EXPRESSION OF SPECIFIC CYTOLYTIC ACTIVITY BY H-2I REGION–RESTRICTED, INFLUENZA VIRUS–SPECIFIC T LYMPHOCYTE CLONES

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Over the past decade a substantial body of evidence has accumulated that supports the existence of functionally distinct subsets of T lymphocytes. This concept has been particularly reinforced by the observations that cytolytic activity by T lymphocytes correlates with recognition of (or restriction by) class I major histocompatibility complex (MHC)[1] gene products while T lymphocyte helper/amplifier function is associated with recognition of class II MHC gene products (1, 2). The association of these discrete functional activities with T lymphocyte populations expressing mutually exclusive cell surface markers further supports this hypothesis (3).

However, the concept that cytolytic function is a property of T lymphocytes recognizing class I MHC gene products has been called into question by observations made with alloreactive T lymphocytes. Several reports (4–7) in the mouse demonstrated the generation of cytolytic T lymphocytes (CTL) directed to H-2I region products in mixed leukocyte cultures. More recently, murine alloreactive CTL lines (8, 9) and clones (10, 11) directed to an I-A product have been described that express the Lyt-1$^+$2$^-$ cell surface phenotype. Similarly, in the human, several groups have described T4$^+$ alloreactive CTL that recognize class II MHC structures (12, 15). These results raise the possibility that cell surface lymphocyte markers such as Lyt-2 in the mouse or T4 in the human correlate with MHC recognition (or restriction) rather than T lymphocyte function (16).

Compared with the data demonstrating class II MHC-alloreactive CTL, there is much less evidence for restriction by class II MHC products in the recognition of nominal antigen by CTL. The earliest suggestion of class II–restricted CTL

[1] *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytolytic T lymphocytes; FBS, fetal bovine serum; [^{3}H]TdR, tritiated thymidine; HAT, hypoxanthine, aminopterin, thymidine; IL-2, interleukin 2; 2-ME, 2-mercaptoethanol; MEM, minimal essential medium; MHC, major histocompatibility complex; PFC, plaque-forming centers; SRBC, sheep red blood cells; TCGF, T cell growth factor; and UV, ultraviolet.

was a report by Wagner et al. (17) describing TNP-specific heterogeneous CTL populations that require I region compatibility for lysis of TNP-conjugated target cells. I region–restricted T lymphocyte clones specific for ovalbumin and cytochrome c, which can destroy, in an H-2-restricted manner, target cells bearing these antigens, recently have been described by Tite and Janeway (18) and Norcross et al. (19), respectively. In at least one instance (18), target cell destruction appeared to be nonspecific and mediated by a soluble factor released as a result of antigen-specific recognition. In the human, class II MHC–restricted T lymphocyte clones with specific cytolytic activity for virus-infected cells have been reported in several viral systems (20–22) but the possible role of soluble cytolytic factors as mediators of lysis has not been fully explored.

In this study we demonstrate the expression of specific cytolytic activity by a panel of murine T lymphocyte clones directed to type A influenza virus and restricted by H-2I-A or I-E subregion–encoded determinants. These clones express the Lyt-1^+2^-, L3T4$^+$ phenotype, proliferate in the absence of exogenous interleukin 2 (IL-2), and release factors that provide help for in vitro B cell responses. The fine specificity of viral antigen recognition by these clones is identical in both proliferation and cytotoxicity assays and is comparable to patterns previously observed with H-2K- or D-restricted antiinfluenza CTL clones (23, 24). Cytolytic activity of the clones is specifically inhibitable by anti-Ia antibody or antibody to the L3T4 molecule. Using L cells that express Ia antigens by DNA-mediated gene transfer, we further demonstrate that two CTL clones are restricted by the E_β^d chain in their recognition of viral antigens. We also present evidence consistent with the concept that these clones mediate their cytotoxic effector function by direct lysis of virus-infected cells rather than release of a nonspecific cytolytic factor. The implications of these findings with regard to the conventional distinction between helper and cytolytic T cell subsets in antiviral immunity are discussed.

Materials and Methods

Animals. CB6F₁/J [BALB/cJ (H-2^d) × C57BL/6J (H-2^b)] and BALB/cByJ mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and used at 7–14 wk of age. B10.D2 (H-2^d), B10.BDR1 (H-2^{g4}), B10.CHR51 (H-2^{w18}), B10.A(5R) (H-2^{i5}), and B10.GD (H-2^{g2}) mice were generously provided by Drs. D. Shreffler and V. Hauptfeld (Department of Genetics, Washington University, St. Louis MO).

Viruses. Influenza virus strains A/JAP/57 [A/Japan/305/57(H2N2), A/AA/67 [A/Ann Arbor/7/67(H2N2)], A/PR/8 [A/Puerto Rico/8/34(H1N1), A/MEL/35 [A/Melbourne/35(H1N1)], A/PC/75 [A/Port Chalmers/75(H3N2), A/HK/68 [A/Hong Kong/1/68(H3N2)], 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 (25).

Influenza A/JAP/57 was concentrated and purified according to standard procedures (26). Briefly, virus was concentrated by adsorption-elution of infectious allantoic fluid from human erythrocytes followed by ultracentrifugation, and then further purified by velocity centrifugation over sucrose gradients.

Purified A/JAP/57 virus was inactivated by exposure to ultraviolet (UV) light, as described elsewhere (27). No residual infectious virus was detectable as measured by absence of plaque-forming centers (PFC) on MDCK cell monolayers (28). No loss of viral hemagglutinating activity, as measured by the hemagglutination titration method of Fazekas de St. Groth and Webster (29), was observed after virus inactivation.

Monoclonal Antibodies. The monoclonal antibodies used in experiments to inhibit cell-

mediated cytotoxicity were Do4, specific for K^d and D^d (30); Do3, anti-I-$E^{d,k}$ (30); MK-D6, anti-I-A^d (31); and GK1.5, anti-L3T4a (32). These monoclonal antibodies were in the form of cell-free sterile culture supernatants. The hybridoma GK1.5 was generously provided by Dr. Frank Fitch (Department of Pathology, The University of Chicago, Chicago, IL). The rat anti-Thy-1 monoclonal antibody T24/40.7 (from the Salk Institute, La Jolla, CA) was concentrated 20-fold by $(NH_4)_2SO_4$ precipitation of culture supernatants, and used at 1:50 dilution to deplete spleen cells of T cells for in vitro anti-SRBC (sheep red blood cells) antibody responses.

Tumor Cell Lines. The P815 (H-2^d) mastocytoma line and the A20-1.11 (H-2^d) B cell lymphoma line (33) were maintained in culture in Dulbecco's modified minimal essential medium (MEM) (Gibco Laboratories, Grand Island, NY) supplemented with antibiotics and 10% fetal bovine serum (FBS).

Construction and Maintenance of L Cell Lines Expressing $E_\alpha^k E_\beta^d$ and $E_\alpha^k E_\beta^k$ Gene Products. L cell (H-2^k) transformants CA36.2.1 and CA36.1.1, expressing products of the $E_\alpha^k E_\beta^d$ genes and the $E_\alpha^k E_\beta^k$ genes, respectively, were established by transfection of Ltk$^-$ cells with DNA corresponding to the indicated gene segments, as described previously (40). These cells were maintained in hypoxanthine, aminopterin, thymidine (HAT) medium. They express high levels of the appropriate transfected gene products as detected by flow cytofluorometry using specific monoclonal reagents (A. E. Lukacher, unpublished observations).

Establishment and Maintenance of T Lymphocyte Clones. T lymphocyte clones were isolated and maintained according to methods described in detail elsewhere (23). The CTL clones A4, A7, 14-1, and 35-6 are all type A influenza virus–specific and restricted by class I MHC products. They were selected by in vitro stimulation of individual primed precursor cells with infectious A/JAP/57 virus (23). The class II MHC–restricted T lymphocyte clones discussed in this report were either established as described for class I–restricted CTL, using infectious A/JAP/57 virus, or by in vitro stimulation of primed precursors using UV light–inactivated A/JAP/57 virus according to the method of Leung and Ada (34).

Assays for Cell-mediated Cytotoxicity. P815 cells, A20-1.11 cells, and the L cell transfectants K_2, CA36.2.1, and CA36.1.1, were used as target cells in ^{51}Cr release assays, which were carried out essentially as described elsewhere (23). Spontaneous release from target cells incubated with medium alone over the 6 h time course of the assay was generally <10% for P815 cells, and <20% for A20-1.11 cells and the L cell transfectants. Release values represent the mean specific ^{51}Cr release from three replicate wells. SEM were always <5% of the mean value and are omitted.

Inhibition of cell-mediated cytotoxicity by monoclonal antibodies was carried out as follows: 1 × 10^4 ^{51}Cr-labeled, A/JAP/57 virus–infected A20-1.11 target cells were added in a 0.05 ml vol to each well of a 96-well round-bottomed microtiter plate. Each well then received 0.1 ml of the indicated dilution in medium of Do4, Do3, or MK-D6 antibody supernatant. After incubation for 20 min at 37°C, a 0.5 ml vol containing 1 × 10^5 CTL clones was added to each well. For blocking of cytotoxicity by GK1.5 antibody, a 0.05 ml vol of 1 × 10^5 effector CTL cells was added to wells containing a 0.1 ml vol of the appropriate dilution of antibody or control medium; the T cells were incubated with the GK1.5 antibody for 20 min at 37°C before the addition of 1 × 10^4 ^{51}Cr-labeled, A/JAP/ 57 virus–infected A20-1.11 target cells in 0.05 ml. Viable effector T cells were used 5 d after routine subculturing and were separated from dead cells according to the method of Davidson and Parish (35) before inclusion in this assay. All antibody dilutions and target cell and effector cell resuspensions were carried out in MEM plus 10% FBS. After a 6 h incubation at 37°C, a 0.1 ml vol was removed from each well and counted in a Beckman 4000 Gamma Counter (Beckman Instruments, Inc., Fullerton, CA).

Assay of Cellular Proliferation. The proliferative response of cloned T cell lines was assessed by [^{3}H]thymidine ([^{3}H]TdR) incorporation, as previously described (24). Results are expressed as the mean cpm ± SEM of quadruplicate cultures at the peak of the proliferative response (day 3 of culture).

Assays for T Cell Helper Activities. Viable T cell clones at day 7 after antigenic

stimulation were separated from erythrocytes and dead cells by sedimentation on Ficoll/ sodium metrizoate (35). 2×10^6 cells of each clone were added to individual wells of six-well-cluster tissue culture plates (Costar, Cambridge, MA) containing 20×10^6 irradiated, uninfected BALB/c spleen cells, inactivated A/JAP/57 virus–pulsed, BALB/c spleen cells, or no spleen cells, in 5 ml of RPMI 1640 medium supplemented with 10% FBS, nonessential amino acids (1×) (Gibco Laboratories), 5×10^{-5} M 2-mercaptoethanol (2-ME), 2 mM glutamine, and antibiotics. After 24 h incubation at 37°C in a humidified atmosphere of 7% CO_2, supernatants were collected and stored at 4°C.

The capacity of these supernatants to promote a primary in vitro anti-SRBC response was examined. CB6F₁/J spleen cells were T cell–depleted by treatment with anti-Thy-1 monoclonal antibody and guinea pig complement (Gibco Laboratories). 8×10^6 T cell–depleted spleen cells were added in 0.2 ml vol RPMI 1640 containing 10% FBS, nonessential amino acids (1×), 5×10^{-5} M 2-ME, 2 mM glutamine, and antibiotics to individual wells of 24-well tissue culture plates (Linbro Chemical Co., Hamden, CT). A 0.3 ml vol of supernatant was added per well. Each well then received 0.03 ml of a 1% SRBC suspension (Colorado Serum Co., Denver, CO). Control cultures received 0.3 ml medium or supernatant of concanavalin A (Con A)-pulsed rat spleen cells. Cultures were fed daily with 0.03 ml/well of a nutritive cocktail (36). At day 5 of culture, wells were harvested and assayed for an anti-SRBC antibody response in a modified Jerne hemolytic plaque assay (37). Results are expressed as the mean PFC ± SEM per culture and the mean PFC ± SEM per 10^6 viable cells from individual cultures assayed in duplicate.

IL-2 activity in the supernatants was assayed using an IL-2-dependent murine T cell clone, 14-7fd (38). 5 d after subculture in 20% T cell growth factor (TCGF)-containing medium, 4×10^4 14-7fd cells were added to each of four replicate wells of flat-bottomed, 96-well microtiter plates (Linbro Chemical Co.) in the presence of 50% culture supernatant. The cells were incubated for 24 h, then pulsed with 1 μCi/well [³H]TdR for 16 h before harvest.

Results

Expression of Cytolytic Activity by Class II MHC–restricted, Influenza Virus–Specific T Cell Clones.   In a previous report (23) we described the isolation and characterization of continuous cloned lines of influenza virus–specific T lymphocytes. Most of the T cell clones selected by this cloning strategy exhibited H-2 K/D–restricted, virus-specific cytolytic activity and possessed the Lyt-1⁺2⁺ phenotype. However, some Thy-1⁺ T lymphocyte clones were isolated that lacked virus-specific cytolytic activity on conventional target cells, e.g., virus-infected P815 mastocytoma cells.

Since P815 cells express H-2K^d and D^d region products but fail to express detectable cell surface H-2I^d region products, it was of interest to examine the capacity of these "noncytolytic" clones to recognize and destroy Ia⁺ virus–infected target cells. Table I shows the results of such an analysis using a panel of influenza virus–specific T lymphocyte clones derived from A/JAP/57-immune mice and tested on P815 target cells and the H-2I^d-positive A20-1.11 B cell lymphoma line. Two conventional CTL clones of BALB/c origin, A4 and A7, efficiently lysed A/JAP/57-infected but not uninfected or B/Lee-infected P815 cells. These clones showed comparable cytolytic effector specificity on A/JAP/57-infected histocompatible A20-1.11 cells. The fine specificity of viral antigen recognition of clones A4 and A7 and their restriction by H-2K^d and L^d, respectively, have been reported previously (28). As shown in Table I, of five clones that did not efficiently lyse A/JAP/57-infected P815 cells, three, D8, G1, and U5, exhibited a high degree of specific lysis on A/JAP/57-infected A20-1.11 cells but failed to

TABLE I

Cytolytic Activity of Influenza Virus-specific T Cell Clones

Clone	Effector/tar-get ratio	Percent specific ^{51}Cr release*					
		P815 targets[‡]			A20-1.11 targets		
		Unin-fected	A/JAP/57	B/Lee	Unin-fected	A/JAP/57	B/Lee
A4	10:1	7	54	10	3	28	3
	1:1	0	29	1	1	13	0
A7	10:1	9	78	12	7	35	8
	1:1	0	49	1	1	21	0
D8	10:1	1	3	2	2	46	0
	1:1	1	0	0	1	23	0
G1	10:1	1	6	2	8	57	6
	1:1	0	0	1	1	32	1
U5	10:1	1	8	3	1	25	0
	1:1	1	1	0	0	14	0
U2	10:1	1	4	1	0	11	0
	1:1	0	2	1	0	7	0
U11	10:1	3	13	4	0	15	2
	1:1	1	3	0	0	5	0

* Cloned T cell lines were examined for cytolytic activity on uninfected and infected ^{51}Cr-labeled target cells 5–6 d after routine subculturing (see Materials and Methods). Assay time was 6 h.

‡ Values are the means of three replicate wells; SEM were <5% of mean values and are omitted. Spontaneous release from P815 target groups was <13% and from A20-1.11 target groups, <14%. 1 × 10⁴ target cells were added per well.

recognize B/Lee-infected A20-1.11 cells or A/JAP/57-infected P815 cells to a significant extent. Two other clones, U2 and U11, exhibited only a marginal degree of lysis on either A/JAP/57-infected A20-1.11 or P815 cells. The ability of clones D8, G1, and U5 to discriminate between the Ia⁺ A20-1.11 cell line and the Ia⁻ P815 cell line suggested that these clones represent CTL restricted by class II MHC products in their recognition of influenza virus–infected cells.

Class II MHC–restricted virus antigen recognition by these clones is supported by several further observations. First, clones D8, G1, U5, U2, and U11 were found, by flow cytofluorometry, to express the Thy-1.2⁺, Lyt-1⁺2⁻, L3T4⁺ surface markers (data not shown), the phenotype characteristic of class II MHC–restricted T cells (16, 32). Second, genetic mapping using congeneic and recombinant inbred mouse strains established that antigen-specific stimulation of these clones to proliferate was restricted by class II MHC genes. As shown in Table II, all five H-2ᵈ clones proliferated in response to A/JAP/57 virus–pulsed B10.BDR1 spleen cells but not to A/JAP/57 virus–pulsed B10.CHR51 spleen cells, thereby mapping their restriction to the H-2Iᵈ region. Clones U5, U2, and U11 recognized viral antigens presented by B10.GD cells, mapping their restriction to the I-Aᵈ subregion. The failure of clones G1 and D8 to proliferate in response to A/

TABLE II

Class II MHC Restriction of Cloned T Cell Proliferation

Strain	MHC Haplotype							Peak proliferative response‡				
	K	Aβ	Aα	Eβ	Eα	S	D	GI‖	D8	U5	U2	U11
BALB/c	d	d	d	d	d	d	d	5,541 ± 554	ND‖	ND	ND	ND
B10.D2	d	d	d	d	d	d	d	ND	4,473 ± 130	9,896 ± 564	3,238 ± 107	8,053 ± 138
B10.BDR1	d	d	d	d	d	d	b	1,422 ± 146	3,178 ± 468	14,918 ± 1,287	2,942 ± 208	4,864 ± 75
B10.CHR51	d	p	p	p	7	W5	W5	29 ± 3	25 ± 2	27 ± 2	34 ± 6	562 ± 21
B10.GD	d	d	d	d/b	b	b	b	43 ± 10	21 ± 3	16,227 ± 401	2,977 ± 128	6,522 ± 373

* Normal spleen cells after 2,000 rad of γ irradiation were pulsed with inactivated A/JAP/57 virus as described (Materials and Methods). 1 × 10^6 spleen cells were cocultured with 1 × 10^4 cloned T cells.

‡ Values are the mean cpm ± SEM of [³H]TdR incorporation from quadruplicate cultures at the peak of the proliferative response (day 3).

‖ 6 d after routine subculturing, cells of each cloned line were resuspended in fresh medium containing 10% TCGF and cultured for an additional 8 d. After this time, replicate cultures of viable cells were examined for proliferation by [³H]TdR incorporation after culturing with stimulator spleen cells in the absence of exogenous IL-2.

‖ Not determined.

TABLE III

Viral Antigen–specific Proliferation of Class II MHC–restricted Clones*

BALB/c stimulator spleen cells‡	Peak proliferative response‖				
	D8	G1	U5	U11	U2
—	160 ± 31	269 ± 35	159 ± 5	116 ± 10	186 ± 15
A/JAP/57 (H2N2)	7,909 ± 352	7,368 ± 192	128,118 ± 10,106	17,010 ± 1,088	5,034 ± 46
A/AA/67 (H2N2)	4,192 ± 269	3,826 ± 241	4,209 ± 379	12,182 ± 634	5,568 ± 124
A/MEL/35 (H1N1)	14,205 ± 536	133 ± 12	358 ± 80	ND‖	5,286 ± 350
A/PR/8 (H1N1)	14,888 ± 716	110 ± 17	170 ± 26	ND	ND
A/PC/75 (H3N2)	22,570 ± 1,960	112 ± 5	184 ± 33	ND	ND
A/HK/68 (H3N2)	23,214 ± 678	107 ± 4	288 ± 24	16,922 ± 416	5,022 ± 285
B/Lee	153 ± 26	175 ± 17	130 ± 75	411 ± 42	654 ± 31

* See footnote * in Table II.

‡ As in Table II, except that normal BALB/c spleen cells, after 2,000 rad irradiation, were either infected with the indicated virus strain or left uninfected. The subtype designations of the type A influenze virus strains appear in parentheses.

‖ As in Table II.

‖ Not determined.

JAP/57 virus–pulsed B10.GD cells tentatively mapped their restriction to the I-E^d subregion. Finally, both the cytolytic and noncytolytic class II MHC–restricted clones proliferated in the absence of exogenous IL-2 after appropriate antigenic stimulation (Tables II and III). This result is consistent with the general finding that class II MHC–restricted T cell clones exhibit antigen-dependent, but exogenous IL-2–independent, growth (39).

Viral Antigen Specificity of Class II MHC–restricted T Cell Clones. Previous studies from this laboratory (23) have demonstrated that class I MHC–restricted, influenza-specific CTL clones exhibit several distinct categories of influenza virus recognition specificity: unique, subtype-specific, and crossreactive. Thus it was of interest to determine whether similar patterns of virus recognition were exhibited by the class II MHC–restricted T cell clones.

Tables III and IV show the viral antigen specificity of the class II–restricted, influenza-specific clones in proliferation and cytotoxicity assays. Clones D8, U11, and U2 proliferated in response to syngeneic spleen cells infected with type A influenza virus strains of serologically distinct subtypes [A/JAP/57(H2N2), A/

TABLE IV

Influenza Virus Specificity of Class II MHC–restricted CTL Clones

Clone	Percent specific ^{51}Cr release from A20-1.11 target cells*					
	Uninfected	B/Lee	A/JAP/57 (H2N2)	A/AA/67 (H2N2)	A/PC/75 (H3N2)	A/PR/8 (H1N1)
D8	1[‡]	1	50	14	56	54
G1	7	8	70	72	14	8
U5	0	1	61	55	1	3

* As in Table I. Effector/target ratio is 5:1.
[‡] As in Table I. Spontaneous release for all target groups was <20%.

MEL/35(H1N1), and A/HK/68(H3N2)], while clones G1 and U5 were directed to an antigenic determinant shared by type A influenza virus strains of the H2N2 subtype [A/JAP/57 and A/AA/67]. None of the clones recognized uninfected or B/Lee virus–infected stimulator cells. Thus, clones D8, U11, and U2 exhibited the crossreactive pattern of type A influenza virus recognition, and clones G1 and U5 displayed the H2N2 subtype–specific pattern. As shown in Table IV, CTL clones D8, G1, and U5 only lysed target cells infected with the influenza virus strains that stimulated their proliferation. The particular viral protein recognized by G1 has been unequivocally mapped to the A/JAP/57 hemagglutinin molecule by the use of Ia$^+$ target cells expressing the products of the transfected A/JAP/57 hemagglutinin gene (Lukacher and Braciale, unpublished observations). Thus, the class II MHC–restricted CTL clones possess identical viral antigenic requirements for both cell-mediated cytotoxicity and cellular proliferation.

Inhibition of Cytolytic Activity of CTL Clones by Monoclonal Antibodies to MHC Determinants and Lymphocyte Surface Markers. The results in Table II provide strong evidence that these clones are I region–restricted in viral antigen recognition, at least in proliferation. To further test the hypothesis that the cytolytic effector function of the D8, G1, and U5 clones was likewise restricted by Ia antigens, monoclonal antibodies specific for class I and class II MHC determinants were used to block the cytolytic activity of these clones toward A/JAP/57 virus–infected A20-1.11 target cells. As shown in Table V, the monoclonal antibody Do4, which is directed to a public determinant on K^d and D^d gene products (30), specifically inhibited the cytolytic function of the class I MHC–restricted CTL clones A4 and 14-1, but did not affect clones D8, G1, and U5. In contrast, the monoclonal antibody Do3, which recognizes the Ia.7 epitope on I-E$^{d, k}$ (30), only blocked target lysis by clones D8 and G1, whose MHC restriction patterns were tentatively mapped to the I-E^d subregion (Table II). Interestingly, the monoclonal antibody MK-D6, which reacts with I-A^d (31), failed to inhibit the cytolytic function of clone U5, which is restricted by I-A^d in its recognition of viral antigens (Table II). As expected, MK-D6 did not interfere with the cytolytic activity of the I-E^d-restricted clones, D8 and G1.

The L3T4 T cell surface marker, defined by the monoclonal antibody GK1.5, has been reported to be involved in antigen recognition by class II–restricted T cells (32). As shown in Table V, the GK1.5 monoclonal antibody completely blocked the cytolytic activity of the L3T4a$^+$ clones D8, G1, and U5, but not of

TABLE V

Inhibition of Cytolytic Activity by Monoclonal Anti-Ia and Anti-L3T4 Antibodies

Monoclonal antibody	Specificity	Dilution[‡]	Percent specific ^{51}Cr release from A/JAP/57 virus–infected A20-1.11 targets*				
			A4	14-1	D8	G1	U5
—	—	—	18 (6)[§]	26 (2)	20 (1)	49 (1)	32 (0)
Do4	K^d, D^d	1/4	3	2	16	41	30
		1/16	5	5	15	43	31
		1/64	6	7	17	42	32
		1/256	11	16	19	42	34
Do3	I-E^d	1/4	17	26	0	1	28
		1/16	17	26	1	3	32
		1/64	17	26	3	15	29
		1/256	22	30	21	44	37
MK-D6	I-A^d	1/4	19	29	28	53	31
		1/16	20	28	28	53	35
		1/64	20	27	25	52	38
GK1.5	L3T4a	1/4	16	23	1	8	0
		1/16	16	24	14	25	6
		1/64	19	27	24	49	30

The effect of the indicated monoclonal antibodies on the cytolytic activity of two class I MHC–restricted CTL clones (A4 and 14-1) and three class II MHC–restricted CTL clones (D8, G1, and U5) was assayed as described (Materials and Methods).

* 1 × 10^4 target cells were added per well. The effector cell/target cell ratio was 10:1. Spontaneous release from target cells was <17%. Assay time was 6 h.

‡ Culture supernatants of the monoclonal antibodies were diluted as indicated before addition to the assay.

§ Values represent the means of three replicate wells; SEM are <5% of mean values and are omitted. Values in parentheses are the percent specific release of ^{51}Cr-labeled uninfected A20-1.11 target cells by the indicated CTL cloned lines.

the class I–restricted CTL clones A4 and 14-1. This is consistent with the concept that the cytolytic effector activity of these clones is restricted by class II MHC products.

Recognition of Class II MHC Product–Expressing L Cell Transfectants by Class II MHC–restricted CTL Clones. The above result strongly suggests that these clones recognize virus in association with class II MHC molecules expressed on the A20-1.11 cells. To definitively demonstrate that a particular class II MHC product restricts viral antigen recognition by these clones, we examined the capacity of these clones to recognize L cells bearing an appropriate class II MHC product introduced by DNA-mediated gene transfer. Two class II MHC gene transfectant lines were used in this analysis: CA36.2.1, which expresses the E$_\alpha^k$E$_\beta^d$ molecule, and CA36.1.1, which expresses the E$_\alpha^k$E$_\beta^k$ molecule. The susceptibility to cell-mediated lysis of these L cell lines and the control L cell line, K$_2$ (transfected with the herpes virus thymidine kinase gene only), was demonstrated by the ability of the H-2D^k-restricted, type A influenza–specific CTL clone 35-6 to destroy A/JAP/57 virus–infected cells of each L cell transfectant, but not

uninfected or B/Lee virus–infected cells (Table VI). Interestingly, the I-E^d-restricted CTL clones, D8 and G1, recognized A/JAP/57-infected target cells of the E$^k_\alpha$E$^d_\beta$ transfectant, but did not lyse B/Lee-infected or uninfected cells of this L cell transfectant. Furthermore, D8 and G1 did not lyse A/JAP/57-infected or uninfected cells of either the control K2 transfectant or of the E$^k_\alpha$E$^k_\beta$ transfectant (Table VI, Exp. 1). As expected, the I-A^d-restricted CTL clone U5 failed to recognize virus-infected or uninfected CA36.2.1 cells. These findings demonstrate that CTL clones D8 and G1 are restricted by the E$^d_\beta$ chain in their recognition of influenza virus antigens on the surface of infected cells.

Helper Function of CTL Clones. In view of the fact that these CTL populations exhibited the same MHC restriction and growth characteristics as T cells of the helper/inducer subset, we examined the capacity of these class II MHC–restricted CTL clones to provide helper activity in an in vitro antibody synthesis assay. As shown in Table VII, culture supernatants taken 24 h after stimulation of these T lymphocyte clones by A/JAP/57 virus–pulsed, irradiated syngeneic spleen cells promoted an in vitro primary anti-SRBC response by T cell–depleted CB6F₁

TABLE VI

CTL Clones D8 and G1 Are Restricted to the E$^d_\beta$

Exp.	CTL clone	Effector/target ratio	Percent specific ^{51}Cr release from target cells*					
			K₂‡		CA36.2.1 (E$^k_\alpha$E$^d_\beta$)		CA36.1.1 (E$^k_\alpha$E$^k_\beta$)	
			Uninfected	A/JAP/57	Uninfected	A/JAP/57	Uninfected	A/JAP/57
1	35-6	2:1	4	19	15	61	4	51
		0.5:1	1	8	4	29	1	20
	D8	2:1	1	3	8	40	3	5
		0.5:1	0	1	1	12	0	1
	G1	2:1	0	0	6	49	0	2
		0.5:1	0	0	0	16	0	0

			CA36.2.1 (E$^k_\alpha$E$^d_\beta$)		
			Uninfected	A/JAP/57	B/Lee
2	35-6	5:1	3	24	3
		1:1	0	8	0
	D8	5:1	5	24	8
		1:1	0	7	1
	G1	5:1	2	33	3
		1:1	0	12	0
	U5	5:1	5	8	6
		1:1	0	0	1

* As in Table I.
‡ As in Table I. Spontaneous release from each L cell target was <19%.
§ As determined by H-2 restriction in proliferation (47 and Table II), clone 35-6 is restricted by H-2D^k, clones D8 and G1 by I-E^d, and clone U5 by I-A^d.

TABLE VII

Production of B Cell Helper Factors and IL-2 by Class II MHC–restricted T Cell Clones

Culture supernatant*		PFC/culture[‡]	PFC/10^6 cells	Proliferative response of an IL-2-dependent line[§]
Clone	Antigen			
None	–	4 ± 4	47 ± 47	47 ± 9
	+	0	0	28 ± 3
D8	–	7 ± 2	46 ± 13	43 ± 8
	+	65 ± 14	181 ± 39	40 ± 2
G1	–	7 ± 2	31 ± 9	48 ± 8
	+	248 ± 0	506 ± 0	35 ± 5
U5	–	88 ± 2	379 ± 9	42 ± 5
	+	2,448 ± 72	3,060 ± 90	52 ± 6
U11	–	0	0	48 ± 13
	+	437 ± 20	840 ± 38	6,845 ± 290

* 24 h supernatants were harvested from cocultures of cells of the indicated cloned line with irradiated BALB/c spleen cells that were either left untreated (–) or pulsed with inactivated A/JAP/57 virus (+).

[‡] 8 × 10^6 T cell–depleted CB6F$_1$/J spleen cells were cultured in the presence of SRBC and 60% T cell clone culture supernatant. After 5 d, each culture was harvested and assayed in duplicate for an anti-SRBC PFC response (Materials and Methods). Values are presented as PFC ± SEM per culture and PFC ± SEM per 10^6 viable lymphocytes. Supernatant of Con A–pulsed rat splenocytes produced 8,784 ± 108 PFC/culture under these conditions.

[§] IL-2 activity was measured using the IL-2-dependent line 14-7fd as an indicator cell. Values are the mean cpm ± SEM of quadruplicate cultures of 14-7fd cells in the presence of 50% supernatant of the indicated clone.

spleen cells. CTL clone U5 provided the greatest amount of helper activity in this assay system, while CTL clones G1 and D8 promoted a significant but much smaller antibody response. The noncytolytic T cell clone U11 (Table I) also generated helper activity after antigenic stimulation. These results demonstrate that the class II MHC–restricted CTL clones secrete an antigen-nonspecific helper activity after specific antigenic stimulation.

These same supernatants were also analyzed for IL-2 activity based on their capacity to stimulate proliferation of the murine IL-2-dependent T cell line 14-7fd (38). Interestingly, only the supernatant of antigen-stimulated clone U11 contained detectable IL-2 activity. After stimulation with the lectin, Con A, however, all of the class II–restricted clones released significant amounts of IL-2 activity (data not shown).

CTL Clones Mediate Effector Activity by Direct Lysis of Target Cells. Since the previous results demonstrated the release of soluble factors by antigen-stimulated, class II MHC–restricted CTL clones, it was of interest to determine whether their cytolytic activity could also be mediated by a lymphokine. To

investigate this possibility, ^{51}Cr-labeled bystander cells (A20-1.11 cells or P815 cells) were cocultured with various numbers of unlabeled A/JAP/57 virus–infected A20-1.11 cells in the presence of clones D8, G1, or U5. None of these CTL clones (Fig. 1) showed any cytolytic effect on either the bystander A20-1.11 or P815 cells. Furthermore, no killing of these bystander cells by clones D8, G1, or U5 was observed even when unlabeled virus-infected A20-1.11 cells were present in 10-fold excess to the bystander cells. That these CTL clones were indeed recognizing the virus-infected A20-1.11 cells was shown by their capacity to lyse ^{51}Cr-labeled, A/JAP/57-infected A20-1.11 target cells. This experiment strongly suggests that these class II MHC–restricted CTL clones carry out their cytolytic effector function by direct lysis of virus-infected cells, and not by release of a soluble cytolytic factor.

Discussion

This report examines the properties of cloned populations of class II MHC–restricted, influenza virus–specific CTL. In addition to antigen-specific cytolytic effector activity, these T cell clones share several characteristics with T cells of the helper/inducer subset: (a) Lyt-1^{+}2^{-}, L3T4^{+} cell surface phenotype; (b) I region–restricted recognition of nominal antigen; (c) proliferation in the absence of exogenous IL-2; and (d) release of factors that help B cell responses. The class II–restricted CTL clones also demonstrated different patterns of type A influenza virus recognition that, for each clone, were identical for both cellular proliferation and cell-mediated cytotoxicity. Furthermore, these clones mediated their cytotoxic effector function by direct lysis of virus-infected cells.

Using L cell lines expressing transfected class II MHC gene products as target cells, we have unambiguously identified the I-E^{d} molecule as the restriction element for two virus-specific CTL clones. Presentation of influenza viral antigens by transfected L cells confirms, at the level of target cell recognition, reports of Malissen et al. (40) and Norcross et al. (19) that expression of Ia antigens at
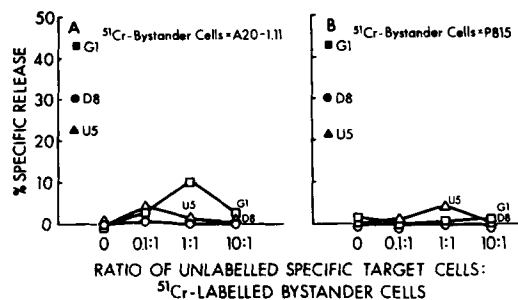

FIGURE 1. Class II MHC–restricted CTL clones do not lyse bystander cells. CTL clones G1 (□), D8 (O), and U5 (△) were tested for cytolytic activity on ^{51}Cr-labeled bystander cells, (A) A20-1.11 cells or (B) P815 cells in the presence of increasing numbers of unlabeled specific target cells (A/JAP/57 virus–infected A20-1.11). The ratio of clones to ^{51}Cr-labeled cells is 2 to 1; 1 × 10^{4} ^{51}Cr-labeled cells were added per well. The cytolytic activity of the CTL clones on ^{51}Cr-labeled specific target cells (A/JAP/57-infected A20-1.11) is indicated by the closed figures (G1 ■, D8 ●, U5 ▲). Cloned cells were used 7 d after routine subculturing, and were separated from dead cells on Ficoll/metrizoate gradients before addition to wells. Spontaneous release was 30% for A20-1.11 cells and 6% for P815 cells. Assay time was 6 h. Values are the means of triplicate wells; SEM, always <5%, are omitted for clarity.

the surface of transfected L cells confers class II MHC–restricted antigen-presenting capacity to these nonlymphoid cells. Furthermore, recognition by the I-E^d-restricted CTL clones of virus-infected cells of an E$_\alpha^k$E$_\beta^d$ but not E$_\alpha^k$E$_\beta^k$ L cell transfectant implicates the E$_\beta^d$ chain as the restriction element for these clones. Localization of the restriction element for these clones, however, is complicated by the finding that the monoclonal antibody Do3, which specifically blocks cytolytic activity by these clones, reacts with specificity Ia.7, a complex determinant on the E$_\alpha$ chain (41). Since others (42, 43) have reported discrepancies between serologically defined epitopes on Ia antigens and epitopes on Ia antigens recognized by T cells, monoclonal antibody–blocking studies may in some instances represent a less satisfactory approach for mapping MHC restriction specificities than recognition of transfected cell lines expressing MHC gene products. Similarly, the failure of the anti-I-A^d monoclonal antibody MK-D6 to block cytolytic activity of an I-A^d-restricted CTL clone may indicate that this clone is restricted by a nonoverlapping epitope on the I-A^d molecule. Thus, recognition of the virus-infected E$_\alpha^k$E$_\beta^d$ transfectant clearly implicates the E$_\beta^d$ chain as the important restriction element in viral antigen recognition by the I-E^d-restricted CTL clones. This restriction element may be solely on the E$_\beta^d$ chain or it may be formed by association with the E$_\alpha$ chain. At present neither possibility can be excluded. In the latter case, it is evident that an E$_\alpha$ chain of either H-2^d or H-2^k origin can associate with the E$_\beta^d$ chain to generate the restriction site.

An important finding is that the class II MHC–restricted CTL clones appear to directly lyse virus-infected cells rather than via the release of a soluble cytolytic factor. These CTL exert no cytolytic effect on uninfected bystander cells after recognition of cells expressing appropriate viral determinants. In this respect, the nature of the cytolytic effector function of the class I–restricted and class II–restricted CTL clones is identical. These observations differ from the recent results of Tite and Janeway (18) who demonstrated that I region–restricted, ovalbumin-specific CTL clones mediate their effector activity through a cytolytic lymphokine. It appears unlikely that the cytolytic effector activity of CTL clones reported here is due to antigen-nonspecific diffusable lymphokines. Further studies are in progress to better define the mechanism of target cell lysis by these class II–restricted, influenza-specific CTL.

In this report we have also demonstrated that the class II–restricted CTL clones secrete a soluble B cell helper activity after specific antigenic stimulation. This finding is in agreement with the observations of Dennert et al. (9, 10) that a class II MHC–alloreactive CTL line can provide nonspecific help for antibody production in vitro. In addition, the class II–restricted CTL clones described here may exhibit heterogeneity in their capacity to help antibody responses. Based on the yield of PFC per culture and total B cell recovery, several of these CTL clones appear to release factors that preferentially induce proliferation of B cells over differentiation to antibody-secreting cells, whereas other clones secrete factors that convert a larger percentage of B cells to antibody-secreting cells. Similar clonal variability was observed for antigen-dependent IL-2 release by the clones. It is not certain whether our failure to detect IL-2 activity in supernatants of the CTL clones reflects an IL-2-independent pathway for proliferation or consumption of released IL-2 by an autocrine mechanism (44). Since

all of these class II–restricted clones can produce IL-2 in response to mitogenic stimulation by Con A, and appear to express cell surface receptors for IL-2 (A. M. Churilla, personal communication), the autocrine pathway for endogenous IL-2 consumption is the most likely explanation for this result.

Among the panel of five class II MHC–restricted T lymphocyte clones examined in this report, specific cytolytic activity was exhibited by three of them. Preliminary analysis of other Lyt-2⁻, L3T4⁺ clones indicates that a significant proportion of these clones exhibit class II–restricted, influenza virus–specific cytolytic activity. Thus it is possible that virus-specific, class II MHC–restricted CTL represent a substantial component of the cellular immune response during viral infection. In the mouse, class II MHC–restricted antiviral CTL may have gone undetected largely due to the use of Ia antigen–negative target cells to analyze T cell cytolytic activity (45). In humans, the use of Ia⁺ lymphoblastoid cell lines as targets allowed our and other laboratories to detect class II MHC–restricted antiviral CTL clones (20–22). Also, it does not appear that cytolytic activity is a property only of class II–restricted, influenza-specific T lymphocytes maintained in long-term culture. In preliminary studies, H-2I region–restricted killing is detectable in cultures of influenza-immune spleen cells shortly after in vitro stimulation with virus (L. A. Morrison, unpublished observations).

Two of the five class II–restricted clones examined in this report, U2 and U11, did not lyse virus-infected A20-1.11 targets. This lack of cytolytic activity was not a consequence of their inability to recognize this target cell since virus-infected A20-1.11 cells could specifically stimulate proliferative responses from both the cytolytic and noncytolytic class II–restricted clones (data not shown). Like the CTL clones, these noncytolytic T cells expressed the Thy-1.2⁺, Lyt-1⁺2⁻, L3T4⁺ cell surface phenotype, proliferated in response to antigen in the absence of exogenous IL-2, secreted soluble nonspecific helper activity upon antigenic stimulation, and exhibited a crossreactive pattern of type A influenza viral antigen recognition. Taken together, these findings suggest that class II–restricted, virus-specific T lymphocytes, like class II–alloreactive T lymphocytes (9), may consist of functionally discrete cytolytic and noncytolytic subsets. This distinction provides a possible explanation for differences observed in the effect of Lyt-1⁺ (L3T4⁺) T lymphocytes on the outcome of viral infection in vivo. Whether a heterogeneous population of Lyt-1⁺ T helper/inducer cells promotes recovery from viral infection (46) or enhances tissue injury as a result of its antiviral effect (34), could depend on the relative proportion of class II–restricted cytolytic and noncytolytic clones in the cell innoculum. The relative proportions of noncytolytic and cytolytic, influenza virus–specific, class II–restricted T lymphocytes generated in response to immunization and infection is currently not known. Experiments are underway to determine their frequencies.

The demonstration in this report of cloned populations of class II–restricted, influenza virus–specific murine T lymphocytes with properties of both T helper lymphocytes and conventional CTL, along with recent findings from other laboratories (8, 10, 18, 19), suggest that the distinction between T lymphocytes of these two subsets is not clear-cut. More importantly, our findings raise questions regarding the relationship of class I– to class II–restricted, virus-specific CTL, both in terms of their in vivo roles in recovery from viral infection and

their requirements for target cell recognition. Studies currently in progress indicate that cloned populations of class II–restricted CTL can, upon adoptive transfer, promote recovery of syngeneic recipient mice from lethal influenza virus pneumonia in a manner similar to that of class I–restricted antiinfluenza CTL clones (28). Moreover, class II–restricted CTL appear to differ markedly from class I–restricted CTL in their requirements for influenza viral antigen presentation in target cell recognition (manuscript in preparation). The availability of virus-specific, class II MHC–restricted CTL clones should enable a detailed characterization of the repertoire of viral antigenic specificities, the regulation of expression of effector functions, and the in vivo activities of this T lymphocyte subpopulation.

Summary

Among murine class II major histocompatibility complex (MHC)–restricted cytotoxic T lymphocyte (CTL) clones specific for type A influenza virus, we have identified both noncytolytic clones and clones exhibiting H-2 I region–restricted cytolytic activity. After appropriate antigenic stimulation, both cytolytic and noncytolytic clones proliferated in the absence of exogenous interleukin 2. All of the clones possess the Thy-1.2$^+$, Lyt-1$^+$2$^-$, L3T4$^+$ phenotype. The class II MHC restriction of viral recognition by the CTL clones was mapped by proliferation using recombinant mouse strains and by inhibition of cytotoxic activity with monoclonal antibodies directed to class II MHC products and L3T4a. The restriction specificity of two CTL clones was unambiguously assigned to the E_β^d chain by using L cell transfectant lines expressing $E_\alpha^k E_\beta^d$ or $E_\alpha^k E_\beta^k$ gene products. Analysis of the viral specificity of the cloned lines revealed subtype-specific and crossreactive patterns of viral antigen recognition; the pattern of viral antigen specificity exhibited by each clone in proliferation and cell-mediated cytotoxicity was identical. Each CTL clone also demonstrated antigen-dependent release of helper factor(s) that promoted in vitro primary anti-SRBC responses. Finally, the cytotoxic effector function of the class II MHC–restricted CTL clones was mediated by direct lysis of virus-infected cells, and not by secretion of a cytolytic lymphokine.

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