QUANTITATIVE ASSESSMENT OF THE POOL SIZE AND SUBSET DISTRIBUTION OF CYTOLYTIC T LYMPHOCYTES WITHIN HUMAN RESTING OR ALLOACTIVATED PERIPHERAL BLOOD T CELL POPULATIONS

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In recent years, it has become widely accepted that functionally distinct T cell subpopulations can be identified by appropriate surface markers. For example, in the mouse, the Lyt-2/3 alloantigen complex has been used to define a functional T cell subset including cells with cytolytic and/or suppressor activity (1). In man, molecules structurally related to Lyt-2/3 antigens have been identified using the monoclonal antibodies OKT8 (2), Leu 2a, b (3), or B9 (4). Initially, functional analysis of T cell populations led to the conclusion that cytolytic T lymphocytes (CTL)[1] were confined to the $T8^+/T4^-$ subset (5). However, this concept was recently challenged by the demonstration that among CTL clones some were $T8^-/T4^+$ and others $T8^+/T4^-$ (6). Based on specificity analysis of a limited number of alloreactive CTL clones, the suggestion has been made that there is a correlation of T4 and T8 expression with recognition of class II and class I major histocompatibility complex (MHC) products, respectively (7).

It should be emphasized that such studies suffer from several limitations in interpretation. First, there is the possibility that CTL clones, because they are selected by their ability to be expanded and maintained in continuous culture, are not representative of the CTL populations used for cloning. More importantly, any meaningful interpretation of these surface phenotype analyses depends on the unproven assumption that expression of T4 and T8 antigens is not altered during the differentiation and/or proliferation steps that are involved in CTL generation and cloning. Indeed, it has been suggested that the Lyt phenotype of functional T cells can change in the course of activation (8). Therefore, a more direct approach to the question of surface phenotype of CTL would be

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[1] *Abbreviations used in this paper:* CTL, cytolytic T lymphocyte; CTL-P, CTL precursor; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FLS, forward light scatter; MHC, major histocompatibility complex; MLC, mixed leukocyte culture; PBMNC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PTL-P, proliferating T lymphocyte precursor; SN, supernatant; TCGF, T cell growth factor.

to determine the frequency of CTL and CTL-precursors (CTL-P) in T cell subsets derived from activated and normal populations, respectively. With the recent development of limiting dilution microculture techniques allowing the development of clonal progeny from single CTL-P, such an approach is now feasible. For example, studies in murine systems using negatively or positively selected T cell subsets have shown that >95% of alloreactive CTP-P are confined to the Lyt-2$^+$ subset (9). Moreover, using a limiting dilution assay that allows the detection of all CTL-P (regardless of their specificity), Wilson et al. (10) reported that almost all Lyt-2$^+$ T cells are CTL-P.

In this work, we have used a similar limiting dilution microculture system to quantitate precursors of CTL and of other proliferating noncytolytic T cells in human T cell subsets positively and negatively selected for the expression of B9 antigen. As reported previously (11), virtually all peripheral blood T cells undergo clonal expansion under the culture conditions used. It will be shown that approximately one-third of all peripheral blood T cells are CTL-P. ~90% of these CTL-P are found in the B9$^+$ subset (which represents 25% of all T cells). Hence, the CTL-P frequency in B9$^+$ cells is virtually 100%, whereas the B9$^-$ T cell subset, which contains 10% of all CTL-P, has a >25-fold lower CTL-P frequency. Similar results were obtained using B9$^+$ and B9$^-$ subsets derived from T cell populations activated in allogeneic mixed leukocyte (MLC) cultures. Frequency analysis of CTL-P and CTL directed against a given set of MHC antigens in these two T cell subsets confirmed these findings.

Materials and Methods

Isolation of Lymphoid Cells.

Purified T cells were isolated from peripheral blood mononuclear cells (PBMNC) as previously described (2). Adherent cells were removed from PBMNC by incubation on plastic petri dishes. Spleen cell suspensions to be used as a source of feeder (or stimulating) cells were frozen and subsequently thawed as needed (13).

Mixed Lymphocyte Cultures.

Cells were suspended in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS). MLC were set up as previously described (14) by culturing purified T cells either in macrowells (10^6 cells/well) or in round-bottom microwells (10^5 cells/well) with equal numbers of irradiated (4,500 rads) allogeneic spleen cells. To evaluate cell proliferation, tritiated thymidine (^{3}HTdR) was added to triplicate microwell cultures 18 h before harvesting. On day 7, cells were harvested and centrifuged on Ficoll-Hypaque gradients, to remove dead cells. The percentage of E-rosetting cells was then determined. When the proportion of E-rosetting cells was >95%, no further purification was performed.

Monoclonal Antibody (mAb).

B9.4 mAb, kindly provided by Dr. Bernard Malissen and Dr. Claude Mawas (Centre d'Immunologie INSERM-CNRS, Marseille-Luminy, France), is derived from BALB/c mice immunized with human anti-HLA-A2 cloned cytotoxic T lymphocytes derived from immunized donors as previously described in detail (15). B9.4 mAb binds to surface molecules that are similar, if not identical, to those identified by Leu 2a, Leu 2b, and OKT8 mAbs. This conclusion is based on the fact that each one of these mAb reacts with the same percentage of peripheral T cells, and that the percentage of positive T cells is not increased when B9.4 mAb is mixed with either one of the other mAb. Moreover, blocking experiments indicate that B9.4 mAb inhibits the binding of Leu 2a, Leu 2b, or

OKT8 mAb to T cells (A. Moretta, manuscript in preparation). Further information on the relationship between antigenic determinants recognized by B9.4 and those recognized by the other mAbs has been provided by experiments in which the sensitivity to trypsin treatment has been evaluated. The antigenic determinants recognized by B9.4 mAb exhibit a trypsin sensitivity similar to those defined by Leu 2b mAb (3) since they are undetectable after treatment of T cells with 30 μg/ml trypsin for 30 min at 37°C (Moretta et al., manuscript in preparation). In contrast, Leu 2a (3) and OKT8 antigenic determinants appear to be more resistant to treatment with this dose of trypsin. B9.4 monoclonal antibody was used under the form of ascitic fluid diluted in RPMI 1640 (Gibco Laboratories, Grand Island, NY) at a final dilution of 1/100 (corresponding to 100 μg/ml of antibody).

Immunofluorescence Staining and Fluorescence-activated Cell Sorter (FACS) Analysis.

The techniques used have been previously described (16). Briefly, samples of 2×10^6 peripheral blood purified T cells or MLC T cells in 100 μl RPMI 1640 medium were incubated with 5 μg of B9.4 monoclonal antibody for 30 min at 4°C. After two washings, the cells were resuspended in 200 μl of medium containing fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG. Control samples were incubated with the FITC-coupled reagent only. After 30 min of incubation at 4°C, all samples were washed three times and analyzed on a flow cytometer (FACS II; B-D FACS system, Becton Dickinson & Co. Sunnyvale, CA). Results are presented as fluorescence histograms with the number of cells on the $Y -$ axis and the fluorescence intensity on the $X -$ axis. Both parameters are expressed in arbitrary linear units. The percentage of positive cells in each histogram is calculated only when the distribution is clearly biphasic. The percentage of cells under each peak will be reduced by the percentage of control samples in the same interval of fluorescence to yield a net value for percent of positive cells.

FACS Sorting. Before sorting cells, the sample tubing of the FACS was flushed sequentially with detergent, sterile distilled water, and 96% ethanol. Then, the tubing was rinsed for 30 min with a solution of gentamycin (50 μg/ml) in PBS. Because the fluorescence profiles of cells stained with B9.4 Mab were clearly biphasic (see Fig. 1), <5% of all cells had to be discarded because their fluorescence intensity coincided with the inflection point of the curve. Sorted cells were collected in sterile plastic tubes whose inside surfaces had been coated with sterile FCS. Once collected, cells were immediately diluted in culture medium.

Microcultures. Cells (MLC T or peripheral blood T cells) were submitted to vigorous mixing (to dissociate possible cell clumps) and then seeded in limiting numbers in round-bottom microwells (Greiner, Nurtingen, Federal Republic of Germany) containing 10^5 irradiated (5,000 rads) feeder cells in a final volume of 0.2 ml RPMI 1640 medium containing 10% heat-inactivated FCS. Supernatant (SN) from cultures of PHA-stimulated human spleen cells (routinely depleted of PHA as described [17]) was used as a source of T cell growth factor (TCGF). SN was added (50% vol/vol) either at the onset of the cell culture (microcultures containing MLC T responder cells) or after 48 h (microcultures containing peripheral blood T lymphocytes responder cells [11]). PHA (1% vol/vol) was added at the onset of the culture in microplates containing peripheral blood T cells (11). Both types of microcultures were then supplemented weekly with 10^5 irradiated feeder cells suspended in 100 μl of TCGF-containing SN. Controls included irradiated feeder cells cultured in the presence of TCGF containing SN or in the presence of both PHA and SN (the first type of control was used for microcultures containing MLC T cells and the second one for microcultures containing peripheral blood T lymphocytes). Plates were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

After 14–18 d of culture each microwell was assessed for growth and cytolytic activities. Proliferation was determined microscopically as previously described (6).

Assay of Cytolytic Activity.

Alloactivated T cell populations derived from bulk cultures in macrowells were tested for cytolytic activity against PHA-induced blasts derived from the stimulator cell popula-

tion and from the responding donor cells as described previously (6). Briefly, varying numbers of MLC T cells were incubated in V-bottom wells of microtiter trays with ^{51}Cr-labeled (5×10^3) target cells (in a final volume of 200 μl) thus ensuring lymphocyte/target cell ratios ranging from 6.25:1 to 50:1. Plates were centrifuged at 100 *g* for 5 min and then incubated for 4 h at 37°C. After incubation the plates were centrifuged at 200 *g* for 5 min. Cytolysis was then assessed by counting the radioactivity of 0.1 ml supernatant for 1 min in a gamma counter. Specific lysis was calculated according to the formula:

$$\text{Percent specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Maximum release was determined by lysing ^{51}Cr-labeled cells in 0.1 N HCl. Spontaneous release from ^{51}Cr-labeled target cells was determined by incubating these cells in culture medium.

Cytolytic activity of individual microcultures in which MLC T cells were plated under limiting conditions was tested against three different types of ^{51}Cr-labeled target cells: (*a*) PHA-induced blasts derived from the stimulator cell population, (*b*) autologous PHA-induced blasts, and (*c*) P815 murine tumor cells in the presence of 1% (vol/vol) PHA (11). To this end, each microculture was split in three 50-μl aliquots. Each aliquot was then tested against a different ^{51}Cr-labeled target cell population (5×10^3/well). In the lectin-dependent cytolytic assay, PHA was added to the ^{51}Cr-labeled P815 cell suspensions immediately before distributing the target cells in microwells (to avoid the formation of cell clumps). Spontaneous release was determined in control microcultures prepared in the same manner as the experimental group, but without the addition of responder cells. Cultures in which ^{51}Cr release exceeded the mean spontaneous release by more than 3 standard deviations were considered positive for cytolytic activity (11). The same procedure was used for evaluating the cytolytic activity of microcultures containing peripheral blood T lymphocytes as responder cells.

Determination of the Frequency of Proliferating and/or Cytolytic T Lymphocyte Precursors.

Minimal estimates of the frequencies of proliferating (PTL-P) or cytolytic (CTL-P) T lymphocytes were obtained by the minimum chi square method from the Poisson distribution relationship between the responding cell number and the logarithm of the percentage of nonresponding (negative) cultures (18).

Results

Distribution of B9 Antigen in Resting and MLC T Cells. The expression of B9 antigen on human peripheral blood T cells was studied by indirect immunofluorescence and cytofluorometric analysis. Freshly isolated (resting) T cells as well as T cells that had been activated in allogeneic mixed leukocyte cultures (MLC) were used. The fluorescence profiles obtained with resting and MLC T cells from a single donor are shown in Fig. 1 *A* and *B*. A bimodal distribution of fluorescence was clearly observed with both populations. ~25% resting T cells and ~30% MLC T cells, were found to express B9 antigen. In a series of six experiments, the percentage of B9$^+$ cells varied between 20 and 30% in resting T cells and between 20 and 60% in MLC T cells. T cell-depleted mononuclear cell populations showed no reactivity with B9 mAb (data not shown). Fig. 1 *C* and *D* also shows a forward light scatter (FLS) analysis of B9$^+$ and B9$^-$ MLC T cells. In populations of regularly shaped cells, FLS is roughly proportional to the square of the mean cellular radius (19). It can be seen that both positive and negative fractions included small, medium, and large cells.

Cytolytic Activity of MLC T Cell Populations Fractionated on the Basis of B9 Antigen

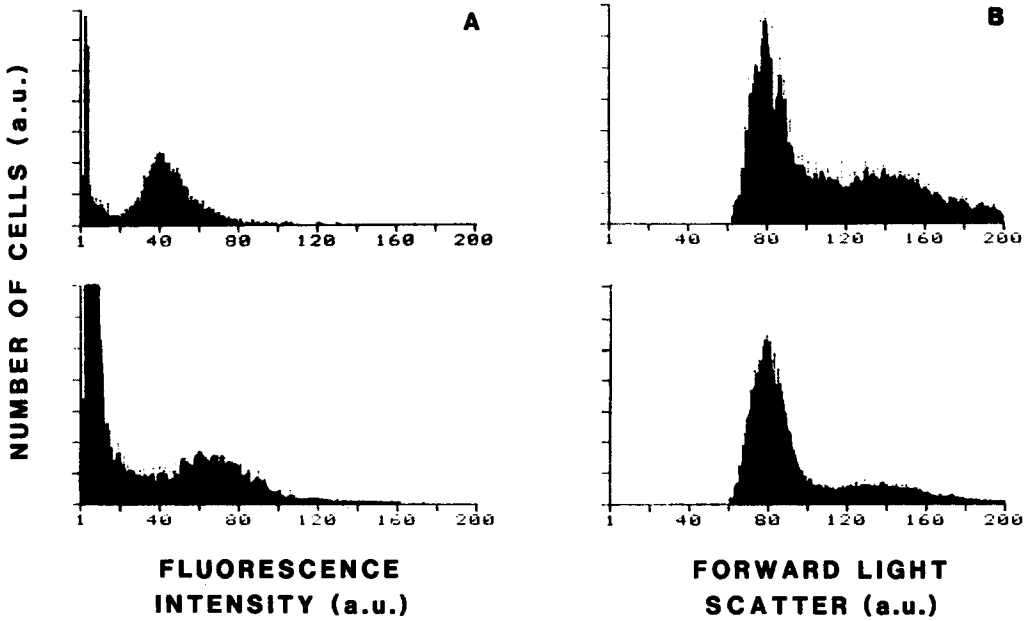

FIGURE 1. (*A*) Fluorescence distribution of B9 antigen in human peripheral blood and MLC-activated T lymphocytes. T cells were stained with optimum concentrations of B9 monoclonal antibody, followed by appropriate fluoresceinated anti-Ig reagents. Samples were run on FACS II gated to exclude nonviable cells. (*B*) Forward light scatter (FLS) analysis of $B9^+$ and $B9^-$ MLC human T lymphocytes.

Expression. T cell populations stimulated for 7 d in MLC were incubated sequentially with B9 mAb and fluoresceinated anti-Ig conjugate, and then sorted into stained and unstained cells on a FACS. Given the clear-cut bimodal fluorescence distribution observed under these conditions (Fig. 1), a precise fractionation of MLC T cells into $B9^+$ and $B9^-$ fractions could readily be achieved. The cytolytic activities of the two fractions as well as the unfractionated MLC T cells were tested on PHA-induced blast cells bearing the stimulating alloantigens in a short-term ^{51}Cr release assay (20). The results of two representative experiments are shown in Fig. 2.

It can be seen that $B9^-$ cell fractions had no detectable cytolytic activity. Moreover, dose-response analysis showed that cytolytic activity was two- to threefold higher in $B9^+$ cell fractions as compared with unfractionated T cell populations.

Frequency Analysis of Proliferating and/or CTL Precursors in MLC T Cell Populations. In an attempt to determine the actual frequency of specific CTL in $B9^+$ and $B9^-$ fractions derived from MLC T cell populations, we used a limiting dilution microculture system similar to that previously established in this laboratory for frequency analysis of alloreactive proliferating and cytolytic T lymphocytes in murine lymphoid cell suspensions (21). Graded numbers of cells from unfractionated MLC populations, and from positively and negatively selected $B9^+$ and $B9^-$ subpopulations were cultured with allogeneic irradiated spleen cells in the presence of a saturating dose of TCGF. After incubation for 14–18 d each

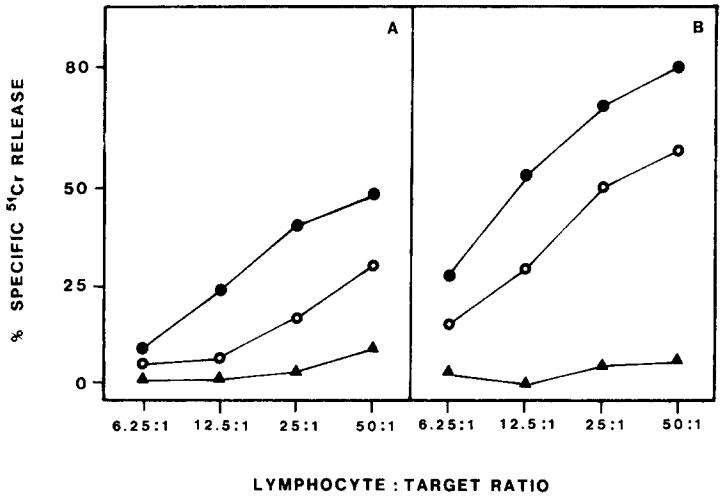

FIGURE 2. Cytolytic activity of MLC-activated human T cells sorted according to B9 fluorescence intensity (see Fig. 1). Unfractionated, $B9^+$ and $B9^-$ MLC T cells were tested against PHA-induced allogeneic blast cells bearing the stimulating alloantigens in a 4-h ^{51}Cr-release assay. The spontaneous release of labeled target cells never exceeded 6% of the total release. O, unfractionated; ●, $B9^+$; and ▲, $B9^-$ MLC-activated T cells.

microculture was scored for proliferation by microscopical examination and then divided into two aliquots for assay of cytolytic activity. One aliquot was tested against ^{51}Cr-labeled PHA-induced blast cells bearing the stimulating alloantigens, whereas the other aliquot was tested against ^{51}Cr-labeled autologous PHA-induced blast cells. The frequencies of proliferating and/or cytolytic cells are calculated by analysis of the Poisson distribution relationship between the number of cells plated per culture and the percentage of negative cultures. The results of such an analysis performed on microcultures tested for cytolytic activity against the relevant allogeneic target cells are shown in Fig. 3. It can be seen that cytolytic microcultures were obtained with relatively low numbers of responding cells from the $B9^+$ fraction, whereas $B9^-$ cells, even at relatively high cell doses, generated few cytolytic microcultures. Since none of the microcultures exhibited cytolytic activity against autologous target cells (data not shown), it can be concluded that the microcultures cytolytically active on allogeneic target cells contained specific alloreactive CTL derived from a single or several precursor cells. The frequencies of such alloreactive CTL precursors (CTL-P) were calculated by the statistical method described by Taswell (18). As summarized in Table I (Exp. 1), the CTL-P frequency was 1 cell in 14 in the unfractionated MLC population, 1 cell in 3.3 in the $B9^+$ fraction and 1 cell in 69 in the $B9^-$ fraction. Similar results were obtained in two additional experiments (Table I, Exp. 2 and 3). Compared with the unfractionated MLC population and the $B9^-$ fraction, the frequency of specific CTL-P was increased 3.5- and 21-fold, respectively, in the $B9^+$ fraction. When the proportion of total cells in each fraction was taken into account, it was calculated that 90% of the specific CTL-P recovered after sorting of the MLC population were found in the $B9^+$ fraction (Table I). In contrast, frequency analysis of the precursors of proliferating T

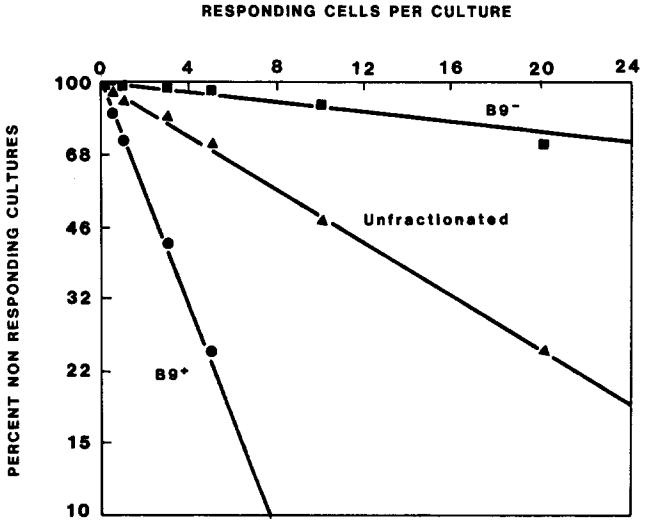

FIGURE 3. Frequency analysis of specific alloreactive CTL-P in MLC T lymphocytes separated according to B9 expression. Responding T cells were stimulated in allogeneic MLC against irradiated human spleen cells. After 7 d of culture, the cells were separated by FACS sorting into $B9^+$ and $B9^-$ fractions. Limiting dilution analysis was performed by culturing varying cell doses (ranging from 1 to 20 cells/well) in the presence of irradiated allogeneic spleen cells and TCGF. After 18 d of culture, each microculture was scored for specific cytolytic activity against ^{51}Cr-labeled PHA-induced blast cells as described in Materials and Methods. Each point is based on a group of 48 microcultures. CTL-P frequencies were calculated as described in Materials and Methods. ▲, unfractionated; ●, $B9^+$; and ■, $B9^-$ T cells.

TABLE I

*Frequency Analysis of Alloreactive CTL-P in MLC T Cell Subsets**

MLC-T population	Percent of total cells			Reciprocal of CTL-P frequency			Percent of the total CTL-P recovered		
	Exp. 1‡	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Unfractionated	100	100	100	14.14	12.65	11.5	—	—	—
$B9^+$	30.3	27.1	35	3.34	3.45	4.2	90	91	90.2
$B9^-$	69.7	72.9	65	69.15	93.45	72.8	10	9	9.8

* T lymphocytes activated in primary allogeneic MLC were sorted according to B9 fluorescence intensity (see Fig. 1) and assayed for their content of specific alloreactive CTL-P as described in Materials and Methods.

‡ Data from Fig. 3.

lymphocytes (PTL-P) in the same populations revealed no significant difference between $B9^+$ and $B9^-$ fractions. For example, in the experiment corresponding to Fig. 3, minimal estimates of the PTL-P frequencies in the $B9^+$, $B9^-$, and unfractionated populations were determined to be 1 cell in 2.2, 1 cell in 2.3, and 1 cell in 2.0, respectively (data not shown). Thus these results clearly indicated that the majority of MLC $B9^+$ lymphocytes that were capable of proliferation were indeed specific alloreactive CTL-P, whereas only a minor fraction of proliferating MLC $B9^-$ cells contained such functional precursors.

From the comparison between PTL-P and CTL-P frequencies in $B9^+$ fractions

(Table I), it is apparent that a few of the proliferating microcultures derived from such cells were devoid of specific cytolytic activity. This finding could be explained by the existence of MLC $B9^+$ cells devoid of lytic potential. Alternatively, such proliferating, apparently noncytolytic microcultures could be derived from CTL-P directed against antigens not expressed by the target cells used. To distinguish between these possibilities, experiments were performed in which the cytolytic activity of microcultures obtained under limiting dilution conditions was determined in a lectin-dependent assay allowing the detection of CTL irrespective of their specificity (11). Fig. 4 shows the results of a representative experiment whereby microcultures derived from unfractionated and fractionated MLC cells as indicated above were assayed for cytolytic activity against mouse P815 tumor target cells in the presence of PHA. Under these conditions, virtually all proliferating microcultures derived from $B9^+$ cells were positive for cytolytic activity. Indeed, comparative analysis of the frequency of PTL-P and total CTL-P among MLC $B9^+$ cells showed no significant difference (Table II). It is thus evident that most, if not all, MLC $B9^+$ cells are (operationally defined) CTL-P. Moreover, it is noteworthy that no increase in CTL-P frequency was observed when microcultures derived from MLC $B9^-$ cells were tested in the lectin-dependent cytolytic assay (Fig. 4 and Table II), thus confirming the presence of low, although significant, numbers of CTL-P in this fraction.

Frequency Analysis of Proliferating and/or CTL Precursors in Resting T Cell Populations. We have recently reported that virtually all peripheral blood T lymphocytes can undergo extensive clonal proliferation under optimal culture conditions in vitro (11). It was therefore of interest to use this microculture system to determine the distribution of CTL-P among resting, $B9^+$ and $B9^-$ T cells. To

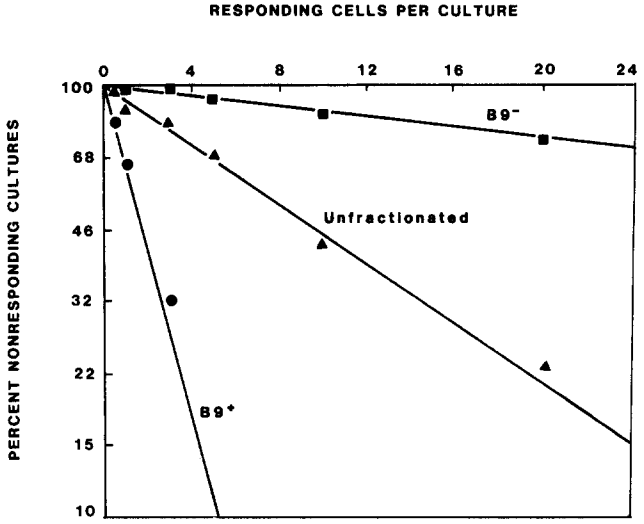

FIGURE 4. Frequency analysis of total CTL-P in MLC T lymphocytes separated according to B9 expression. The experimental protocol was the same as that described in Fig. 3, with the exception that each microculture was assayed for lectin-dependent lysis against ^{51}Cr-labeled P815 murine tumor target cells in the presence of PHA (see Materials and Methods). Each point is based on a group of 48 microcultures. CTL-P frequencies were calculated as described in Materials and Methods. ▲, unfractionated; ●, $B9^+$; and ■, $B9^-$ T cells.

TABLE II

*Frequency Analysis of Total PTL-P and CTL-P in MLC T Cell Subsets**

MLC-T population	Percent of total cells			Reciprocal of PTL-P frequency			Reciprocal of CTL-P frequency			Percent of the total CTL-P recovered		
	Exp. 1	Exp. 2	Exp. 3‡	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Unfractionated	100	100	100	2.31	2.36	2.27	9.5	9.1	12	—	—	—
$B9^+$	24	25	18	2.25	2.31	2.28	2.25	2.32	2.28	90.1	91.4	87.2
$B9^-$	76	75	82	2.28	2.36	2.27	64.82	74.10	70.9	9.9	8.6	12.8

* T lymphocytes activated in primary allogeneic MLC were sorted according to B9 fluorescence intensity (see Fig. 1) and assayed for their content of total PTL-P and CTL-P as described in Materials and Methods.

‡ Data from Fig. 4.

TABLE III

*Frequency Analysis of Total PTL-P and CTL-P in Peripheral Blood T Cell Subsets**

Experiment number	Cell source	Frequency (95% confidence limits)	
		Proliferation	P815 + PHA (LDCC)
1	Unfractionated peripheral blood	0.94 (0.82–1.06)	0.32 (0.25–0.38)
	T cells	0.95 (0.79–1.11)	0.96 (0.79–1.13)
	$B9^+$ T cells	1.05 (0.93–1.17)	0.012 (0.009–0.015)
	$B9^-$ T cells		
2‡	Unfractionated T cells	1.02 (0.92–1.12)	0.34 (0.23–0.45)
	$B9^+$ T cells	1.00 (0.86–1.14)	1.01 (0.82–1.20)
	$B9^-$ T cells	1.03 (0.94–1.12)	0.026 (0.014–0.037)

* Peripheral blood T lymphocytes from two different donors were sorted according to B9 fluorescence intensity (see Fig. 1) and cultured under optimal conditions, which allows the clonal expansion of all human T cells (see Materials and Methods). After 18 d of culture total PTL-P and CTL-P were determined (see Materials and Methods).

‡ Data from Fig. 5.

this end, peripheral blood T cells were stained with B9 mAb and sorted into $B9^+$ and $B9^-$ fractions on the FACS. Graded numbers of cells from each fraction as well as unfractionated T cells were plated in the presence of irradiated spleen (feeder) cells and PHA. After 48 h, TCGF-containing supernatant was added to each microculture. After incubation for 18 d, microcultures were assessed for proliferation and cytolytic activity. In agreement with our previous report (11), the frequency of proliferating cells in unfractionated T cells was essentially 100% (Table III). Moreover, this high cloning efficiency was unaffected by the staining and/or sorting procedure since both $B9^+$ and $B9^-$ fractions exhibited a similar clonogenic potential (Table III).

The cytolytic activity of the limiting dilution microcultures was assayed using lectin-dependent assay system. Fig. 5 shows semi-log plots of the data obtained in a representative experiment. In all groups of responder cells, there was a linear relationship between the number of responder cells cultured and the log of the proportion of noncytolytic cultures. The results of CTL-P frequency estimates are summarized in Table III. In unfractionated resting T cells, the frequency of CTL-P was ~0.33 or 1 cell in 3. The CTL-P frequency was

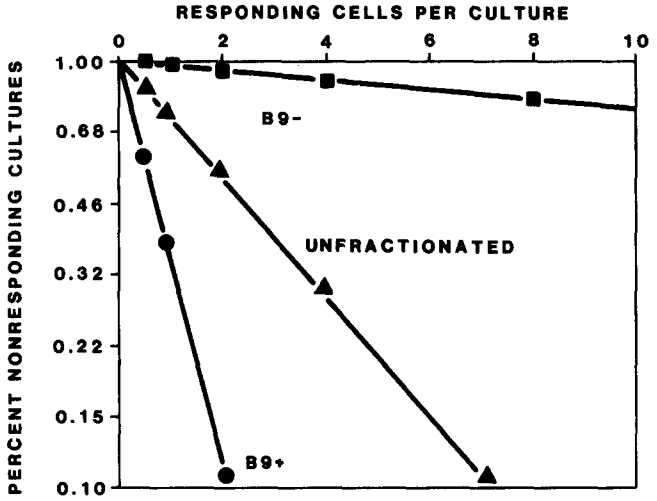

FIGURE 5. Frequency analysis of total CTL-P in peripheral blood T lymphocytes separated according to B9 expression. Peripheral blood T lymphocytes were separated by FACS sorting into $B9^+$ and $B9^-$ fractions. Limiting dilution analysis was performed by culturing different cell doses (ranging from 0.5 to 8 cells/well) in the presence of irradiated allogeneic spleen cells and PHA. TCGF was added 48 h later. At the end of the culture period each microculture was assayed for lectin-dependent cytolytic activity against ^{51}Cr-labeled P815 murine tumor target cells in the presence of PHA. Each point is based on a group of 48 microcultures CTL-P frequencies were determined as described in Materials and Methods. ▲, unfractionated; ●, $B9^+$; and ■, $B9^-$ T cells.

increased threefold in the $B9^+$ fraction, thus indicating that essentially all $B9^+$ cells in peripheral blood are CTL-P. In contrast, the CTL-P frequency in the $B9^-$ fraction (which represented 70–80% of the total T cells) was very low (1/38; 1/83).

In view of these results, it was of interest to determine the frequency of CTL-P directed against a given alloantigen set in unfractionated and fractionated peripheral blood T cells. To this end, microcultures were established with graded numbers of responder cells from the various populations tested above, irradiated allogeneic spleen cells (without PHA) and an appropriate source of TCGF. After 18 d, the cytolytic activity of each microculture was tested against PHA-induced blast cells bearing the stimulating alloantigens. Minimal estimates of specific alloreactive CTL-P obtained under these conditions are shown in Fig. 6 and Table IV. In a series of three experiments, mean CTL-P frequencies were determined to be 1/2,340, 1/573, and 1/14,266 in unfractionated, $B9^+$ and $B9^-$ cells, respectively. Thus, there was a 25-fold difference in CTL-P frequency between $B9^+$ and $B9^-$ resting T cells.

Discussion

In this work, we have combined a high efficiency microculture cloning system with positive selection on a FACS to assess directly the B9 phenotype of human CTL and their precursors in normal and MLC T cell populations. From limited structural analysis, it appears that the molecules bearing the B9 antigen are related to those defined by monoclonal antibodies of the OKT8 and Leu-2 series

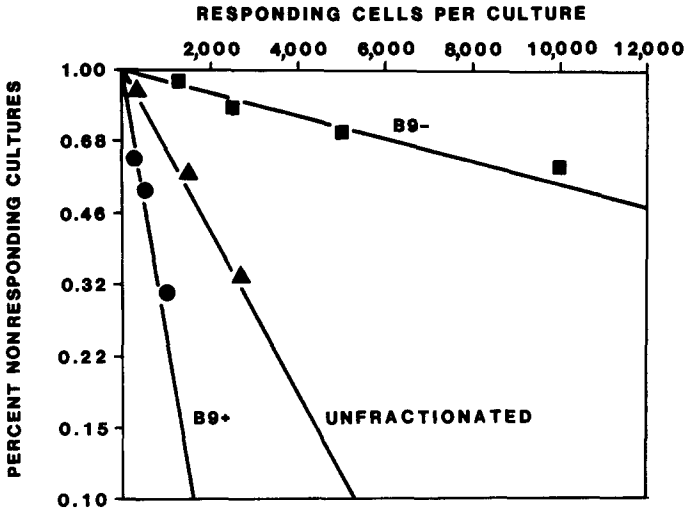

FIGURE 6. Frequency analysis of specific alloreactive CTL-P in peripheral blood T lymphocytes separated according to B9 expression. The experimental protocol was the same as that described in Fig. 5, with the exception that the responder cells were cultured in the presence of irradiated allogeneic spleen cells and TCGF (without PHA). After 18 d of culture, each group of microculture was tested for specific cytolytic activity against ^{51}Cr-labeled PHA-induced blast cells bearing the stimulating alloantigens. Each point is based on a group of 48 microcultures. CTL-P frequencies were calculated as described in Materials and Methods. ▲, unfractionated; ●, $B9^+$; and ■, $B9^-$ T cells.

TABLE IV

*Frequency Analysis of Alloreactive CTL-P in Peripheral Blood T Cell Subsets**

Cell population	Reciprocal of CTL-P frequency		
	Exp. 1‡	Exp. 2	Exp. 3
Unfractionated T cells	2,800	2,200	2,010
$B9^+$ T cells	710	550	460
$B9^-$ T cells	16,400	14,800	11,600

* Peripheral blood T lymphocytes from three different donors were sorted according to B9 fluorescence intensity (see Fig. 1) and assayed for their content of alloreactive CTL-P as described in the Materials and Methods.
‡ Data from Fig. 6.

(4, 22). Preliminary results concerning the sensitivity of the antigenic determinant defined by the B9.4 monoclonal antibody to mild trypsin treatment suggest that this determinant is closely related, if not identical, to that defined by the Leu 2b monoclonal antibody (A. Moretta, unpublished observation). In peripheral blood T cells, 25% cells were B9 positive as assessed by indirect immunofluorescence and flow microfluorometry. The percentage of $B9^+$ cells was more variable in T cell populations that had been stimulated in allogeneic MLC, ranging from 20 to 60%.

In the first part of this study, our aim has been to determine the frequency and B9 phenotype of the CTL that are present in MLC populations. Although a direct approach to this question would be to examine individual CTL, the

methods available for detecting CTL at the single cell level are not easily amenable to the study of their surface phenotype. Therefore, we turned to the more practical approach of determining the surface phenotype of the MLC cells that produce a CTL progeny upon appropriate stimulation in limiting dilution microcultures. Although this has not been formally proven in a human system, previous studies in the mouse using similar microculture systems indicate that CTL can give rise to a cytolytic progeny upon stimulation in microcultures (23). We therefore assume that the (operationally defined) CTL-P in MLC populations correspond to the CTL themselves. It should be emphasized that, under the culture conditions used, the frequency of MLC T cells that underwent extensive proliferation was as high as 40–60%. Given this high cloning efficiency, we conclude that the CTL-P frequencies obtained by limiting dilution analysis of MLC T cells are representative of the actual CTL frequencies in these populations.

Two different target cell systems were used to analyze the cytolytic activity of the limiting dilution microcultures, established with MLC T cells, namely PHA-induced blast cells derived from the lymphoid population used as the source of (allogeneic) stimulators in MLC, and murine (P815) tumor cells in conjunction with PHA. With the latter system, CTL activity can be detected regardless of the antigenic specificity of the effector cells (11, 24). Thus, by using these two assays systems, we have been able to compare directly the frequencies of CTL-P specifically directed against the MHC alloantigens of the stimulators with those of all CTL-P present in MLC populations. For simplicity, these frequencies are further referred to as frequencies of specific and total CTL-P, respectively.

The results of several independent experiments indicated that the frequency of total CTL-P in MLC T cell populations was ~1 in 10. The large majority (≥90%) of these CTL-P were found in the $B9^+$ subset. Moreover, the frequency of total CTL-P in MLC $B9^+$ cells was always identical to that of proliferating cells, whereas <1 in 20 proliferating $B9^-$ cells produced a cytolytic progeny. As evidenced by the frequency analysis of specific CTL-P, the great majority of the CTL-P detected in the lectin-dependent assay were directed against the stimulating alloantigens. Taken together, these results thus suggest that most $B9^+$ MLC cells, all of which appear to have a cytolytic potential, are specific CTL, whereas the frequency of such effector cells is much lower in $B9^-$ MLC cells.

In order to relate these findings to the characterization of CTL-P in unprimed T cell populations, we took advantage of the availability of a recently developed microculture system that allows clonal expansion of virtually every peripheral blood T lymphocyte (11). Using this system, we demonstrated previously that 1 peripheral blood T cell in 3 is a CTL-P. The work presented here confirms and extends these findings to the distribution of the peripheral blood CTL-P in the two subsets defined by the B9.4 monoclonal antibodies. As in the studies concerning the surface phenotype of MLC CTL-P, the relationship between B9 phenotype and CTL-P frequencies was examined by positive selection using the FACS to separate $B9^+$ and $B9^-$ cells in peripheral blood T cell populations. It should be emphasized that the high cloning efficiency observed with unseparated T lymphocytes was unaffected by the staining and/or sorting procedure. Since the frequency of proliferating cells was essentially 100% with both $B9^+$ and $B9^-$

cells, a quantitative assessment of the CTL-P distribution in these two subsets could readily be obtained. The results demonstrate that ≥90% of total CTL-P in peripheral blood express the $B9^+$ phenotype. It is of interest that this value is comparable to that of total CTL-P in mouse spleen that are Lyt-2^+ (10).

Our finding that the frequency of total CTL-P in the $B9^+$ subset is essentially 100% was somewhat unexpected in view of the reports indicating that this subset also contains T suppressor cells. Although it cannot be excluded that a small fraction of T cells is unable to grow under our culture conditions, the demonstration that most, if not all, $B9^+$ cells in peripheral blood have a cytolytic potential suggests that this subset contains very few cells with suppressor activity, or, alternatively, CTL-P may exhibit suppressor activity.

While the vast majority of CTL-P were included in the $B9^+$ subset, a small, although significant, number of them was detectable in the $B9^-$ subset. This finding cannot be explained by a contamination of $B9^-$ cells by $B9^+$ cells since cytofluorometric analysis of the few cytolytic microcultures derived from $B9^-$ cells clearly indicated that these cultures contained only $B9^-$ cells (A. Moretta, unpublished observation). If one assumes that the $B9^-$ subset is similar to the $OKT4^+$ subset, our results are then consistent with the recent demonstration of CTL clones expressing the $T4^+/T8^-$ phenotype (6, 7). As shown here, CTL-P expressing this phenotype are relatively rare (<10% of total CTL-P pool in peripheral blood).

In line with recent reports concerning the frequency of alloreactive CTL-P in murine lymphoid populations (25), comparison of the frequencies of CTL-P directed against a set of MHC alloantigens in unprimed T cells and day 7 MLC T cells revealed that these frequencies increased ≥200-fold following in vitro stimulation in bulk cultures (Tables I and IV). It is of interest that the magnitude of this increase was similar among $B9^+$ and $B9^-$ CTL-P despite the 25-fold difference in CTL-P frequency between the two subsets.

In conclusion, this work provides for the first time a quantitative assessment of the size and subset distribution of the total CTL-P pool in unprimed and in vitro primed peripheral blood human T cells. In view of the high cloning efficiency of the microculture system used, it is now possible to perform similar studies concerning noncytolytic functional T cells. Studies along these lines should provide a more accurate understanding of the relationship between expression of surface markers and functional activities of human T cells.

Summary

In order to directly assess the distribution of cytolytic T lymphocytes (CTL) and their precursors (CTL-P) in the two major subsets of human T cells, we have used limiting dilution microculture systems to determine their frequencies. The two subsets were defined according to their reactivity (or lack thereof) with B9.4 monoclonal antibody (the specificity of which is similar, if not identical, to that of Leu 2b monoclonal antibody). Both $B9^+$ and $B9^-$ cells obtained by sorting peripheral blood resting T cells using the fluorescence-activated cell sorter (FACS) were assayed for total CTL-P frequencies in a microculture system that allows clonal growth of every T cell. As assessed by a lectin-dependent assay, ~30% of peripheral blood T cells were CTP-P. In the $B9^+$ subset (which

represents 20–30% of all T cells), the CTL-P frequency was close to 100%, whereas the $B9^-$ subset had a 25-fold lower CTL-P frequency. It is thus evident that 90% and 10% of the total CTL-P in peripheral blood are confined to the $B9^+$ or $B9^-$ T cell subsets, respectively. Analysis of the subset distribution of CTL-P directed against a given set of alloantigens confirmed these findings.

CTL-P frequencies were also determined in $B9^+$ and $B9^-$ subsets derived from T cells that had been activated in allogenic mixed leucocyte cultures (MLC). ~10% of MLC T cells were CTL-P. This frequency was increased 3.5-fold in the $B9^+$ subset, whereas the $B9^-$ subset contained only a small, although detectable number of CTL-P. Moreover, the great majority of the (operationally defined) CTL-P in MLC T cell population were found to be directed against the stimulating alloantigens, thus indicating a dramatic increase in specific CTL-P frequencies following in vitro stimulation in bulk cultures.

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