Brief Definitive Report

SIMULTANEOUS EXPRESSION OF H-2-RESTRICTED AND ALLOREACTIVE RECOGNITION BY A CLONED LINE OF INFLUENZA VIRUS-SPECIFIC CYTOTOXIC T LYMPHOCYTES*

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Alloreactive T lymphocytes comprise an extremely high proportion of the total pool of mature T lymphocytes (1, 2), i.e., from 1-10% of peripheral T cells can be stimulated by a single major histocompatibility complex (MHC) haplotype difference (3, 4). In contrast, the frequency of T cells directed to non-MHC foreign antigen is several orders of magnitude lower (4). To explain the high frequency of alloreactive T cells in the face of apparent T cell responsiveness to the rest of the antigenic universe, it has been postulated that these two T cell subsets are overlapping (2, 5). Indeed several different lines of evidence have emerged which support this notion (5-7). Further support of this hypothesis has come from recent studies employing cloned continuous T cell lines (8, 9).

Recently during an analysis of the specificity of cloned continuous lines of murine cytotoxic T lymphocytes (CTL) directed against type A influenza viruses, we isolated a cloned virus-specific CTL line with apparent alloreactive cytotoxicity (10). In this report we demonstrate that this cloned CTL line of $(H\text{-}2^b \times H\text{-}2^d)F_1$ origin is not only cytotoxic for influenza virus-infected target cells expressing the $H\text{-}2^d$ haplotype, but is also specifically cytotoxic for cells expressing the $H\text{-}2K^k$ alloantigen. Cold target inhibition analysis demonstrates that both H-2-restricted and alloreactive cytotoxicity are mediated by the same cell population. Furthermore, analysis of the proliferative response of this clone in vitro indicates that both influenza virus-infected cells expressing the $H\text{-}2^d$ haplotype and uninfected cells of the $H\text{-}2^k$ haplotype are able to specifically stimulate proliferation of this line. The significance of these observations is discussed.

Materials and Methods

Animals. BALB/cByJ ($H\text{-}2^d$), C57BL/6J ($H\text{-}2^b$), CBA/J ($H\text{-}2^k$), and (C57BL/6 × BALB/c)F_1 ($H\text{-}2^b \times H\text{-}2^d$) mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. B10.A (5R) ($H\text{-}2K^bD^d$), B10.LG ($H\text{-}2K^dD^f$), B10.BDR ($H\text{-}2K^dD^b$), B10.M ($H\text{-}2^f$), B10.BAR4 ($H\text{-}2K^kD^b$), and B10.OH ($H\text{-}2K^dD^k$) mice were generously provided by Dr. Vera Hauptfeld (Department of Genetics, Washington University, St. Louis, Mo.).

Viruses. Influenza virus strans A/PR/8 (A/Puerto Rico/8/34) (H_0N_1), A/JAP/57 (A/Japan/305/57) (H_2N_2), A/AA/67 (A/Ann Arbor/7/67) (H_2N_2), and B/Lee were grown as described (11).

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Cloned CTL Lines. CTL lines 14-2, 11-1, and 14-13 were isolated and maintained as described in detail elsewhere (10).

Target Cells and Assays for Cell-mediated Cytotoxicity. P815 (H-2^d) and L929 (H-2^k) cell lines, maintained in vitro, and concanavalin A (Con A)-stimulated splenic lymphoblasts were used as targets. Infection of target cells, ^{51}Cr-release assays, and determinations of percent specific ^{51}Cr release were carried out as described previously (11). Cold target inhibition assays were performed as described previously (11).

Assay of Cellular Proliferation. The proliferative response of CTL line 14-2 was assessed by [^{3}H]thymidine ([^{3}H]TdR) incorporation at 3, 4, and 5 d of culture. After routine subculture (10) a portion of line 14-2 was transferred at 0.5×10^6 viable cells/ml to medium containing 10% T cell growth factor TCGF only and cultured at 37°C for 4–5 d. 1×10^4 viable cells were then cultured at 37°C in individual wells of 96-well microtiter plates with 10^6 irradiated stimulator spleen cells of the indicated type in medium supplemented with 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 10% (vol:vol) crude TCGF of rat spleen origin. At 3, 4, and 5 d of culture, the proliferative response was assessed by [^{3}H]TdR incorporation (1 μCi/well) in a 4-h assay. Results are the mean incorporation from quadruplicate cultures.

Results

Table I demonstrates the expression of alloreactive cytotoxicity for uninfected L929 (H-2^k) target cells and virus-specific cytotoxicity for A/Jap/57-infected P815 (H-2^d) target cells by the cloned CTL line 14-2. This clone which was derived from an A/JAP/57-immune CTL precursor of (H-2^b × H-2^d)F_1 origin was shown previously to be restricted in virus recognition to the K end of the H-2^d haplotype and recognizes only viral antigenic determinants expressed on A/JAP/57-infected target cells (10). Also included in Table I are the patterns of cytotoxicity exhibited by two other cloned CTL lines, 11-1, 14-13, likewise of (H-2^b × H-2^d)F_1 origin. Although both of these lines are restricted in influenza virus recognition to the H-2^d haplotype, and one line, 11-1, has an identical pattern of virual antigen and H-$2K^d$ restriction, neither line exhibited detectable cytotoxicity for L929 cells.

In light of the capacity of clone 14-2 to lyse L929 target cells it was of interest to determine if other cells expressing the H-2^k locus products were likewise susceptible to lysis, and if so, whether the recognition was specific for the K or D end of the H-2^k haplotype. The results of this analysis are shown in Table II. In addition to L929 fibroblasts, clone 14-2 efficiently lysed lymphoblast targets of the H-$2K^kD^k$ (CBA/J) and H-$2K^kD^b$ (B10.BAR4) haplotypes. No significant lysis was observed on H-$2K^dD^k$ (B10.OH) target cells, parental H-2^b and H-2^d target cells, or target cells of the unrelated H-2^f (B10.M) haplotype. These findings indicate that clone 14-2 recognizes determinants encoded in the H-$2K^k$ region.

To directly determine if killing of virus-infected H-2^d targets and uninfected H-2^k targets was mediated by a single population of CTL the cytotoxic activity of line 14-2 was tested in a cold target competition protocol using unlabeled virus-infected P815 cells and unlabeled L929 cells as inhibitors. As Table III demonstrates both competitor cells efficiently inhibited the killing of labeled infected P815 cells and labeled L929 cells. This finding indicates that the same cell population is mediating both H-2-restricted and alloreactive cytotoxicity.

We have observed that influenza virus-specific CTL lines isolated in the presence of both TCGF and influenza-infected syngeneic spleen cells require both TCGF and a specific antigenic stimulus for optimal proliferation and continued propagation (T. J. Braciale, M. E. Andrew, and V. L. Braciale, manuscript in preparation). Further-

TABLE I

*Expression of Virus-specific and Alloreactive Cytotoxicity by a Cloned Continuous Line of Influenza Virus-specific CTL**

CTL line	Effector:target cell ratio§	Percent specific ^{51}Cr release from target cells‡					
		P815 (H-2^d)					L929 (H-2^d) Uninfected
		Uninfected	A/PR/8 (H_0N_1)	A/JAP/57 (H_2N_2)	A/AA/67 (H_2N_2)	B/Lee	
14-2	0.5:1	0	0	15	0.6	0	8
	1:1	0	0	26	1	0	14
	2.5:1	0	0.6	54	2	0	27
11-1	0.5:1	0	2	23	4	2	0
	1:1	3	6	36	6	3	0
	2.5:1	8	9	50	10	11	0
14-13	0.5:1	0	34	17	23	1	0.4
	1:1	0	49	36	42	2	0.8
	2.5:1	1	65	47	53	4	0.3

* CTL lines were examined for cytotoxic activity on uninfected and infected ^{51}Cr-labeled target cells 3–5 d after routine subculturing in the presence of 10% TCGF and infected irradiated syngeneic spleen cells. Assay time was 6 h.

‡ Values are the means from four replicate wells with spontaneous release subtracted. Spontaneous release from all target groups was <20%. SEM, <5% of mean values in all cases, are omitted.

§ 1×10^4 target cells were added per well.

TABLE II

*H-2-End Restriction of Alloreactive Cytotoxicity**

Effector:target cell ratio§	Percent specific ^{51}Cr release from target cell‡						
	L929 (K^kD^k)	CBA/J (K^kD^k)	B10.BAR 4 (K^kD^b)	B10.OH (K^dD^k)	B10.M (K^fD^f)	C57BL/6 (K^bD^b)	BALB/c (K^dD^d)
1:1	21	28	22	1	3	1	0
5:1	48	72	45	0	0	1	0
10:1	56	81	60	0	4	0	0

* As in Table I except that the assay time was 5 h.

‡ As in Table I. In addition to L929 fibroblasts, the target cells were Con A-induced lymphoblasts from the indicated mouse strain. Spontaneous release values ranged from 15.5% (L929 fibroblasts) to 38% (CBA/J lymphoblasts).

§ As in Table I.

more, for a given line the pattern of H-2-restricted viral antigen recognition expressed at the level of target cell recognition was identical to the antigenic requirements for cell proliferation. This finding provided an opportunity to examine the capacity of cells expressing the H-2^k haplotype to stimulate proliferation of clone 14-2. Results of such an analysis using [^{3}H]TdR incorporation as an assay of proliferation are shown in Table IV. Significant proliferation of the clone was observed only with A/Jap/57-infected stimulator cells that expressed H-2K^d-region products and with uninfected H-2K^k stimulator cells. Thus the pattern of antigen recognition exhibited by clone 14-2 at the target cell level was likewise reflected in the antigenic requirement for proliferation of the line.

The finding that irradiated cells of the H-2^k haplotype could stimulate proliferation of line 14-2 suggested that the line could be propagated in the presence of H-2^k cells. The line was therefore subcultured in the presence of irradiated CBA spleen cells plus 10% TCGF and was efficiently propagated for up to 60 d (8–10 passages). When tested for cytotoxic specificity at the end of this period, the line maintained an identical pattern of virus-specific and alloreactive cytotoxicity as observed above

TABLE III

*Inhibition of H-2-restricted and Alloreactive Cytotoxicity by Cold Target Competitors**

Unlabeled competitor cells	Competitor:target cell ratio§	Percent specific ^{51}Cr release from labeled targets‡	
		P815 infected	L929 uninfected
—	—	51‖	28‖
P815 uninfected	1:1	53	28
	5:1	53	27
	10:1	54	28
	20:1	51	23
P815 infected	1:1	46	17
	5:1	32	6
	10:1	24	3
	20:1	17	2
L929 uninfected	1:1	49	20
	5:1	39	10
	10:1	34	7
	20:1	26	4

* Cold target inhibitions were carried out as described (11) using line 14-2 as the source of effector cells.

‡ As in Table I.

§ As in Table I. The effector cell:target cell ratio was 2.5:1.

‖ Values are the percent specific ^{51}Cr release in the absence of unlabeled competitors.

TABLE IV

*Stimulation of Cellular Proliferation by Syngeneic Virus-infected and Allogeneic Stimulator Cells**

Experiment 1		Experiment 2		Experiment 3	
Stimulator cells	[^{3}H]TdR uptake‡	Stimulator cells	[^{3}H]TdR uptake‡	Stimulator cells	[^{3}H]TdR uptake‡
—§	997 ± 262	(H-2^b × H-2^d)F$_1$ + A/JAP/57	7,939 ± 70	(H-2^b × H-2^d)F$_1$ + A/JAP/57	33,500 ± 1,991
(H-2^b × H-2^d)F$_1$‖	362 ± 204	BALB/c (H-2^d) + A/JAP/57	21,539 ± 1,500	CBA (H-2^k)‖	38,044 ± 951
(H-2^b × H-2^d)F$_1$ + A/JAP/57	15,774 ± 1,389	C57BL/6 (H-2^b) + A/JAP/57	468 ± 135	B10.BR (H-2^k)‖	38,790 ± 3,912
(H-2^b × H-2^d)F$_1$ + A/PR/8	594 ± 174	B10.A(5R) (H-2K^{b}D^d) + A/JAP/57	241 ± 20	B10.BAR4 (H-2K^{k}D^b)‖	48,004 ± 2,593
(H-2^b × H-2^d)F$_1$ + A/AA/67	610 ± 117	B10.LG (H-2K^{d}D^f) + A/JAP/57	5,155 ± 736	B10.OH (H-2K^{d}D^k)‖	653 ± 138
(H-2^b × H-2^d)F$_1$ + B/Lee	684 ± 199	B10.BDRI (H-2K^{d}D^b) + A/JAP/57	7,839 ± 86		
BALB/c (H-2^d) + A/JAP/57	33,844 ± 999	CBA (H-2^k)‖	23,539 ± 1,938		
C57BL/6 (H-2^b) + A/JAP/57	647 ± 52				
CBA (H-2^k)‖	33,118 ± 2,594				

* The proliferative response of CTL line 14-2 was examined as described in Materials and Methods.

‡ Values are the mean cpm ± SEM of [^{3}H]TdR incorporation for quadruplicate determinations taken at the peak of the response (day 4).

§ Indicates proliferative response in the presence of TCGF alone.

‖ Indicates irradiated uninfected stimulator spleen cells.

(Table I). In light of this result, line 14-2 passaged in the presence of A/JAP/57-infected irradiated F$_1$ spleen cells was recloned under limiting-dilution conditions in the presence of irradiated uninfected CBA spleen cells and TCGF. Six such subclones were expanded in the presence of irradiated CBA spleen cells and tested for cytotoxic specificity. All subclones retained the same specificity as the parental line (not shown).

Discussion

Our results demonstrate that a cloned line of CTL that exhibit H-2 restricted recognition of an influenza viral determinant can simultaneously express alloreactiv-

ity. Cold target inhibition data provide direct evidence that both H-2-restricted and alloreactive recognition is mediated by the same cell. Further support for the clonal origin of this CTL population is that this line of $(H\text{-}2^b \times H\text{-}2^d)F_1$ is restricted in virus recognition to the K end of the $H\text{-}2^d$ haplotype and, in contrast to heterogeneous populations of anti-influenza CTL (11), is directed exclusively to a determinant on the immunizing (selecting) influenza strain. Also, the findings that this line can be continuously propagated in response to either the appropriate viral plus self-MHC structure or the appropriate alloantigen without any change in cytotoxic specificity and that subclones derived from this line retained both H-2-restricted and alloreactive specificity strongly suggest that a homogeneous CTL population mediates both types of recognition.

These results are in agreement with the observations of von Boehmer et al. (8) who reported the expression of specific alloreactive cytotoxicity for $H\text{-}2D^d$ by a cloned line of $H\text{-}2D^b$-restricted male (H-Y) antigen-specific CTL. Our finding that the relevant alloantigen can specifically stimulate proliferation of this clone in the absence of viral antigen suggests that this cross-reactivity between viral antigen plus self-MHC products and a specific alloantigen also extends beyond the level of target cell recognition to stimulation of cell division and presumably to specific CTL activation. This conclusion is supported by the recent results of Sredni and Schwartz (9) who showed that a clone of noncytotoxic T cells directed to a soluble antigen (dinitrophenyl-ovalbumin) in conjunction with self-MHC products ($H\text{-}2I^k$) could likewise be stimulated to proliferate by a specific alloantigen ($H\text{-}2I^s$).

Our results as well as those of von Boehmer et al. and Sredni and Schwartz provide strong evidence that there are specific T cells that can simultaneously recognize alloantigen as well as foreign antigens restricted by self-K/D- or I-region products. These observations made at the clonal level therefore directly support previous observations made with heterogeneous T cell populations (5-7). Mechanisms to explain this overlap between MHC-restricted and alloreactive T cells have been discussed elsewhere (2, 6, 7, 9). Perhaps a fundamental issue raised by all of these observations is the degree of overlap between the H-2-restricted and alloreactive T cell populations. Experiments are now in progress to determine the degree of this overlap.

Summary

Based on theoretical considerations and several types of experimental evidence with heterogeneous cell populations it has been proposed that alloreactive T cells and major histocompatibility complex (MHC)-restricted T cells directed to foreign non-NHC antigens represent overlapping subsets. In this report we provide direct evidence for this hypothesis at the clonal level. We have isolated a cloned continuous influenza virus-specific cytotoxic T cell (CTL) line derived from a single $(H\text{-}2^b \times H\text{-}2^d)F_1$ CTL precursor which simultaneously exhibits H-2-restricted cytotoxicity of influenza A/Japan/305/57 virus in association with $H\text{-}2K^d$ and alloreactive cytotoxicity for $H\text{-}2K^k$ alloantigen. Cold target inhibition data demonstrate that both MHC-restricted and alloreactive recognition is mediated by the same cell population. In addition to cross-reactivity at the target cell level, we show that this cloned CTL line can be specifically stimulated to proliferate either by A/Japan/305/57 virus-infected cells expressing $H\text{-}2K^d$ or by uninfected cells of the $H\text{-}2K^k$ haplotype.

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