HETEROGENEITY AND SPECIFICITY OF
CLONED LINES OF INFLUENZA-VIRUS-
SPECIFIC CYTOTOXIC T LYMPHOCYTES*

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An essential element in our current understanding of the structure of the immunoglobulin molecule, the organization of the immunoglobulin gene complex, and the regulatory influences on B lymphocyte function has been the availability of monoclonal antibody populations derived from myeloma protein-producing plasmacytomas. In contrast, progress in the study of the antigen recognition structures on T lymphocytes and the factors that regulate T lymphocyte function has proceeded more slowly, probably in part as a result of the necessity of using heterogeneous T lymphocyte populations for these analyses. However, recent technological advances in the long-term culturing and maintenance of T lymphocytes in vitro (1) now offer the possibility of analyzing homogeneous populations of T lymphocytes derived from individual T lymphocyte precursors. Several different groups of investigators have isolated continuous cloned lines of T lymphocytes with functional properties characteristic of antigen-specific proliferating T lymphocytes (2, 3), alloreactive cytotoxic and amplifier T lymphocytes (4), helper T lymphocytes for humoral responses (5, 6), and major histocompatibility complex (MHC)[1]-restricted cytotoxic T lymphocytes (CTL) directed to hapten and male (H-Y) antigens (7, 8).

We have employed this technology in an analysis of the cytotoxic T lymphocyte (CTL) response to type A influenza viruses and have derived a series of continuous cloned lines of H-2-restricted CTL directed to influenza virus. In this report we examine the patterns of H-2 restriction and the specificity of viral antigen recognition exhibited by lines obtained from $(H-2^b \times H-2^d)F_1$ and parental $(H-2^b, H-2^d)$ precursors. We show that lines of F_1 origin are restricted in their recognition to infected target cells of either parental haplotype. Similarly CTL lines of both F_1 and parental origin appear to be restricted to either the H-2K or the H-2D end of the appropriate responding H-2 haplotype. Analysis of the viral antigen specificity of these lines reveals several distinct patterns of H-2-restricted viral recognition, suggesting both heterogeneity and specificity in the CTL response to influenza virus at the clonal level. Data on the expression by these lines of the Thy-1.2, Lyt-1, and Lyt-2 antigenic markers is also presented.

Materials and Methods

Animals. Male BALB/cByJ (H-2^d), C57BL/6J (H-2^b), and CB6F$_1$/J (BALB/cJ × C57BL/6J) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine and used at 7–14 wk of age.

Viruses. Influenza virus strains A/PR/8 (A/Puerto Rico/8/34 [H$_0$N$_1$]), A/JAP/57 (A/Japan/305/57 [H$_2$N$_2$]), A/AA/67 (A/Ann Arbor/7/67 [H$_2$N$_2$]), A/JAP/BEL (A/Japan/305/57 × A/Bellamy/42 [H$_2$N$_1$]), A/X7-F$_1$, (A/NWS/43 × A/RI/5/57 [H$_0$N$_2$]), A/PC/75 (A/Port Chalmers/75 [H$_3$N$_2$]), and B/Lee were grown in the allantoic cavity of 10-d-old embryonated hen's eggs and stored as infectious allantoic fluid as previously described (9).

Cell Lines. The P815 (H-2^d), L929 (H-2^k), and MC57G (H-2^b) cell lines were maintained in culture in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with antibiotics and 10% newborn bovine serum (K. C. Biologicals, Inc., Lenexa, Kans.). The continuous fibroblast cell line HTGSV (H-2 K^{d}D^b) was established by transforming kidney cells from mouse strain B10.HTG with simian virus 40 (SV-40) virus and was kindly provided by Dr. Peter Doherty of the Wistar Institute, Philadelphia, Pa. These lines served as target cells in cytotoxicity assays.

Immunizations. Mice at 7–12 wk of age were innoculated i.v. with 100–300 hemagglutinating units of infectious A/JAP/57 or A/PR/8 virus as infectious allantoic fluid as described (9). Mice were used as responder spleen cell donors 3 or more wk after immunization.

In Vitro Secondary and Tertiary Responses. Spleen cell suspensions from immune donors were prepared as described previously (9). Usually, 100 × 10^6 immune spleen cells were cultured with 20 × 10^6 virus-infected stimulator cells in 75-cm^2 tissue culture flasks containing 40 ml of minimal essential medium supplemented with 10% fetal bovine serum (FBS) (K. C. Biologicals, Inc.), antibiotics, and 5 × 10^5M 2-mercaptoethanol (2-ME). Stimulator cells were normal syngeneic spleen cells that had received 2,000 rad of irradiation from a cesium source before virus infection. Infection of stimulator cells was carried out as previously described (9). Cultures were maintained in a humid atmosphere of 7% CO$_2$ at 37°C until harvesting.

Tertiary cultures were established by harvesting secondary cultures at 15–18 d of incubation. Dead cells were removed according to the method of Davidson and Parish (10). Viable cell recovery at this time was usually 8–10% of starting cell number. Viable cells were restimulated as above at a responder cell:stimulator cell ratio of 1:10. Usually, 5× 10^5 viable responder cells were cultured with 5 × 10^6 virus-infected syngeneic irradiated spleen cells in 2 ml of medium in multiwell tissue culture plates (24-well plates; 76-033-05; Linbro Chemical Co., Hamden, Conn.).

At the indicated times after secondary or tertiary stimulation (see below) viable cultured cells were used as a source of clonal precursors for CTL-line generation.

Establishment and Maintenance of CTL Lines. Two general cloning methods were employed: limiting-dilution and colony formation in soft agar. In the limiting-dilution approach, spleen cells obtained directly from immune mice or viable cells taken from secondary or tertiary cultures at various times after in vitro stimulation (Table I) were cultured in individual wells of 96-well flat-bottomed microtiter plates (76-003-05; Linbro Chemical Co.) with 1 × 10^6 syngeneic, virus-infected, irradiated (2,000 rad) stimulator spleen cells in 0.2 ml of medium. This medium was Iscove's modified Dulbecco's medium (430-2200; Grand Island Biological Co.) supplemented with 10% FBS (Sterile System Inc., Logan, Utah), 5 × 10^{-5} M 2-ME and 10% (vol:vol) crude rat T cell growth factor (TCGF). Precursor cells were simultaneously cultured at five to seven different cell concentrations in replicates of 48–96 wells. Cell concentration ranges were from 10^3 to 10^4 cells/well for spleen cells directly from primed donors; 10^2 to 10^4 cells/well for cultured cells taken 48 h after in vitro secondary stimulation; 0.5 to 20 cells/well for cultured cells taken 15–18 d after in vitro secondary stimulation and on day 3 of in vitro tertiary stimulation. Clusters of proliferating cells usually appeared in the cloning wells after 6–10 d of incubation. Estimates of precursor frequency (Table I) were based on visual observation of the number of wells containing proliferating cell clusters at each responder cell dilution and were computed by the Poisson method (11). Proliferating cell clusters were chosen for further expansion only when the frequency of positive wells at a given responder cell dilution was <20% (usually <10–15%). Also, wells containing more than one distinct cell cluster at these limiting-dilutions were not selected for further expansion. These

TABLE I

Isolation of CTL Lines

CTL line	Haplotype of origin	Selecting virus*	Day of iso-lation after stimulation in vitro‡	Isolation procedure	Precursor frequency (proliferat-ing cells)§
11-1	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	18	Soft agar	ND‖
13-1	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	18	Soft agar	ND
14-1	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	0	Limiting-dilution	1/9,600
14-2	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	0	Limiting-dilution	1/9,600
14-7	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	2	Limiting-dilution	1/2,100
14-10	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	18	Limiting-dilution	1/24
14-12	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	18	Limiting-dilution	1/24
14-13	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	18	Limiting-dilution	1/24
14-15	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	3 (3°)	Limiting-dilution	1/5
14-16	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	3 (3°)	Limiting-dilution	1/5
14-17	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	3 (3°)	Limiting-dilution	1/5
20-2	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	18	Soft agar	ND
20-15	$(H\text{-}2^b \times H\text{-}2^d)F_1$	A/JAP/57	18	Soft agar	ND
8-1	$H\text{-}2^d$	A/PR/8	3 (3°)	Limiting-dilution	ND
8-4	$H\text{-}2^d$	A/PR/8	3 (3°)	Limiting-dilution	ND
12-12	$H\text{-}2^b$	A/PR/8	3 (3°)	Limiting-dilution	ND

* Indicates type A influenza strain used for priming in vivo, subsequent in vitro stimulation, and, in the case of isolation by limiting-dilution, used for infection of stimulator cells during cloning in microwells.
‡ Viable splenic lymphocytes were isolated directly from immune donors (0), at 2 d (2), or 18 d (18) after in vitro secondary stimulation or at 3 d after in vitro tertiary stimulation [3 (3°)].
§ Values are the reciprocal of the splenic lymphocyte number added per microwell at which 37% of total culture wells were negative for proliferating cell clusters according to the Poisson distribution. Frequencies of proliferating colonies after soft-agar cloning were not determined.
‖ Not determined.

measures were taken to insure that the expanded lines were the progeny of individual clonal precursors.

Clones were expanded by transferring the contents of positive wells to individual wells of 24-well tissue culture plates (76-033-05; Linbro Chemical Co.) containing 5×10^6 irradiated (2,000 rad) syngeneic, virus-infected stimulator spleen cells in 2 ml of Iscove's medium supplemented with 10% FBS, 5×10^{-5} M 2-ME and 10% TCGF. After 5–7 d, the contents of wells containing proliferating cells (usually 30–50% of wells seeded) were transferred to individual wells of six-well cluster tissue culture plates (3506; Costar, Data Packaging, Cambridge, Mass.) containing 20×10^6 irradiated syngeneic virus-infected stimulator spleen cells in 5 ml of Iscove's medium supplemented as above. The cell lines were then maintained by serial passage every 3–5 d in six-well tissue culture plates containing irradiated syngeneic infected stimulator spleen cells in the presence of medium supplemented with 10% TCGF. For routine passage and maintenance, the cell lines were seeded at a concentration of 2×10^4–5×10^4 cells/ml and reached stationary concentrations of 1×10^5–1×10^6 cells/ml 3–5 d later.

Cloning in soft agar was carried out as described by von Bohmer et al. (7) using viable cells derived from in vitro secondary stimulations 15–21 d after culturing. Colonies of 20–50 proliferating cells were visible 7–10 d after plating in soft agar. Colonies were picked with a drawn-out pasteur pipette and transferred to individual wells of 96-well microtiter plates containing 1×10^6 irradiated syngeneic, virus-infected stimulator spleen cells in 0.2 ml RPMI-1640 (K. C. Biologicals, Inc.) supplemented with 10% FBS, 5×10^{-5} M 2-ME, 25 mM Hepes buffer (Sigma Chemical Co., St. Louis, Mo.), and 10% TCGF. Wells containing proliferating cell clusters were then expanded and maintained as described above for the limiting-dilution method except that supplemented RPMI-1640 medium was used instead of Iscove's medium.

Production of TCGF. Rat TCGF was prepared according to the method of Lafferty and Woolnough (12) using spleen cells from Sprague-Dawley or Lewis rats. Rat spleen cells were

cultured in serum-free minimal essential medium (MEM) containing 5 mg/ml concanavalin A (Con A) (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) at a concentration of 5×10^6 nucleated cells/ml in 75-cm^2 tissue culture flasks and were maintained in the horizontal position. After 2 h of incubation at 37°C, when an adherent cell monolayer was formed, the Con A-containing medium was carefully removed and the monolayer washed three times with warmed Hanks' balanced salt solution. Fresh, serum-free MEM supplemented with 10^{-4} M 2-ME was added and the flasks were incubated at 37°C for 18–24 h. The supernates were collected, freed of cellular debris by centrifugation (3,000 g) and sterile filtered. The crude TCGF was stored at −20°C until used. No further purification of TCGF was undertaken.

Cell Surface Phenotyping. Cloned T cell lines were tested for the expression of the murine Lyt-1, Lyt-2, and Thy-1.2 surface antigens using rat monoclonal antibodies directed against these antigens that were secreted by the hybridoma cell lines 53-7.313 (anti-Lyt-1), 53-6.72 (anti-Lyt-2) and 30-H12(r 4) (anti-Thy-1.2) (13) provided by Dr. N. L. Warner, University of New Mexico, Albuquerque, N. Mex.). Cells were typed by indirect rosette formation using the rosette method of Parish and McKenzie (14). Cloned cell lines were grown to stationary phase (3–5 d of culture) and freed of dead cells by centrifugation through Ficoll-Isopaque (10). Cells, at a concentration of 1×10^5–5×10^5 cells/ml in 0.05 ml of MEM that contained 10% newborn bovine serum (NBBS) and 0.1% sodium azide, were incubated 30 min on ice with 0.1 ml of medium containing a predetermined dilution of the appropriate monoclonal antibody in 12-× 75-mm glass tubes. The cells were then washed three times with 3 ml of cold serum-containing medium with 0.1% sodium azide and resuspended in 0.05 ml of this medium at a concentration of 1×10^5–5×10^5 cells/ml. Cells were tested for the presence of bound rat immunoglobulin (monoclonal antibody) by the addition of an equal volume of a 2% (vol:vol) suspension of sheep erythrocytes (Colorado Serum Co., Denver, Colo.) that had been coated with the IgG fraction of hyperimmune goat anti-rat immunoglobulin antiserum (Gateway Immunosera Co., St. Louis, Mo.) by the chromic chloride method (14).

Assay for Cell-mediated Cytotoxicity. The ^{51}Cr-release cytotoxicity assay was carried out as described (9). Briefly 1×10^4 ^{51}Cr-labeled uninfected or infected P815, MC57G, HTGSV, or L929 target cells were added to individual wells of 96-well, flat-bottomed microtiter culture plates in a 0.1-ml vol of medium. Effector cells from cloned CTL lines in a 0.1-ml vol were added to the wells. The plates were incubated for 6–7 h at 37°C and harvested as described (9). The percent specific ^{51}Cr release was obtained from the formula:

$$\frac{\text{tests counts} - \text{spontaneous release}}{\text{total counts} - \text{spontaneous release}} \times 100.$$

Spontaneous release from target cells incubated with medium alone usually ranged from 10 to 20% of total counts. Total release was determined by target cell solubilization in 1% Triton X-100 (Sigma Chemical Co.). Release values represent the mean percent specific ^{51}Cr release from four replicate wells. SEM were always <5% of the mean value and have been omitted from Tables. All assays were carried out at three to four different effector cell:target cell ratios. Representative results from one ratio are usually presented in the various Tables.

Results

Establishment of Cloned Lines of Influenza Virus-specific CTL. Two methods were employed to isolate cloned lines of virus specific CTL: limiting-dilution and colony formation in soft agar. Continuous lines of CTL could be established by either method. The pedigrees of sixteen cell lines analyzed in this report are shown in Table I.

By the limiting-dilution method, cloned CTL lines could be established by culturing in microwells either limiting numbers of spleen cells taken directly from immune donors or limiting numbers of viable lymphocytes taken at various times after in vitro secondary or tertiary stimulation of heterogeneous immune cell populations. As

expected, the frequency of responding (proliferating) cultures increased substantially (>100-fold) after stimulation and prolonged culturing of primed spleen cells in vitro (Table I). In all cases, wells containing proliferating cells were chosen for expansion and further analysis only when <20% (usually <10%) of the cloning wells were positive for proliferating cell clusters. This step was taken to optimize the probability that the proliferating cell lines were the progeny of individual precursors.

In experiments employing soft agar cloning, a significant proportion of expanded colonies (~50%) exhibited patterns of H-2-restricted cytolysis that indicated that they were derived from more than one precursor. For example, lines derived by soft agar cloning of (H-2^b × H-2^d)F$_1$ virus-specific CTL precursors often exhibited cytolytic activity on infected target cells of both the H-2^b and the H-2^d parental haplotypes. This finding necessitated recloning of these cell lines by limiting-dilution. Continuous lines produced by recloning of soft agar-derived lines did exhibit H-2-restricted cytolytic activity directed to either one of the two parental haplotypes.

The cell lines were maintained in culture for at least 1 mo before measurement of cytotoxic properties. All lines were maintained in continuous culture for at least 2 mo and several lines have been maintained in culture for >4 mo. One line, 11-1, has been maintained in continuous culture for >8 mo without any change in cytotoxic specificity, morphology, or growth potential. All lines required the continual presence of TCGF for maintenance of proliferation. Optimal proliferation of cultured cell lines also requires the presence of syngeneic irradiated spleen cells infected with the influenza strain used for immunization of the precursor donor mice. The maximum level of cellular proliferation was variable from line to line (cell densities at stationary phase ranging from 1 × 10^5 to 1.5 × 10^6 cells/ml) and appeared to be an intrinsic property of a given cell line. This intrinsic proliferative capacity appeared to be independent of cytotoxic specificity or the cell surface phenotype of a given line.

Patterns of H-2 Restriction Exhibited by Cloned Virus-specific CTL Lines.   Early studies on the properties of H-2-restricted CTL demonstrated that the CTL response of F$_1$ animals consists of two distinct populations of CTL: one directed to viral antigens in conjunction with H-2 molecules of one parental haplotype, and the other recognizing viral antigens in association with H-2 molecules of the other parental haplotype (15). Although this phenomenon was initially demonstrated by adoptive transfer of immune F$_1$ cells into acutely infected amplifier mice of the parental haplotypes, the clonal progeny of individual virus-specific CTL precursors of F$_1$ origin should exhibit cytotoxic activity exclusively on virus-infected target cells expressing H-2 antigens of one but not the other parental haplotype. Table II shows the pattern of cytotoxicity on virus-infected P815 (H-2^d) and MC57G (H-2^b) target cells exhibited by twelve continuous cloned influenza CTL lines of (H-2^b × H-2^d)F$_1$ origin. All lines exhibited substantial cytotoxicity only on infected target cells. 10 lines were directed to target cells expressing the H-2^d haplotype, and two lines killed exclusively infected target cells of the H-2^b haplotype. None of the F$_1$ lines were cytotoxic on both parental target cells, although cytotoxicity on both parental target cells is readily observed with a heterogeneous population of virus-specific F$_1$ CTL (Table II). Two continuous lines of parental origin, 8-4 from BALB/c (H-2^d), and 12-12 from C57BL/6 (H-2^b), exhibited appropriate cytotoxic activity on histocompatible infected target cells. Two other lines, 8-1 of BALB/c origin, and 14-16 of F$_1$ origin, failed to exhibit any

TABLE II

*H-2 Restriction of Cytotoxicity Exhibited by Cloned Lines of Influenza Virus-specific CTL**

CTL line	Haplotype of origin	P815 (H-2^d)		MC57G (H-2^b)	
		Uninfected	Infected	Uninfected	Infected
11-1	(H-2^b × H-2^d)F$_1$	1§	68	1	1
13-1	(H-2^b × H-2^d)F$_1$	0	6	12	46
14-1	(H-2^b × H-2^d)F$_1$	4	71	1	0
14-2	(H-2^b × H-2^d)F$_1$	0	64	9	6
14-7	(H-2^b × H-2^d)F$_1$	0	75	2	0
14-10	(H-2^b × H-2^d)F$_1$	0	84	0	0
14-12	(H-2^b × H-2^d)F$_1$	0	90	0	0
14-13	(H-2^b × H-2^d)F$_1$	0	82	0	1
14-15	(H-2^b × H-2^d)F$_1$	0	76	0	1
14-16	(H-2^b × H-2^d)F$_1$	0	4	0	0
14-17	(H-2^b × H-2^d)F$_1$	0	85	0	1
20-2	(H-2^b × H-2^d)F$_1$	0	55	0	1
20-15	(H-2^b × H-2^d)F$_1$	0	0	0	23
8-1	H-2^d	1	2	0	0
8-4	H-2^d	9	76	12	12
12-12	H-2^b	0	0	0	42
Bulk‖	(H-2^b × H-2^d)F$_1$	2	85	2	29

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

‡ 1 × 10^4 target cells were added per well. The effector cell:target cell ratio is 1:1. All assays were simultaneously carried out at three to four effector:target ratios.

§ Values are the means from four replicate wells with spontaneous release substracted. Spontaneous release from all target groups was <20%. SEM—always <5% of mean values—are omitted.

‖ Indicates heterogeneous population of CTL generated 5 d after in vitro stimulation of immune spleen cells from a pool of two F$_1$ donors.

significant cytotoxicity on either target cell type. The possible significance of this finding is discussed below.

Another property of virus-specific CTL previously observed in heterogeneous populations is restriction of recognition to viral antigens in association with either the H-2K-end products or the H-2D-end products of the H-2 gene complex (15). Clonal progeny of individual CTL precursors would likewise be expected to recognize infected target cells expressing either the H-2K- or H-2D-region products of the appropriate haplotype. A limited analysis of the H-2-end preference of the cloned lines described here was carried using a SV-40-transformed continuous fibroblast line, HTGSV, derived from the mouse strain B10.HTG (H-2K^d,D^b), which expresses H-2K products of the d haplotype and H-2D products of the b haplotype. The pattern of the cytotoxic activity of the cloned continuous F$_1$ and parental CTL lines on the HTGSV, as well as the two parental lines (P815 and MC57G), and a third-party target of the H-2^k haplotype (L929 cells) is shown in Table III. Of 11 lines exhibiting cytotoxicity for the infected targets of the H-2^d haplotype (P815), 8 showed significant cytotoxicity on

TABLE III

H-2 K/D-End Restriction of Cytotoxicity Exhibited by Cloned Lines of Influenza Virus-specific CTL*

CTL line	P815 (H-2K^d, D^d)		MC57G (H-2K^b, D^b)		HTGSV (H-2K^d, D^b)		L929 (H-2K^k, D^k)	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
11-1	1	88	3	2	0	31	0	0
13-1	1	5	11	53	0	7	0	0
14-1	11	88	3	4	2	68	1	0
14-2	8	85	1	6	0	61	55	42
14-7	1	80	3	0	0	56	2	0
14-10	2	88	0	3	0	40	0	0
14-12	2	89	1	3	0	40	1	0
14-13	0	82	0	1	0	1	0	0
14-15	1	90	1	2	0	33	0	0
14-17	0	85	1	1	1	0	0	0
20-2	3	75	5	7	2	0	0	0
20-15	0	0	0	39	0	37	0	0
8-4	9	69	12	12	10	11	3	5
12-12	0	0	1	42	0	12	—	—
(H-2^b × H-2^d)F$_1$‖	2	85	2	29	5	67	—	—

Percent specific ^{51}Cr release from target cells‡§

* As in Table II.
‡ As in Table II.
§ As in Table II except that effector cell:target cell ratio is 5:1.
‖ As in Table II.

infected HTGSV (H-2K^d, D^b) indicating restriction to the H-2K end of the d haplotype. The remaining three H-2^d-restricted lines (14-13, 14-17, and 20-2), which exhibited significant cytotoxicity on infected P815, failed to recognize infected HTGSV, indicating probable restriction to the H-2D end of the d haplotype. Of the three H-2^b-restricted lines, only one showed significant cytotoxicity on HTGSV, implying restriction of this clone to the D end of the b haplotype. Because suitable target cells which segregated the H-2K^b and H-2D^d antigens were not available, the pattern of H-2-end restriction exhibited by these lines remains tentative. With one notable exception, line 14-2, the lines showed no cytotoxic activity on uninfected or infected targets of the unrelated L929 target cells of the H-2^k haplotype. Line 14-2 exhibited significant cytotoxic activity on both infected and uninfected L929 cells and did so in several independent assays.

Pattern of Influenza Virus Recognition Exhibited by Cloned Virus-specific CTL Lines. Previous studies on the CTL response to influenza virus in heterogeneous CTL populations demonstrated a high degree of serologic cross-reactivity among serologically distinct type A influenza viruses. Experiments involving cold target competition (9, 16) and specific restimulation of CTL precursors with purified influenza hemagglutinin (17) revealed the presence of at least two distinct subpopulations of influenza virus-specific CTL, one cross-reactive of all type A influenza viruses, the other subtype-specific. Related studies also demonstrated differences in the recognition of serologically related viruses by the subtype-specific CTL subpopulation (17). Because this series of observations indicated a considerable degree of

heterogeneity in the CTL response to type A influenza viruses, it was of interest to determine if this heterogeneity was as well-reflected in the pattern of viral antigen recognition by CTL derived from individual precursors. The CTL lines were therefore tested for cytotoxicity on appropriate target cells infected with the immunizing influenza strain, i.e., A/JAP/57 (H_2N_2) or A/PR/8 (H_0N_1) and with appropriate strains representative of major type A influenza subtypes, i.e., H_0N_1, H_2N_2, H_3N_2. Also included were uninfected target cells and target cells infected with the unrelated influenza B strain B/Lee. Table IV shows the results of this analysis. Of 12 lines stimulated and selected with A/JAP/57 virus, 6 lines were cytotoxic exclusively for A/JAP/57 virus-infected cells and showed no cytotoxic activity either on the serolog-

TABLE IV

Influenza Virus Specificity of Cloned CTL Lines*

CTL line	Selecting virus strain	Effector: target ratio	Percent specific ^{51}Cr release from target cells‡					
			P815 (H-2^d)					
			Uninfected	A/PR/8 (H_0N_1)	A/JAP/57 (H_2N_2)	A/AA/67 (H_2N_2)	A/PC/75 (H_3N_2)	B/Lee
11-1	A/JAP/57	0.5:1	0.6§	0	57	0	0.5	0
		1:1	1	1	68	1	1	1
14-1	A/JAP/57	0.5:1	2	5	57	4	7	6
		1:1	4	7	71	9	10	10
14-1	A/JAP/57	0.5:1	0	0	46	1	3	1
		1:1	0	1	64	2	3	2
14-7	A/JAP/57	0.5:1	0	1	52	48	0	0
		1:1	0	1	75	64	2	2
14-10	A/JAP/57	0.5:1	0	1	67	1	2	1
		1:1	0	3	84	3	3	2
14-12	A/JAP/57	0.5:1	0	2	76	1	0	2
		1:1	0	2	90	3	3	3
14-13	A/JAP/57	0.5:1	0	57	53	54	60	1
		1:1	0	78	68	70	76	2
14-15	A/JAP/57	0.5:1	0	2	54	2	1	2
		1:1	0	2	76	1	5	2
14-17	A/JAP/57	0.5:1	0	55	67	68	73	2
		1:1	0	73	80	81	85	0
20-2	A/JAP/57	0.5:1	0	4	34	28	1	1
		1:1	0	4	55	44	2	1
8-4	A/PR/8	0.5:1	3	53	45	54	57	12
		1:1	9	76	69	72	76	23
			MC57G (H-2^b)					
			Uninfected	A/PR/8 (H_0N_1)	A/BEL/42 (H_0N_1)	A/JAP/57 (H_2N_2)	A/PC/75 (H_3N_2)	B/Lee
13-1	A/JAP/57	0.5:1	8	33	—	37	26	9
		1:1	12	43	—	46	36	13
20-15	A/JAP/57	1:1	0	0	—	23	9	0
		5:1	0	0	—	39	26	0
12-12	A/PR/8	2.5:1	1	42	62	1	0	1

* As in Table II.
‡ As in Table II.
§ As in Table II.

ically related A/AA/67 (H_2N_2) virus-infected targets or on cells infected with serologically distinct type A viruses of other subtypes, i.e., A/PR/8 (H_0N_1) or A/PC (H_3N_2). Two lines (14-7 and 20-2) were subtype-specific, exhibiting cytotoxicity exclusively on A/JAP/57 and A/AA/67 virus-infected targets. Three lines (14-13, 14-17, and 13-1) were cross-reactive, exhibiting significant cytotoxicity on target cells infected with viruses representative of all three type A influenza subtypes. One line, 20-15, selected for recognition of A/JAP/57, demonstrated cytotoxicity on A/JAP/57 and A/PC virus-infected targets with no cytotoxicity on serologically related A/AA/67 virus-infected targets (data not shown) or on serologically unrelated A/PR/8 virus-infected targets. Of the two lines selected for reactivity to influenza A/PR/8 virus, one line (12-12) exhibited subtype specificity with significant cytotoxic activity demonstrable only on target cells infected with A/PR/8 virus and the serologically cross-reactive A/BEL/42 influenza strain. The other line (8-4) was cytotoxic for target cells infected with all of the type A influenza strains.

In an attempt to further define the fine specificity of CTL lines for a particular influenza viral antigen, two lines, 14-1 and 14-7, selected for recognition of A/JAP/57 virus, were examined for cytotoxic activity on target cells infected with recombinant influenza strains A/JAP/BEL (H_2N_1) and A/X7-F_1 (H_0N_2). These two recombinant virus strains possess respectively either the hemagglutinin (A/JAP/BEL) or the neuraminidase (A/X7-F_1) of the prototype A/JAP/57 along with a serologically unrelated type A influenza neuraminidase or hemagglutinin. As Table V shows, both CTL lines exhibited optimum cytotoxic activity on A/JAP/BEL virus-infected target cells but little cytotoxic activity on A/X7-F_1 targets. This finding implicates the influenza A/JAP/57 hemagglutinin as the specific viral antigen recognized by these two CTL lines.

Cell Surface Phenotypes of Cloned Virus-specific CTL Lines. 13 cell lines were examined for the cell surface expression of the Thy-1.2, Lyt-1, and Lyt-2 markers using monoclonal antibodies directed to these antigens in a rosette assay (Materials and Methods). As Table VI shows, all lines expressed the Thy-1.2 antigens but the magnitude of Thy-1.2 expression as measured by the percentage of rosetting cells in a given line was variable from line to line, ranging from 18 to 88%. All lines with

TABLE V

Fine Specificity of Viral Antigen Recognition Exhibited by Cloned Lines of Influenza Virus-specific CTL*

CTL line	Effector:target ratio §	Percent specific ^{51}Cr release from P815 cells infected with‡					
		A/PR/8 (H_0N_1)	A/JAP/57 (H_2N_2)	A/JAP/BEL (H_2N_1)	A/X7-F_1 (H_0N_2)	A/PC/75 (H_3N_2)	B/Lee
14-1	0.5:1	1‖	54	58	2	2	2
	1:1	2	72	82	4	3	3
	5:1	9	85	88	11	9	8
14-7	0.5:1	0	46	50	0	0	0
	1:1	0	65	73	1	0	0
	5:1	1	84	89	5	3	2

* As in Table II.
‡ As in Table II.
§ 1 X 10⁴ target cells per well.
‖ As in Table II.

TABLE VI

Cell Surface Phenotype of Cloned Lines of Influenza Virus-specific CTL*

CTL line	Reactivity of cell line with monoclonal antibody directed to		
	Lyt-1	Lyt-2	Thy-1.2
11-1	−	+ (80)‡	+ (30)
14-1	+ (37)	+ (23)	+ (32)
14-2	−	+ (18)	+ (16)
14-7	+ (10)	+ (74)	+ (14)
14-10	−	+ (83)	+ (41)
14-12	−	+ (93)	+ (66)
14-13	+ (50)	+ (91)	+ (70)
14-15	−	+ (89)	+ (68)
14-17	+ (54)	+ (93)	+ (88)
20-2	−	+ (89)	+ (55)
8-4	−	+ (23)	+ (88)
8-1	+ (73)	−	+ (34)
14-16	+ (44)	−	+ (88)

* CTL lines at 3–5 d after subculturing were examined for the expression of the indicated surface marker by indirect rosette formation using monoclonal rat antibodies preparations and anti-rat Ig-coated sheep erythrocytes. (Materials and Methods).

‡ Cells with three or more erythrocytes bound to their surface were scored as positive in the rosette assay. In most analyses, positive cells had >10 erythrocytes bound. Treatment of the cells with indicator erythrocytes alone (without antibody pre-treatment) uniformly gave ≤1% rosettes formation. Values in parenthesis are the percentage of cells scoring positive for the expression of the indicated surface marker.

cytotoxic activity expressed the Lyt-2 surface antigen, but again there was some variability from line to line in the percentage of cells expressing the Lyt-2 phenotype. 4 of 13 cytotoxic lines examined also expressed detectable levels of Lyt-1 antigen. The remaining nine lines had no detectable Lyt-1 expression as measured by the binding of monoclonal anti Lyt-1 in the rosette assay. It is also of interest that the two lines, 8-1 and 14-16, that failed to demonstrate any cytotoxic activity on infected target cells (Table II) typed positive for the expression of the Lyt-1 antigen but expressed no detectable Lyt-2 antigen.

Discussion

The findings reported here demonstrate that continuous cloned lines of H-2-restricted virus-specific cytotoxic T lymphocytes can be isolated and maintained in the presence of TCGF and stimulator cells. More importantly, our results confirm, at the clonal level, several observations made previously with heterogeneous populations of H-2-restricted CTL. The finding that cloned virus-specific CTL derived from (H-2^b × H-2^d)F_1 precursors are restricted in their recognition either to the H-2^b or the H-2^d parental haplotype confirms the observation of Doherty et al. (15) that H-2-restricted CTL responses in F_1 animals are composed of CTL populations restricted to either parental haplotype (15). Also the finding, albeit tentative, that cloned virus-specific CTL lines appear to be restricted to either the H-2K or the H-2D of the responding haplotype likewise is consistent with previous observations made with heterogeneous CTL populations (15).

Another finding described here that supports previous observations concerns the heterogeneity and specificity of influenza virus-specific CTL. As discussed above (Results) several lines of evidence suggested that the CTL response to influenza virus showed considerable heterogeneity from the standpoint of viral antigen recognition. At the clonal level, it is evident that CTL clones with quite different specificities of influenza recognition can be defined. The three major types of recognition patterns which are apparent at the clonal level are: (a) unique clones reacting exclusively with the immunizing (selecting) virus strain, (b) subtype-specific clones directed to determinants shared by viruses of the same subtype as the immunizing virus, and (c) cross-reactive clones that recognize determinants shared by representative type A influenza viruses of all subtypes. No attempt was made in this report to further define the fine specificity of viral antigen recognition by these CTL lines. It will be of particular interest to examine the capacity of the unique and subtype-specific lines to recognize viral antigenic determinants on target cells infected with other serologically related influenza strains. This approach has been particularly useful in defining the repertoire of the antibody response to influenza virus at the clonal level (18). Such an approach at the level of T cell recognition may provide useful information both on the degree of diversity of H-2-restricted CTL recognition and on the specificity of this recognition.

All lines examined in this report expressed the Thy-1.2 antigen and were therefore presumably of T cell origin. Of the lines which exhibited cytotoxic activity on virus-infected target cells, all lines so-far tested likewise typed positive for the expression of the Lyt-2 antigen. Most of these lines (7 of 11) lacked detectable Lyt-1 antigen on their surfaces and tentatively can be classified as expressing the $Thy-1.2^+$, $Lyt-1^-2^+$ surface antigen phenotype. Four cytotoxic lines had both Lyt-1 and Lyt-2 antigens readily demonstrable on their surfaces. Data on the percentage of cells expressing these two markers in the case of two lines (Table VI, 14-13 and 14-17) and preliminary data from subclones derived from these four lines indicate that this $Lyt-1^+2^+$ phenotype is stable and not due to contamination of $Lyt-1^-2^+$ cells with progeny of a noncytotoxic $Lyt-1^+2^-$ cell during initial cloning. This observation appears to be consistent with recent evidence suggesting that most T lymphocytes independent of functional activity may express the Lyt-1 antigen but in quantitatively different amounts (19). Our failure to detect the Lyt-1 antigen on the majority of cytotoxic lines may very well reflect the insensitivity of the typing assay in detecting small quantities of this antigen, which could be expressed on these lines. Similarly the variability from line to line and within a line in the percentage of cells expressing a given cell surface marker may, as well, reflect quantitative difference in expression of these markers. It is not as yet clear if this variability is an intrinsic property of a given line or is a result of differences in cell cycle, culture conditions, etc. A detailed analysis of cell surface phenotype using more sensitive quantitative methods will be necessary to clarify this point.

During the course of this study, two lines were isolated which failed to exhibit any virus-specific cytotoxic activity. Both of these lines expressed the $Thy-1.2^+$, $Lyt-1^+2^-$ surface antigen phenotype. It is possible that these cells could lack cytotoxic activity because they fail to recognize virus in association with the H-2 K/D products on the tumor cell lines used as target cells. A more likely possibility is suggested by the $Lyt-1^+2^-$ phenotype of these cells i.e., they represent cloned lines derived from noncytotoxic T cell precursors belonging to the helper/amplifier T cell subset. Although precursor

T lymphocyte cloning in the presence of TCGF may preferentially select for cytotoxic T lines ([20]; and T. J. Braciale, unpublished observations) methods similar to those employed here have been used to select cloned lines of T cells with antibody helper activity (6). Data on the virus specificity and functional properties of these noncytotoxic lines should help in determining the T lymphocyte-subset origin of these lines.

In addition to showing virus specificity and H-2 restriction all lines examined, save one, showed no reactivity to target cells expressing H-2-K/D product of a third-party haplotype, i.e., L929 cells (H-2^k). One line of F$_1$ origin, 14-2, although it recognized H-2^d target cells infected with the immunizing virus (A/JAP/57) exclusively and was restricted to infected target cells expressing the H-2K-end products of the d haplotype, also showed significant cytotoxic activity on both infected and uninfected third-party L929 cells. This cross-reactivity was not associated with a particular pattern of virus recognition and H-2 restriction because several other F$_1$ lines showed identical patterns of viral and H-2 recognition. It is possible that the simultaneous expression of H-2-restricted, virus-specific cytotoxicity and apparent alloreactivity by this line is a result of biclonality of the line, i.e., the line is derived from two F$_1$ precursors, one H-2K^d restricted and virus specific, and the other alloreactive for the k haplotype. In this connection, it should be emphasized that this line was selected on infected F$_1$ stimulator cells and under conditions where ~10% of cloning wells were positive. Furthermore at no time during selection or passage was this line or its progenitor(s) exposed to cells bearing the H-2^k haplotype. Another, more interesting, possibility is that this line is monoclonal in origin and is simultaneously H-2 restricted and alloreactive in cytotoxic potential. The possibility that H-2-restricted and alloreactive CTL are overlapping populations has been hypothesized previously (21–23). Accordingly, the third-party target cell and haplotype chosen for specificity analysis (L929 cells [H-2^k]) would have fortuitously represented the appropriate allogeneic target haplotype for line 14-2 but not for the other cytotoxic lines. Experiments are now in progress to further confirm the clonality of this line and to further examine this hypothesis.

This report has focused on the specificity of target cell recognition exhibited by continuous lines of CTL isolated under clonal conditions in the presence of TCGF. The finding that these lines are distinguishable according to their patterns of H-2 and viral recognition suggests that they are most likely clonal in origin. A critical issue raised by these findings is the extent to which these lines isolated and maintained in the presence of TCGF reflect the properties of virus-specific CTL generated in vivo. Preliminary data on the proliferative response of several lines indicate that optimum proliferation and maintenance of these lines requires continued specific antigenic stimulation in addition to an exogenous source of TCGF. If these preliminary findings are substantiated, then the continuous cloned CTL lines could prove to be a useful in vitro model for analyzing the various influences which regulate the activity of MHC-restricted CTL. Equally important, the availability of such lines offers the possibility of examining at the structural level the molecules involved in antigen recognition and cellular regulation.

Summary

Continuous lines of murine cytotoxic T lymphocytes (CTL) directed to type A influenza viruses have been generated in vitro by stimulation of individual CTL

precursors in the presence of T cell-growth factor TCGF and syngeneic virus-infected stimulator cells. The cloned CTL lines are H-2 restricted in their target cell recognition and exhibit distinct patterns of influenza virus recognition. All CTL lines appear to be restricted in target cell recognition to either the H-2K or the H-2D end of the appropriate H-2 haplotype. Likewise, CTL lines of F_1 origin are restricted in recognition exclusively to one of the parental haplotypes. All CTL lines examined express the Thy-1.2 and the Lyt-2 surface antigen markers. 4 of 11 cytotoxic lines examined also expressed detectable levels of the Lyt-1 surface antigen. These findings confirm at the clonal level previous observations on the H-2K/D restriction of virus-specific CTL and also demonstrate heterogeneity among H-2-restricted CTL both from the standpont of viral antigen recognition and cell surface phenotype.

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