

SPECIFICITY STUDIES OF CYTOLYTIC T LYMPHOCYTES
DIRECTED AGAINST MURINE LEUKEMIA
VIRUS-INDUCED TUMORS

Analysis by Monoclonal Cytolytic T Lymphocytes*

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The immune response to tumors induced by murine leukemia viruses (MuLV)¹ in mice includes the generation of MuLV-immune cytolytic T lymphocytes (CTL), which are specific for the immunizing MuLV-induced tumor cells (1-4) and are actively involved in the immune rejection of the MuLV-induced tumor in vivo (5-8). The antigens recognized by MuLV-immune CTL on the tumor cell surface, however, have not yet been clearly identified. A problem in defining the specificities of MuLV-immune CTL has been the large degree of heterogeneity among CTL populations generated in vivo after the appearance of a tumor.

Two surface-membrane components have been shown to play a major role in the recognition of the tumor target cell by MuLV-immune CTL: (a) viral proteins expressed as constitutive elements of the tumor cell membrane (1-4, 9-11) and (b) H-2 transplantation antigens (2-4, 12, 13). Studies (1, 2) concerning infection with Friend MuLV, Moloney leukemia-sarcoma virus (MSV), or Rauscher MuLV indicated that MuLV-immune CTL recognized an antigen analogous to the serologically defined cross-reactive antigen expressed by cells infected with Friend, Moloney, or Rauscher MuLV (FMR) (14). More recent studies have suggested that Friend MuLV-immune CTL (9) and MSV-immune CTL (10) recognize an antigenic determinant expressed on the viral glycoprotein gp70, coded by the *env* gene of the infecting virus and expressed on the tumor cell membrane. In contrast, other studies (3, 4, 15) concerning the immune response to Gross MuLV-induced tumors suggested that in the Gross MuLV model the principal antigen recognized by CTL was analogous to the serologically defined Gross virus-associated cell-surface antigen (GCSA; 16), coded by the *gag* gene of Gross MuLV. Extensive analyses (1-4) have shown that CTL generated against Friend, Moloney, or Rauscher MuLV-induced tumors, on the one hand, and Gross MuLV-induced tumors, on the other hand, can distinguish FMR-like antigens from GCSA-like antigens.

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¹ Abbreviations used in this paper: CTL, cytolytic T lymphocyte; FMR, cross-reactive antigen expressed by cells infected with Friend, Moloney, or Rauscher MuLV; GCSA, Gross virus-associated cell-surface antigen; IL-2, interleukin 2; LU, lytic unit; MLTC, syngeneic mixed leukocyte tumor cell culture; MSV, Moloney leukemia-sarcoma virus; MuLV, murine leukemia virus.

The involvement of *H-2* transplantation antigens in the recognition of tumor target cells by MuLV-immune CTL has also been established and has been submitted to immunogenetic analysis (2-4, 12, 13, 17). Two genes located in the major histocompatibility gene complex, *H-2K* and *H-2D*, code for the *H-2* antigens that are recognized in conjunction with virus-induced antigens by MuLV-immune CTL. Two independent subpopulations of MuLV-immune CTL have been identified, one subpopulation recognizing viral antigens in association with *H-2K* antigen, and the other, with *H-2D* antigen (3, 4, 13, 15, 17). Moreover, a recent study (17) showed that individual MuLV-induced tumors were capable of affecting the relative proportions of *H-2K*- and *H-2D*-specific CTL subpopulations as a result of quantitative variations in the amounts of *H-2K* and *H-2D* antigens expressed on the tumor cell surface.

This report concerns a specificity analysis of CTL elicited by immunization with syngeneic Friend or Gross MuLV-induced tumors in BALB/c (*H-2^d*) and BALB.B (*H-2^b*) congenic mice. Analytical studies performed with monoclonal CTL cultures indicated that MuLV-immune CTL were composed of highly heterogeneous subpopulations of CTL. Various categories of CTL clones were identified, including a majority of clones tightly restricted in their cytolytic activity to the infecting MuLV and to their autologous *H-2* haplotype. The remaining CTL clones had decreasing degrees of specificity; in fact, a group of Lyt-2.2-positive CTL clones showed no discernible pattern of cytolytic specificity and were capable of attacking a large number of target cells, including uninfected lymphoblasts.

Materials and Methods

Mice. All mice were bred at Hôpital Cochin, Paris, from breeding pairs originally provided by Dr. F. Lilly (Albert Einstein College of Medicine, New York). The inbred *H-2*-congenic strains BALB/c (*H-2^d*) and BALB.B (*H-2^b*) were used.

Tumor Cells. Continuous leukemia cell lines induced by Gross, Friend, Rauscher, or Moloney MuLV in various strains of mice (Table I) were maintained as stationary suspension cultures in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. B.GV cells were induced by Gross MuLV (18), and HFL/b cells were induced by Friend MuLV (19) in BALB.B (*H-2^b*) mice. RBL-5 cells were induced by Rauscher MuLV in C57BL/6 (*H-2^b*) mice (20). C.GV and C.GV-300 cells were induced by Gross MuLV (17, 18); HFL/d cells were

TABLE I
Description of the Tumor Cell Lines Used

Nomenclature	Expression of MuLV-induced antigens		Inducing virus	<i>H-2</i> haplotype	Mouse strain of origin	Described in reference number
	FMR	GCSA				
B.GV	-	+	Gross MuLV	<i>b</i>	BALB.B	18
HFL/b	+	-	Friend MuLV	<i>b</i>	BALB.B	19
RBL-5	+	-	Rauscher MuLV	<i>b</i>	C57BL/6	20
C.GV	-	+	Gross MuLV	<i>d</i>	BALB/c	17, 18
C.GV-300	-	+	Gross MuLV	<i>d</i>	BALB/c	18
HFL/d	+	-	Friend MuLV	<i>d</i>	BALB/c	19
LSTRA	+	-	Moloney MuLV	<i>d</i>	BALB/c	21
K.GV	-	+	Gross MuLV	<i>k</i>	BALB.K	18

induced by Friend MuLV (19); and LSTRA cells were induced by Moloney MuLV (21) in BALB/c (*H-2^d*) mice. K.GV cells were induced by Gross MuLV (18) in BALB.K (*H-2^k*) mice.

Immunofluorescent Staining. The surface phenotype of cloned CTL was established by immunofluorescence techniques, as described elsewhere (22). Direct immunofluorescence was used to identify surface immunoglobulin with fluorescein-labeled rabbit IgG anti-mouse IgG and IgM (N. L. Cappel Laboratories Inc., Cochranville, PA). Indirect immunofluorescence was used to detect Thy-1.2, Lyt-1.2, and Lyt-2.2 antigens with mouse monoclonal antibodies directed against these antigens (New England Nuclear, Boston, MA).

Lymphocyte Cultures and CTL Clones. MuLV-specific CTL were generated in syngeneic secondary mixed leukocyte-tumor cell cultures (MLTC) from spleen cells of primed mice, as described elsewhere (3, 11). CTL specific for *H-2* alloantigens were generated in primary mixed leukocyte cultures (11). CTL were maintained in continuous culture by repeated stimulation with x-irradiated tumor cells as a source of antigen (23). In some experiments, conditioned medium containing interleukin 2 (IL-2; 24) was added to the lymphocyte cultures. Conditioned medium came from rat spleen cell cultures incubated with 5 $\mu\text{g/ml}$ concanavalin A at 37°C for 36 h.

CTL clones were derived from lymphocytes harvested in MLTC and distributed in multiwell plates at limiting dilutions of 0.5 and 1.0 cells per well in 30% IL-2-conditioned medium and 1×10^6 x-irradiated (3,000 rad) syngeneic spleen feeder cells. Starting on day 4 after distribution, the microplate cultures were submitted to daily microscopic inspection, and those cultures that presented single proliferating cell clusters were defined as lymphocyte clones. Cloning efficiency ranged from 64 to 77%. Each CTL culture was fed every 4–5 d with conditioned medium, and after 4 wk the clones were transferred into upright tissue culture flasks and expanded by the repeated addition of 30% conditioned medium, 3×10^6 x-irradiated (3,000 rad) spleen feeder cells, and 1×10^6 x-irradiated (5,000 rad) tumor cells as a source of antigen. Each CTL clone was assayed for cytotoxicity in triplicate on a panel of ^{51}Cr -labeled tumor target cells. A CTL clone was considered positive if it induced the release of at least three times the background value of spontaneous ^{51}Cr release from each individual tumor target cell.

^{51}Cr Release Cytotoxicity Assay. Cell-mediated antitumor cytolytic activity was detected using a modification (3) of the method of Brunner et al. (25). All assays were performed with 10,000 ^{51}Cr -labeled tumor target cells and were terminated after 6-h incubation at 37°C. Spontaneous release values varied between 4 and 15% of the total incorporated label. Cytolytic activity was sometimes expressed in terms of lytic units (LU), with one LU being the number of lymphocytes necessary to lyse 50% of the target cells during the incubation period of the assay (7, 11, 23); results were expressed in terms of LU per 10^6 lymphoid cells.

Inhibition Assays. In some experiments, CTL specificity for target antigens was analyzed by addition of varying numbers of competitor target cells to the ^{51}Cr release cytotoxicity assay (3, 17). The percentage of inhibition of CTL-mediated cytotoxicity was calculated according to the formula:

$$\frac{\text{control} - \text{experimental}}{\text{control}} \times 100,$$

where the specific cytotoxic activity was detected in positive control wells and the experimental cytotoxicity values were obtained from wells containing competitor target cells.

Results

Specificity of CTL Generated against MuLV-induced Tumors in Syngeneic MLTC. The results summarized in Fig. 1 confirm and extend previous results (3) concerning MuLV-specific CTL generated in secondary MLTC. BALB.B (*H-2^b*) and BALB/c (*H-2^d*) CTL generated against syngeneic Gross MuLV-induced tumor cells were most efficient in killing tumor cells of the same *H-2* haplotype induced by Gross MuLV (Fig. 1 A and C). Tumor cells induced by other MuLV (i.e., Rauscher MuLV-induced RBL-5 cells and Friend MuLV-induced HFL/b and HFL/d cells) were generally spared from lysis. However, an unexpected cross-reactivity of high intensity was

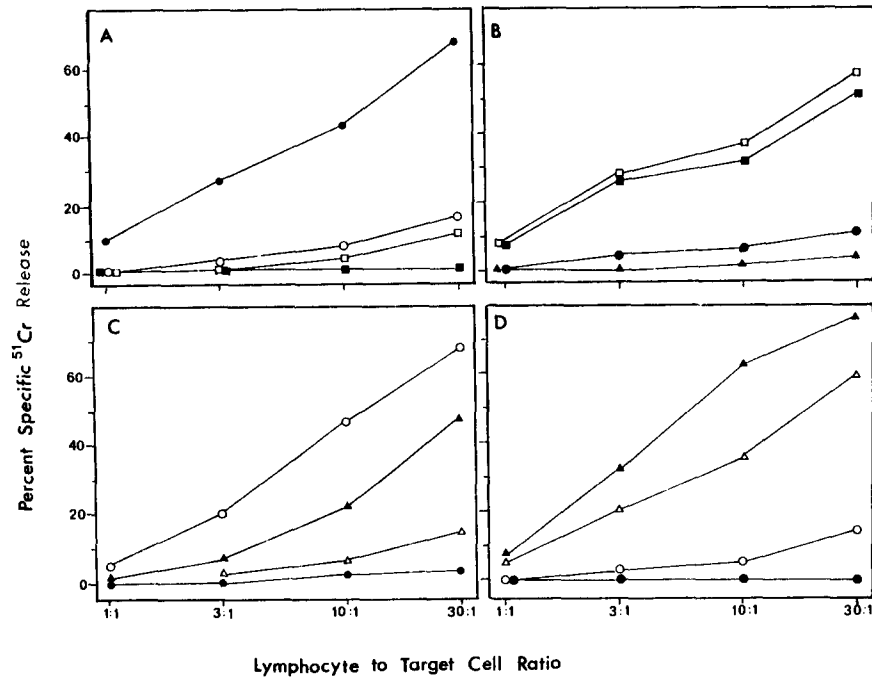


FIG. 1. Specificity of lysis mediated by BALB.B ($H-2^b$) and BALB/c ($H-2^d$) CTL directed against different MuLV-induced tumors. (A) BALB.B anti-B.GV CTL; (B) BALB.B anti-HFL/b CTL; (C) BALB/c anti-C.GV-300 CTL; (D) BALB/c anti-LSTRA CTL. CTL were generated in syngeneic secondary MLTC, harvested after 6 d in culture, and assayed on 10^4 ^{51}Cr -labeled tumor cells in a 6-h cytotoxicity assay at various lymphocyte-to-target cell ratios. The tumor cells used as targets were Gross MuLV-induced B.GV (●, $H-2^b$) and C.GV-300 (○, $H-2^d$) cells; Friend MuLV-induced HFL/b (■, clone B2, $H-2^b$) and HFL/d (Δ, $H-2^d$) cells; Rauscher MuLV-induced RBL-5 cells (□, $H-2^b$); and Moloney MuLV-induced LSTRA cells (▲, $H-2^d$).

observed when BALB/c anti-C.GV-300 and BALB/c anti-C.GV CTL (specific for Gross MuLV) were assayed on syngeneic LSTRA tumor cells (induced by Moloney MuLV). On the other hand, analysis of BALB.B and BALB/c CTL directed against MuLV-induced tumors of the FMR group (Fig. 1 B and D) revealed a high degree of avidity among these CTL for tumor target cells syngeneic at $H-2$ and positive for FMR antigen (i.e., RBL-5, HFL/b, LSTRA, and HFL/d tumor cells). FMR-positive, $H-2$ -different tumor target cells were spared, as were tumor cells induced by Gross MuLV (i.e., B.GV and C.GV-300 cells).

The data summarized in Fig. 1 indicated the existence of cross-reactivities of variable intensities; the strongest cross-reactivity was observed with Gross MuLV-specific BALB/c CTL (Fig. 1 C) assayed on Moloney MuLV-induced LSTRA tumor cells. This cross-reactivity was further analyzed by competitive inhibition of cytotoxicity: BALB/c anti-C.GV CTL were mixed with ^{51}Cr -labeled C.GV-300 cells (Fig. 2 A) or LSTRA cells (Fig. 2 B), and increasing amounts of unlabeled tumor cells of different origins were added. The results suggested the existence of multiple CTL subsets that recognized MuLV-induced antigens of different identities. One CTL subset killed C.GV-300 cells only, whereas a second CTL subset appeared to recognize both C.GV-300 and LSTRA cells (Fig. 2 A). A third subset of BALB/c anti-C.GV CTL lysed both C.GV-300 and LSTRA target cells and seemed to escape from

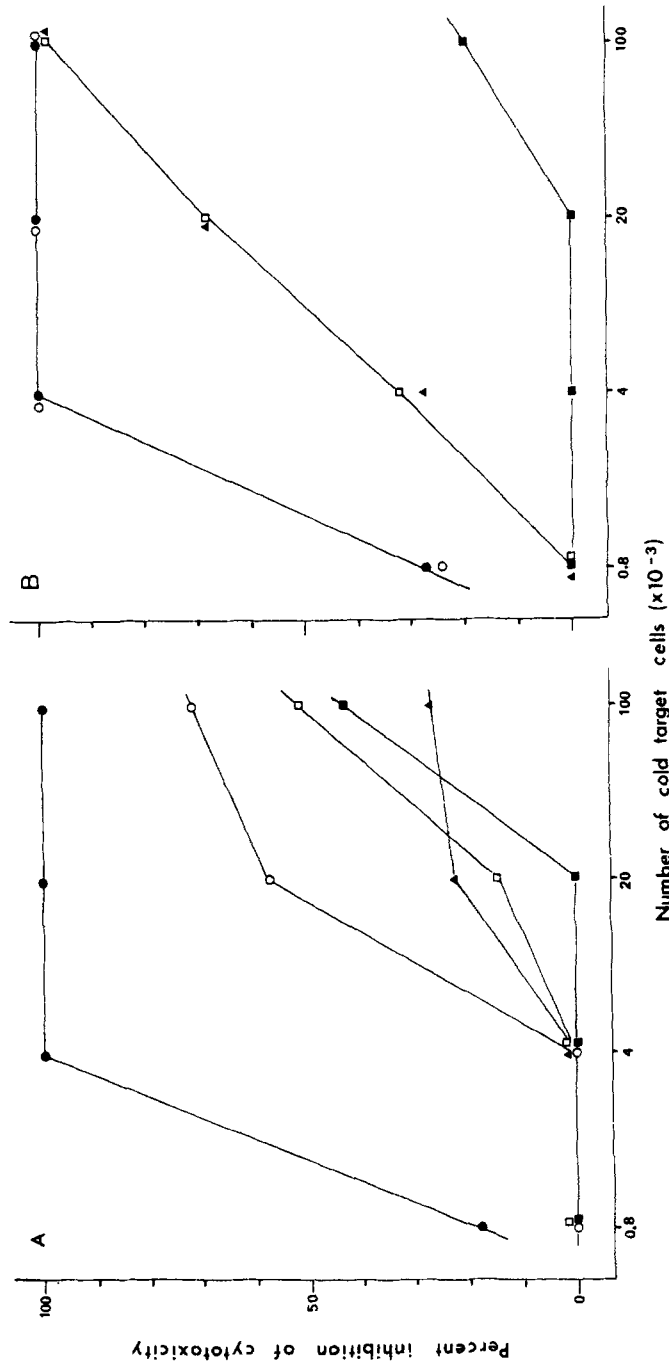


FIG. 2. Analysis by competitive inhibition of the cytotoxic activity of BALB/c anti-C.GV CTL from syngeneic secondary MLTC, as detected on 10^4 ^{51}Cr -labeled (A) C.GV:300 and (B) LSTRA tumor target cells in a 6-h assay. Increasing numbers of unlabeled tumor cells were added to the cell mixture, and the degree of inhibition of cytotoxicity was calculated at the end of the assay. The tumor cells used were Gross MuLV-induced C.GV:300 cells (●, $H-2^b$), B.GV cells (□, $H-2^b$), and K.GV cells (▲, $H-2^b$); Moloney MuLV-induced LSTRA cells (○, $H-2^d$); and Friend MuLV-induced HFL/d cells (■, $H-2^d$).

restriction by *H-2* because the lysis of LSTRA cells was partially inhibited by Gross MuLV-induced B.GV (*H-2^b*) cells and K.GV (*H-2^k*) cells (Fig. 2B).

Establishment of MuLV-specific CTL in Continuous Culture. In preparation for cloning, the best conditions for proliferation and long-term culture of MuLV-specific CTL were determined. Two independent attempts were made to establish BALB.B anti-B.GV CTL in continuous culture without losing their cytotoxic activity directed against B.GV tumor cells. Spleen cells from BALB.B mice previously immunized with an inoculum of B.GV tumor cells were set in continuous culture by repeated exposure to x-irradiated B.GV tumor cells and culture reinitiation every 10 d (Fig. 3A), according to a protocol developed previously (23). Lymphocyte proliferation reached a peak equivalent to 180% viable cell recovery 5 d after the second addition of B.GV cells and subsequently attained plateau values of 100% viable cell recovery. Cytolytic activity was detectable until day 40; however, starting on day 50 in culture, the B.GV-specific CTL subpopulation degenerated and eventually died out, leaving a T lymphocyte population that proliferated in the presence of tumor antigen (i.e., B.GV cells) but that had low cytolytic activity.

An alternative approach, originally described by Ryser et al. (26) and Baker et al. (27), involved the repeated addition of x-irradiated B.GV cells to B.GV-immune lymphocytes in the presence of conditioned medium containing IL-2 (24). Regular

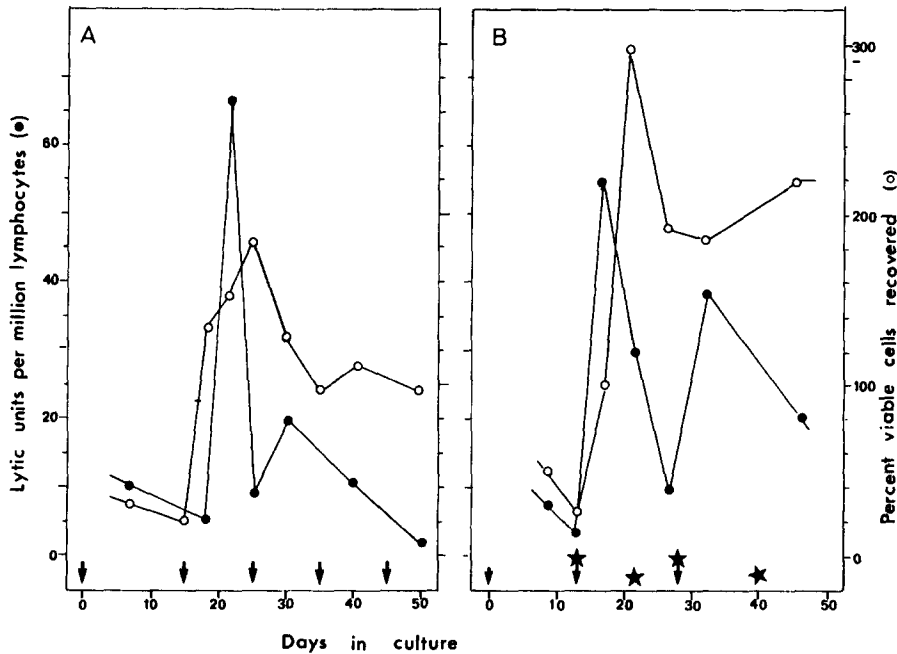


FIG. 3. Long-term culture of BALB.B anti-B.GV CTL by multiple antigenic stimulation in MLTC (A) or by multiple stimulation with antigen and IL-2-conditioned medium (B). Arrows indicate the addition of x-irradiated B.GV tumor cells as a source of antigen upon culture reinitiation. Stars indicate the addition of 30% IL-2-conditioned medium at the time of culture reinitiation. Cell proliferation was determined by viable cell counts in 0.1% trypan blue. Cytotoxic activity was detected using 10^4 ^{51}Cr -labeled B.GV tumor target cells in a 6-h cytotoxicity assay at various lymphocyte-to-target cell ratios. LU were calculated from the cytotoxicity curves and were standardized to LU per 10^6 viable lymphocytes recovered from MLTC.

culture reinitiation with the combined addition of tumor antigen and IL-2 resulted in sustained cellular proliferation (i.e., 188–300% viable cell recoveries (Fig. 3 B) as well as in the indefinite survival of B.GV-specific CTL. This procedure was consequently chosen to maintain MuLV-specific CTL clones in vitro.

Generation and Specificities of CTL Clones Lymphocyte populations from secondary MLTC were restimulated by the addition of x-irradiated tumor cells and IL-2-conditioned medium and subsequently cloned by limiting dilution at 0.5 or 1.0 cells per microplate well. Cultures that presented single proliferating cell clusters upon daily microscopic inspection were defined as cell clones. After expansion and antigenic restimulation, each clone was tested for cytotoxicity on a panel of ^{51}Cr -labeled tumor target cells. Fig. 4 shows the results obtained when 25 lymphocyte clones from BALB.B anti-B.GV spleen cells were assayed for cytotoxicity on ^{51}Cr -labeled B.GV cells. A high degree of heterogeneity was observed with respect to the cytolytic

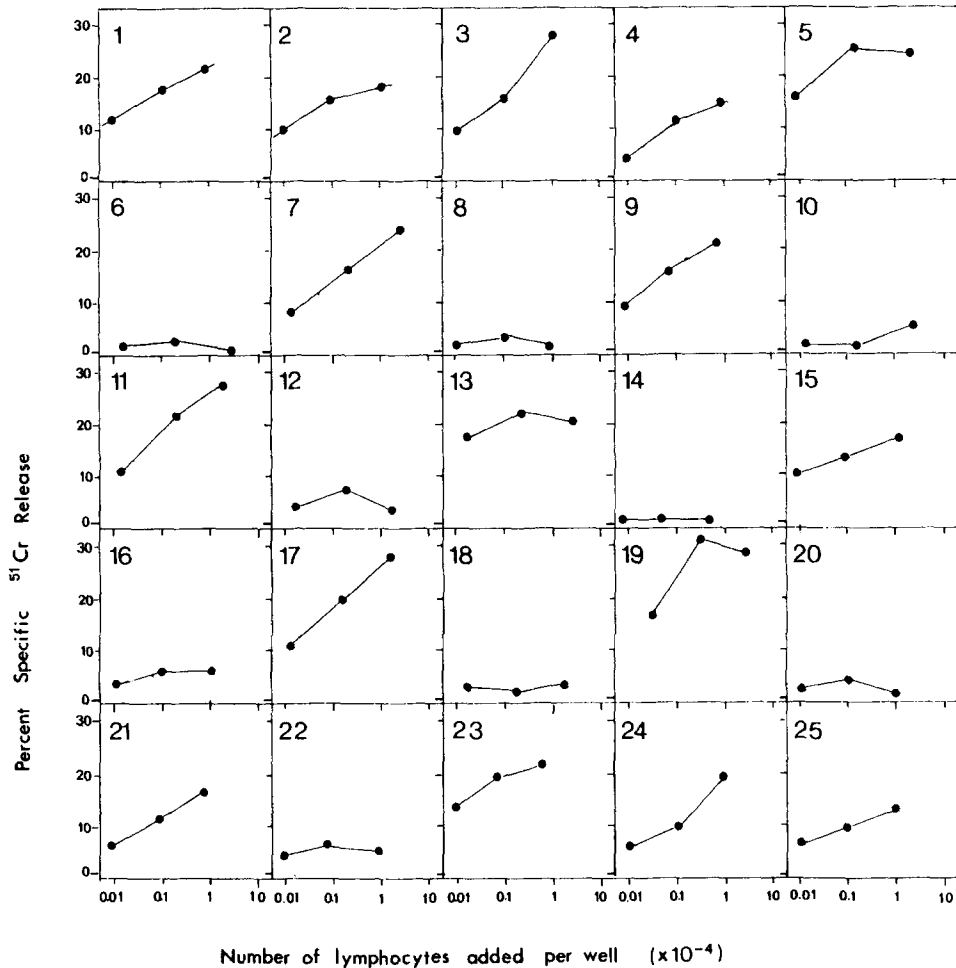


FIG. 4. Cytolytic activity of 25 lymphocyte clones derived from BALB.B anti-B.GV CTL generated in syngeneic secondary MLTC. Cytotoxicity was detected on 10^4 ^{51}Cr -labeled B.GV cells in a 6-h assay; lymphocytes were tested at various cell concentrations, as indicated.

potential of these clones because they ranged from clones with barely detectable cytolytic activity (i.e., clones 6, 8, 10, 18, and 22) to cytotoxic clones whose activity was detected at ratios as low as 100 lymphocytes per 10,000 tumor target cells (i.e., clones 1, 2, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 23). The cytotoxic activity of these lymphocyte clones varied in intensity from one assay to the other and required the addition of x-irradiated B.GV tumor cells and IL-2-conditioned medium for its maximum expression.

Similar patterns of reactivity were observed with CTL clones obtained from BALB.B (*H-2^b*) lymphocytes primed against syngeneic Friend MuLV-induced HFL/b tumor cells and with BALB/c (*H-2^d*) lymphocytes primed against Gross MuLV-induced C.GV cells. Table II summarizes the results obtained with 92 lymphocyte clones assayed independently on a panel of ⁵¹Cr-labeled tumor target cells. The clones were distributed into different groups according to their specificities. Thus, group I included the majority of CTL clones (i.e., 52–64%): these CTL attacked the tumor cell against which they were initially primed and not any of the other tumor cells assayed. The CTL clones included in group II (i.e., 5–16%) were specific for the priming MuLV-induced antigen in apparent association with an *H-2* public specificity shared between *H-2^b* and *H-2^d* antigens. Group III included six CTL clones that attacked tumor cells induced by Gross, Rauscher, Friend, and Moloney MuLV, and they were *H-2* restricted. Group IV included only one CTL clone that was obtained from BALB.B anti-B.GV lymphocytes; this clone was *H-2^b* restricted and recognized RBL-5 and HFL/b tumor cells of the FMR family but failed to recognize the immunizing Gross MuLV-induced B.GV cells. The cytolytic clones included in group V showed a total lack of target specificity; it was noteworthy that 33% of the

TABLE II
Viral and H-2 Specificities of CTL Clones

Initial lymphocyte culture	Clone group	Cytotoxic activity detected on ⁵¹ Cr-labeled								Frequency*
		B.GV Gross	C.GV-300 Gross	K.GV Gross	RBL-5 Rauscher	HFL/b Friend	LSTRA Moloney	HFL/d Friend		
		MuLV <i>H-2^b</i>	MuLV <i>H-2^d</i>	MuLV <i>H-2^k</i>	MuLV <i>H-2^b</i>	MuLV <i>H-2^b</i>	MuLV <i>H-2^d</i>	MuLV <i>H-2^d</i>		
BALB.B anti-B.GV (<i>H-2^b</i>) anti-Gross MuLV	I	+	-	-	-	-	-	-	0.64 (16/25)	
	II	+	+	-	-	-	-	-	0.12 (3/25)	
	III	+	-	-	+	+	-	-	0.08 (2/25)	
	IV	-	-	-	+	+	-	-	0.04 (1/25)	
	V	+	+	+	+	+	+	+	0.12 (3/25)	
	VI	-	-	-	-	-	-	-	0	
BALB.B anti-HFL/b (<i>H-2^b</i>) anti-Friend MuLV	I	-	NT‡	NT	+	+	-	-	0.52 (13/25)	
	II	-	NT	NT	+	+	+	+	0.16 (4/25)	
	III	+	NT	NT	+	+	-	-	0.12 (3/25)	
	IV	+	NT	NT	-	-	-	-	0	
	V	+	NT	NT	+	+	+	+	0.16 (4/25)	
	VI	-	NT	NT	-	-	-	-	0.04 (1/25)	
BALB/c anti-C.GV (<i>H-2^d</i>) anti-Gross MuLV	I	-	+	-	NT	NT	-	-	0.57 (24/42)	
	II	+	+	-	NT	NT	-	-	0.05 (2/42)	
	III	-	+	-	NT	NT	+	+	0.02 (1/42)	
	IV	-	-	-	NT	NT	+	+	0	
	V	+	+	+	NT	NT	+	+	0.33 (14/42)	
	VI	-	-	-	NT	NT	-	-	0.02 (1/42)	

Each CTL clone was tested for cytotoxicity three or four independent times on the same panel of ⁵¹Cr-labeled target cells in 6-h assays.

* Number of clones positive per total number of clones assayed.

‡ Not tested.

clones obtained from BALB/c anti-C.GV lymphocytes fell into this category. Finally, among the 92 lymphocyte clones presented in Table II, only two lacked any detectable cytotoxic activity (group VI). Although the intensity of lysis varied from one cytotoxicity assay to another, the specificity pattern of lysis of these CTL clones was conserved throughout a period of 4–6 mo. These results thus established the existence of multiple independent CTL subsets present among MuLV-specific lymphocytes harvested from syngeneic MLTC.

Surface Membrane Markers. Immunofluorescent analysis of the surface markers of 16 CTL clones selected from all groups showed that 100% of the cells were negative for surface IgG or IgM but strongly positive for Thy-1.2 antigen, thus confirming their T lymphocyte nature. In addition, these clones expressed Lyt-2.2 antigen and were weakly positive for Lyt-1.2 antigen, as indicated by immunofluorescent inspection using monoclonal anti-Lyt-2.2 and anti-Lyt-2.1 antibodies. Consequently, the surface phenotype of these lymphocyte clones was $\text{IgG}^- \text{IgM}^- \text{Thy-1.2}^+ \text{Lyt-1.2}^+ \text{Lyt-2.2}^+$, irrespective of the specificity of lysis of the clone.

Discussion

The present communication provides direct evidence as to the highly heterogeneous constitution of CTL populations generated against MuLV-induced tumors in BALB/c and BALB.B mice. This heterogeneity was previously suggested by studies concerning the specificity of MuLV-immune CTL (2, 3, 15, 17). Figs. 1 and 2 provide further examples of heterogeneity among Gross MuLV-, Friend MuLV- and Moloney MuLV-immune lymphocytes. Although major specificity was apparently restricted to tumor target cells expressing GCSA or FMR antigen and belonging to the same *H-2* haplotype as the CTL, weak cytotoxic reactions were often observed that crossed these restriction barriers. An exceptionally high cross-reactivity was observed among BALB/c CTL sensitized against syngeneic Gross MuLV-induced C.GV or C.GV-300 leukemia cells when these CTL were assayed on Moloney MuLV-induced LSTRA cells. The results obtained by competitive inhibition of CTL activity using unlabeled tumor cells (Fig. 2) suggested that LSTRA cells were recognized by a particular subset of BALB/c anti-C.GV CTL.

The systematic study of cloned MuLV-specific CTL established the existence of a large degree of heterogeneity among lymphocytes recovered from syngeneic MLTC. Heterogeneity was observed both with respect to the relative intensities of target cell lysis mediated by cloned CTL (Fig. 4) as well as to the specificity of lysis displayed by CTL clones (Table II). Studies by other laboratories concerning cloned T lymphocytes have also revealed a high degree of heterogeneity among CTL in other models of immunity, including syngeneic MSV-induced tumors (28), syngeneic influenza virus-infected cells (29), syngeneic hapten-conjugated lymphocytes (30), and *H-2* alloantigens (31). Moreover, Baker and collaborators (27) obtained monoclonal cytolytic T cell lines from lymphocytes of C57BL/6 (*H-2^b*) origin sensitized against an allogeneic (*H-2^d*) Friend MuLV-induced tumor; specificity analysis of these monoclonal CTL lines indicated the coexistence of alloantigen-specific cytotoxic lymphocytes and of lymphocytes specific for Friend MuLV-induced antigens expressed on syngeneic target cells.

The majority of the CTL clones studied here (i.e., 52–64%) were restricted to tumor target cells syngeneic with respect to *H-2* and positive for the immunizing MuLV-

induced antigen (GCSA- or FMR-related). A limited degree of heterogeneity was observed with respect to the *H-2* specificity of these CTL clones because they were themselves distributed among *H-2K-* and *H-2D-*specific CTL, as described in detail recently (17). However, clones showing diverse degrees of cross-reactivities with unrelated MuLV-induced tumor cells were also detected. Some of these cross-reactivities (clone groups II-IV, Table II) could be explained by the existence of cross-reactive or "public" antigens on *H-2* and viral proteins, such as have been described for antibody specificities (32, 33). Alternatively, some of these cross-reactive clones could be directed against endogenous virus antigens (33) expressed concomitantly with FMR or GCSA antigen or against *H-2* alloantigens. The CTL clones in the present study, however, did not show clear-cut specificities for *H-2* alloantigens, such as have been reported for cloned influenza virus-specific CTL (34) and for CTL obtained in other syngeneic models of immunity (27, 35, 36).

CTL clones in group V (Table II) lacked any discernible pattern of specificity and attacked all tumor target cells with which they were presented. These lymphocytes could be related to the CTL responsible for autoimmune, nonspecific cytolysis, which is particularly evident among BALB/c mice (37). In this context, CTL clones in group V from BALB/c anti-C.GV lymphocytes killed bacterial lipopolysaccharide (LPS)-induced lymphoblasts from normal uninfected BALB/c mice; CTL clones included in the other groups were negative when tested on the same blasts (data not shown). The presence of an unusually high number of these "autoreactive" clones among BALB/c anti-C.GV lymphocytes could explain the high degree of killing of Moloney MuLV-induced LSTRA tumor cells observed with these lymphocytes before cloning (Figs. 1 and 2). In this same context, Haas and colleagues (30) isolated CTL clones from hapten-immune spleen lymphocytes that lacked discernible specificities and were capable of killing a large number of different haptened and nonhaptened target lymphoblasts. Similarly, Baker et al. (27) isolated cytotoxic CTL clones with an apparent lack of target cell specificity after cloning T lymphocytes sensitized against an allogeneic Friend MuLV-induced tumor.

Upon immunofluorescent analysis, the lymphocyte clones presented in Table II were seen to be negative for surface IgG and IgM, which indicated they were not B cells. The T cell nature of these cloned lymphocytes was confirmed by inspection with monoclonal antibodies: 100% of the cells observed were highly positive for Thy-1.2 and Lyt-2.2 antigens and weakly positive for Lyt-1.2 antigen. The presence of these surface markers favored the classification of these cells as CTL (38) and invalidated the hypothesis that some of these clones might consist of natural killer cells because natural killer cells have been shown to be negative for both Lyt-1 and Lyt-2 antigens (39).

An interesting observation was that, among the 92 lymphocyte clones considered, only two lacked any discernible cytotoxic activity. This indicated that the protocols chosen to generate the lymphocyte clones was highly selective for cytolytic T lymphocytes. The positive selection for CTL probably occurred during the phase of multiple stimulation with antigen in MLTC before cloning. Previous studies (23) showed that after two successive stimulations with tumor antigen in MLTC, 83% of all cells were T lymphocytes (as opposed to 35% in fresh spleen). Subsequent studies by Brunner et al. (40) indicated that the proportion of MuLV-specific CTL precursors was significantly increased in MLTC after repeated stimulation with syngeneic tumor cells.

Finally, the combined addition of IL-2 and tumor antigen to long-term MLTC (26) resulted in the selection of a proliferating lymphocyte population that was highly enriched in tumor-immune CTL. Because CTL appear to be one of the lymphocyte subpopulations that respond preferentially to the growth-stimulating activity of IL-2 (24, 27, 31), cloning by limiting dilution at very low cell concentrations in IL-2-conditioned medium probably resulted in a drastic selection of proliferating CTL. The possibility of obtaining long-lived cloned populations of MuLV-immune CTL with a defined and constant specificity should render possible the biochemical definition of the principal target antigens recognized by these lymphocytes.

Summary

The specificities of cloned cytolytic T lymphocytes (CTL) were studied for the analysis of CTL populations generated against murine leukemia viruses (MuLV) in *H-2* congenic BALB/c (*H-2^d*) and BALB.B (*H-2^b*) mice. In particular, CTL generated in response to tumors induced by Gross MuLV and Friend MuLV were studied; these tumors express virus-induced antigens that do not cross-react and that can be distinguished from each other. The systematic study of 92 CTL clones clearly indicated that MuLV-immune CTL were highly heterogeneous with respect to both the intensities of target cell lysis that they mediated and to their specificity of recognition of MuLV-induced tumor target cells. Various categories of CTL clones were identified, ranging from CTL clones that were tightly *H-2* restricted and specific for the immunizing tumor to CTL clones that displayed no discernible patterns of specificity and that attacked a large number of different target cells. In addition, the surface markers of these cloned CTL were defined, and the best conditions for their prolonged maintenance in culture were determined. The present data indicate that future efforts in the definition of target antigens recognized by tumor-specific CTL should be performed with monoclonal lymphocytes.

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