QUANTITATION AND CLONAL ISOLATION OF CYTOLYTIC T LYMPHOCYTE PRECURSORS SELECTIVELY INFILTRATING MURINE SARCOMA VIRUS-INDUCED TUMORS

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In the past, many attempts have been made to correlate tumor immunity and/or tumor rejection with the presence of cytolytic T lymphocytes (CTL)[1] within the tumor mass (1, 2). Most of these attempts have failed, with some notable exceptions (3–9). One of the reasons for the failures may have been the lack of sensitive assay systems for the detection of small numbers of CTL and their precursors. In most tumor systems, CTL are at best formed in small numbers and appear only transiently, or they may be present in the form of noncytolytic CTL precursors (CTL-P) or memory CTL-P, which can only be detected in secondary responses.

Fewer difficulties in detecting CTL have been encountered in studies of the Moloney sarcoma-leukemia virus (MSV-MoLV)-induced tumor system. This system is characterized by the formation of highly immunogenic tumors that regress spontaneously. Before and at the time of tumor regression, CTL specific for MoLV-associated cell surface antigens appear not only in lymphoid organs and blood of the tumor-bearing animals (10), but also in the tumor itself (4–7). The detection of these CTL, however, required a 20-h ^{51}Cr release assay using highly sensitive lymphoma target cells, suggesting the presence of relatively few cytolytic effector cells. On the other hand, cells from lymphoid organs (11) and tumors (12) of such animals gave secondary type CTL responses when stimulated in vitro with syngeneic tumor cells, demonstrating the presence of (memory) CTL-P able to rapidly generate CTL readily detectable in a 6-h assay.

Kinetic studies of CTL formation in the MSV-MoLV system have revealed peak CTL activities in lymph nodes and blood at the time of maximum tumor diameter, whereas intratumoral cells showed peak activity at the onset of tumor regression (5). However, no accurate studies comparing the relative frequency of CTL and/or CTL-P in blood and tumor were carried out, i.e., the question of the possible selective accumulation of CTL and their precursors in the tumor was not resolved.

Methods facilitating the detection, quantitation, and clonal analysis of antigen-

[1] *Abbreviations used in this paper:* CTL, cytolytic T lymphocyte; CTL-P, CTL precursor cell; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MSV, Moloney sarcoma virus; MoLV, Moloney leukemia virus; MLC, mixed leukocyte culture; 2° MLC SN, secondary MLC supernate; MLTC, mixed leukocyte-tumor cell culture; micro-MLTC, MLTC microculture system; PBL, peripheral blood leukocytes; TCGF, T cell growth factor.

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reactive T lymphocytes have recently been described. They are based on the use of T cell growth factor (TCGF) or interleukin 2 as a nonspecific stimulant able to induce and/or maintain the in vitro proliferation of antigen-activated T cells, including CTL (13). The efficacy of TCGF in promoting in vitro the proliferation of small numbers of CTL infiltrating solid 3-methylcholanthrene-induced tumors was recently demonstrated (9).

In our own studies of CTL generation in vivo and in vitro in the MSV-MoLV system, we have shown that small (limiting) numbers of tumor-specific CTL-P could respond under clonal conditions to antigenic stimulation in mixed leukocyte-tumor cell microcultures (micro-MLTC), provided that TCGF and accessory cells in the form of syngeneic irradiated spleen cells were added (14, 15). Not only CTL-P but also mature CTL responded with high cloning efficiency, i.e., CTL behaved operationally like CTL-P (15). Thus it was possible to obtain minimal estimates of the frequency of CTL and of their precursors contained in various cell populations, and to study the progeny derived, at least theoretically, from single CTL-P.

In the present study, we have applied this technique to the determination of CTL-P frequencies in cells infiltrating primary MSV-MoLV-induced tumors at the onset of tumor regression. It will be shown that the tumors contained higher frequencies of MoLV-specific CTL-P than either blood or spleen of the same tumor-bearing animals, demonstrating selective accumulation of CTL-P in the tumor mass at the time of rejection.

Materials and Methods

Mice. Inbred C57BL/6 mice were provided by the animal colony maintained at the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland. The original breeding pairs were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Tumors. MSV tumors were induced in 4-wk-old female C57BL/6 mice by intramuscular injection in the thigh of 0.1 ml of a cell-free tumor homogenate containing the MSV-MoLV complex.

Tumor Cell Lines. MBL-2 (H-2^b), a MoLV-induced lymphoma, was maintained by serial passage of the ascitic form in female C57BL/6 mice. LSTRA (H-2^d), a MoLV-induced lymphoma, P815 (H-2^d) and EL4 (H-2^b), both chemically induced tumors, were maintained in vitro in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS).

Lymphoid Cells. 10 d after MSV-MoLV injection, spleens, blood, and tumors of groups of five mice were collected and a pool of lymphoid cells was prepared as described previously (5). Briefly, spleens were cut into large fragments, suspended in DMEM, and homogenized in Tenbroeck glass homogenizers (Bellco Glass, Inc., Vineland, N. J.). Debris was allowed to sediment for 20 min at unit gravity, and cells in the supernate were washed twice in DMEM. Peripheral blood leukocytes (PBL) were obtained by mixing 1 vol of blood with 2 vol of 2.4% poly-vinyl-pyrolidone containing 5 IU/ml heparin. After sedimentation of the erythrocytes at unit gravity for 45 min, the supernates were centrifuged at 1,400 *g* for 10 min and the cells in the pellet were washed twice with DMEM (5×10^6–8×10^6 cells/ml blood were recovered under these conditions). Tumors were finely minced with scalpels, the fragments disrupted in a Tenbroeck homogenizer, and the debris in the resulting cell suspension (30 ml/tumor) was allowed to sediment for 20 min at unit gravity. The supernates were then centrifuged at 600 *g* for 10 min, the pellet was washed in DMEM, and the cells were suspended in 3 ml DMEM containing 100 μg/ml DNase I (Worthington Biochemical Corp., Freehold, N. J.) and 10 mM Hepes. After 30 min incubation at 37°C, the cells were washed once in 10 ml DMEM, resuspended in 1 ml trypsin (0.5 mg/ml; Difco Laboratories, Detroit, Mich.), and incubated for 5 min at 37°C. After two washes, the cells were resuspended in DMEM containing 10% FBS (10^7 viable leukocytes were recovered per tumor under these conditions).

Micro-MLTC. As described previously (14), micro-MLTC were prepared in DMEM supplemented with additional amino acids (16), 5×10^{-5} M 2-mercaptoethanol, 10% FBS (culture medium), to which 12–23% (vol:vol) secondary mixed leukocyte culture supernate (2° MLC SN) as a source of TCGF (17) was added. 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 (stimulator) MBL-2 tumor cells in a final volume of 0.2 ml in round-bottomed microwells (Greiner, Nürtingen, Federal Republic of Germany). After 7 d of culture, cell growth was assessed microscopically and 100-μl aliquots of effector cells were removed to measure cytolytic activity.

Maintenance of CTL Clones. CTL clones derived from microcultures were transferred to and maintained in 1.6-ml cultures that contained 5×10^6 irradiated (2,000 rad) syngeneic spleen cells, and 3×10^5 irradiated (5,000 rad) syngeneic (stimulator) MBL-2 tumor cells in culture medium supplemented with 25% 2° MLC SN in 16-mm multiwell plates (Costar, Data Packaging, Cambridge, Mass.; 15). After initial expansion, clones were passaged every 4–5 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% dimethyl sulfoxide and 50% FBS.

Assay for Cytolytic Activity. Cytolytic activity of effector cells was determined using a ^{51}Cr release assay (18). Briefly, aliquots of effector cells were mixed with either 2,000 or 10,000 ^{51}Cr-labeled tumor cells (as indicated) in a final volume of 200 μl in round-bottomed microplate wells and incubated for 3.5 h at 37°C. The assay plates were then centrifuged, 100 μl of the fluid was removed, and the amount of released ^{51}Cr was determined in a well-type gamma counter. Controls included maximal release of ^{51}Cr represented by freeze-thawed target cells, and spontaneous release of ^{51}Cr from labeled targets in the absence of effector cells.

Frequency of CTL-P. The method used for the calculation of CTL-P frequencies is described in detail elsewhere (19). Briefly, 24 replicate microcultures prepared with various numbers of responding cells were assessed for cytolytic activity. Positive cultures were defined as those in which the ^{51}Cr release values exceeded by 3 SD the spontaneous release values. Minimum estimates of CTL-P frequencies were calculated by analysis of the Poisson distribution relationship between the number of responding cells per microculture and the percentage of nonresponding cultures. Experimental values were fitted to the zero order term Poisson equation by the minimum χ^2 method.

Fluorescent Staining and Flow Cytometric Analysis. Rat monoclonal antibodies directed against Thy-1.2 (AT83) and Lyt-2 (53.6.7) were kindly provided by Dr. F. Fitch and Dr. J. Ledbetter, respectively. Purified IgG from BALB/c mice immunized with rat immunoglobulin was labeled with fluorescein isothiocyanate as described elsewhere (20).

For fluorescent staining, lymphoid cells (0.5×10^6–1×10^6 in 100 μl) were first incubated with anti-Thy-1.2 (2 μg) or anti-Lyt-2 (undiluted hybridoma culture supernate). Samples were then washed and incubated with ~20 μg of the fluoresceinated mouse anti-rat immunoglobulin. All manipulations were carried out at 4°C.

All samples were run on a flow cytometer (FACS II; B-D FACS Systems, Mountain View, Calif.) gated to exclude nonviable cells as described in detail elsewhere (21). Fluorescence histograms representing 1×10^4–4×10^4 viable cells were accumulated and arbitrarily normalized.

Results

Frequency Estimation of CTL-P in Tumor, Spleen, and Blood Obtained on Day 10 after MSV-MoLV Injection. The frequency of CTL-P contained in cell suspensions obtained from pooled tumor tissue, spleen, or blood from groups of C57BL/6 mice given an intramuscular injection of MSV-MoLV complex 10 d earlier was determined by limiting dilution analysis. Various limiting numbers of responder cells from each pool, 3×10^4 irradiated syngeneic (MBL-2) lymphoma stimulator cells, and 10^6 irradiated syngeneic spleen accessory cells were cultured for 7 d in 200 μl micro-MLTC in the presence of 12–33% 2° MLC SN as a source of TCGF. The microcultures were then scored microscopically for the presence of proliferating cells, and the CTL activity of

each of the 24 replicate cultures was assayed individually by testing a fraction (e.g., 100 μl) against 2,000 ^{51}Cr-labeled MBL-2 target cells. The remaining cells were maintained for up to several days at 37°C to be available for selection and expansion. Results of a representative experiment (Fig. 1) show that as few as 25 cells obtained from the tumors generated positive microcultures with high cytolytic activity against syngeneic MBL-2 tumor target cells, and that increasing numbers of responder cells resulted in increasing numbers of positive microcultures. It can also be seen that 5- to 10-fold higher numbers of responder cells from spleen or blood had to be added to the microcultures to obtain similar numbers of positive cultures. When minimum estimates of CTL-P frequencies were calculated by analysis of the Poisson distribution relationship between the percentage of nonresponding cultures per group and the number of responding cells per microculture (Fig. 2), the frequency of CTL-P as determined by the negative slope of the linear regression curves was found to be 1/67 for the tumors, 1/457 for the spleens, and 1/515 for the PBL, i.e., seven- to eightfold higher in the tumors as compared with spleen and blood.

In five additional experiments identical to the one described in Figs. 1 and 2, frequency estimates were performed on pools of tumor-derived cells, spleen cells, and PBL of mice injected 10 d earlier with MSV-MoLV. Table I summarizes the results

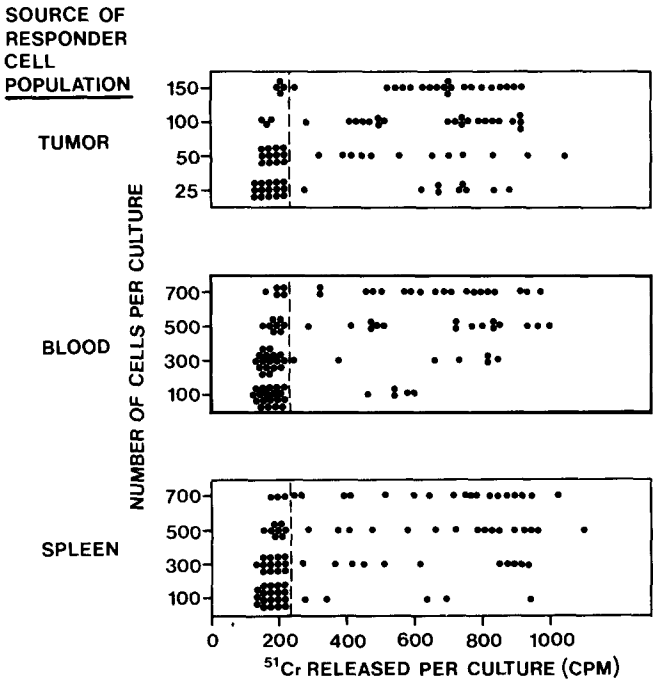

FIG. 1. Cytolytic activity of individual micro-MLTC prepared with (responder) cells obtained from tumors, blood, or spleen of C57BL/6 mice injected 10 d earlier with MSV-MoLV complex. Limiting numbers of responder cells were cultured in micro-MLTC with 3×10^4 irradiated syngeneic MBL-2 lymphoma cells, 10^6 irradiated syngeneic spleen cells, and 12–33% (vol:vol) 2° MLC SN. After 7 d incubation, sets of 24 replicate microcultures were assayed for cytolytic activity against 2,000 ^{51}Cr-labeled MBL-2 target cells in a 3.5-h test. The dotted line represents the mean spontaneous ^{51}Cr-release plus 3 SD, determined in groups of 24 control cultures containing no responder cells, but syngeneic tumor, syngeneic spleen, and 2° MLC SN. This defines the lower limit of positive cytolytic activity. Maximal ^{51}Cr release was 1,254 cpm.

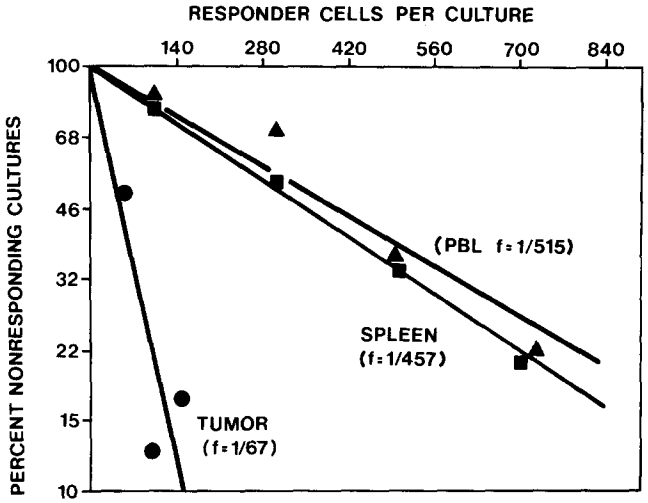

FIG. 2. Minimum estimate of the frequency of CTL-P specific for syngeneic MBL-2 tumor cells in cells obtained from tumors, blood, and spleen of C57BL/6 mice injected 10 d earlier with MSV-MoLV complex. The frequency was calculated by fitting the experimental values presented in Fig. 1 to the zero order term Poisson equation by the minimum χ^2 method. The nonresponding cultures are those that are negative for cytolytic activity against MBL-2 target cells.

TABLE I

*Frequency of MoLV-specific CTL-P in Spleen, PBL, and Tumor of C57BL/6 Mice on Day 10 after MSV-MoLV Injection**

Experiment	Spleen	PBL	Tumor
1	1/918	1/876	1/102
2	1/944	1/552	1/141
3	1/936	1/360	1/98
4‡	1/457	1/515	1/67
5	1/1,031	1/242	1/53
6	1/936	1/468	1/85
Mean	1/806	1/430	1/83

* Micro-MLTC were prepared with limiting numbers of responder cells from spleens, PBL, and tumors as described for Fig. 1, 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 of CTL-P by the minimum χ^2 method.

‡ Data from Fig. 2.

of all six experiments. It can be seen that the tumors consistently contained higher frequencies of CTL-P than either spleens or PBL, and that the difference was on the average 5- to 10-fold. These results suggest selective accumulation of tumor-specific CTL-P in tumor tissue.

T Cell Surface Markers on Tumor-derived Cells, Spleen Cells, and PBL. One explanation for the increased frequency of CTL-P in tumor tissue could be the selective accumulation (either specific or nonspecific) of a T cell subpopulation with the CTL-P surface phenotype. Because CTL-P directed against alloantigens (21, 22) or against MSV-associated antigens (K. T. Brunner, unpublished data) express both Thy-1 and Lyt-2 alloantigens, we therefore determined the proportion of cells in spleen, PBL, or tumor

bearing these markers. In particular, unselected lymphoid cells from each tissue were stained with monoclonal rat antibodies directed against Thy-1.2 or Lyt-2 followed by fluoresceinated mouse anti-rat immunoglobulin. The murine second-step reagent was chosen to eliminate problems with background staining of surface immunoglobulin-positive B cells in the lymphoid suspensions. Samples were then run on a FACS II that was gated to exclude nonviable cells, and fluorescence histograms were obtained.

The results of one of two similar experiments performed are shown in Fig. 3. It can be seen that discrete biphasic fluorescence distributions were obtained with both reagent combinations on all three cell populations tested. Quantitatively, the monoclonal anti-Thy-1.2 reagent was found to stain 13% of spleen cells, 35% of PBL, and 30% of tumor-infiltrating cells in the experiment shown in Fig. 3. Using the anti-Lyt-2 reagent, 5% of spleen cells, 13% of PBL, and 17% of tumor-derived cells were stained. The results of both experiments are summarized in Table II. The relatively low proportion of T cells obtained in spleen may simply reflect a dilution of T cells due to the virus-induced splenic hyperplasia regularly observed in such animals.

Based on the surface phenotype results, CTL-P frequencies in spleen, PBL, and

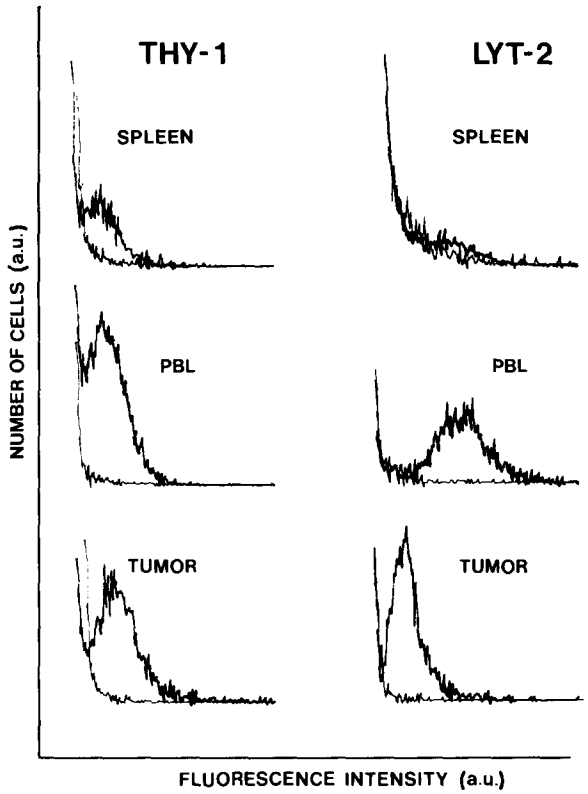

FIG. 3. T cell surface markers of MSV-MoLV immune cells. Suspensions of cells from spleen, blood, or tumors of C57BL/6 mice injected 10 d earlier with MSV-MoLV complex were incubated with rat monoclonal antibodies directed against Thy-1.2 or Lyt-2, followed by fluoresceinated mouse anti-rat Ig. Stained samples were run on a FACS II gated to exclude nonviable cells. Fluorescence histograms are in each case compared with control samples stained with the anti-Ig reagent alone. (The decreased intensity of anti-Lyt-2 fluorescence in the tumor cell population results from the trypsin treatment).

TABLE II

*CTL-P Frequencies in Spleen, PBL, and Tumor-derived Cells Normalized according to T Cell Content**

Cell population	Surface phenotye‡		CTL-P/10^4 cells§		
	Thy-1^+	Lyt-2^+	Total	Thy-1^+ only	Lyt-2^+ only
Spleen	13; 11	5.1; 4.5	10; 11	77; 100	196; 244
PBL	35; 29	13; 12	41; 21	117; 72	315; 175
Tumor	30; 22	17; 11	190; 115	633; 523	1,118; 1,045

* Data are from two independent experiments, corresponding to experiments 5 and 6 in Table I.
‡ Percent positive cells as determined by flow microfluorometry.
§ CTL-P frequencies for each of the cell populations analyzed by flow microfluorometry are expressed relative to the content of Thy-1^+ or Lyt-2^+ cells.

tumor were recalculated to account for the relative T cell content in these tissues. Such an analysis (Table II) revealed that CTL-P in tumor tissue were still five- to eightfold enriched as compared with spleen or PBL when only Thy-1^+ cells were considered, and four- to sixfold enriched when the calculation was based only on Lyt-2^+ cells. It is noteworthy that, in absolute terms, about 1/9 Lyt-2^+ cells in the tumor-derived population behaved operationally as a CTL-P, vs. an average of 1/45 and 1/41 for spleen and PBL, respectively. These phenotypic data thus confirmed that CTL-P were in fact significantly enriched among tumor-infiltrating lymphoid cells.

Expansion of Clonal CTL Populations Isolated from Tumors and Determination of Cytolytic Activity and Specificity. It was shown in a previous study that MoLV-specific CTL populations obtained under limiting dilution (clonal) conditions from spleens of regressor mice could be expanded by stimulation with irradiated syngeneic tumor cells in 1.0-ml cultures containing irradiated syngeneic spleen cells and TCGF in the form of 2° MLC SN (15). Such expanded populations could then be recloned by the limiting dilution technique and/or studied for cytolytic activity and specificity.

Following this approach, highly cytolytic microculture populations obtained from limiting numbers of tumor-derived cells were selected in several experiments, and the cells expanded. In a typical experiment, 6 of 12 microcultures selected for expansion showed optimal proliferation, generating between 1.2×10^6 and 2.1×10^6 cells/culture (average 1.8×10^6) in 5 d. Fig. 4 shows the comparative cytolytic activity of these expanded isolates against syngeneic MBL-2 lymphoma target cells. It can be seen that five of the six populations showed high activity, leading to 50% lysis at lymphocyte:target cell ratios of 0.5:1 to 1.1:1.

In an experiment designed to study the specificity of tumor-derived CTL clones, micro-MLTC containing limiting numbers of responder cells were screened microscopically on day 7 of incubation for proliferation, and 13 positive cultures were randomly selected for expansion. Of these cultures, five responded optimally in two successive restimulations. These five putative clones (A1, C5, E1, D4, and C12) were then tested for cytolytic activity against four tumor target cell types, i.e., MBL-2 (H-2^b), the syngeneic stimulating MoLV-derived lymphoma; LSTRA (H-2^d), an allogeneic MoLV-derived lymphoma; EL4 (H-2^b), a syngeneic non-MoLV-derived lymphoma, and P815 (H-2^d), an allogeneic non-MoLV-derived tumor. To confirm the sensitivity of CTL-mediated lysis of these different target cells, a spleen-derived C57BL/6 anti-MSV clone (C1) that had been selected for cross-reactivity with H-2^d

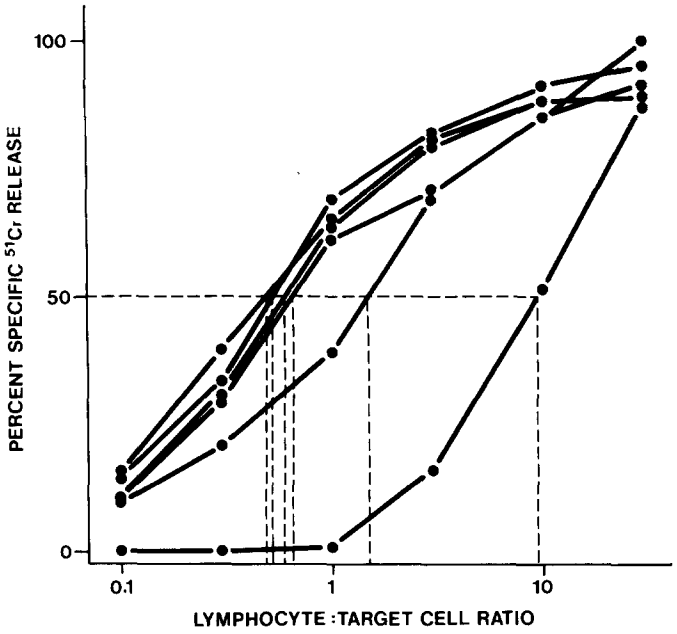

FIG. 4. Cytolytic activity of tumor-derived MoLV-specific CTL clones. The cytolytic activity of six clones was assessed against 10,000 ^{51}Cr-labeled MBL-2 tumor target cells in a 3.5-h assay.

alloantigens (14, 15) was included in the assay. As shown in Fig. 5, all five tumor-derived populations showed exquisite specificity for the syngeneic MoLV-derived tumor target, i.e., all showed strong cytolytic activity (50% lysis at lymphocyte:target cell ratios ranging from 0.6:1 to 3.2:1) against the syngeneic MoLV-derived MBL-2 targets only. The cross-reactive clone C1 showed strong cytolytic activity for P815 and MBL-2 target cells and weaker activity for LSTRA, but, as expected, it showed no activity for the syngeneic non-MoLV-derived target EL4. As described previously, the same EL4 cells are readily lysed by CTL directed against H-2^b alloantigens (23).

Discussion

The present report demonstrates that MoLV-specific CTL-P contained in MSV-MoLV induced tumors can be isolated, quantitated, and clonally analyzed by applying a sensitive limiting dilution micro-MLTC technique. Our results obtained in applying this technique show that tumor-infiltrating cells collected on day 10 after MSV-MoLV injection (i.e., approximately at or close to the onset of tumor regression) contained on the average fivefold higher CTL-P frequencies than either PBL or spleen cells.

Previous studies in this system (5) had shown that tumor-derived cells were heterogeneous, containing 52% lymphocytes (including 23% Thy-1$^+$ cells). In the present report, monoclonal antibodies directed against Thy-1 or Lyt-2 alloantigens were used in combination with flow cytometric technology to further quantitate T cell subsets in the tumor-derived cell population. We found that, on the average, ~26% of tumor-derived cells expressed Thy-1 and 14% expressed Lyt-2, as compared with 32% Thy-1$^+$ and 13% Lyt-2$^+$ in the circulating peripheral blood. Thus it appears

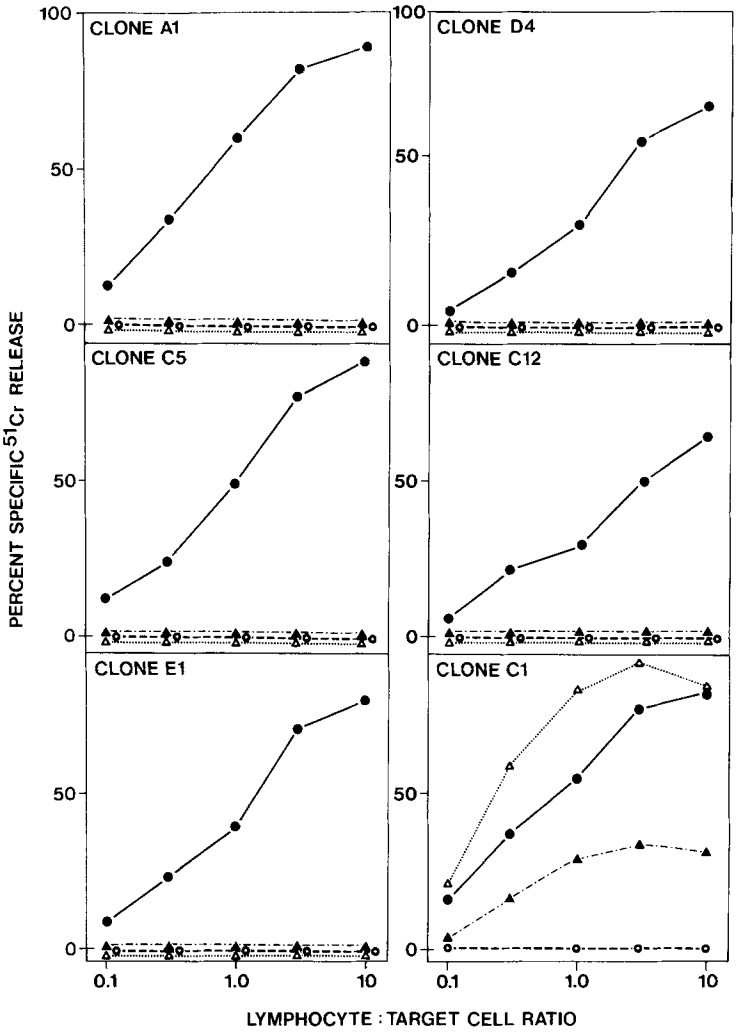

FIG. 5. Specificity of five tumor-derived CTL clones (A1, C5, E1, D4, and C12) reactive with MoLV-associated cell surface antigens. Micro-MLTC were prepared as indicated in Fig. 1, and 5 of 13 randomly selected cultures were expanded and assayed against 10,000 ^{51}Cr-labeled MBL-2 (●), LSTRA (▲), EL4 (○), and P815 (△) target cells in a 3.5-h assay. A spleen-derived MoLV-specific CTL clone (Cl) showing cross-reactivity with P815 and LSTRA target cells was assayed in parallel.

that the proportion of total T cells expressing Lyt-2 is slightly higher among tumor-derived cells (54%) than among either PBL (39%) or spleen cells (40%). Because CTL-P reactive against alloantigens (21, 22) or MSV-associated antigens (K. T. Brunner, unpublished data) are entirely contained within the Lyt-2^+ subpopulation of T cells, it was considered important to base a comparison of CTL-P frequencies in tumor-derived cells and PBL (or spleen) on their content in Lyt-2^+ cells. When this was done (Table II), the average CTL-P frequencies per Lyt-2^+ T cell were found to be 1/9 and 1/41 for tumor-infiltrating cells and PBL, respectively, i.e., the frequency was significantly (4.6-fold) higher in the tumor-derived population. These data thus demonstrate a selective accumulation of CTL-P in the tumor mass.

Earlier studies in the same system had shown that day 12 tumor-derived lymphoid cell populations had higher cytolytic activities than regional lymph node cells from the same animals, suggesting a selective accumulation of CTL in the tumors (5). However, PBL collected on day 9 were more active than either spleen or regional lymph node cells, and a direct comparison of tumor-derived lymphoid cells and PBL from the same tumor-bearing animals was not carried out. Furthermore, CTL assays at the population level cannot distinguish between CTL with different cytolytic activities and thus do not allow formal conclusions as to the frequency of CTL. Although the precursor frequency assay used in the present study circumvents this problem, it should nevertheless be noted that such assays provide minimum estimates of the frequency of cells of the CTL lineage regardless of their state of differentiation, i.e., they detect noncytolytic CTL-P as well as cytolytic CTL independently of their killing efficiency (15, 24). It may therefore be assumed that the CTL that can be detected in tumor-infiltrating cells by a direct lytic assay are comprised in the operationally defined CTL-P determined by the limiting dilution assay.

Our results showing lysis of syngeneic MoLV-derived target cells and lack of lysis of MoLV-induced allogeneic or of syngeneic and allogeneic unrelated tumor target cells by five (clonal) tumor-derived CTL populations (Fig. 5) confirm that H-2 restriction of clonal MoLV-specific CTL populations is not only valid for spleen-derived (15, 25) but also for tumor-derived C57BL/6 effector cells. Because the five CTL populations were randomly selected, it may be assumed that their specificity was representative for the majority of the tumor-reactive CTL-P contained in the tumor mass. In this context, it is worth mentioning that a minority of the MoLV-specific CTL-P in C57BL/6 regressor spleens had been found to cross-react with MoLV-induced allogeneic (H-2^d) tumor cells or normal (uninfected) allogeneic (H-2^d) target cells (14, 15). It remains to be determined whether such cross-reactive CTL are present in tumor-infiltrating cell populations. The five CTL populations tested for specificity against four target cell types were obtained from an experiment in which limiting numbers (25, 50, 100, and 150) of responding cells were placed in sets of 24 micro-MLTC, and in which 17–37.5% of the microcultures were positive. On statistical grounds, the probability that the five selected cultures were clonal thus ranged from 79 to 91%. However, the clonality of these populations was not considered to be an issue for the present considerations, and no effort was made to reclone these populations.

The approach used to isolate CTL-P directly from tumors by placing limiting numbers of lymphoid cells isolated from homogenized and enzyme-treated tumor tissue into micro-MLTC has several advantages. First, the extraction procedure is simple and gives high yields of relatively pure leukocytes; second, only tumor-specific CTL-P proliferate under the microculture conditions used; third, the frequency of tumor-associated CTL-P (including CTL) can be estimated directly; fourth, large numbers of clonal CTL populations with the capability of continued proliferation can readily be obtained for further study. In this context it was interesting to observe that individual clones showed variable growth potential, some clones being lost after one or several restimulations. In conclusion, direct isolation of CTL clones from tumor tissue, especially if shown to be applicable to other tumor systems, should greatly facilitate further studies of the role of CTL in tumor immunity, particularly concerning the selective localization of CTL (and of potential helper T cells) in tumors, the

specificity repertoire of tumor-associated CTL, and the homing pattern and functional activity of clonal CTL populations studied in transfer experiments.

Summary

A limiting dilution mixed leukocyte-tumor cell microculture system was used to quantitate cytolytic T lymphocytes and their precursors (CTL-P), which infiltrate tumors induced by injection of Moloney sarcoma-leukemia virus (MSV-MoLV) complex into C57BL/6 mice. Leukocyte populations obtained from tumors collected on day 10 after virus injection were found to contain significantly higher frequencies of operationally defined (tumor-specific) CTL-P than either peripheral blood leukocytes (PBL) or spleen cells from the same animals. When these frequencies were normalized according to the content of Lyt-2^+ T cells in each tissue, average CTL-P frequencies were found to be 1/9 in tumor-infiltrating cells vs. 1/41 in PBL. These results directly demonstrate selective accumulation of CTL-P in the tumor mass.

A number of clonal isolates obtained from tumor-infiltrating leukocyte populations were expanded and studied for cytolytic activity and specificity. Of 11 isolates, 10 were found to have high cytolytic activity, leading to 50% lysis of the syngeneic MoLV-derived tumor target cells in 3.5 h at lymphocyte:target cell ratios ranging from 0.5:1 to 3.2:1. Furthermore, five randomly selected clones showed H-2 restriction by their selective lytic activity against MoLV-derived syngeneic MBL-2 target cells and their lack of activity against either MoLV-derived allogeneic (LSTRA) tumor cells or against syngeneic (EL4) or allogeneic (P815) target cells unrelated to MoLV.

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