

ANTIGENIC SPECIFICITY OF THE CYTOLYTIC
T LYMPHOCYTE RESPONSE TO
MURINE SARCOMA VIRUS-INDUCED TUMORS

III. Characterization of Cytolytic T Lymphocyte Clones Specific for
Moloney Leukemia Virus-associated Cell Surface Antigens

BY ARTHUR WEISS, K. THEODOR BRUNNER, H. ROBSON MACDONALD, AND
JEAN-CHARLES CEROTTINI

*From the Department of Immunology, Swiss Institute for Experimental Cancer Research; and the Unit of
Human Cancer Immunology, Ludwig Institute for Cancer Research, 1066 Epalinges, Switzerland*

The inoculation of Moloney sarcoma virus (MoSV)¹-murine leukemia virus (MoLV) complex into immunocompetent mice results in the development of a sarcoma at the site of injection that spontaneously regresses (1). Temporally related to the regression of these tumors is the appearance of cytolytic T lymphocytes (CTL) specific for MoLV-associated cell surface antigens. MoLV-specific CTL activity has been detected in the spleens and tumors of mice at the time of the regression of these sarcomas (1-4) and has been generated *in vitro* by stimulating spleen cells from normal mice or from mice that have rejected their sarcomas (regressors) with irradiated MoLV-induced lymphomas in mixed leukocyte-tumor cell cultures (MLTC) (5). Studies that examined the specificity of CTL activity have demonstrated H-2 restriction of recognition of MoLV-associated cell surface antigens (6). More specifically, lysis of H-2^b MoLV-derived target cells requires identity at the H-2D^b locus of the CTL population and target cells, whereas lysis of H-2^d MoLV-derived targets requires identity at H-2K^d (7). Further characterization of these effector cells has suggested that they express the Lyt-1⁻2⁺ phenotype (8). However, the interpretation of studies that examined the specificity and nature of MoLV-specific CTL has been limited because heterogeneous cell populations that contained the relevant CTL in unknown concentrations were used.

The application of limiting-dilution analysis toward the study of the clonal progeny of CTL-precursors (CTL-P) has been reported by several groups (9-12) and has recently been applied by this laboratory to the study of homogeneous MoLV-specific CTL populations (13). In initial experiments that employed a MLTC microculture system (micro-MLTC), conditions required for the detection of CTL-P progeny at the clonal level were defined, and preliminary studies of CTL-P frequency and specificity were carried out (13). Interestingly, these specificity studies suggested a

¹ *Abbreviations used in this paper:* CTL, cytolytic T lymphocyte(s); CTL-P, CTL precursor cell(s); DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; LU, lytic unit(s); MoSV, Moloney sarcoma virus; MoLV, Moloney leukemia virus; MLC, mixed leukocyte culture; 2° MLC SN, secondary MLC supernate(s); MLTC, mixed leukocyte-tumor cell culture(s); micro-MLTC, MLTC microculture system(s); SN, supernate(s); TCGF, T cell growth factor.

degree of heterogeneity of the clones generated with C57BL/6 regressor spleen cells. However, further analysis of such clones was limited by the relatively small number of cells available at any one time.

In the studies reported here, advantage was taken of the high frequency of operational CTL-P generated after *in vitro* stimulation of regressor spleen cell populations with MoLV-infected spleen cells. Large numbers of homogeneous CTL were obtained from such populations by first selecting and subsequently expanding clones obtained under limiting-dilution conditions. These expanded clones were then analyzed in detail for specificity, phenotypic stability, and Lyt phenotype.

Materials and Methods

Mice. Adult C57BL/6 (H-2^b) mice used in these experiments were bred in the animal facilities at the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland. Original breeding pairs were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Tumor Cell Lines. MBL-2 (H-2^b), an MoLV-induced lymphoma, and RBL-5 (H-2^b), a Rauscher leukemia virus-induced lymphoma, were maintained by serial passage of the ascitic forms in C57BL/6 mice. LSTRA (H-2^d), an MoLV-induced lymphoma, and P-815 (H-2^d) and EL-4 (H-2^b), chemically induced tumors, were maintained *in vitro* in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS).

Generation of CTL in Mass Culture. Regressor spleen cells were obtained from 3- to 4-wk-old C57BL/6 mice 14 d after an intramuscular injection of 0.1 ml of MoSV-MoLV complex (5). Mass cultures were prepared with 30×10^6 regressor (responder) spleen cells and 6×10^6 MoLV-infected syngeneic (stimulator) spleen cells (mice were injected with a MoLV preparation within 24 h of birth, and spleen cells from these mice were used at 4 wk of age)² in 20 ml of Dulbecco's modified Eagle's medium supplemented with additional amino acids (14), 5×10^{-5} M 2-mercaptoethanol, and 2% FBS (culture medium), in upright 50-ml tissue culture flasks (Nunc, Roskilde, Denmark). After 7 d of culture at 37°C in a humidified atmosphere of 5% CO₂ in air, an aliquot of cells was tested for cytolytic activity; the remaining cells, from several flasks, were pooled and frozen at -80°C in culture medium that contained 10% dimethyl sulfoxide (DMSO) and 10% FBS.

Micro-MLTC. Micro-MLTC were prepared in culture medium supplemented with 10% FBS and 25% (vol:vol) secondary mixed leukocyte culture (MLC) supernate (SN) (2° MLC SN) as a source of T cell growth factor (15). Each culture contained limiting numbers of responder cells, 1×10^6 irradiated (2,000 rad) syngeneic spleen cells, and 3×10^4 irradiated (5,000 rad) syngeneic (stimulators) MBL-2 tumor cells in a final vol of 0.2 ml in round-bottomed microwells (Greiner, Nürtingen, Federal Republic of Germany) (13). After 7 d of culture, cell growth was assessed microscopically and aliquots were removed to measure cytolytic activity.

Maintenance of CTL Clones. CTL clones derived from microcultures were transferred to and maintained in 1-ml cultures that contained 5×10^6 irradiated (2,000 rad) syngeneic spleen cells and 3×10^6 irradiated (5,000 rad) MBL-2 tumor cells in culture medium supplemented with 10% FBS and 25% MLC SN in 16-mm multiwell plates (Costar, Data Packaging, Cambridge, Mass.). After initial expansion, clones were passaged every 3-5 d by transferring 5×10^4 cells to fresh cultures. Aliquots of cells were periodically frozen at -80°C in culture medium that contained 10% DMSO and 10% FBS.

Assay for Cytolytic Activity. Cytolytic activity of effector cells was determined using a ⁵¹Cr-release assay (16). Briefly, aliquots of effector cells were mixed with either 2,000 or 5,000 ⁵¹Cr-labeled tumor cells (as indicated) in a final vol of 200 μl in round-bottomed microplates and incubated for 3.5 h at 37°C. The assay plates were then centrifuged, and 100 μl of the SN fluid was removed and the amount of released ⁵¹Cr determined in a well-type gamma counter. Controls included maximal release of ⁵¹Cr as the result of freezing and thawing targets, and

² Collavo, D., P. Zanovello, G. Biasi, and L. Chieco-Bianchi. T lymphocyte tolerance and early appearance of virus-induced cell surface antigens in Moloney-murine leukemia virus neonatally infected mice. Manuscript submitted for publication.

spontaneous release of ^{51}Cr from labeled targets in the absence of effector cells. Percent-specific release or lytic units (LU) were calculated as previously described (16); 1 LU was equivalent to the number of lymphocytes required to produce 50% specific ^{51}Cr release.

Frequency of CTL-P. The method used for the calculation of CTL-P is described in detail elsewhere.³ Briefly, 24 replicate microcultures prepared with varying numbers of responding cells were assessed for cytolytic activity. Positive cultures were defined as those in which the ^{51}Cr release values exceeded by 3SD the spontaneous release values. Minimal estimates of CTL-P frequencies were calculated by analysis of the Poisson distribution relationship between the number of responding cells/microculture and the percentage of nonresponding cultures. Experimental values were fit to the zero-order-term Poisson equation by the maximum log likelihood method.³

Alloantisera and Monoclonal Antibodies. Hyperimmune C57BL/6 anti-DBA/2 (anti-H-2^d) and DBA/2 anti-C67BL/6 (anti-H-2^b) alloantisera were produced by intraperitoneal injection of recipient mice with 5×10^7 allogeneic spleen cells three to six times at 3-wk intervals. 7 d after the last injection, mice were exsanguinated and serum collected, filtered, and heat-inactivated.

Ascitic fluids that contained monoclonal antibodies produced by hybridomas B22-249.R1 and H141-30, specific for H-2D^b, were obtained from G. Hämmerling, Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany. Their reactivity patterns are described in detail elsewhere (17). Serum that contained monoclonal antibody produced by the hybridoma B8-24-1, specific for H-2K^b, was obtained from G. Köhler, Basel Institute for Immunology, Basel, Switzerland.

Concentrated culture SN that contained rat monoclonal antibodies directed against nonpolymorphic determinants of either Lyt-1 (53-7.3) or Lyt-2 (53-6.7) molecules were used in the analysis of the Lyt phenotype of MoLV-specific CTL clones. The reactivity pattern of these antibodies has previously been described (18).

Fluorescein-labeled rabbit anti-rat IgG was purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands).

Fluorescent Staining and Flow Cytometric Analysis. Indirect immunofluorescence was performed on Ficoll-Hypaque-purified lymphoid cell samples of $0.3\text{--}1 \times 10^6$ cells. Cells in a 0.1-ml vol were incubated with appropriate concentrations of rat monoclonal antibody directed against Lyt-1 or Lyt-2 at 4°C for 30 min. After washing, cell pellets were resuspended for 30 min at 4°C in 0.1 ml of medium that contained fluoresceinated rabbit anti-rat IgG. Control samples were incubated in the fluorescein conjugate only. Samples were then finally centrifuged over a layer of FBS and analyzed on a flow cytometer (FACS II; B. D. FACS Systems, Mountain View, Calif.).

Flow cytometric analysis was performed as described in detail elsewhere.⁴ Briefly, the FACS II, modified to allow three parameter analysis, was routinely gated for viable cells by a combination of narrow-angle forward light scatter and perpendicular light scatter. Fluorescence was the third parameter measured. Approximately 30,000 gated events were accumulated for each determination.

Results

Production of MoLV-Specific CTL Clones. Preliminary studies had shown that the frequency of MoLV-specific CTL-P increased dramatically (from $\sim 1/600$ up to $1/2$) after in vitro stimulation of C57BL/6 regressor spleen cells with syngeneic irradiated MoLV-induced MBL-2 lymphoma cells (or with MoLV-infected spleen cells). This suggested the use of in vitro stimulated cells as the starting population in attempts to derive MoLV-specific CTL clones, rather than the regressor spleen cells used in the previous studies (13). Limiting numbers of cells (three cells/well) obtained at the peak

³ Taswell, C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. Manuscript submitted for publication.

⁴ MacDonald, H. R., R. K. Lees, B. Sordat, P. Zaech, J. L. Maryanski, and C. Bron. Age-associated increase in expression of the T cell surface markers Thy-1, Lyt-1 and Lyt-2 in congenitally athymic (nu/nu) mice: analysis by flow microfluorometry. Manuscript submitted for publication.

of the CTL response (day 7) of C57BL/6 regressor spleen cells stimulated with MoLV-infected C57BL/6 spleen cells were cultured in micro-MLTC with 1×10^6 syngeneic irradiated spleen (accessory) cells and 3×10^4 irradiated MBL-2 tumor (stimulator) cells, and 2° MLC SN as a source of T cell growth factor. After 7 d, each culture was assessed microscopically for growth and an aliquot was tested for cytolytic activity against ^{51}Cr -labeled MBL-2 target cells. In one such experiment, 55 out of 96 microcultures were positive for growth and all 55 demonstrated cytolytic activity. Cells from six highly cytolytic microcultures were selected for expansion and transferred to 1-ml cultures that contained 5×10^6 irradiated C57BL/6 spleen cells, 3×10^5 irradiated MBL-2 cells, and 25% 2° MLC SN. Under these conditions, all six of these putative clonal isolates grew and were subsequently recloned 1 wk later by limiting-dilution in micro-MLTC with 0.3 responding cells/well (other attempts at expansion and recloning of micro-MLTC populations were similarly successful). All clones derived by this procedure were cytolytic for MBL-2 targets. These clones and others have been maintained in culture for >4 mo by passage of 5×10^4 cells every 3–5 d in 1-ml cultures that contained irradiated C57BL/6 syngeneic spleen cells, irradiated MBL-2 tumor cells, and 2° MLC SN.

To date, no clone has lost its high level of cytolytic activity or its cytolytic specificity. Fig. 1 compares the cytolytic activity of four of the derived clones. Although some degree of heterogeneity was seen, the levels of activity (50% lysis at effector:target cell ratios of 0.3:1 to 3:1) were similar to or higher than those observed with mass cultures like those used to establish the clones (data not shown).

Karyotypic analysis of four of the clones carried for 4 mo in culture revealed the normal complement of mouse chromosomes, and no aberrant chromosomes were observed (M. Cianfriglia. Unpublished data.).

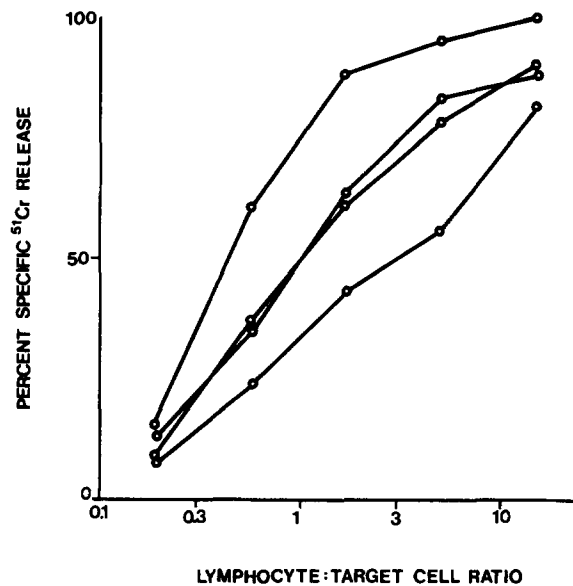


FIG. 1. Cytolytic activity of MoLV-specific CTL clones. The cytolytic activity of four MoLV-specific C57BL/6 CTL clones was assessed against 5,000 ^{51}Cr -labeled MBL-2 tumor cells in a 3.5-h assay.

Kinetics and Requirements for Growth of MoLV-specific CTL Clones. To examine the growth requirements of MoLV-specific CTL clones, cultures were prepared with 5×10^4 cells from each of three clones using various supplements added to standard culture medium. Cell recovery and cytolytic activity against MBL-2 target cells were assessed on days 2, 3, 4, and 6. Fig. 2 illustrates the pattern of growth of a clone (C5.2) in the presence of the indicated culture supplements, and Table I presents the cytolytic activity recovered from cultures of three clones on days 3 and 6. It is apparent from

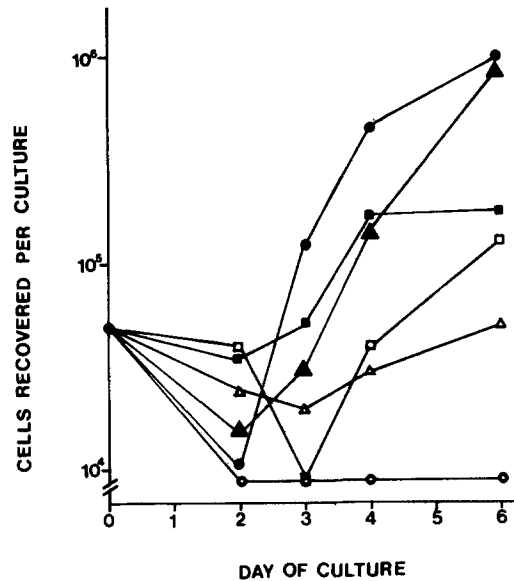


FIG. 2. Kinetics and requirements for growth of MoLV-specific CTL clones. Cultures were prepared with 5×10^4 C5.2 cells in 1 ml of culture medium only (○), with 25% 2° MLC SN (■), with SN plus irradiated C57BL/6 spleen cells (□), with SN plus irradiated MBL-2 (▲), with SN plus irradiated spleen and irradiated MBL-2 cells (●), with irradiated spleen and irradiated MBL-2 cells (△). Viable cell recovery was assessed in replicate cultures on the days indicated.

TABLE I
*Requirements for the Generation of Cytolytic Activity in Cultures Prepared with MoLV-specific CTL Clones**

Culture supplement	Clone A.2.2		Clone 5.2		Clone F9.F8D	
	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
	<i>LU/culture (LU/10⁶)</i>					
None	<1	<1	<1	<1	<1	<1
Irr. spleen + MBL-2	3.3	1.5	6.7	5.7 (114)	14.3	7.4 (148)
SN	3.1	3.7 (74)	4.0	11.9 (66)	21.3	100 (156)
SN + MBL-2	7.1	25.6 (128)	30.0	86.6 (94)	66.7	31.2 (26)
SN + irr. spleen	2.3	4.0 (67)	5.4	31.3 (238)	17.2	57.1 (101)
SN + MBL-2 + irr. spleen	7.1	100 (182)	33.3	313 (332)	103	143 (108)

* Cultures were prepared with 5×10^4 cells from the indicated CTL clone in 1 ml of culture medium plus the indicated culture supplement, i.e., irradiated syngeneic MBL-2 lymphoma cells (MBL-2); irradiated syngeneic spleen cells (irr. spleen), and 2° MLC SN (SN). After the indicated period of culture, cytolytic activity against ⁵¹Cr-labeled MBL-2 target cells was assessed. LU were calculated from dose-response curves, 1 LU equals the number of lymphocytes required to produce 50% specific ⁵¹Cr release. The number of LU/10⁶ lymphocytes or LU/culture was determined as described previously (16).

these data that there is an absolute requirement for 2° MLC SN, but optimal cell recovery and cytolytic activity were also dependent upon the presence of syngeneic irradiated spleen cells and stimulating antigen (MBL-2). In general, peak cell recovery (ranging from 5×10^5 to 3×10^6 /culture) was observed by day 4 and appeared to depend to some extent on the length of a lag period that all clones underwent after transfer. After the 6th d in culture, cell recovery and lytic activity diminished rapidly (data not shown).

Frequency Analysis of CTL-P in MoLV-specific Clones. Limiting-dilution analysis was used to determine the frequency of CTL-P in three clones. The frequency varied from 33–100%, suggesting a homogeneous population of cells with a high plating efficiency (Fig. 3).

Lyt Antigen Phenotype of MoLV-specific CTL Clones. Flow cytometric analysis that employed monoclonal rat anti-Lyt-1 and anti-Lyt-2 antibodies was used to assess the Lyt phenotype of several of the MoLV-specific CTL clones. Fig. 4 compares the fluorescence profiles of three clones with those obtained with normal C57BL/6 thymocytes. It can be seen that both Lyt-1 and Lyt-2 antigens were expressed on the surface of the cells of all three clones. It should be noted that the relative amplification used to detect fluorescence on thymocytes was twice that used for the CTL clones. Thus, the Lyt-1 density of the CTL clones is at least equivalent to that on thymocytes, and Lyt-2 appears even more abundant. There was, however, heterogeneity in the density of Lyt antigens among the various clones, the quantity of Lyt-1 on clone B11.1 being twice that seen on A2.2 or C5.2. The stability of this difference in antigen density with time was confirmed in determinations 2 mo apart.

Specificity of MoLV-specific CTL clones. Previous studies in the MoLV system had demonstrated that CTL contained in mass MLTC populations preferentially lysed syngeneic MoLV-derived targets. However, significant lysis of allogeneic MoLV-derived tumor cells could also be observed at relatively high lymphocyte:target cell ratios (6). Studies in micro-MLTC under limiting-dilution conditions further showed

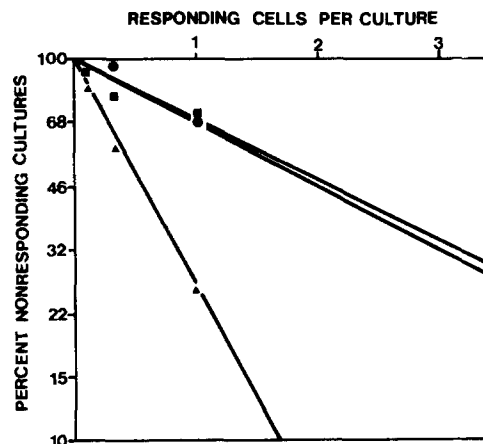


FIG. 3. Minimal estimate of the frequency of CTL-P in MoLV-specific CTL clones. Micro-MLTC were prepared with limiting numbers of cells from each CTL clone and assessed for cytolytic activity against ^{51}Cr -labeled MBL-2 cells after 7 d. The percent negative cultures at each cell dose was used to calculate the frequency (f) of CTL-P by the method of maximum log likelihood as described in Materials and Methods. ■, E4.2 ($f = 1/2.7$); ●, A2.2 ($f = 1/2.6$); ▲, B11.1 ($f = 1/0.7$).

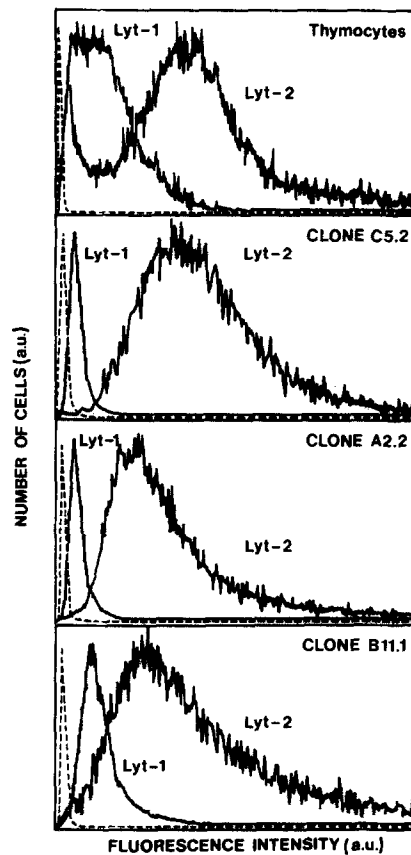


FIG. 4. Analysis of Lyt phenotype of MoLV-specific CTL clones by flow microfluorometry. Normal C57BL/6 thymocytes or the indicated CTL clones were incubated with rat monoclonal antibodies specific for Lyt-1 or Lyt-2 antigens followed by fluoresceinated rabbit anti-rat IgG. Samples were run on an FACS II gated to exclude nonviable cells. Fluorescence histograms were arbitrarily normalized. The dotted curve in each panel represents cells stained with the fluoresceinated conjugate only. a.u., arbitrary units.

that, in the C57BL/6 system, a small proportion of clones were able to lyse syngeneic and allogeneic MoLV-derived target cells (13). These results suggested some degree of heterogeneity among MoLV-specific CTL. To further characterize such cross-reactive MoLV-specific C57BL/6 CTL, micro-MLTC were prepared with limiting numbers of responding cells (two to four cells/culture) from 7-d mass cultures of C57BL/6 regressor spleen cells previously stimulated with MoLV-infected syngeneic spleen cells. After 7 d, such microcultures were split and assayed against four tumor target-cell types, i.e., MBL-2 (H-2^b), the syngeneic stimulating MoLV-derived tumor; LSTRA, an allogeneic MoLV-derived tumor; EL-4 (H-2^b), a syngeneic non-MoLV-derived lymphoma; and P-815 (H-2^d), an allogeneic non-MoLV-derived tumor. The results of one such experiment are illustrated in Fig. 5, where the percent specific ⁵¹Cr release of positive cultures against MBL-2 is compared with that obtained with each of the other three target cells. Of a total of 168 cultures, 31 demonstrated significant cytolytic activity against MBL-2. Whereas the vast majority of these putative clones

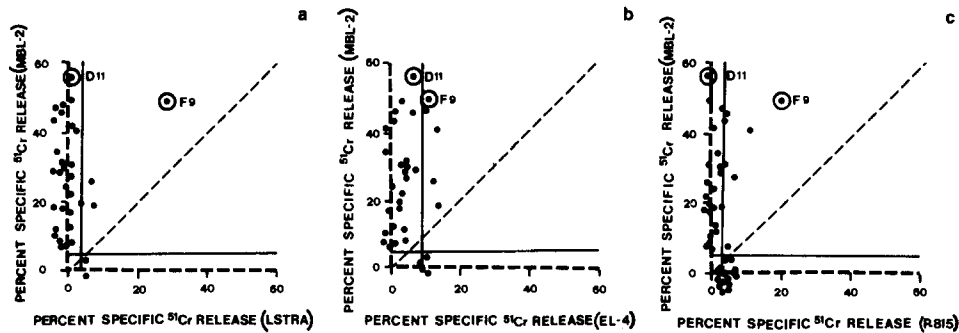


FIG. 5. Specificity analysis of individual micro-MLTC. A limiting number of cells (two/culture) from a mass culture prepared with C57BL/6 regressor spleen cells were cultured in micro-MLTC with stimulating MBL-2 cells as described in Materials and Methods. After 7 d, each of 168 individual microcultures was split and assessed for cytolytic activity against 2,000 ^{51}Cr -labeled target cells, which included the stimulating MoLV-derived syngeneic tumor (MBL-2), an allogeneic MoLV-derived tumor (LSTRA), a syngeneic non-MoLV-derived tumor (EL-4), or an allogeneic non-MoLV-derived tumor (P-815). Two microcultures (circled) were selected for further specificity studies after subcloning (Fig. 6).

demonstrated a high degree of specificity, lysing only MBL-2 targets, a smaller number demonstrated varying degrees of cross-reactivity with the other target cells. The remaining cells in two of these cultures (circled), one specific (D11) and one cross-reactive against LSTRA and P-815 (F9), were subcloned in micro-MLTC, expanded, and maintained in 1-ml cultures as described earlier in this report.

The specificity of these 2 expanded CTL clones (and 12 others selected in a similar manner) was assessed using the 4 tumor targets employed in the initial screening procedure. At least three patterns of reactivity were observed, as illustrated by the three representative clones shown in Fig. 6. Two of these (D11.F1S and F9.F8D) are subclones from microcultures described in Fig. 5. All three clones demonstrated high levels of cytolytic activity toward the syngeneic MoLV-derived tumor, MBL-2. However, although D11.F1S had no significant cytolytic activity against the other three targets tested (Fig. 6a), A2.2 lysed LSTRA, though to a lesser extent, but again had no significant activity against EL-4 or P-815 (Fig. 6b). In contrast, F9.F8D lysed both P-815 and LSTRA in addition to the syngeneic MoLV-derived lymphoma (Fig. 6c). As shown in Table II, of 14 selected clones, 6, 2, and 4 clearly exhibited the patterns exemplified by clones D11.F1S, A2.2, and F9.F8D, respectively. The likelihood that the F9.F8D pattern corresponds to clones that cross-react with H-2^d alloantigens was supported by the ability of DBA/2 (H-2^d), but not C57BL/6 spleen cells, to effectively function as cold target inhibitors when F9.F8D was assayed against MBL-2 or P-815, and by the ability of F9.F8D to lyse DBA/2, but not C57BL/6 concanavalin A blast target cells (data not shown).

Stability of Cytolytic Activity of MoLV-specific CTL Subclones. Three clones (A5, E6, and C3) selected for their representative specificity patterns were subcloned by stimulating limiting numbers of cells from each clone in a series of micro-MLTC. From each parental clone, three randomly selected subclones were expanded and tested at different lymphocyte:target cell ratios for cytotoxicity against the four target cell types used for the initial selection. As shown in Fig. 7, all three subclones of each parental clone closely resembled each other in its specificity pattern. This demonstrates a high degree of stability of activity and specificity of the subclones. Occasional

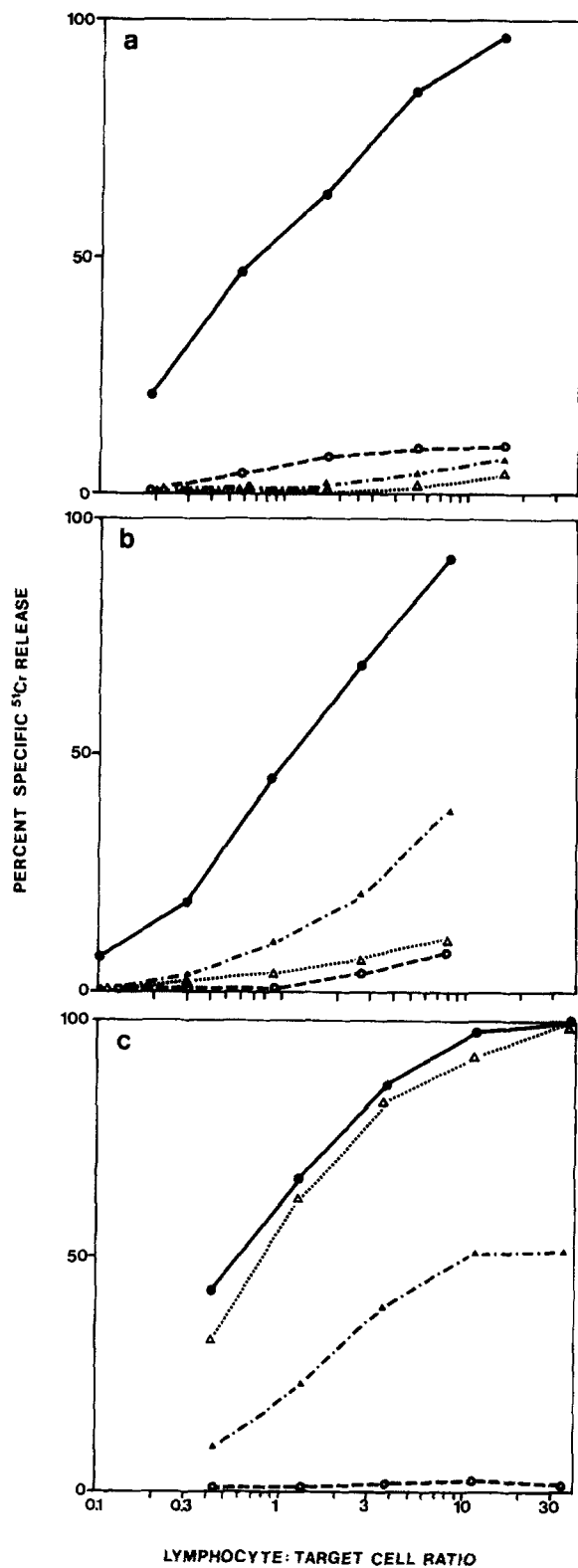


FIG. 6. Specificity patterns of CTL clones reactive with MoLV-associated cell surface antigens. Micro-MLTC were prepared as indicated in Fig. 5, and selected cultures were recloned by limiting-dilution and expanded. The cytolytic activity of clones D11.F1S (a), C5.2 (b), or F9.F8D (c) against the indicated ^{51}Cr -labeled target was assessed in a 3.5-h ^{51}Cr release assay using 5,000 target cells/well. ●, MBL-2; ○, EL-4; ▲, LSTRA; △, P-815.

TABLE II
Reactivity Pattern of MoLV-specific CTL Clones

Target cells	H-2 haplo-type	MoLV derived	CTL clone*															
			A2.2	B4.2	BH.2	C5.2	E4.2	E10	F10.1	D11.F1S	F9.F8D	A5.6	E6.6	C3.1	B4.7	D2.7		
MBL-2	b	Yes	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	
RBL-5	b	Yes	+++	++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
EL-4	b	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
LSTRA	d	Yes	++	-	-	++	+	-	+	+	++	+	+	+	+	++	+	
P-815	d	No	+	-	-	-	+	-	-	-	++++	+	+	+	+	+	+	

* Percent-specific ⁵¹Cr release during a 3.5-h assay at a lymphocyte:target-cell ratio of 3:1. +++++ = ≥75%; ++++ = 50-74%; +++ = 25-49%; ++ = 10-24%; + = <10%.
 † ND, not done.

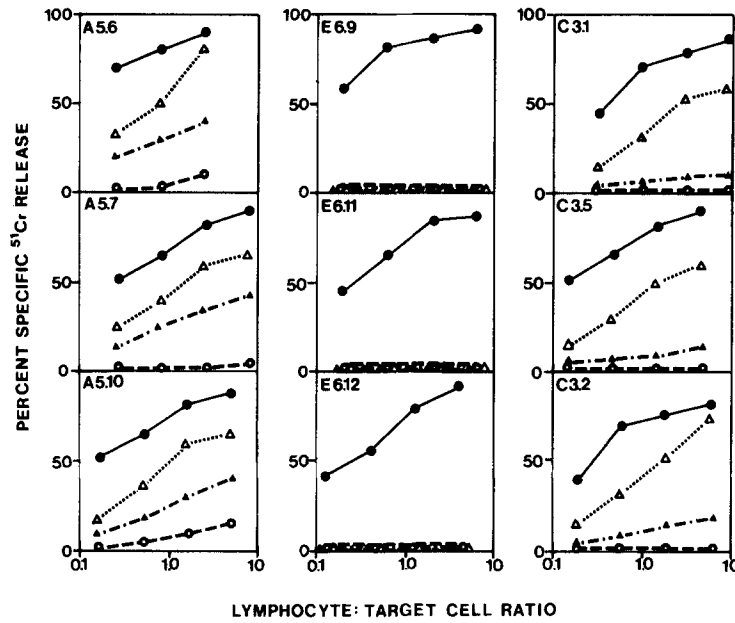


FIG. 7. Stability of specificity and cytolytic activity of MoLV-specific CTL subclones. The cytolytic activity of each of three expanded subclones derived from three clones was assessed against 5,000 ^{51}Cr -labeled MBL-2 (●), EL-4 (○), LSTRA (▲), or P-815 (△) cells.

restesting of individual subclones up to 4 mo after initial isolation confirmed this stability (data not shown).

H-2D^b Restriction of MoLV-specific CTL Clones. Previous reports (7) suggested that CTL of the H-2^b haplotype demonstrate H-2D^b-restricted recognition of MoLV-antigens; however, this restriction has not been examined at the clonal level. Therefore, we investigated whether lysis of MBL-2 target cells by the CTL clones reported here could be inhibited by alloantisera or monoclonal antibodies directed against H-2 determinants. Using conventional alloantisera, it was found that anti-H-2^b serum was a potent inhibitor of CTL activity, whereas anti-H-2^d serum had no detectable inhibitory activity (data not shown).

To further characterize the H-2 restriction of these CTL clones, monoclonal antibodies reactive with determinants encoded by H-2D^b or H-2K^b were used as inhibitors in the CTL assay. All of the six MoLV-specific CTL clones tested were inhibited by both of the anti-H-2D^b monoclonal antibodies (B22-249.R1 and H141-30), whereas the anti-K^b monoclonal antibody (B8-24-1) had no effect (Fig. 8). Control experiments established that the anti-K^b monoclonal antibodies effectively inhibited alloreactive B10A (4R) anti-C57BL/6 (i.e., anti-K^b) CTL.⁵ Thus, based on the limited number of MoLV-specific clones examined, these observations support previous mass culture results (7) of H-2D^b-restricted lysis in the MoLV system.

Discussion

Previous studies had shown the feasibility to isolate CTL clones specific for MoLV-associated cell surface antigens by stimulation of regressor spleen cells in micro-MLTC

⁵ Weiss, A., H. R. MacDonald, J.-C. Cerottini, and K. T. Brunner. Inhibition of cytolytic T lymphocyte clones reactive with Moloney leukemia virus associated antigens by monoclonal antibodies: a direct approach to the study of H-2 restriction. *J. Immunol.* In press.

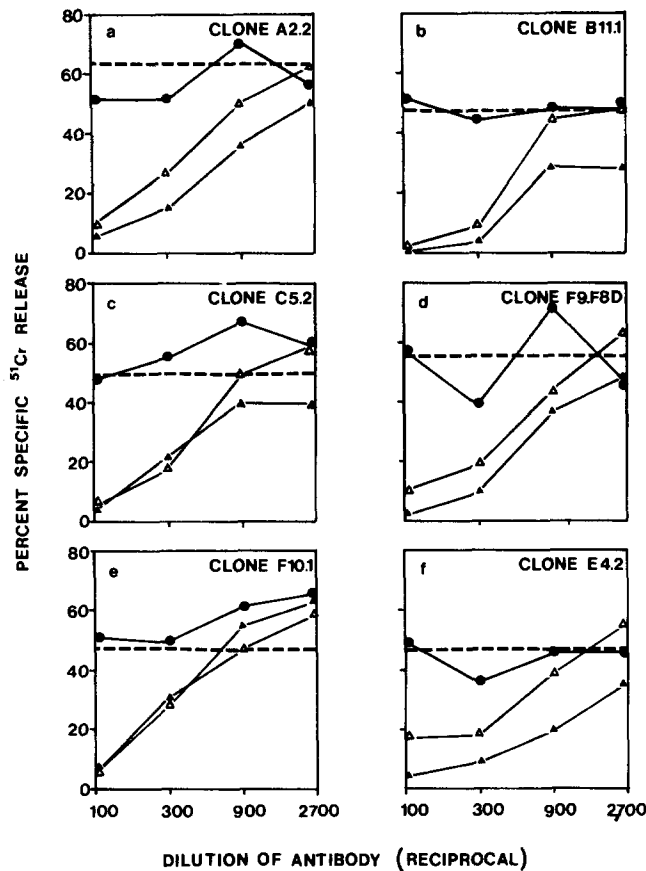


FIG. 8. Inhibition of cytolytic activity of MoLV-specific CTL clones by monoclonal anti-H-2 antibodies. ^{51}Cr -labeled MBL-2 (2,000/culture) were incubated for 30 min at 22°C in the presence of the indicated dilution of B22-249.R1, anti H-2D^b (▲); H141-30, anti H-2D^b (△); or B8-24-1, anti H-2K^b (●). The cytolytic activity of clones A2.2 (a), B11.1 (b), C5.2 (c), F9.F8D (d), F10.1 (e), or E4.2 (f) was then assessed at a ratio of one lymphocyte:target cell in a 3.5-h assay. Dotted lines correspond to cytolysis in the absence of inhibitor.

under limiting-dilution conditions (13). However, the study of the specificity of such clones had been hampered by the limited number of cells generated. Our study shows that CTL clones obtained under limiting-dilution conditions can be expanded readily and maintained in culture for extended periods of time under appropriate culture conditions, i.e., in the presence of antigen (stimulating tumor or infected spleen cells), accessory cells (irradiated syngeneic spleen cells), and a source of T cell growth factor (TCGF) (2° MLC SN). The application of this technique provided large numbers of homogeneous CTL for detailed analysis of Lyt phenotype, specificity, and phenotypic stability.

Regressor spleen cells stimulated in mass culture with MoLV-infected spleen cells were used as a starting population to generate CTL clones. Limiting-dilution analysis indicated that the frequency of CTL-P in such populations had increased dramatically after *in vitro* stimulation, i.e., was between 15 and 50% (K. T. Brunner. Unpublished observations.). This allowed the use of very low responder cell numbers (2–3/micro-

culture), thus increasing the probability that CTL contained in individual microcultures were derived from single precursor cells (i.e., were monoclonal) even at a relatively high percentage of positive cultures. Such putative clones were then selected, expanded, and recloned by limiting-dilution. Results comparing the cytolytic patterns of original and recloned populations in most instances suggested clonality of the original isolates (Fig. 7).

Our study as well as those of Glasebrook and Fitch (19, 20) suggest an important role of antigen in the maintenance of specificity and high cytolytic activity during expansion of CTL clones in TCGF-containing medium. As reported by Nabholz et al. (21), maintenance of cloned CTL lines in TCGF without additional antigen often led to loss of cytolytic activity, loss of specificity, and appearance of chromosomal aberrations. In contrast, such changes have not been observed with the MoLV-specific CTL clones reported here, at least within the 4-mo period during which they have been maintained in culture. Furthermore, the culture conditions used in our study appear to allow the isolation of large numbers of expanded CTL clones, insofar as 16 of 20 attempts to expand selected clones have been successful.

As assessed by flow microfluorometry using monoclonal antibodies, the MoLV-specific CTL clones tested expressed both Lyt-1 and Lyt-2 antigens. These findings are in apparent disagreement with those of Leclerc and Cantor (8), who reported that the cytolytic activity of MoLV-specific CTL populations was unaffected by treatment with anti-Lyt-1 alloantiserum and complement. However, it is likely that this discrepancy can be explained by a relative resistance of CTL to lysis by anti-Lyt-1 antibody plus complement. Indeed, although early studies suggested that alloreactive CTL did not express Lyt-1 antigens, recent data from Nakayama et al. (22) and from this laboratory⁶ clearly indicate that such CTL are Lyt-1⁺2⁺. Thus, it is evident that the MoLV-specific clones reported here have the same Lyt phenotype as alloreactive CTL.

In previous studies, the specificity analysis of CTL clones derived from regressor spleens had demonstrated cross-reactivity of a small percentage of the clones (7%) with allogeneic tumor target cells (13). Our studies confirm and extend these findings. Using four (two additional) target cell types, clones with three patterns of specificity were observed. A large majority of the clones was restricted to the syngeneic tumor, and a small (undetermined) percentage either cross-reacted with a MoLV-derived tumor or (apparently) with H-2^d alloantigens. 14 clones (Table II) were selected and analyzed for clonality and phenotypic stability after expansion and recloning. As shown in a representative experiment (Fig. 7), a high degree of phenotypic stability was observed, with only minor differences in activity and specificity patterns between three subclones derived from each of three different cloned parent populations. Several of these clones were retested over a period of 4 mo, and all were found to maintain their high cytolytic activity and their specificity (A. Weiss. Unpublished results.).

In the studies reported here, interesting clones were selected on the basis of their specificity pattern. Certainly, these clones are not likely to represent the whole repertoire of MoLV antigen-reactive CTL-P in the C57BL/6 mouse, nor has any

⁶ Cerottini, J.-C., and H. R. MacDonald. Limiting dilution analysis of alloantigen-reactive T lymphocytes. V. Lyt phenotype of cytolytic T lymphocyte precursors reactive against normal and mutant H-2 antigens. *J. Immunol.* In press.

attempt been made to determine the frequency of clones that express any given phenotype. Studies in which individual CTL clones are tested for cytolytic activity against syngeneic target cells infected with other viruses and against various allogeneic target cells are required to further analyze the specificity repertoire of CTL in this system.

The restriction of MoLV-specific CTL for target cells that bear syngeneic H-2 determinants has been well documented (6, 7, 23, 24). In particular, Gomard et al. (7) reported that in the H-2^b and H-2^d haplotypes, CTL recognition was restricted to the H-2D^b and H-2K^d regions, respectively. The studies reported here (and other studies)⁵ that employed monoclonal anti-H-2K^b and anti-H-2D^b antibodies support and extend to the clonal level the conclusions of Gomard et al. (7) concerning the restriction of MoLV-specific CTL in the C57BL/6 mouse. All of 6 clones tested (and in other experiments 44 of 51 additional ones)⁵ were inhibited by anti-H-2D^b monoclonal antibodies, whereas none were inhibited by anti-H-2K^b monoclonal antibodies. It remains to be determined whether the basis for such a high degree of restriction to H-2D^b is related to the selective incorporation of H-2D^b molecules into the virion envelope (25). In view of a recent report that suggested that intact virus could substitute for tumor cells in the generation of MoLV-specific CTL in mass culture (26), experiments are in progress to examine if viral preparations can be used as a source of antigen for the isolation and maintenance of CTL clones.

Summary

Cytolytic T lymphocyte (CTL) clones specific for Moloney leukemia virus (MoLV)-derived tumor cells were generated by placing limiting numbers of C57BL/6 responder cells into mixed leukocyte-tumor cell microcultures. Under appropriate conditions (presence of stimulating tumor cells, accessory cells, and T cell growth factor), such cloned CTL could readily be expanded to provide large numbers of homogeneous, highly cytolytic CTL populations for further characterization. Using four target-cell types, three specificity patterns were observed: one reactive with the syngeneic MoLV-derived tumor only, one cross-reactive with an allogeneic MoLV-derived tumor, and one cross-reactive with normal allogeneic cells. Subclones derived from these three types of clones exhibited a high degree of stability in terms of lytic activity and specificity over a 4-mo period of observation. Three clones analyzed by flow cytometry using monoclonal antibodies were all found to be of the Lyt-1⁺2⁺ phenotype. Furthermore, lysis of target cells by all of six clones tested was inhibited by anti-H-2D^b (but not by anti-H-2K^b) monoclonal antibodies, demonstrating H-2D^b-restriction at the clonal level.

We would like to thank Dr. D. Collavo for providing the MoLV preparation used for the infection of neonatal mice, Doctors G. Hämmerling and G. Kohler for providing the monoclonal antibodies, Doctors H. Engers and M. Nabholz for stimulating discussions, P. Corthésy and C. Horvath for their excellent technical assistance, C. Taswell for providing the computer program used for the statistical evaluation of the data, and Mme Cesco for typing the manuscript.

Received for publication 10 July 1980.

References

1. Levy, J. P., and J.-C. Leclerc. 1977. The murine sarcoma virus-induced tumor: exception or general model in tumor immunology? *Adv. Cancer Res.* **24**:1.

2. Holden, H. T., J. S. Haskill, H. Kirchner, and R. B. Herberman. 1976. Two functionally distinct anti-tumor effector cells isolated from primary murine sarcoma virus-induced tumors. *J. Immunol.* **117**:440.
3. Plata, F., and B. Sordat. 1977. Murine sarcoma virus (MSV)-induced tumors in mice. I. Distribution of MSV immune cytotoxic T lymphocytes *in vivo*. *Int. J. Cancer.* **19**:205.
4. Gillespie, G. Y., C. B. Hansen, R. G. Hoskins, and S. W. Russell. 1977. Inflammatory cells in solid murine neoplasms. IV. Cytolytic T lymphocytes isolated from regressing or progressing Moloney sarcomas. *J. Immunol.* **119**:564.
5. Plata, F., J.-C. Cerottini, and K. T. Brunner. 1975. Primary and secondary *in vitro* generation of cytolytic T lymphocytes in the murine sarcoma virus system. *Eur. J. Immunol.* **5**:227.
6. Plata, F., V. Jongeneel, J.-C. Cerottini, and K. T. Brunner. 1976. Antigenic specificity of the cytolytic T lymphocyte (CTL) response to murine sarcoma virus (MSV)-induced tumors. I. Preferential reactivity of *in vitro* generated secondary CTL with syngeneic tumor cells. *Eur. J. Immunol.* **6**:823.
7. Gomard, E., V. Duprez, T. Reme, M. J. Colombani, and J. P. Levy. 1977. Exclusive involvement of *H-2D^b* or *H-2K^d* product in the interaction between T-killer lymphocytes and syngeneic *H-2^b* or *H-2^d* viral lymphomas. *J. Exp. Med.* **146**:909.
8. Leclerc, J.-C., and H. Cantor. 1980. T cell mediated immunity to oncornavirus-induced tumors. I. Ly phenotype of precursor and effector cytolytic T lymphocytes. *J. Immunol.* **124**: 846.
9. Skinner, M. A., and J. Marbrook. 1976. An estimation of the frequency of precursor cells which generate cytotoxic lymphocytes. *J. Exp. Med.* **143**:1562.
10. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatibility antigen-activated cytolytic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J. Exp. Med.* **145**: 508.
11. Teh, H.-S., E. Harley, R. A. Phillips, and R. G. Miller. 1977. Quantitative studies on the precursors of cytotoxic lymphocytes. I. Characterization of a clonal assay and determination of the size of clones derived from single precursors. *J. Immunol.* **118**:1049.
12. Ryser, J.-E., and H. R. MacDonald. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. I. Comparison of precursor frequencies for proliferative and cytolytic responses. *J. Immunol.* **122**:1691.
13. Brunner, K. T., H. R. MacDonald, and J.-C. Cerottini. 1980. Antigenic specificity of the cytolytic T lymphocyte (CTL) response to murine sarcoma virus-induced tumors. II. Analysis of the clonal progeny of CTL precursors stimulated *in vitro* with syngeneic tumor cells. *J. Immunol.* **124**:1627.
14. Cerottini, J.-C., H. D. Engers, H. R. MacDonald, and K. T. Brunner. 1974. Generation of cytotoxic T lymphocytes *in vitro*. I. Response of normal and immune mouse spleen cells in mixed leukocyte culture. *J. Exp. Med.* **140**:703.
15. Ryser, J.-E., J.-C. Cerottini, and K. T. Brunner. 1978. Generation of cytolytic T lymphocytes *in vitro*. IX. Induction of secondary CTL responses in primary long-term MLC by supernatants from secondary MLC. *J. Immunol.* **120**:370.
16. Brunner, K. T., H. D. Engers, and J.-C. Cerottini. 1976. The ⁵¹Cr release assay as used for the quantitative measurement of cell-mediated cytotoxicity *in vitro*. In *In Vitro Methods in Cell-Mediated and Tumor Immunity*. B. R. Bloom and J. R. David, editors. Academic Press, Inc., New York. p 423.
17. Lemke, H., G. J. Hämmerling, and U. Hämmerling. 1979. Fine specificity analysis with monoclonal antibodies of antigens controlled by the major histocompatibility complex and by the Qa/TL region in mice. *Immunol. Rev.* **47**:175.
18. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* **47**:63.

19. Glasebrook, A. L., and F. W. Fitch. 1979. T-cell lines which cooperate in generation of specific cytolytic activity. *Nature (Lond.)* **278**:171.
20. Glasebrook, A. L., and F. W. Fitch. 1980. Alloreactive cloned T cell lines. I. Interaction between cloned amplifier and cytolytic T cell lines. *J. Exp. Med.* **151**:876.
21. Nabholz, M., A. Conzelmann, O. Acuto, M. North, W. Haas, H. Pohlitz, H. von Boehmer, H. Hengartner, J.-P. Mach, H. Engers, and J. P. Johnson. 1980. Established murine cytolytic T-cell lines as tools for somatic cell genetic analysis of T-cell functions. *Immunol. Rev.* **51**:125.
22. Nakayama, E., H. Shiker, E. Stockert, H. F. Oettgen, and L. J. Old. 1979. Cytotoxic T cells: Lyt phenotype and blocking of killing activity by anti-Lyt antisera. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1977.
23. Chesebro, B., and K. Wehrly. 1976. Studies on the role of the host immune response in recovery from Friend virus leukemia. II. Cell-mediated immunity. *J. Exp. Med.* **143**:85.
24. Schrader, J. W., and G. M. Edelman. 1976. Participation of the H-2 antigens of tumor cells in their lysis by syngeneic T cells. *J. Exp. Med.* **143**:601.
25. Bubbers, E. J., and F. Lilly. 1977. Selective incorporation of H-2 antigenic determinants into Friend virus particles. *Nature (Lond.)* **266**:458.
26. Taniyama, T., and H. T. Holden. 1979. In vitro induction of T-lymphocyte-mediated cytotoxicity by infectious murine type C oncornaviruses. *J. Exp. Med.* **150**:1367.