

# DIFFERENCES IN ANTIGEN PRESENTATION TO MHC CLASS I- AND CLASS II-RESTRICTED INFLUENZA VIRUS-SPECIFIC CYTOLYTIC T LYMPHOCYTE CLONES

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The immune response to many viral infections is characterized by the induction of a response from both the cytolytic and helper/amplifier T lymphocyte (Th) subsets. Classically described antiviral CTL are restricted by class I MHC gene products in their recognition of viral and other foreign antigens, while Th are restricted by MHC class II gene products (1, 2). These two T lymphocyte subsets are also believed to differ in their effector functions (2), cell surface phenotypes (3), and requirements for foreign antigen recognition (4, 5).

Although alloreactive CTL responses to MHC class II antigens are well-documented (6-11), T lymphocytes that exhibit cytolytic activity and are restricted by MHC class II molecules in nominal antigen recognition have only recently been appreciated as a distinct T lymphocyte subpopulation. They were first described in the recognition of hapten-modified cells (12), and later in the recognition of virus-infected cells in the human (13-15). Since then, there have been many additional reports of foreign antigen-specific, class II-restricted CTL (16-18).

We have recently described a series of cloned T lymphocytes derived from mice infected with type A influenza virus that are restricted in influenza viral antigen recognition by H-2I region gene products and, like conventional H-2K/D-restricted CTL, specifically and directly lyse virus-infected target cells in vitro in an MHC-restricted fashion (19). These class II-restricted, influenza-specific CTL, along with a panel of influenza-specific, class I-restricted CTL, provided us with a unique opportunity to examine the recognition of influenza viral antigens expressed on infected cells by cloned T lymphocyte populations with identical functional activities but different MHC class restrictions. In this study we examine the role of viral antigen presentation in target cell recognition by these two CTL types. We have observed that like conventional class I-restricted CTL, class II-restricted CTL lysed infected histocompatible target

This work was supported by grants AI-15608, AI-15353, and HL-33391 from the U.S. Public Health Service, IN-36 from the American Cancer Society, NIH Training Grant in Cellular and Molecular Biology GM07067 (L. Morrison), Medical Scientist Training Program grant 5T32GM07200 (A. Lukacher), and by research support from Brown and Williamson Tobacco Corp., Philip Morris, Inc. and The United States Tobacco Company.

cells, yet these H-2I region-restricted CTL also recognized target cells exposed to noninfectious virion preparations or purified hemagglutinin (HA)<sup>1</sup> polypeptide. In addition, target cell sensitization for recognition by these H-2I region-restricted CTL was inhibited by the lysosomotropic agent chloroquine, but was unaffected by inhibition of viral protein synthesis in the target cells. Finally, unlike H-2K/D region-restricted CTL, the H-2I region-restricted CTL did not recognize the protein product of the influenza HA gene introduced into the target cells using a recombinant vaccinia virus expression vector. These results suggest that potentially important differences may exist in the character of the antigenic determinants recognized by the majority of MHC class I- and class II-restricted, influenza-specific CTL. The potential implications of these results for CTL recognition and antiviral immunity are discussed.

### Materials and Methods

**Animals.** Female CB6F1/J (BALB/cJ [H-2<sup>d</sup>] × C57BL/6J [H-2<sup>b</sup>]) and BALB/cByJ mice, and male CBA/J (H-2<sup>k</sup>) mice were purchased from the Jackson Laboratory, Bar Harbor, ME, and used at 7–14-wk-old.

**Viruses.** Influenza virus strains A/JAP/57 (A/Japan/305/57 [H2N2]), and B/Lee were grown in the allantoic cavity of 10-d-old embryonated chicken eggs and stored as infectious allantoic fluid as previously described (20). Purified A/JAP/57 virus (21) at a concentration  $1.67 \times 10^5$  hemagglutinating units (HAU)/ml in PBS was subjected to inactivation by exposure to UV light, as described elsewhere (22). The inactivated preparation contained no residual infectious virus as assayed by methods previously described (19).

Vaccinia virus (VV), as an infected cell sonicate and a highly purified ( $2.8 \times 10^{10}$  PFU/ml) preparation of vaccinia virus/influenza HA (VV/HA) recombinant (23), were the generous gifts of Dr. B. Moss (National Institutes of Health, Bethesda, MD). The recombinant virus was constructed by inserting into the VV genome a DNA copy of the gene encoding influenza virus A/JAP/305/57 (H2) HA.

Purified HA (80 µg/ml) was isolated from A/JAP/305/57 virions by Triton X-100 extraction and ion exchange chromatography.

**Cell Lines.** The B cell lymphoma line A20-1.11 (24) (H-2<sup>d</sup>) and the P815 (H-2<sup>d</sup>) mastocytoma line were maintained in culture in DME (Gibco Laboratories, Grand Island, NY) supplemented with antibiotics, 1% glutamine, and 10% FCS.

The L cell (H-2<sup>k</sup>) transformant CA36.2.1, transfected with the  $E_k^k$  and the  $E_d^d$  genes by DNA-mediated gene transfer (25), was kindly provided by Dr. B. Malissen (Le Centre d'Immunologie, Marseille, France), and was maintained in continuous culture using the selective medium HAT supplemented with antibiotics, 10% FCS, and 1% glutamine. This line expresses high levels of the transfected gene products as determined by flow cytometry using specific monoclonal reagents (Lukacher, A., unpublished observations).

**Cloned T Lymphocyte Lines.** All cloned CTL populations were derived from primed CTL precursors isolated from the spleens of donor mice immunized  $\geq 3$  wk previously with infectious A/JAP/57 virus ( $3.5 \times 10^7$  infectious virus units per mouse) (20). The CTL clones were isolated and established as previously described (26, 27). Under the cloning conditions used only immune T lymphocyte precursors routinely give rise to continuous in vitro CTL clones (26). The influenza type A-specific, MHC class I-restricted clones 14-1, 14-7, 14-13, A4, 35-6, and 36-1 were maintained by weekly subculture with fresh A/JAP/57-infected splenocytes of the appropriate haplotype. The influenza type A-specific, MHC class II-restricted clones G1, U-12, D8, V-4, and U-5 were restimulated weekly with inactivated virus-pulsed splenocytes in media and conditions otherwise identical to the class I-restricted CTL, as detailed elsewhere (19). These

<sup>1</sup> *Abbreviations used in this paper:* HA, hemagglutinin; HAU, hemagglutinating units; NP, nucleoprotein; VV, vaccinia virus; VV/HA, vaccinia virus-A/JAP/57 hemagglutinin gene recombinant expression vector.

class II-restricted CTL can be driven to proliferate in response to syngeneic, virus-infected splenocytes in the absence of exogenous growth factors (19).

*Assays for Cell-mediated Cytotoxicity.* P815 cells, A20-1.11 cells, and the L cell transfectant CA36.2.1 were used as target cells in  $^{51}\text{Cr}$ -release assays, which were carried out essentially as described elsewhere (26). Briefly,  $^{51}\text{Cr}$ -labeled target cell groups were washed twice and treated either with infectious virus, inactivated virus, purified HA, or left uninfected in serum-free medium for 10 min at 4°C, then incubated 1 h at 37°C. Subsequently, groups were washed twice, and  $10^4$  cells were placed in individual wells of round-bottom 96-well microtiter plates in a volume of 0.1 ml DME plus 10% FCS. T cell clones, 4–6 d after routine subculture, were added in 0.1-ml vol per well. After incubation for 6 h at 37°C and 7%  $\text{CO}_2$ , 100  $\mu\text{l}$  supernatant from each well as removed and the radioactivity counted. Values for percent specific release represent the mean counts per minute of quadruplicate cultures. SEM fell below 5% of the mean values in all instances and are omitted.

For experiments requiring addition of emetine, CA36.2.1 target cells were washed once in medium alone or in medium containing  $10^{-5}$  M emetine dihydrochloride (Sigma Chemical Co., St. Louis, MO). Target cell groups were first virus-infected, then labeled with  $^{51}\text{Cr}$ , each in the absence or presence of emetine. After labeling, cells were washed once with or without emetine, and once without emetine. All target groups were diluted in medium without emetine before addition to the assay.

*Cytotoxicity Assays in the Presence of Chloroquine.* In studies of the impact of the lysosomotropic agent chloroquine on CTL recognition it was noted that the effect of this agent was slowly reversible. Thus, target cells pretreated with chloroquine ( $5 \times 10^{-5}$  M) at the time of exposure to infectious or inactivated virus and subsequently maintained in the absence of the agent during the cytotoxicity assay became increasingly susceptible to lysis by class II-restricted CTL with increasing time after removal of the drug. To circumvent this problem of chloroquine reversibility the following modifications were made when chloroquine was used in the assay: A20-1.11 target cells were first incubated with  $^{51}\text{Cr}$ , then washed in medium alone or containing  $5 \times 10^{-5}$  M chloroquine (Sigma Chemical Co.). Each target group was resuspended in 0.4 ml media alone or containing  $6.25 \times 10^{-5}$  M chloroquine, and 0.1 ml of infectious allantoic fluid, inactivated virus or purified HA in PBS (30 HAU/ $5 \times 10^5$  cells), or PBS alone, was added to each group. The final chloroquine concentration during infection was thus  $5 \times 10^{-5}$  M. After infection, cells were washed twice with or without  $5 \times 10^{-6}$  M chloroquine, and diluted before addition to the assay in medium alone or containing  $10^{-5}$  M chloroquine; 1:1 dilution of target cells in the assay wells yielded a final chloroquine concentration of  $5 \times 10^{-6}$  M.

## Results

We have recently described the properties of a panel of cloned CTL that are restricted in influenza virus recognition by MHC class II-encoded gene products (19). Most of these clones proliferate in the absence of an exogenous source of the growth factor IL-2, express the L3T4 marker, and produce, after antigenic stimulation, soluble factors that augment a primary in vitro antibody response to SRBC in a noncognate fashion (19). These phenotypic properties resemble the properties of T lymphocytes of the helper/amplifier subset. Since influenza-specific Th can proliferate in response to noninfectious forms of the influenza virion (28–31), we wished to determine whether the class II-restricted CTL could recognize and destroy target cells exposed to noninfectious forms of the virus. Experiment I in Table I shows the results of a standard  $^{51}\text{Cr}$ -release cytotoxicity assay in which cells of the Ia<sup>+</sup> B lymphoma line A20-1.11 (H-2<sup>d</sup>) were infected with A/JAP/57 influenza virus or pulsed with UV light-inactivated purified influenza A/JAP/57 virions, then used as target cells for a panel of class I- and class II-restricted, anti-influenza CTL clones. As previously reported

TABLE I  
*CTL Recognition of Target Cells Sensitized with  
 Noninfectious Influenza Virus*

Exp.	Clone	E/T ratio	Percent specific <sup>51</sup> Cr-release* using A20-1.11 targets		
			Uninfected	UV-A/JAP pulsed <sup>‡</sup>	A/JAP infected
1	14-1	2:1	0 <sup>§</sup>	5	49
		8:1	0	9	65
	14-7	2:1	0	0	49
		8:1	0	0	60
	14-13	2:1	0	0	44
		8:1	0	0	50
	A4	2:1	0	0	49
		8:1	0	0	55
	G1	2:1	0	31	48
		8:1	1	46	65
	U-5	2:1	0	22	34
		8:1	1	34	44
	U-12	2:1	1	29	45
		8:1	0	43	62
V-4	2:1	0	43	49	
	8:1	2	56	67	
2	35-6	2:1	0	1	17
		8:1	2	3	33
	36-1	2:1	0	1	18
		8:1	1	0	40
	G1	2:1	3	20	20
		8:1	9	36	34
	U-12	2:1	0	24	22
		8:1	3	40	38

\* Cloned T cell lines were examined for cytolytic activity on uninfected, infected, or inactivated virus-pulsed, <sup>51</sup>Cr-labeled target cells 5-6 d after routine subculturing (see Materials and Methods). 10<sup>4</sup> target cells were added per well. Spontaneous <sup>51</sup>Cr-release from A20-1.11 target groups was <10%, and from CA36.2.1 target groups <15% over an assay time of 6 h.

<sup>‡</sup> 300 HAU of purified, UV light-inactivated A/JAP/57 virus per 5 × 10<sup>5</sup> cells was used to pulse the A20-1.11 and CA36.2.1 target cells.

<sup>§</sup> Values are the means of four replicate wells; SEM were <5% of mean values and are omitted.

TABLE II  
Antigen Dose Dependence of Target Cell Sensitization by Inactivated Influenza Virus

Exp.	Clone	Percent specific <sup>51</sup> Cr-release from CA36.2.1 targets*							
		Uninfected	A/JAP infected	UV-virus pulsed (HAU/5 × 10 <sup>5</sup> cells)					
				2,500	1,000	100	10	1	0.1 <sup>‡</sup>
1	U-12	4 <sup>§</sup>	47	52	52	51	42	18	10
	G1	4	73	88	63	50	27	6	7
2	D8	15	71	ND	77	62	39	27	28
	G1	18	76	ND	75	75	70	41	33

\* As in Table I. Spontaneous <sup>51</sup>Cr-release from all target groups was <24% in Exp. 1 and <20% in Exp. 2. E/T ratio is 8:1 in Exp. 1 and 4:1 in Exp. 2.

<sup>‡</sup> Indicates dose of purified, UV light-inactivated A/JAP/57 virus in HAU used to treat 5 × 10<sup>5</sup> target cells.

<sup>§</sup> As in Table I.

(19), both the K/D and the I region-restricted clones recognize A/JAP/57-infected target cells. The class I-restricted CTL clones 14-1, 14-7, 14-13, and A4 failed to lyse A20-1.11 cells treated with UV-inactivated virus, a result which is consistent with previous findings (22, 32), suggesting that class I-restricted anti-influenza CTL preferentially recognize target cells expressing newly synthesized viral polypeptides on their surface. In contrast, the class II-restricted CTL clones G1, U-5, U-12, and V-4 lysed target cells pulsed with UV-inactivated virus, and lysed them with an efficiency comparable to that of infected target cells. Furthermore, this capacity of class II-restricted CTL to recognize inactivated virus-pulsed target cells was not due to a unique property of the A20-1.11 lymphoma targets since two I-E<sub>β</sub><sup>d</sup>-restricted CTL clones, G1 and U-12, could efficiently lyse UV-inactivated virus-pulsed CA36.2.1 fibroblast target cells (Table I, Exp. 2). The CA36.2.1 line is an L cell (H-2<sup>k</sup>) line displaying the products of the E<sub>α</sub><sup>k</sup> and E<sub>β</sub><sup>d</sup> genes expressed by DNA-mediated gene transfer (25). As observed for A20-1.11 targets, two MHC class I (H-2K<sup>k</sup>)-restricted CTL clones, 35-6 and 36-1, lysed infected L cell transfectant targets but not inactivated virus-pulsed targets. This capacity of MHC class II-restricted CTL to recognize Ia<sup>+</sup> target cells treated with noninfectious virions did not appear to be solely a property of a selected panel of cloned CTL; similar results were obtained with heterogeneous populations of immune effectors generated by in vivo immunization with infectious A/JAP/57 virus followed by in vitro restimulation and short term in vitro culture of immune splenocytes with virus-infected syngeneic splenocyte stimulators (Morrison, L. A., et al., manuscript in preparation).

The capacity of noninfectious virus to sensitize the Ia-expressing target cells for class II-restricted CTL recognition was dependent on the dose of virus used to pulse the targets. As shown in Table II, treatment of the Ia<sup>+</sup> CA36.2.1 transfectant with increasing amounts (HAU) of inactivated virions resulted in a greater target cell vulnerability to lysis. Indeed, efficient target cell sensitization routinely occurred with 10 HAU of UV-inactivated virus and in some experiments (Table II, Exp. 2), lysis was detected with as little as 0.1–1.0 HAU of

TABLE III  
*Effect of Inhibition of Protein Synthesis on Sensitization of Target Cells*

Clone	E/T ratio	Percent specific <sup>51</sup> Cr-release from CA36.2.1 targets* with or without emetine					
		Uninfected		UV-A/JAP <sup>‡</sup> pulsed		A/JAP infected	
		- <sup>‡</sup>	+	-	+	-	+
35-6	2:1	1 <sup>‡</sup>	0	2	1	20	3
	8:1	3	1	6	4	44	10
36-1	2:1	1	0	0	0	12	0
	8:1	2	1	0	1	32	2
G1	2:1	1	0	15	16	10	12
	8:1	4	3	32	33	23	27
U-12	2:1	1	0	15	16	10	12
	8:1	2	1	42	37	31	30
D8	2:1	1	1	12	13	9	15
	8:1	4	6	23	25	18	29

\* As in Table I. Spontaneous <sup>51</sup>Cr-release from all target groups was ≤16% over an assay time of 4 h.

<sup>‡</sup> UV light-inactivated A/JAP/57 virus (1,000 HAU/5 × 10<sup>5</sup> cells) was used to pulse the target cells.

<sup>‡</sup> Infection and <sup>51</sup>Cr-labeling of target cells was carried out in the absence (-) or presence (+) of 10<sup>-5</sup> M emetine-HCl. The assay was carried out without emetine.

<sup>‡</sup> As in Table I.

noninfectious virus. Target cells could not be sensitized for class I-restricted, CTL-mediated lysis with even 3,000 HAU of inactivated virus.

Because the H-2I-restricted CTL could recognize target cells treated with either infectious or noninfectious virus, it was likely that *de novo* viral protein synthesis was not required to sensitize target cells for recognition by these class II-restricted CTL. To establish this point we examined the effect of the protein synthesis inhibitor emetine on the capacity of infectious virus to sensitize target cells for class I- and class II-restricted CTL recognition. Emetine at a concentration of 10<sup>-5</sup> M completely inhibited nascent protein synthesis in Ia<sup>+</sup> CA36.2.1 targets (as measured by [<sup>35</sup>S]methionine incorporation). This concentration, however, also dramatically inhibited CTL function when the <sup>51</sup>Cr-release assay was carried out in the presence of emetine. Because emetine is an irreversible inhibitor of protein synthesis (33), exposure of the CTL clones to emetine was obviated by pretreating the L cell transfectant targets with the drug during incubation with infectious or inactivated virus (see Materials and Methods). Using this protocol, we observed that the H-2K<sup>k</sup>-restricted CTL clones 35-6 and 36-1 lysed infected CA36.2.1 cells, but not infected target cells treated with emetine at the initiation of infection (Table III). Infected target cells treated with emetine after the onset of viral protein synthesis (4-6 h after infection) were susceptible

TABLE IV  
Target Cell Sensitization by Purified Influenza Hemagglutinin

Clone	E/T ratio	Percent specific <sup>51</sup> Cr-release from CA36.2.1 targets*			
		B/Lee infected	UV-A/JAP <sup>‡</sup> pulsed	HA pulsed	A/JAP infected
35-6	2:1	2 <sup>§</sup>	3	3	21
	8:1	7	9	9	39
36-1	2:1	0	1	1	20
	8:1	2	2	2	43
G1	2:1	2	31	23	24
	8:1	11	52	43	47
U-12	2:1	0	28	22	32
	8:1	2	42	38	47

\* As in Table I. Spontaneous <sup>51</sup>Cr-release from all target groups was ≤17%.

<sup>‡</sup> 30 HAU of purified, UV light-inactivated A/JAP/57 virus, or isolated HA protein (80 μg/ml) were used to treat 5 × 10<sup>5</sup> target cells.

<sup>§</sup> As in Table I.

to lysis by class I-restricted CTL (data not shown). In contrast, the class II-restricted CTL clones G1, U-12, and D8 efficiently lysed L cell targets treated with either infectious or UV-inactivated virus preparations regardless of emetine pretreatment. These results indicated that nascent viral protein synthesis during viral infection is required to sensitize target cells for class I-restricted, CTL-mediated cytolysis, but not for lysis by class II-restricted CTL. In this connection it should be noted that the infectious A/JAP/57 virus inoculum used to infect the target cells contained ~300 HAU of virion particles. As shown in Table II there is sufficient viral antigen in such a preparation to account for target cell sensitization by the input virions without *de novo* viral protein synthesis.

The demonstration of target cell sensitization with intact, noninfectious virions for H-2I region-restricted CTL recognition raised the possibility that isolated viral polypeptides might also render target cells susceptible to lysis. CTL were therefore examined for their capacity to recognize Ia<sup>+</sup> target cells treated with purified A/JAP/57 HA (Table IV). Two class II-restricted CTL clones, G1 and U-12, did indeed lyse target cells treated with isolated HA; these cells were lysed to a degree comparable to target cells treated with UV-inactivated or infectious A/JAP/57 virus. Other influenza-specific, H-2I region-restricted CTL clones such as D8 (Table II) lysed target cells exposed to intact inactivated virions but not cells treated with purified HA (Morrison, L., unpublished observations). These clones appear to be directed to virion polypeptides other than the HA (e.g., nucleocapsid protein). Also included in the analysis in Table IV were two MHC class I-restricted CTL clones, 35-6 and 36-1, that are HA-specific as defined by their recognition of L cell targets displaying the A/JAP/57 HA by DNA-mediated gene transfer (34). Once again, these HA-specific, class I-restricted CTL failed to lyse purified HA- or inactivated virus-pulsed target cells but did recognize A/JAP/57-infected cells expressing the newly synthesized HA polypeptide. Furthermore, as observed for inactivated virus-treated cells, the

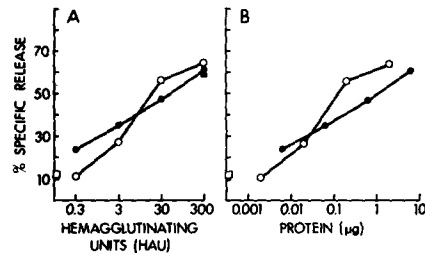


FIGURE 1. Comparison of the efficiency of target cell sensitization by UV-inactivated A/JAP/57 virus and purified A/JAP/57 HA. Clone G1 was tested for cytolytic activity against CA36.2.1 target cells pulsed with titrated amounts of purified, inactivated virions (○), or HA protein (●).  $1 \times 10^4$  CA36.2.1 targets were added per well; ratio of clones to targets is 4:1. In A, equivalent amounts of hemagglutinating activity are compared. These results are presented in terms of total protein content of the two preparations in B: 300 HAU of inactivated virions is equivalent to 1.3  $\mu\text{g}$  protein; 300 HAU of purified HA is equivalent to 8.1  $\mu\text{g}$  protein. Percent lysis of target cells infected with 300 HAU infectious A/JAP/57 virus (■), or of uninfected target cells (□) is indicated. Clone G1 was used 5 d after routine subculture. Assay time was 6 h. Values are the means of quadruplicate wells, and SEM, always  $<5\%$ , are omitted for clarity.

capacity of purified HA to render target cells susceptible to lysis by H-2I region-restricted CTL was directly dependent on the amount of HA used to pulse the target cells (Fig. 1a). Sensitization with purified HA, however, was less efficient than sensitization with intact, inactivated virions when activity per microgram protein was determined (Fig. 1b).

Recognition of soluble protein antigens by MHC class II-restricted Th has been well documented (5, 35–41). The lysosomotropic agent chloroquine has been reported to have an inhibitory effect on soluble protein antigen presentation for Th recognition (5, 42–45). In view of the several functional similarities between MHC class II-restricted CTL and Th, and the capacity of class II-restricted CTL to recognize Ia<sup>+</sup> target cells exposed to noninfectious virions or isolated HA in a manner analogous to presentation of protein antigens for H-2I region-restricted Th recognition, it was of interest to examine the effect of chloroquine on target cell sensitization by noninfectious virus. In preliminary studies we have observed that chloroquine in concentrations  $10^{-5}$  M in the cytotoxicity assay directly inhibits the ability of class I- and class II-restricted CTL to lyse target cells already expressing viral proteins. This agent also retards early events in influenza virus replication (46). To circumvent these problems, a two step protocol was devised using a chloroquine concentration of  $5 \times 10^{-5}$  M during the initial target cell interaction with virus, followed by a lower (CTL noninhibitory) chloroquine concentration ( $5 \times 10^{-6}$  M) maintained throughout the period of the cytotoxicity assay. As shown in Table V, the ability of class II-restricted CTL to recognize target cells pulsed with UV-inactivated A/JAP/57 virus or purified HA polypeptide is completely abolished by treatment of the target cells with chloroquine. Chloroquine treatment did not inhibit or diminish the level of expression of Ia molecules on the surfaces of the target cells during the 4–6-h period of the assay, as determined by cytofluorometric analysis (not shown). In control experiments where  $^{51}\text{Cr}$ -labeled A20–1.11 cells were exposed to the inactivated virus preparation, then incubated at  $37^\circ\text{C}$  for 4 h before



TABLE V  
*Effect of Chloroquine on Target Cell Sensitization by Inactivated Virus and Purified HA Protein*

Clone	Percent specific <sup>51</sup> Cr-release from A20-1.11 targets*				
	Uninfected	UV-JAP‡	UV-JAP + chloroquine‡	HA	HA + chloroquine
14-1	2 <sup>†</sup>	17	10	1	0
14-7	0	3	2	0	0
G1	2	54	2	43	1
U-12	0	45	0	38	0

\* As in Table I. Spontaneous <sup>51</sup>Cr-release from all target groups was <10%. E/T ratio is 5:1.

‡ As in Table IV.

§ Target cells were exposed to antigen in the absence or presence of  $5 \times 10^{-5}$  M chloroquine. Chloroquine was then maintained at a lower concentration ( $5 \times 10^{-6}$  M) throughout the course of the assay as described (see Materials and Methods).

† As in Table I.

chloroquine treatment and use as targets in the cytotoxicity assay, no inhibitory effect of chloroquine on the magnitude of target cell lysis was observed (not shown). Thus, recognition of these "soluble" influenza viral constituents by these MHC class II-restricted CTL appear to be chloroquine sensitive in a manner analogous to Th recognition of soluble protein antigens.

The ability of H-2I region-restricted CTL to recognize target cells exposed to infectious virus in the absence of *de novo* viral protein synthesis (Table III) implies that virions in an infectious virus inoculum can sensitize target cells for recognition by these CTL in a manner analogous to sensitization by noninfectious virion preparations. In light of the profound inhibitory effect of chloroquine on target cell sensitization using noninfectious virus, we next examined the capacity of infectious A/JAP/57 virus to render target cells susceptible to lysis by H-2I region-restricted CTL in the presence of chloroquine. The results of this analysis are shown in Table VI. Both the class II-restricted, HA-specific CTL clones G1 and U-12, and the class I-restricted, HA-specific CTL clones 14-1 and 14-7 (26), efficiently lysed control infected A20-1.11 targets maintained in the absence of chloroquine. Importantly, chloroquine had no effect on target cell sensitization for class I-restricted CTL recognition; clones 14-1 and 14-7 lysed the A/JAP/57-infected, chloroquine-treated cells as efficiently as the control infected cells. High levels of the influenza HA were detectable by hemadsorption on infected, chloroquine-treated target cells by 4-6 h after infection at 37°C. In marked contrast, chloroquine completely abolished sensitization of the target cell population for recognition by the class II-restricted CTL clones G1 and U-12. Again, in control experiments where infected target cells were first incubated for 4 h at 37° and then exposed to the chloroquine regimen, no inhibitory effect of chloroquine on sensitization was observed for either H-2K/D or H-2I region-restricted CTL clones. Therefore these chloroquine-treated, infected target cells that expressed high levels of the viral HA molecule on their surfaces as detected by hemadsorption (HA levels indistinguishable from those expressed by control infected cells), were efficiently lysed by class I-restricted CTL. The same cells,

TABLE VI  
*Effect of Chloroquine on Target Cell Sensitization by Infectious Virus*

Clone	Percent specific <sup>51</sup> Cr-release from A20-1.11 targets*		
	Uninfected	A/JAP infected	A/JAP infected + chloroquine <sup>‡</sup>
14-1	4 <sup>§</sup>	64	66
14-7	2	66	62
G1	4	68	14
U-12	2	67	7

\* As in Table I. Spontaneous <sup>51</sup>Cr-release from all target groups was <10%. E/T ratio is 5:1.

<sup>‡</sup> Target cells were exposed to infectious A/JAP/57 virus (10–50 infectious units per cell) in the absence or presence of  $5 \times 10^{-5}$  M chloroquine. Chloroquine was then maintained at a lower concentration ( $5 \times 10^{-6}$  M) throughout the course of the assay as described (see Materials and Methods).

<sup>§</sup> As in Table I.

however, failed to serve as adequate targets for HA-specific, class II-restricted CTL. This finding suggests that the presentation of the antigenic moieties on virus-infected cells to H-2I region-restricted CTL is also chloroquine-sensitive.

One interpretation of the results presented in Tables V and VI is that the H-2I region-restricted CTL efficiently recognize only exogenously introduced influenza viral constituents expressed on the target cells. In the case of virus infected cells, virions in the infectious inoculum would serve as the source of the viral antigens. According to this interpretation, the H2I region-restricted CTL would not recognize newly synthesized viral polypeptides expressed on the virus-infected cell. The failure of HA-specific, class II-restricted CTL to recognize the HA on chloroquine-treated, infected target cells would appear to be consistent with this interpretation. Alternatively, chloroquine could be selectively inhibiting the presentation of newly synthesized viral polypeptides on the infected target cells. An approach to distinguish between these possibilities was to permit synthesis and expression of the relevant viral polypeptide in the target cell in the absence of chloroquine and without introduction of the viral antigen in the input virion preparation, since the latter antigen source could ultimately be presented by the target cells. To achieve this end we introduced the gene encoding the influenza A/JAP/57 HA into A20-1.11 target cells using a recombinant VV expression vector and tested the capacity of these cells to be recognized by HA-specific, class I- and class II-restricted CTL clones (Table VII). None of the clones lysed uninfected target cells or target cells infected with the parent VV. All of the CTL clones efficiently lysed A/JAP/57-infected target cells. Strikingly, only the class I-restricted, HA-specific CTL clones 14-1 and 14-7 recognized and destroyed cells infected with the VV/HA. The class II-restricted, HA-specific CTL clones G1 and U-12 showed no capacity to recognize the influenza HA introduced and displayed on the cell surface via the recombinant vaccinia expression vector. As reported previously (23), target cells infected with the recombinant VV were found to express high levels of cell surface A/JAP/57 HA which were comparable to levels displayed on influenza virus-infected cells. Thus, the failure of I-region-restricted CTL to recognize these target cells was

TABLE VII  
*CTL Response to Target Cells Infected with Purified VV/HA  
 Recombinant Virus*

Clone	Percent specific <sup>51</sup> Cr-release from A20-1.11 targets*			
	Un- infected	Vaccinia infected	VV/HA <sup>‡</sup> infected	A/JAP infected
14-1	2 <sup>§</sup>	1	49	69
14-7	0	0	77	68
G1	3	3	4	68
U-12	1	1	0	69

\* As in Table I. Clones were examined for cytolytic activity 4 d after routine subculturing. Spontaneous <sup>51</sup>Cr-release from all target groups was <12%. E/T ratio is 5:1.

<sup>‡</sup> Indicates target cells infected with VV/HA. 25  $\mu$ l of highly purified VV/HA ( $\sim 10^{10}$  PFU/ml) was used for infection.

<sup>§</sup> As in Table I.

not readily attributable to quantitative differences in HA expression on vaccinia- and influenza-infected targets. Furthermore, VV infection itself did not significantly inhibit presentation to MHC class II-restricted CTL, for concomitant exposure of target cells to parental VV and inactivated A/JAP/57 virions resulted in strong sensitization for class II-restricted lysis (data not shown). These studies used a highly purified preparation of the HA recombinant VV which contained no detectable influenza HA in the virion preparation. In companion studies (not shown) crude vaccinia preparations consisting of homogenates of infected cell cultures, which contained the influenza HA gene product, readily sensitized target cells for class II-restricted CTL recognition.

### Discussion

This report examines viral antigen presentation for target cell recognition by influenza virus-specific CTL restricted by either class I (H-2K/D region) or class II (H-2I region) MHC gene products. For this analysis we used a panel of well-characterized cloned CTL of defined viral specificity and H-2 restriction. We have observed clear-cut differences between MHC class I- and class II-restricted CTL in the antigen presentation requirements for target cell sensitization and recognition.

The observation that class II-restricted CTL could efficiently lyse target cells treated with noninfectious influenza virion or purified HA preparations provided the initial evidence for a divergence between MHC class I- and class II-restricted CTL in viral antigen recognition. This distinction was reinforced by the finding that inhibition of nascent viral synthesis in infected target cells abolished target cell recognition by class I-restricted CTL but did not affect target cell recognition by class II-restricted CTL. Thus, while H-2K/D region-restricted CTL preferentially recognize target cells displaying viral polypeptides as a result of *de novo* viral protein synthesis in virus-infected cells, H-2I region-restricted antiviral CTL could, like most H-2I region-restricted T lymphocytes (47, 48), also recognize and respond to the soluble form of antigens displayed by antigen-pulsed presenting cells. These initial findings suggested, then, that class II-

restricted CTL were capable of recognizing either newly synthesized viral polypeptides, or exogenous viral polypeptides derived from the input infectious virions and subsequently, displayed on the target cell surface.

A crucial and unexpected result that did not support this viewpoint emerged when the effect of chloroquine on class I- and class II-restricted CTL recognition was examined. This lysosomotropic amine has been shown to inhibit antigen presentation to MHC class II-restricted Th cells (5, 42-45). Our results parallel these observations in that recognition of target cells pulsed with inactivated virus or purified HA by class II-restricted, HA-specific CTL clones was completely abolished by chloroquine treatment. Surprisingly, recognition of infected target cells by these class II-restricted CTL clones was also dramatically inhibited. Chloroquine treatment, however, had no effect on the expression or recognition of newly synthesized cell surface HA by HA-specific, class I-restricted CTL. The concentration of chloroquine used in the assay ( $5 \times 10^{-6}$  M) did not appear to inhibit influenza HA synthesis or migration of the nascent polypeptide to the cell surface, since abundant HA was readily detectable on the infected cell surface by 4 h after infection. These results raised the possibility that the class II-restricted, HA-specific CTL recognized only target cells sensitized by exogenously introduced HA molecules.

Since the mechanism by which chloroquine inhibits MHC class II-restricted T lymphocyte recognition is not precisely defined, and since this agent could have multiple effects on target cell physiology, the results of the studies with chloroquine could be open to other interpretations. As an alternative approach to the issue of exogenous antigen and class II-restricted CTL recognition we employed a recombinant vaccinia virus expression vector to sensitize target cells. In these experiments, in which the HA gene but not the HA polypeptide was introduced into the target cell by the vaccinia vector, newly synthesized HA protein was demonstrable on the infected cell surface, and these HA-expressing target cells were readily lysed by class I-restricted CTL (49). These same targets were not, however, recognized by HA-specific, class II-restricted CTL clones.

Taken together, the findings reported here raise the possibility that important differences exist in viral antigen presentation and recognition by MHC class I- and class II-restricted T lymphocytes. Although this report has focused for the most part on CTL directed to the influenza HA, comparable differences appear to be evident for class I- and class II-restricted T lymphocytes directed against influenza virion polypeptides (Morrison, L., unpublished observations). More importantly, these results are in keeping with our previous *in vivo* observations in the mouse where differences in *in vivo* CTL responses to infectious and noninfectious virus preparations were noted (22). As mentioned above, the present data suggest that HA-specific, class II-restricted CTL preferentially recognize target cells displaying exogenously introduced HA. This HA could be in an altered or processed form on the cell surface as a result of intracellular trafficking through an acid endocytic compartment. At present, we have no direct biochemical evidence for a processing event. Presentation of the HA on the cell surface as an intact molecule is equally possible. Class I-restricted, HA-specific CTL, on the other hand, appear to preferentially recognize HA displayed on the target cell surface as a result of *de novo* polypeptide synthesis. Whether

this is the intact native HA expressed as an integral membrane constituent or a truncated portion of the molecule is not as yet known and is under investigation.

Studies of class I-restricted CTL recognition in several other antigen systems appear to differ from those reported here. These include observations with soluble hapten-protein conjugates (50), the nonstructural SV40 virus T antigen (51, 52), a genetically engineered chimeric HA molecule (53), as well as the internal influenza virus nucleoprotein (NP) (54, 55), and polymerase gene products (56). In these systems either exogenously introduced antigens (50, 53) or nonstructural and internal viral polypeptides can serve as target antigens for H-2K/D region-restricted CTL. On the basis of their studies of class I-restricted CTL recognition of the NP antigen, Townsend et al. (54, 55) have speculated that MHC class I-restricted CTL, like class II-restricted T lymphocytes, may primarily recognize degraded or fragmented forms of viral antigens displayed on the target cell surface. The observations reported here are not entirely consistent with such a proposal. Notably, the lysosomotropic agent chloroquine fails to inhibit target cell sensitization for class I-restricted CTL recognition under conditions where class II-restricted CTL recognition is completely abolished. Likewise, isolated influenza HA fails to sensitize target cells for recognition by HA-specific, class I-restricted CTL, though exposure to it readily makes target cells vulnerable to class II-restricted CTL recognition and lysis. This sensitization step for class II-restricted CTL recognition is also chloroquine inhibitable. In the least, our results suggest that if class I-restricted CTL recognize forms of antigen other than the native molecule, presentation for class I-restricted CTL recognition occurs by a mechanism distinctly different from that required for class II-restricted CTL. In this connection, it should be emphasized that the intrinsic properties of disparate molecules like the integral membrane HA glycoprotein and soluble, predominantly intracellular nonglycosylated NP protein may play a key role in dictating the form of antigen recognized by CTL.

An alternate, highly speculative hypothesis, which could account for our results and those of others (50-55, 57), is that class II-restricted antiviral CTL recognize predominantly antigenic epitopes exposed during (or which potentially survive) intracellular processing events associated with endocytosis of input viral polypeptides prior to their reexpression on the cell surface. Indirect evidence supporting this view has come from studies on H-2I region-restricted T lymphocyte recognition of soluble protein and viral antigens (30, 58, 59). In contrast, class I-restricted antiviral CTL would be preferentially directed against antigenic epitopes displayed on viral polypeptides in their native conformation. Thus the apparent requirement for nascent HA protein synthesis in the recognition of target cells by HA-specific, class I-restricted CTL reflects the presence of the target HA epitopes for these CTL only on the newly synthesized and expressed native HA molecule. The failure of exogenous HA to sensitize target cells for recognition by these class I-restricted CTL could be explained by the loss of the relevant HA epitopes during trafficking of the exogenously introduced protein through an endocytic compartment. The possible importance of antigen conformation in class I-restricted CTL recognition has been suggested by studies of Sherman (60) with alloreactive CTL directed to MHC class I gene products.

Furthermore, according to this hypothesis certain antigens might sensitize target cells directly without *de novo* protein synthesis if the epitopes on these antigens could survive transit through an endocytic compartment and retain a conformation necessary for H-2K/D region-restricted CTL recognition upon reemergence at the cell surface. This model can also account for the results of Tevethia et al. (51) and Gooding and O'Connell (52), who show class I-restricted CTL recognition of cells transfected with fragments of the gene encoding the SV40 T antigen, and more recent results of Townsend et al. (55) using NP gene fragments, since these newly synthesized truncated antigen molecules might still retain appropriate conformational epitopes. Similarly, this paradigm affords an explanation for the finding that Sendai virus virions with cleaved, active fusion proteins can directly sensitize target cells for class I-restricted CTL recognition without *de novo* viral protein synthesis, while Sendai virus without an active fusion protein fails to sensitize target cells (57). In this instance, direct fusion of the Sendai virion surface polypeptide into the target cell cytoplasmic membrane would maintain the conformational stability of the target epitopes, whereas endocytosis of the fusion-negative virions would result in loss of the relevant target epitopes before reemergence of viral antigens on the cell surface. Experiments are now in progress to further examine this hypothesis.

Perhaps the most important issue raised by the observations in this report concerns the function of MHC class II-restricted antiviral CTL in recovery from viral infection. The failure of the H-2I region-restricted CTL to recognize chloroquine-treated, infected target cells, or target cells infected with the HA recombinant VV, suggests that these CTL do not recognize viral polypeptides expressed on the cell surface as a direct result of viral infection. Preliminary analysis of the *in vivo* effector activity of the HA-specific CTL clone G1, however, indicates that this clone can efficiently promote recovery of lethally infected recipients after adoptive *in vivo* transfer (Lukacher, A., unpublished observations). Furthermore, this clone can reduce pulmonary influenza virus titer with an efficiency and time course comparable to class I-restricted CTL. Thus, these class II-restricted CTL likely play a positive role in viral clearance and recovery from viral infection. The mechanism through which these cells exert their *in vivo* antiviral effect and the target cells for these CTL *in vivo* remain to be elucidated.

In conclusion, in this report we have analyzed a panel of MHC class I- and class II-restricted, influenza-specific CTL which appear to exhibit distinctly different requirements for viral antigen presentation in target cell recognition. These differences may reflect differences in the structure of the antigen receptors used by class I- and II-restricted CTL, or may reflect intrinsic differences in the structure of MHC class I and II molecules and in the range of interaction between viral antigens and these two classes of MHC molecules. Since the structure and organization of the antigen receptors on these CTL subsets can now be analyzed at the molecular level it should be possible to relate antigen recognition to antigen receptor structure. An understanding of the basis for the difference in antigen presentation requirements for recognition between class I- and class II-restricted CTL should elucidate requirements for induction of these

two CTL subsets *in vivo*. This would have obvious implications for future viral vaccine design.

### Summary

We have examined requirements for antigen presentation to a panel of MHC class I- and class II-restricted, influenza virus-specific CTL clones by controlling the form of virus presented on the target cell surface. Both H-2K/D- and I region-restricted CTL recognize target cells exposed to infectious virus, but only the I region-restricted clones efficiently lysed histocompatible target cells pulsed with inactivated virus preparations. The isolated influenza hemagglutinin (HA) polypeptide also could sensitize target cells for recognition by class II-restricted, HA-specific CTL, but not by class I-restricted, HA-specific CTL. Inhibition of nascent viral protein synthesis abrogated the ability of target cells to present viral antigen relevant for class I-restricted CTL recognition. Significantly, presentation for class II-restricted recognition was unaffected in target cells exposed to preparations of either inactivated or infectious virus. This differential sensitivity suggested that these H-2I region-restricted CTL recognized viral polypeptides derived from the exogenously introduced virions, rather than viral polypeptides newly synthesized in the infected cell. In support of this contention, treatment of the target cells with the lysosomotropic agent chloroquine abolished recognition of infected target cells by class II-restricted CTL without diminishing class I-restricted recognition of infected target cells. Furthermore, when the influenza HA gene was introduced into target cells without exogenous HA polypeptide, the target cells that expressed the newly synthesized protein product of the HA gene were recognized only by H-2K/D-restricted CTL. These observations suggest that important differences may exist in requirements for antigen presentation between H-2K/D and H-2I region-restricted CTL. These differences may reflect the nature of the antigenic epitopes recognized by these two CTL subsets.

We thank Catherine Hamby for secretarial assistance in preparation of the manuscript, and Dr. Bernard Moss for his generous gift of vaccinia-HA recombinant virus.

*Received for publication 18 October 1985 and in revised form 23 December 1985.*

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