among these hemagglutinins that was the converse of the serologic hierarchy. These results are discussed in terms of possible differences and similarities in the specificity repertoire of cytotoxic T cells and antibodies. Possible implications of these findings from the standpoint of cytotoxic T-cell induction are also discussed.

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