

DIRECT DEMONSTRATION OF THE CLONOGENIC POTENTIAL  
OF EVERY HUMAN PERIPHERAL BLOOD T CELL  
Clonal Analysis of HLA-DR Expression and Cytolytic Activity\*

BY ALESSANDRO MORETTA, GIUSEPPE PANTALEO, LORENZO MORETTA,  
JEAN-CHARLES CEROTTINI, AND MARIA CRISTINA MINGARI

*From the Ludwig Institute for Cancer Research, Lausanne Branch, Epalinges, Switzerland; and Istituto di Patologia Medica, University of Bari, Italy*

The recent development of culture techniques allowing the cloning of T lymphocytes has proved extremely useful for analyzing the functional heterogeneity of these cells (1, 2). In most instances, such clonal analyses have been applied to cell populations containing T lymphocytes activated by stimulation in vitro with mitogen or antigen. Under optimal culture conditions, the cloning efficiency of in vitro stimulated T cells appears to be relatively high (3, 4). However, because of the possibility of a selection of rapidly proliferating T cells during culture, the clones derived from in vitro stimulated T cell populations are not necessarily representative of the starting T cell populations. On the other hand, initial attempts to directly clone normal, unstimulated T cells in semisolid media (5-7) have resulted in very low cloning efficiencies, ranging from 1 in 500 (8) to 1 in 1000 (9).

Recently, Chen and colleagues (10) described a limiting dilution microculture technique that allows the majority of murine T lymphocytes to undergo clonal expansion. Along the same lines, we have investigated the culture conditions necessary to obtain a clonal proliferation from every single human T cell in resting, nonstimulated populations. In the present study, we have performed a limiting dilution analysis of human peripheral blood T cells cultured in microcultures containing phytohemagglutinin (PHA)<sup>1</sup> and a suitable source of T cell growth factor (TCGF). It will be shown that under appropriate culture conditions, clones containing  $5-30 \times 10^4$  cells can be obtained within 14-20 d from virtually all T cells. Moreover, by using this microculture system, we have assessed directly the frequency of T lymphocytes in peripheral blood that give rise to a clonal progeny expressing Ia antigens. Finally, this technique has been used to quantitate the potential pool size of cytolytic T lymphocyte precursors (CTL-P) in peripheral blood and to analyze the relationship between the CTL-P and the precursors of effector T cells exhibiting a natural killer (NK)-like activity.

\* Recipient of a short-term fellowship from the European Molecular Biology Organization. Supported in part by grants 81.01421.96 and 82.00332.96 PFCCN from CNR.

<sup>1</sup> Abbreviations used in this paper: Con A, concanavalin A; CTL-P, cytolytic T lymphocyte precursor; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; NK, natural killer; PBMNC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PTL-P, proliferating T lymphocyte precursors; SN, supernatant; TCGF, T cell growth factor; TdR, thymidine.

### Materials and Methods

**Isolation of Lymphoid Cells.** Lymphocytes were isolated from peripheral blood or spleen as previously described (11). T cells were isolated by rosette formation with neuraminidase-treated sheep erythrocytes (E) and subsequent serial centrifugations (at least two) over Ficoll/Hypaque gradients (11). Only cell suspensions containing >98% E-rosetting cells were used. Human spleens were removed for technical reasons during abdominal surgery. Four different spleens were used, and in all instances no damage of the organ was observed. When used as a source of feeder cells, each of the four spleens gave comparable results. Cell suspensions derived from spleens were frozen and subsequently thawed as needed (3, 12).

**Microcultures.** Cells (peripheral blood mononuclear cells [PBMNC] or purified T cells) were submitted to vigorous vortex mixing (to dissociate possible cell clumps) and then seeded in limiting numbers in round-bottomed microwells (Greiner, Nurtingen, Federal Republic of Germany) containing  $10^5$  irradiated (5,000 rad) feeder cells in a final volume of 0.2 ml. Spleen cell suspensions to be used as a source of feeder cells were irradiated with 5,000 rad. Under these conditions, no proliferative response of these cells was observed, even in the presence of PHA and TCGF (Fig. 1). The culture medium was RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS) and 1% (vol/vol) PHA (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). The PHA in lyophilized form was rehydrated with 10 ml sterile distilled water. Subsequently, 2  $\mu$ l of such rehydrated PHA were added to each microwell. Supernatant (SN) from cultures of PHA-stimulated human spleen cells (routinely depleted of PHA as described [13]) was used as a source of TCGF. SN was added (50% vol/vol) either at the onset of the cell culture or after 48 h. The microcultures were then supplemented weekly with  $10^5$  irradiated feeder cells suspended in 100  $\mu$ l of TCGF containing SN.

In control culture containing only PHA (or TCGF), the lectin (or SN) was added at the onset of the culture and then weekly, together with irradiated feeder cells. All plates were

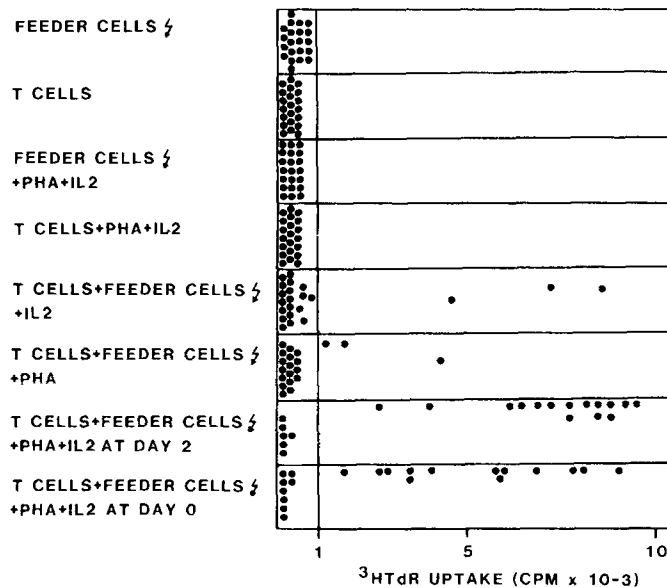


FIG. 1. Microculture conditions necessary for optimal proliferation of individual peripheral blood human T cells. Groups of 21 microwells containing purified T cells seeded at 1 cell/well were supplemented with TCGF and/or PHA in the presence or in the absence of irradiated feeder cells. TCGF was added either at the onset of the culture or after 48 h. Controls included feeder cells cultured alone or in the presence of PHA and/or TCGF. After 14 d, microwells were assessed for proliferation by thymidine uptake. The solid line represents 3 SD above the mean [ $^3\text{H}$ ]TdR uptake value in a group of 21 control microwells containing all supplements, but not responding cells.

centrifuged at 100 *g* for 5 min and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

*Single-cell Micromanipulation.* Purified T cell populations were resuspended at 10<sup>4</sup> cells/well. Single cells were isolated in a drawn out glass pipette under microscopical examination according to the method of Zagury et al. (14) and plated directly into round-bottomed microwells containing irradiated feeder cells and PHA. TCGF containing SN was added 48 h later.

*Screening of Microcultures.* After 14–21 d of culture each microwell was assessed for growth and/or cytolytic activities. Proliferation was determined either microscopically (3) or by tritiated thymidine ([<sup>3</sup>H]TdR) uptake as previously described (15). Cultures in which [<sup>3</sup>H]TdR (0.1 μCi/well, sp act 9 mCi/mM) uptake exceeded by >3 SD the mean uptake detected in control cultures (containing 10<sup>6</sup> irradiated spleen cells but no responder cells) were defined as positive. Cytolytic activity was assayed according to the procedure described in detail elsewhere (16). Briefly, the cells were resuspended with a micropipette and then divided into two 100-μl aliquots. One aliquot was tested for cytolytic activity against murine P815 tumor target cells in the presence of PHA, whereas the other was tested for cytolytic activity against human K562 target. To this end, the cell suspensions were transferred into V-bottomed wells of microtiter trays to which 5 × 10<sup>3</sup> <sup>51</sup>Cr-labeled target cells were added (the final volume per well was 200 μl). In the lectin-dependent cytolytic assay, 1% (vol/vol) PHA was added to the <sup>51</sup>Cr-labeled P815 cell suspensions immediately before distributing the target cells in microwells (to avoid the formation of cell clumps). Microplates were centrifuged at 100 *g* for 5 min and then incubated for 4 h at 37°C. The plates were then centrifuged again (200 *g* for 5 min) and 100 μl of supernatant was removed for measurement of <sup>51</sup>Cr release as previously described (17). Spontaneous release was determined in control microcultures prepared in the same manner as the experimental groups, but without addition of responder cells (4). Cultures in which <sup>51</sup>Cr release exceeded the mean spontaneous release by >3 SD were considered positive for cytolytic activity.

*Determination of the Frequency of Proliferating and/or CTL-P.* Minimal estimates of precursor frequency 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).

*Immunofluorescence Staining and Fluorescence-activated Cell Sorter (FACS) analysis.* The techniques used have been described in previous reports (17). Samples of 2 × 10<sup>4</sup> cloned T cells in 50 μl RPMI 1640 medium were incubated with 5 μg of D1/12 (anti-Ia) monoclonal antibody (19) for 30 min at 4°C. After two washings, the cells were resuspended in 50 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 incubation at 4°C, all samples were washed three times and analyzed on a flow cytometer (FACS II; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA). Results are presented as fluorescence histograms with the number of cells on the Y axis and the fluorescence intensity expressed in arbitrary linear units, on the X axis (3).

## Results

*Establishment of Culture Conditions for Optimal T Cell Cloning.* Most of the T cell cloning procedures are based on the use of TCGF derived from mixed lymphocyte culture (MLC) or lectin-activated lymphocyte populations (1, 20). Since TCGF receptors are primarily expressed in activated T cells (21), we first determined whether activation of single resting T cells with PHA would provide suitable conditions for their subsequent response to TCGF. To this end, T cells were prepared from human peripheral blood and plated at 1 cell/well on the average into cultures containing 1% (vol/vol) PHA and 1 × 10<sup>5</sup> human irradiated allogeneic spleen cells as a source of filler cells. A saturating dose of TCGF containing supernatant was added at the onset of the cultures or 48 h later. At weekly intervals, cultures were supplemented with fresh TCGF and irradiated filler cells. Control cultures included irradiated filler cells

cultured alone or in the presence of TCGF and PHA, T cells cultured alone or in the presence of both TCGF and PHA, and T cells cultured in the presence of irradiated filler cells together with either PHA or TCGF (added at the onset of the cultures). Cultures were scored for proliferation at day 14, either by measuring the uptake of [ $^3\text{H}$ ]TdR or by microscopical examination.

The results of a representative experiment in which cell proliferation was evaluated by [ $^3\text{H}$ ]TdR uptake are shown in Fig. 1. No proliferating wells were detected in microcultures containing irradiated filler cells or T cells cultured either alone or in the presence of PHA and TCGF. A few proliferating wells were obtained in microcultures containing both T cells and irradiated filler cells cultured with either PHA or TCGF. In contrast, addition of both PHA and TCGF to such cultures resulted in large numbers of positive wells, especially in cultures in which TCGF was added after 48 h. Similar results were obtained in six other experiments. In all instances, the highest clonal efficiency was observed when single T cells were cultured with PHA and irradiated filler cells for 2 d before the addition of TCGF. These culture conditions were therefore used in all subsequent experiments. It is noteworthy that, under the same culture conditions, virtually no clones were obtained from mononuclear cell fractions extensively depleted of E-rosetting cells (data not shown); in addition, the very few clonal isolates obtained from such populations had T cell characteristics as judged by surface markers analysis (data not shown), thus indicating that only T cells undergo clonal expansion under our experimental conditions.

*Frequency Analysis of Proliferating Peripheral Blood Human T Cells.* The actual frequencies of proliferating cells in unfractionated PBMNC and T cells were determined by limiting dilution analysis. In these experiments, graded numbers of cells were plated in groups of 54 microcultures and cell growth was assessed microscopically from day 14 to day 21. The frequency of proliferating cells was then calculated by analysis of the Poisson distribution relationship between the number of cells plated per culture and the percentage of nonproliferating cultures. One example of such an analysis is shown in Fig. 2. In this experiment, proliferation was observed in 9/54 (17%), 12/54 (22%), and 24/54 (44%) of the cultures established with 0.25, 0.5, and 1 unfractionated mononuclear cell, respectively. Under the same conditions, 13/54 (24%), 22/54 (40%), and 35/54 (64%) of the microcultures containing similar numbers of purified T cells were scored as positive. From these data, the frequencies of proliferating cells were determined by the minimum chi square method described by Taswell (18). In PBMNC this frequency was 0.58 or 1 cell in 1.70, whereas in purified T cells it was 1.05 or 1 cell in 0.95 (Table I, experiment 1). These results were confirmed in four additional experiments using peripheral blood from different individuals (Table I, experiments 2–5). The frequencies of proliferating cells ranged between 0.52 and 0.73 in PBMNC and 0.98 and 1.11 in purified T cell populations. Finally, a direct assessment of the cloning efficiency obtained under the culture conditions mentioned above was obtained by using a micromanipulation technique as described by Zagury et al. (14). A total of 96 single cells from purified T cell populations were isolated in a drawn-out glass pipette and seeded directly into microwells containing filler cells and PHA. TCGF was added after 48 h. As assessed microscopically, 92 of the 96 microcultures contained proliferating cells on day 14 (data not shown), thus confirming that the cloning efficiency of normal T cells was close to 100% under the experimental conditions used. In addition, cell counts performed in individual micro-

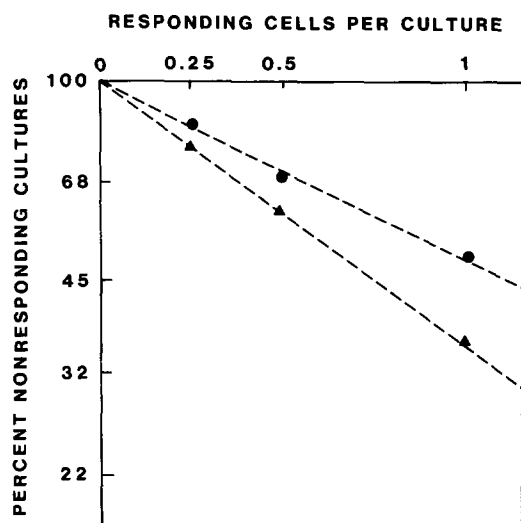


FIG. 2. Limiting dilution analysis of the frequency of proliferating cells in peripheral blood mononuclear cells (●) or purified T cell populations (▲) stimulated under optimal conditions as described in Materials and Methods. After 18 d of culture, each microculture was scored microscopically for proliferation. The regression line was fitted to the data by the minimum  $\chi^2$  method (18).

TABLE I  
*Precursors Frequency of Proliferating Cells in Human PBMNC and Purified T Cells*

Experiment number	Cell source*	Frequency (95% confidence limits)‡
1	PBMNC	0.52 (0.34–0.70)
	Purified T cells	1.05 (0.80–1.30)
2	PBMNC	0.58 (0.44–0.72)
	Purified T cells	0.98 (0.74–1.23)
3	PBMNC	0.58 (0.41–0.75)
	Purified T cells	1.00 (0.78–1.22)
4	PBMNC	ND§
	Purified T cells	1.11 (0.87–1.36)
5	PBMNC	0.73 (0.48–0.98)
	Purified T cells	1.05 (0.74–1.37)

\* Cells were derived from five donors.

‡ Frequency was calculated as described in Materials and Methods.

§ Not done.

cultures indicated that the number of viable lymphocytes after 14–20 d ranged between  $5 \times 10^4$  and  $3 \times 10^5$  per microwell.

*All Peripheral Blood T Cells Express HLA-DR Antigens upon Clonal Expansion.* Having defined the conditions under which virtually all normal T cells undergo clonal proliferation, we first applied this technique to the enumeration of T cells that express HLA-DR (Ia) upon activation (22). To this end, 264 microcultures were established using low (0.25 cell/well) numbers of purified T cells. After 18 d of culture (including weekly addition of TCGF and irradiated filler cells), the cultures were scored microscopically for growth and the positive ones (63 in this experiment) were harvested

and tested by indirect immunofluorescence and cytofluorometry for expression of Ia antigens using mouse monoclonal antibodies against nonpolymorphic determinants (19). All of the 63 putative clones analyzed were found to be positive, although the staining intensity varied from one clone to another. The distribution of the 63 clones according to their mean fluorescence intensity is shown in Fig. 3, and the staining

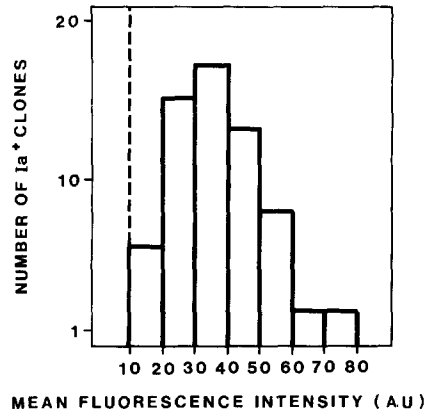


FIG. 3. Distribution of 63 T cell clones stained for HLA-DR antigens according to mean fluorescence intensity. The clones were obtained from microcultures in which T cells were plated at 0.25 and 0.5 per well and stimulated under optimal conditions as described in Materials and Methods. Each cloned population was stained with D1.12 monoclonal antibody followed by fluoresceinated goat anti-mouse conjugate and analyzed by flow cytofluorometry. The dotted line defines the lower limit of fluorescence intensity considered as positive. Under the same conditions, the fluorescence intensity of the same clones incubated with the second reagent alone was  $<7$  arbitrary units.

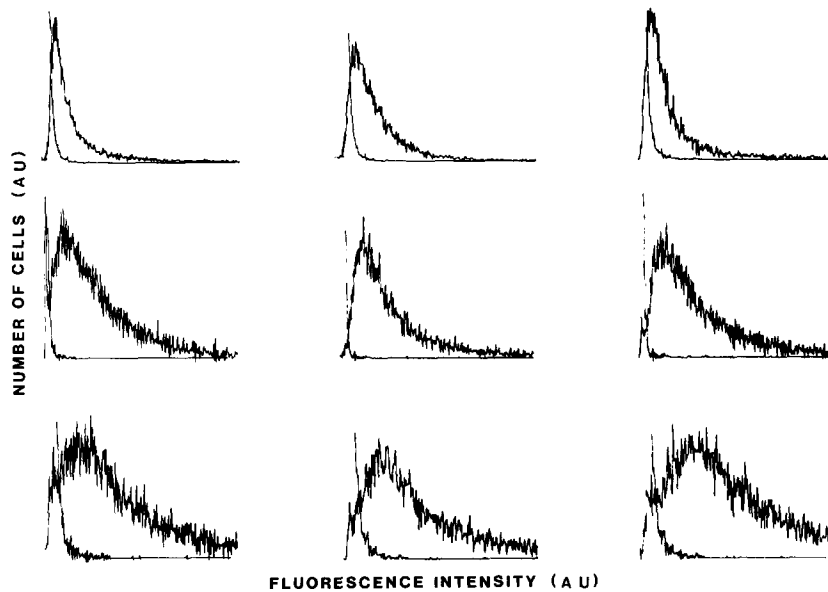


FIG. 4. Fluorescence distribution of nine human T cell clones representative of the various fluorescence patterns observed after staining for HLA-DR antigen. The first three clones have a mean fluorescence distribution comprised between 15 and 20 arbitrary units (AU). The mean fluorescence intensity in clones 4-6 was between 20 and 50 AU and in clones 7-9 was  $>50$  AU. The fluorescence intensity of the same clones incubated with the second reagent alone was  $<7$  AU.

patterns of 9 clones representative of the various fluorescence intensities observed are shown in Fig. 4. It is evident that the staining pattern was relatively heterogeneous even within individual clones. 10 clones were chosen randomly for further expansion in macrowells in the presence of TCGF, but in the absence of filler cells. When analyzed for expression of Ia antigens after 7–14 d of culture, all of the clones expressed Ia antigens in similar or even larger amounts than the original clonal isolates (data not shown). Since under these conditions Ia-positive filler cells were virtually absent, it appears unlikely that the Ia molecules detected on the cell surface of the analyzed clones were passively adsorbed (23) rather than actively produced by the cloned cells.

*Analysis of the Frequencies of CTL-P in Human Peripheral Blood.* The same limiting dilution culture system was used to evaluate the CTL-P frequency in peripheral blood T cell populations. To this end, purified T lymphocytes were cultured at different cell doses (ranging from 0.25 to 10 cells/well). After 18 d of culture, each well was scored for cell proliferation microscopically and was then split into two aliquots. One aliquot was assayed against  $^{51}\text{Cr}$ -labeled P815 target cells in the presence of PHA, whereas the other was assayed against  $^{51}\text{Cr}$ -labeled K562 target cells. As shown in Fig. 5, the frequency of proliferating T lymphocytes in this experiment was 0.98 or 1 cell in 1.01, whereas the frequency of cytolytic precursors was 0.307 or 1 cell in 3.2. Similar frequencies were obtained in three other experiments, the results of which are summarized in Table II. Since lectin-dependent cytolysis allows detection of CTL irrespective of their specificity (24, 25), these results thus indicate that about one-third of peripheral blood T lymphocytes in humans are CTL-P. As shown in Fig. 5, the frequency of T cells developing into effector cells able to kill K562 target cells was 1/6.32.

Moreover, analysis of the lytic activities exhibited by microcultures established at low cell doses (0.25 and 0.5 cell/well) showed that 24 of the 47 cultures exhibiting lectin-dependent lysis were also positive against K562 target cells (Fig. 6). No culture

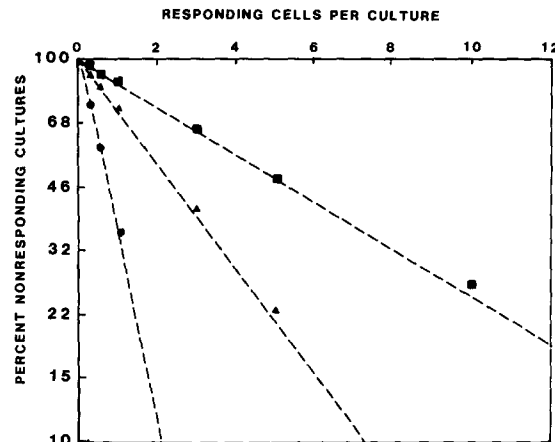


FIG. 5. Frequency analysis of the precursors of proliferating T cells with cytolytic potential. After 18 d of culture, each microculture was scored microscopically for growth (●) and then was split into two aliquots which were tested against either murine P815 target cells in the presence of PHA (▲) or human K562 target cells (■). Each point is based on a group of 54 microcultures. The regression lines were fitted to the data by the minimum  $\chi^2$  method.

TABLE II  
Precursors Frequencies of Effector Cells Mediating Lectin-dependent Lysis or Lysis of K562 Target Cells in Human Peripheral Blood T Cells

Experiment number*	Cell source‡	Frequency (95% confidence limits)§	
		P815 + PHA	K562
1	PBMNC	0.23 (0.13-0.33)	0.12 (0.05-0.19)
	Purified T cells	0.31 (0.25-0.37)	0.16 (0.12-0.20)
2	Purified T cells	0.30 (0.19-0.42)	0.15 (0.07-0.24)
3	Purified T cells	0.32 (0.26-0.38)	0.14 (0.12-0.16)
4	Purified T cells	0.32 (0.29-0.35)	0.16 (0.13-0.19)
5	PBMNC	0.22 (0.12-0.31)	0.10 (0.03-0.16)
	Purified T cells	0.34 (0.30-0.38)	0.16 (0.11-0.21)

\* The experiments are those reported in Table I.

‡ Cells were derived from five individuals.

§ Frequency was calculated as described in the Materials and Methods.

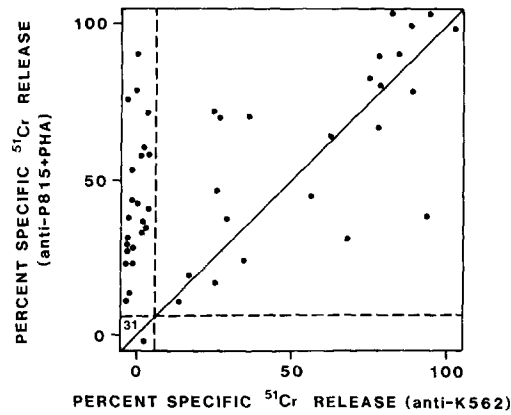


FIG. 6. Comparison of the cytolitic activities of clonal cultures derived from peripheral blood human T cells. After 18 d of culture, a total of 77 microcultures established with 0.25 cell/well were split into two aliquots and tested against both murine P815 target cells in the presence of PHA and human K562 target cells. Dotted lines represent 3 SD above the mean spontaneous  $^{51}\text{Cr}$  release of each target.

was found with lytic activity against K562 target cell alone. Given the high probability that these cultures were monoclonal, these results indicate that the effector T cells that mediate lysis of K562 target cells were derived from CTL-P. However, only 50% of the CTL-P appeared to give rise to a progeny exhibiting both lectin-dependent and NK-like cytolitic activities.

### Discussion

The present report provides direct evidence that virtually every T lymphocyte from human peripheral blood can undergo clonal expansion under suitable culture conditions. In contrast to the cloning procedures most often used in previous studies (1-4, 26), the microculture system described herein does not require stimulation of T cells in bulk cultures before cloning and therefore limits the risk of deriving clones that are not representative of the original cell populations. Moreover, it is clear that this liquid



culture system is much more efficient than the soft agar technique used in previous studies of colony formation by human peripheral blood T lymphocytes (5–9, 26). The basic feature of our system involves the combined use of a T cell activator, such as PHA, and an appropriate source of TCGF. Similar approaches have been used previously in murine culture systems. For example, Larsson (25) described a limiting dilution system in which single spleen T cells were activated with concanavalin A (Con A) and expanded in TCGF containing medium. Under the conditions used by the author, the frequency of proliferating T lymphocyte precursors (PTL-P) in nylon wool-purified spleen cells was one in five. More recently, Chen et al. (10), using a refined culture system, demonstrated that up to 100% of individual splenic T cells formed extensive clones when cultured in presence of Con A and a source of TCGF. In both human and murine culture systems, few if any individual resting T lymphocytes underwent proliferation in the presence of TCGF alone, thus confirming that normal resting T cells do not express TCGF receptors (21). If one assumes that the main effect of a T cell mitogen is to induce the expression of TCGF receptors (20, 21), then the results of Chen and colleagues (10) and the present study indicate that Con A or PHA, respectively, can induce TCGF reactivity in virtually all T cells of mouse or human origin. Although it cannot be excluded that a minimal fraction of T cells is unable to grow under our culture conditions, it is clear that the system reported here can be used to determine the frequency of the precursors of the various functional T cells in lymphoid cell populations. As a first step to this aim, we performed a limiting dilution analysis of the frequency of CTL-P in peripheral blood T cell populations. Lectin-dependent cytolysis was used to detect all cultures with cytolytic activity regardless of their specificity (24, 25). Our results clearly indicate that about one-third of all human peripheral blood T cells can form a measurable cytolytic clone. It is of interest that similar values have been found recently in murine spleen T cell populations (10). In these studies, virtually all the precursors of the effector cells mediating lectin-dependent cytolysis were confined within the *Lyt-2*<sup>+</sup> subset, in agreement with reports concerning the *Lyt-2* phenotype of CTL-P. Although a similar analysis of CTL-P frequencies in human T cell subsets has yet to be done, preliminary experiments from our group suggest that most, if not all, CTL-P are restricted to the cell subset defined by a monoclonal antibody directed against human CTL surface structures (27) (A. Moretta, manuscript in preparation). Since our culture system allows the clonal expansion of every T cell, we were able to investigate the relationship between CTL-P and the precursors of effector T cells mediating lysis of K562 target cells. From the data obtained in microcultures established with less than one cell on the average, it appears that approximately one-half of all CTL-P gave rise to a progeny which mediated lysis of K562 target cells, in addition to lectin-dependent cytolysis, whereas the remaining half developed into CTL devoid of activity against K562 target cells. In contrast, no activity against K562 target cells was detected in the proliferating microcultures devoid of lectin-dependent lytic activity. It is thus evident that under the culture conditions used for clonal expansion, lytic activity against K562 target cells is correlated exclusively, although partially, with CTL activity. Further experimentation is in progress to determine whether CTL with or without anti K562 activity are phenotypically different.

Finally, the usefulness of our culture system for the analysis of surface markers expressed by activated T cells (28) is illustrated by our results concerning the

expression of HLA-DR (Ia) antigens in individual clones. Previous studies at the population level have indicated that HLA-DR antigens became detectable on human T cells after stimulation with antigen or mitogen (22, 29, 30). In addition, all of the 36 T cell clones (including 8 CTL clones) derived from MLC (3) or pokeweed mitogen-activated populations (31) were found to express Ia antigens. In all of these experimental systems, however, T cells undergo a selection process which may favour the expansion of a limited number of cells capable of expressing Ia antigens. The present study clearly indicates that every resting T lymphocyte is potentially capable of expressing HLA-DR molecules upon activation and clonal expansion. It is evident, however, that the amount of Ia antigens expressed by individual cells may vary not only between but also within clones. It is therefore likely that the apparent conflicting data mentioned above simply reflect differences in sensitivity between the detection systems used by the various investigators.

In conclusion, the present study demonstrates that we have now a general assay for testing the clonal progeny of every T cell for a variety of cell functions. Therefore it is now possible to determine unequivocally whether cells with given functional capacities are indeed confined to subpopulations which can be defined by surface markers. A useful application of the system described herein will be to study, at the clonal level, the actual composition of functional T cell populations infiltrating tissues in autoimmune diseases, viral and parasitic infections, graft rejection, and solid tumors.

### Summary

In an attempt to determine the clonogenic properties of human peripheral blood T cells, we have developed a limiting dilution microculture system using phytohemagglutinin (PHA) as T cell activator and supernatant from PHA-stimulated spleen cultures as a source of T cell growth factors. The frequencies of cells capable of extensive proliferation under these culture conditions were 0.52–0.73, 0.98–1.11, and <0.02 in peripheral blood mononuclear, E-rosette-positive, and E-rosette-negative cell populations, respectively. The clonogenic potential of virtually all T cells was confirmed in experiments using single cells isolated by micromanipulation. Clone size ranged between 5 and  $30 \times 10^4$  cells on day 14 of culture. The same microculture system was used to determine the precursor frequency of all cytolytic T lymphocytes (CTL-P). As assessed by a lectin-dependent  $^{51}\text{Cr}$  release assay, the CTL-P frequency in purified T cell populations ranged between 0.30 and 0.34. In comparison, the precursor frequency of T cells capable of lysing K562 target cells was ranging between 0.14 and 0.16. Parallel analysis of individual clonal cultures for both lytic activities showed that 50% of the clones exhibiting lectin-dependent lysis were also active against K562 target cells. All of the proliferating clones expressed HLA-DR antigens, although to a varying degree as assessed by flow cytofluorometry. Given the high cloning efficiency of this culture system, it appears now possible to determine the precursor frequencies of the various classes of functional cells in T cell populations.

We would like to thank Dr. H. R. MacDonald for helpful discussion, P. Zeach for performing cytofluorometric analysis, C. Horvath for help in micromanipulation techniques, and M. Zappella for typing the manuscript.

*Received for publication 20 September 1982 and in revised form 19 October 1982.*

## References

1. Moller, G., editor. 1981. T cell clones. *Immunol. Rev.* **54**:1.
2. Glasebrook, A. L., and F. W. Fitch. 1982. Murine T cell clones reactive with alloantigens: an overview. In *Isolation, Characterization and Utilization of T Lymphocyte Clones*. C. G. Fathman, and F. W. Fitch, editors. Academic Press. N. Y. 265.
3. Moretta, L., M. C. Mingari, P. R. Sekaly, A. Moretta, B. Chapuis, and J. C. Cerottini. 1981. Surface markers of cloned human human T cells with various cytolytic activities. *J. Exp. Med.* **154**:569.
4. Maryanski, J. L., H. R. MacDonald, and J. C. Cerottini. 1980. Limiting dilution analysis of alloantigen-reactive T lymphocytes. IV. High frequency of cytolytic T lymphocyte precursor cells in MLC blasts separated by velocity sedimentation. *J. Immunol.* **124**:42.
5. Rozenszajn, L. A., D. Shoham, and Y. Kalechman. 1975. Clonal proliferation of PHA-stimulated human lymphocytes in soft agar cultures. *Immunology.* **29**:1041.
6. Fibach, E., E. Gerassi, and L. Sachs. 1976. Induction of colony formation in vitro by human lymphocytes. *Nature (Lond.)*. **259**:127.
7. Shen, J., F. Wilson, M. Shifrine, and M. E. Gershwin. 1977. Select growth of human T lymphocytes in single phase semisolid culture. *J. Immunol.* **119**:1299.
8. Pistoia, V., R. Ghio, W. Canonica, M. Colombatti, and L. Moretta. 1981. Comparison of the colony-forming capacities of human T lymphocyte subpopulations. *Clin. Immunol. Immunopathol.* **21**:289.
9. Lindahl-Kiessling, K., and I. Karlberg. 1982. Unicellular or multicellular origin of human T lymphocyte colonies in soft agar? *Scand. J. Immunol.* **15**:525.
10. Chen, W., R. Scollay, and K. Shortman. 1983. Limit-dilution assay and clonal expansion of all T cells capable of proliferation. *J. Immunol. Methods*. In press.
11. Moretta L., S. R. Webb, C. E. Grossi, P. M. Lydyard, and M. D. Cooper. 1977. Functional analysis of two human T cell subpopulations: help and suppression of B-cell responses by T cells bearing receptors for IgM or IgG. *J. Exp. Med.* **146**:184.
12. Moretta A., M. Colombatti, and B. Chapuis. 1981. Human spleen as a source of T cell growth factor. *Clin. Exp. Immunol.* **44**:262.
13. Moretta, A., R. S. Accolla, and J. C. Cerottini. 1982. IL-2-mediated T cell proliferation in humans is blocked by a monoclonal antibody directed against monomorphic determinants of HLA-DR antigens. *J. Exp. Med.* **155**:599.
14. Zagury, D., J. Bernard, P. Jeannesson, N. Thiernes, and J. C. Cerottini. 1979. Studies on the mechanism of T cell-mediated lysis at the single effector cell level. I. Kinetic analysis of lethal hits and target cell lysis in multicellular conjugates. *J. Immunol.* **123**:1604.
15. Moretta A., M. C. Mingari, M. Colombatti, and L. Moretta. 1981. Fc receptors on human T lymphocytes: loss of Fc $\mu$  and expression of Fc $\gamma$  receptors by T cells stimulated in mixed lymphocyte reactions. *Scand. J. Immunol.* **13**:447.
16. Moretta, A., G. Corte, M. C. Mingari, and L. Moretta. 1982. Human T cell subpopulations defined by a monoclonal antibody. II. Evidence for cell cooperation in the response to alloantigens and generation of cytolytic cells. *J. Immunol.* **128**:20.
17. Moretta A., M. C. Mingari, B. F. Haynes, R. P. Sekaly, L. Moretta, and A. S. Fauci. 1981. Phenotypic characterization of human cytolytic T lymphocytes generated in mixed lymphocyte culture. *J. Exp. Med.* **153**:213.
18. Taswell, C. 1981. Limiting dilution assays for the determinations of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* **126**:1614.
19. Accolla, R. S., R. Tosi, N. Tanigaki, A. L. Carmagnola, N. Gross, and S. Carrel. 1980. Human Ia isotypes. In *8th International Histocompatibility Workshop Newsletters*. 46.
20. Moller, G., editor. 1980. T cell growth factors. *Immunol. Rev.* **51**:337.
21. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factor receptors. *J. Exp. Med.* **154**:1455.

22. Winchester, R. J., and H. G. Kunkel. 1980. The human Ia system. *Adv. Immunol.* **28**:22.
23. Yu, D. T. Y., J. M. McCune, S. M. Fu, R. J. Winchester, and H. G. Kunkel. 1980. Two types of Ia-positive T cells. Synthesis and exchange of Ia antigens. *J. Exp. Med.* **152**:89s.
24. Bevan, M. J., and M. Cohn. 1975. Cytotoxic effects of antigen and mitogen-induced T cells on various targets. *J. Immunol.* **114**:559.
25. Larsson, E. L. 1982. One third of murine splenic T cells are cytotoxic precursors induced to grow by leucoagglutinin and TCGF. *Scand. J. Immunol.* **15**:515.
26. Paul, W. E., B. Sredni, and R. H. Schwartz. 1981. Long-term growth and cloning of non-transformed lymphocytes. *Nature (Lond.)*. **294**:697.
27. Malissen, B., N. Rebai, A. Liabeuf, and C. Mawas. 1982. Human cytotoxic T cell structures associated with expression of cytolysis. I. Analysis at the clonal level of the cytolysis inhibiting effect of 7 monoclonal antibodies. *Eur. J. Immunol.* **12**:737.
28. Moretta, L., M. C. Mingari, A. Moretta, and A. S. Fauci. 1982. Human lymphocyte surface markers. *Semin. Hematol.* **19**:273.
29. Ko, H. S., S. M. Fu, R. J. Winchester, D. T. Y. Yu, and H. G. Kunkel. 1979. Ia determinants on stimulated human T cells. Occurrence on mitogen and antigen-activated T cells. *J. Exp. Med.* **150**:246.
30. Evans, R. L., T. J. Faldetta, R. E. Humphreys, D. M. Pratt, E. J. Yunis, and S. F. Schlossman. 1978. Peripheral human T cells sensitized in mixed lymphocyte culture synthesize and express Ia-like antigens. *J. Exp. Med.* **148**:1440.
31. Mingari, M. C., G. Melioli, A. Moretta, G. Pantaleo, and L. Moretta. Surface markers of cloned human T cells with helper or suppressor activity on Pokeweed mitogen-driven B cell differentiation. 1982. *Eur. J. Immunol.* **12**:900.