PRECURSOR FREQUENCY ANALYSIS OF LYMPHOKINE-SECRETING ALLOREACTIVE T LYMPHOCYTES

Dissociation of Subsets Producing Interleukin 2, Macrophage-activating Factor, and Granulocyte-Macrophage Colony-stimulating Factor on the Basis of Lyt-2 Phenotype

By ANNE KELSO* and H. ROBSON MACDONALD

From The Department of Immunology, The Swiss Institute for Experimental Cancer Research, and the Ludwig Institute for Cancer Research, Lausanne Branch, CH-1066 Epalinges, Switzerland

Murine T lymphocytes have been shown to release a variety of lymphokines upon antigenic or mitogenic stimulation (1). These comprise factors such as interleukin 2 (IL-2;[1] also known as T cell growth factor) (2) and B cell helper factor (3), which act directly on lymphocytes, and others, including macrophage-activating factor (MAF) (4), granulocyte-macrophage colony-stimulating factor (GM-CSF) (5), and interferon-γ (6), which act on other leukocytes. However, it remains to be clarified whether all T lymphocytes can secrete these activities or whether different lymphokines are produced by distinct lymphocyte subsets. Although recent studies from several laboratories (7–9), including our own (10, 11), have demonstrated lymphokine production by cloned T cell lines, the limited number of clones tested to date and the possibility of selection imposed by extended culture make it difficult to extrapolate these results to the normal T cell population.

Analysis of the frequency of normal T lymphocytes that respond to alloantigens or mitogens can now be carried out in limiting dilution microcultures supplemented with irradiated "feeder" cells and a source of IL-2 (12). In our own studies (13), we used this method to quantitate precursors of cytolytic T lymphocytes (CTL-P) and of other proliferating noncytolytic T cells that respond to allogeneic stimulation. We now report the extension of this approach to the simultaneous assessment of the frequencies of precursors of T lymphocytes producing three independent lymphokines, IL-2, MAF, and GM-CSF, in the same series of microcultures. Furthermore, by combining the limiting dilution system with positive selection on a fluorescence-activated cell sorter (FACS), it has been possible to assess directly the Lyt-2 phenotype of these precursors of lymphokine-secreting T cells.

* Supported by a grant from the Swiss National Foundation for Scientific Research.

[1] *Abbreviations used in this paper:* CTL-P, cytolytic T lymphocyte precursor; EL-4 SN, EL-4 thymoma supernatant; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-2, interleukin 2; MAF, macrophage-activating factor; 2° MLC SN, secondary mixed leukocyte culture supernatant; SN, supernatant(s).

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/11/1366/14 $1.00
Volume 156 November 1982 1366–1379

Materials and Methods

Mice. Adult female C57BL/6 (H-2^b, Mls^b) and DBA/2 (H-2^d, Mls^a) mice were obtained from the animal colony of the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland.

Cell Suspensions. Spleen cell suspensions were prepared in Dulbecco's modified Eagle's medium supplemented with additional amino acids, 10 mM Hepes, 5×10^5 M 2-mercaptoethanol (culture medium), and 2% heat-inactivated fetal calf serum (FCS) as described (14). Spleen cells were depleted of T lymphocytes or Lyt-2-bearing cells by treatment with monoclonal rat anti-Thy-1.2 or anti-Lyt-2 reagents (AT83 and 3.168.81, provided by Dr. F. W. Fitch, University of Chicago, Chicago, IL) and complement, as described elsewhere (15). Positive selection of Lyt-2^+ and Lyt-2^- lymphocytes was performed, as previously described (15), by staining nylon wool column-purified spleen cells (16) (recovery 20–32%) with rat monoclonal anti-Lyt-2 antibody (53-6.7, provided by Dr. J. A. Ledbetter, Genetic Biosystems Inc., Seattle, WA) and fluorescein-conjugated mouse anti-rat Ig; cells were sorted on the basis of fluorescence using a FACS II (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) gated to exclude nonviable cells.

Mixed Leukocyte Microcultures. Microcultures were prepared, as detailed elsewhere (13), in culture medium supplemented with 10% FCS and 0.5% IL-2-containing supernatant prepared from an EL-4 thymoma subline, provided by Dr. J. J. Farrar (NIH, Bethesda, MD) (EL-4 SN) (17). Limiting numbers of C57BL/6 responder cells were cultured (24–32 microcultures per cell dose) with $0.5–1 \times 10^6$ 2,000 rad-irradiated T cell-depleted DBA/2 spleen stimulator cells in 200 μl in round-bottomed microwells (Greiner, Nürtingen, Federal Republic of Germany) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 7–9 d, cultures were scored microscopically for growth. Microcultures were divided into two aliquots, one for cytolytic assay and the other for restimulation to prepare SN for lymphokine assay. Microcultures were restimulated by washing twice to remove EL-4 SN and adding $0.5–1 \times 10^6$ irradiated T cell-depleted DBA/2 spleen cells in 200 μl culture medium with 5% FCS. After 24-h incubation, plates were centrifuged and 150 μl of SN removed.

Cytolytic Assay. Cytolytic activity was determined, as previously described (14), by incubation of an aliquot of each microculture (50 μl or 100 μl) with 2×10^3 ^{51}Cr-labeled P815 tumor cells in 200 μl for 3.5 h. Plates were centrifuged and 100 μl of supernatant was removed for counting of gamma emission.

Assay for IL-2. IL-2 activity in microculture SN was measured in either of two ways. (*a*) Based on the assay originally described by Gillis et al. (2), 4×10^3 cells of a cloned IL-2-dependent T cell line (either BD.2.10, provided by Dr. M. Nabholz, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland, or CTLL, provided by Dr. K. A. Smith, Dartmouth Medical School, Hanover, NH) were washed extensively and incubated with 30 μl SN in a total of 100 μl culture medium with 5% FCS at 37°C in flat-bottomed microwells (3596; Costar, Data Packaging, Cambridge, MA). After 24 h, 1 μCi methyl-[^{3}H]-thymidine (2.0 Ci/mmole; Amersham International Ltd., Amersham, England) was added for a further 4–5 h. Cells were harvested onto filter paper strips using an automated cell harvester (Dynatech Produkte AG, Kloten, Switzerland) and counted in a liquid scintillation β counter. (*b*) 2×10^3 cells of an H-2D^d-reactive CTL clone L3C5 (18), whose proliferation is dependent on IL-2, were incubated with 50 μl SN in a total of 100 μl culture medium with 5% FCS at 37°C for 3 d. Cytolytic activity, which is proportional to L3C5 cell number and hence to IL-2 concentration (19), was then measured by incubation of the surviving cells with 2×10^3 ^{51}Cr-labeled P815 tumor cells for 3.5 h, as described above.

Assay for MAF. MAF activity was measured, as described elsewhere (11), in a ^{51}Cr release assay for tumoricidal activity of bone marrow-derived macrophages (20). Briefly, monolayers prepared by seeding 5×10^4 C57BL/6 macrophages in flat-bottomed microwells (Costar, Data Packaging) were cultured for 24 h with test microculture SN (50 μl) or serial dilutions of pooled SN in a total of 200 μl culture medium with 5% FCS and 100 ng/ml lipopolysaccharide (*Escherichia coli* 055:B5, Difco Laboratories, Detroit, MI). The monolayers were washed once, and 10^4 ^{51}Cr-labeled P815 tumor cells were added in 200 μl culture medium with 5% FCS for a further 20 h. Plates were then centrifuged and 100 μl of SN harvested for counting of gamma emission. MAF titers of pooled SN were expressed as the reciprocal of the SN dilution that

produced 50% specific ^{51}Cr release, standardized by reference to a control secondary mixed leukocyte culture SN (2° MLC SN) (21) defined as 100 U/ml.

Assay for GM-CSF. GM-CSF activity was measured by the differentiation and proliferation of granulocytes and macrophages from bone marrow in a modification of the liquid culture system of Marchal and Milon (22). Triplicate wells of Terasaki microtest plates (Greiner) received 10^3 adult BALB/c tibial bone marrow cells and 1 μl of test microculture SN in a total of 10 μl culture medium containing 20% heat-inactivated horse serum. Pooled SN were assayed by culturing bone marrow cells with serial dilutions of SN in groups of 30 wells per SN dose. After 5–7 d incubation at 37°C, plates were scored microscopically for growth. GM-CSF titers for pooled SN were expressed as the reciprocal of the SN dilution that produced growth in 50% of the test wells, standardized by reference to a control 2° MLC SN (100 U/ml). Histochemical staining has shown that the cells that proliferate in these cultures are of the granulocyte and macrophage lineages (D. Heumann, personal communication).

As GM-CSF may be produced in vitro in low but detectable quantities by non-T spleen cells, a limiting number of indicator bone marrow cells (10^3) and a low concentration of microculture SN (10%) were cultured so that the upper limit (mean plus 3 SD) of control well counts was usually only 50–100 cells/well, which facilitated scoring of test wells. The average frequency of responding bone marrow progenitors in this system at optimal GM-CSF concentrations is 1/356 (mean of seven determinations, range 1/502–1/273). The mean probability that a well did not receive a progenitor from 10^3 cells is therefore 0.07, and the probability that the three test wells for each SN did not receive a progenitor is 0.0003. Under these conditions, the sensitivity of the CSF assay was similar to that of the IL-2 and MAF assays in that ~0.3–1% 2° MLC SN gave 50% of the maximal response for each lymphokine.

Statistical Methods. For all cytolytic and lymphokine assays, positive microcultures were defined as those in which activity exceeded that in control wells (no responder cells) by more than 3 SD above the mean. In the GM-CSF assay, the mean cell number in control wells was calculated from the highest cell count from each triplicate, and test SN were considered positive when they stimulated growth in at least one of the triplicate microtest wells. Minimal estimates of precursor frequency for each activity were obtained by the minimum Chi^2 method from the Poisson distribution relationship between the responding cell number and the logarithm of the percentage of nonresponding cultures (23).

Results

Precursor frequencies of lymphokine-secreting cells in normal spleen populations were determined in a modification of the limiting dilution microculture system previously established in this laboratory for frequency analysis of alloreactive cytolytic and proliferating T lymphocytes (13). Graded numbers of C57BL/6 responder spleen cells were cultured with irradiated DBA/2 stimulator spleen cells in the presence of a saturating dose of IL-2-containing supernatant (EL-4 SN). After 7–9 d incubation, an aliquot of each microculture was removed for assay of cytolytic activity. The remaining cells were washed extensively to remove residual IL-2 and GM-CSF present in the EL-4 SN and restimulated for 24 h by addition of irradiated T cell-depleted DBA/2 spleen cells. Supernatants were then removed for assays of the lymphokines IL-2, MAF, and GM-CSF.

The requirement for restimulation of limiting dilution microcultures for the efficient detection of lymphokine-secreting cells could be examined directly in the case of MAF because it is not present in the EL-4 SN used as a source of IL-2 for primary stimulation. Fig. 1 presents an experiment in which MAF activity in primary 7-d microculture SN was compared with those taken 24 h later after washing and restimulation of the same cultures with irradiated spleen cells. The figure shows, first, that MAF activity could be detected in supernatants of some microcultures that had received responder lymphocytes but not in control wells containing stimulator cells

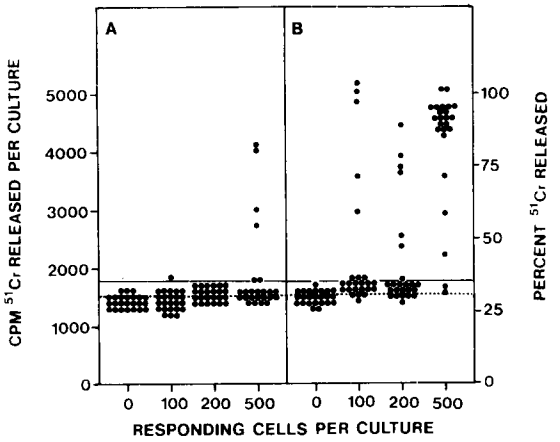

FIG. 1. Comparison of MAF activity in limiting dilution microcultures before and after restimulation. Groups of 24 replicate microcultures were established from the indicated numbers of C57BL/6 spleen responder cells with irradiated DBA/2 spleen stimulator cells and a source of IL-2. After 7 d incubation, microculture supernatants were removed for MAF assay (A). The cells in each microwell were washed and restimulated with irradiated T cell-depleted DBA/2 spleen cells, and supernatants were collected for MAF assay 24 h later (B). Each dot represents the activity in a single microculture. Positive cultures were defined as those wherein activity exceeded the mean activity in control wells (no responders, ...) by more than 3 SD (——).

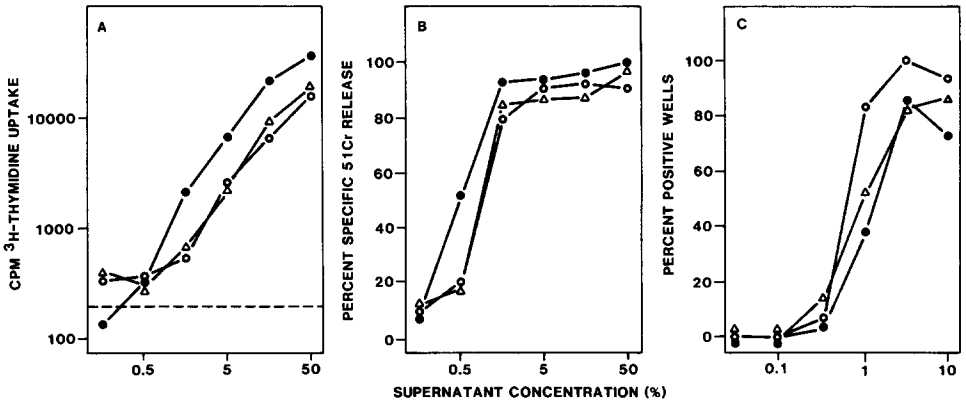

FIG. 2. Kinetics of lymphokine release after restimulation of limiting dilution microcultures. Microcultures were established from 1,000 nylon wool column- and FACS-passed (Table I) C57BL/6 spleen responder cells. After 7 d incubation, cultures were restimulated, and supernatants were collected 24 h (●), 48 h (Δ), and 72 h (○) later. Supernatants from groups of 12 microcultures were pooled and titrated in the assays for IL-2 (A), MAF (B), and GM-CSF (C). IL-2 activity was assayed on the CTLL line; background incorporation in the absence of SN was 201 cpm (-----). No activity in any assay was detected in SN from microcultures that did not receive responder cells, with the exception of the 72-h SN in the GM-CSF assay (25% positive wells at 10% SN concentration).

alone. In addition, it is apparent that the percentage of positive microcultures at each responder cell dose was significantly higher 24 h after restimulation than in the 7-d cultures; estimation of the frequency of precursors of MAF-secreting cells from these data by the minimum Chi^2 method (23) indicated that restimulation increased the apparent frequency by more than sixfold, from 4.6 to 30 precursors per 10^4 cells.

The kinetics of lymphokine release after restimulation was then examined in

microcultures established from 1,000 C57BL/6 spleen cells enriched for T lymphocytes by passage over nylon wool columns. Supernatants from groups of 12 microcultures were collected 24, 48, and 72 h after restimulation, pooled, and titrated in the three lymphokine assays. As shown in Fig. 2, the supernatants produced sigmoidal dose-response curves in each assay, with comparable thresholds of detection of the three activities (~0.5–1% supernatant concentration). These assays indicated that maximal activities of each factor were already present 24 h after restimulation and that, in the case of MAF and GM-CSF, the activities did not vary significantly in supernatants collected at different times. As IL-2 activity was approximately threefold lower after 72 h than 24 h in this experiment, and as previous studies had shown that IL-2 disappears rapidly from the supernatant of 2° MLC after 24–48 h (21), a 24-h restimulation period was chosen for the subsequent precursor frequency determinations.

The relationship between the precursor frequencies of cells producing IL-2, MAF, and GM-CSF and the Lyt-2 phenotype of the responder cells was examined using positively and negatively selected Lyt-2^+ and Lyt-2^- subpopulations in a series of experiments, one of which is presented in detail in Figs. 3 and 4. In this experiment, responder C57BL/6 splenic T lymphocytes were separated before culture into Lyt-2^+ and Lyt-2^- fractions by staining of nylon wool column-purified cells with a monoclonal anti-Lyt-2 antibody and fluorescein-conjugated anti-Ig and sorting into stained and unstained cells using a FACS. Fig. 3 shows data obtained for individual microcultures of unselected and separated responder cells in the quantitative assays for CTL, IL-2, and MAF. In each assay, increasing the number of responder cells increased the percentage of positive microcultures and produced cultures where activity reached 80–100% of maximal levels. It can be seen that the MAF assay was particularly well-suited to this experimental approach as the activity of positive microcultures generally clustered at 80–100% of total releasable cpm with few weakly positive cultures.

Supernatants from the same microcultures were also tested for GM-CSF activity. These results and the data shown in Fig. 3 are presented in Fig. 4 as semilogarithmic plots of the percentage of nonresponding microcultures against the number of responder cells cultured. Minimal estimates of the frequencies of precursors for each function in each responder population can be made from the slope of these curves provided they fit the Poisson distribution (23). It can be seen from these frequency estimates that the majority of precursors for CTL were found in the Lyt-2^+ responder population, whereas those for IL-2 production were present in the Lyt-2^- population. Compared with the unselected population, the frequency of CTL-P was increased approximately threefold in the Lyt-2^+ fraction that represented 33% of the total cells, and the frequency of precursors for IL-2 production was increased 1.5-fold in the Lyt-2^- fraction (67%). In contrast, precursors of MAF- and GM-CSF-producing cells were found in significant numbers in both the Lyt-2^+ and Lyt-2^- fractions, but at three- to fourfold lower frequency in the Lyt-2^+ than the Lyt-2^- population.

The lower precursor frequency of MAF- and GM-CSF-secreting cells among Lyt-2^+ compared with Lyt-2^- cells raised the possibility that culture and/or assay conditions might have been less efficient for the detection of lymphokine-secreting Lyt-2^+ than Lyt-2^- cells. A control experiment was therefore performed in which multiple microcultures were established with 1,000 FACS-passed unselected, Lyt-2^+ or Lyt-2^- responder cells. After 7 d, when all microwells contained visible lymphocyte

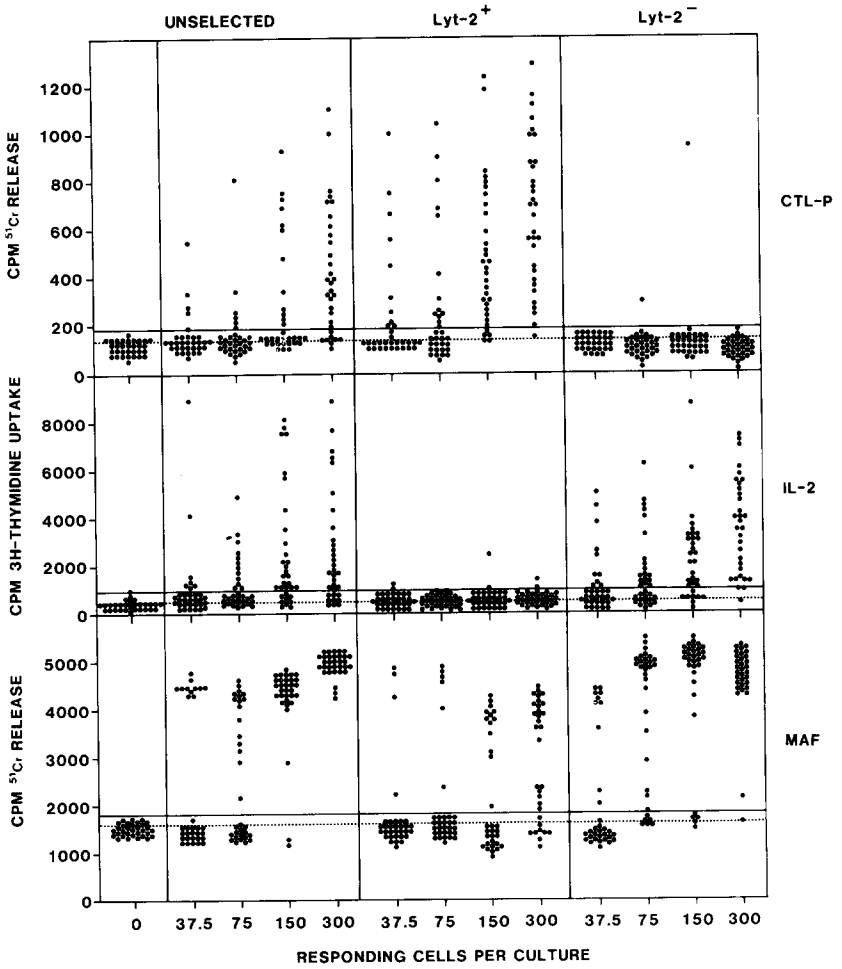

FIG. 3. Cytolytic, IL-2, and MAF activity in limiting dilution microcultures of T lymphocytes separated according to Lyt-2 phenotype. Nylon wool column-purified C57BL/6 spleen cells were stained with a rat monoclonal anti-Lyt-2 antibody and fluorescein-conjugated mouse anti-rat Ig and passed on a FACS gated to exclude nonviable cells. Cells were sorted into Lyt-2$^+$ (33%) or Lyt-2$^-$ (67%) fractions or collected without regard to fluorescence intensity (unselected) and cultured at the indicated numbers in groups of 32 microcultures. After 7 d incubation, microcultures were split and one aliquot tested for cytolytic activity against ^{51}Cr-labeled P815 tumor cells (top panels). The other aliquot was restimulated and SN collected after 24 h for assay of IL-2 (using the BD.2.10 line; middle panels) and MAF (lower panels).

growth, the cultures were washed and restimulated; supernatants were then harvested and pooled from groups of 12 microwells after 24, 48, and 72 h and titrated in the IL-2, MAF, and GM-CSF assays, as described above in Fig. 2. It can be seen from Table I that for each responder population, approximately equivalent MAF and GM-CSF activities were present in supernatants collected at the three time points. IL-2, on the other hand, was only detected in supernatants from the unselected and Lyt-2$^-$ populations, in accordance with the precursor frequency results presented above, and was higher in 24-h supernatants than in those collected after 48 or 72 h. These results substantiate the choice of a 24-h restimulation period for the optimal detection of

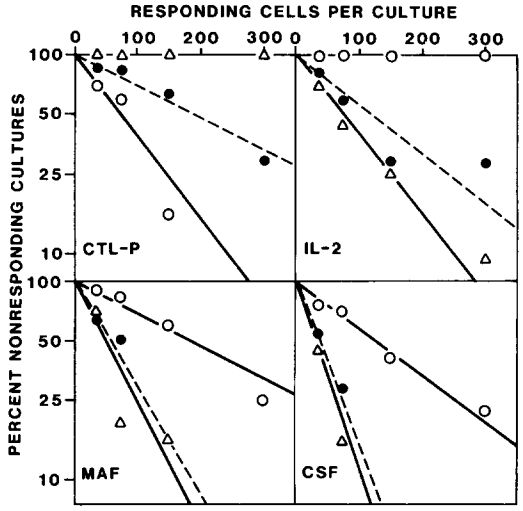

FIG. 4. Comparison of precursor frequencies of CTL and lymphokine-secreting cells in T lymphocyte populations separated according to Lyt-2 phenotype. The data from Fig. 3 and results of GM-CSF assays performed with the same microculture supernatants are plotted in accordance with Poisson statistics by the minimum Chi^2 method for the three responder populations: unselected cells (●), Lyt-2^+ cells (○), and Lyt-2^- cells (△).

TABLE I

Kinetics of Lymphokine Release after Restimulation of Microcultures Established from Spleen Cells Separated According to Lyt-2 Phenotype

Responder population*	Lymphokine titer‡								
	IL-2			MAF			GM-CSF		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Unselected	3.3	1.3	1.1	127	72	69	56	74	119
Lyt-2^-	35.0	24.5	5.5	576	709	656	322	329	361
Lyt-2^+	<0.1	<0.1	<0.1	50	62	58	34	57	30
None	<0.1	<0.1	<0.1	<1	<1	<1	<2	<2	4

* Nylon wool column-purified C57BL/6 spleen cells separated into Lyt-2^+ (32%) and Lyt-2^- (68%) populations or similarly treated without separation (unselected) were cultured in limiting dilution microcultures at 1,000 cells per well. After 7 d, cultures were washed and restimulated with irradiated T cell-depleted DBA/2 spleen cells. Supernatants were collected after 24, 48, or 72 h and pooled from groups of 12 replicate cultures. Results for the unselected population in the same experiment were presented graphically in Fig. 2.

‡ IL-2 assays were performed using the CTLL line as indicator cells. Lymphokine titers are expressed as the reciprocal of the SN dilution that produced 50% of the maximal response (IL-2: 44,700 cpm with EL-4 SN; MAF: 5,100 cpm ^{51}Cr release from P815 target cells incubated with 0.5 N HCl, corrected for background release of 31%; GM-CSF: 29 out of 30 wells positive for growth on day 5).

lymphokines released by both Lyt-2^+ and Lyt-2^- populations. However, they also indicate that the average quantitites of MAF and GM-CSF produced by the Lyt-2^+ population were about 10-fold less than those produced by equivalent numbers of Lyt-2^- responder cells.

Table II summarizes the results of the experiment described in Figs. 3 and 4 (number 1) and three others. The first two were performed with nylon wool-purified, positively selected Lyt-2^+ and Lyt-2^- cells, as described above. In the third and fourth

TABLE II

*Summary of Precursor Frequencies of CTL and Lymphokine-producing Cells in Spleen Cells Separated According to Lyt-2 Phenotype**

Activity	Precursor frequency (per 10^4 cells)									
	Unselected				Lyt-2$^-$				Lyt-2$^+$	
	1‡	2	3	4	1	2	3	4	1	2
CTL	37	51	25	31	1.8	0.7	0.6	1.4	99	46
IL-2	58	38	32	23	93	98	40	26	2.5	2.0
MAF	123	66	30	85	135	87	45	95	38	25
GM-CSF	185	36	NT§	111	217	57	NT	118	56	18

* Limiting dilution microcultures were established from C57BL/6 spleen responder cells, either with positive selection in experiments 1 and 2 (33% and 35% Lyt-2$^+$ cells respectively), or with negative selection by anti-Lyt-2 antibody and C treatment in experiments 3 and 4.

‡ Experiment number.

§ Not tested.

experiments, the responder population consisted of unfractionated spleen cells with or without depletion of Lyt-2$^+$ lymphocytes by treatment with antibody and complement. Essentially the same results were obtained in each case. Whereas the precursors of CTL and IL-2-producing cells were found almost exclusively in the Lyt-2$^+$ and Lyt-2$^-$ fractions, respectively, the precursors of MAF- and GM-CSF-producing cells were present in both of these subpopulations. As noted above, the precursor frequencies of cells releasing MAF and GM-CSF were three- to fourfold lower in the Lyt-2$^+$ than the Lyt-2$^-$ population.

Discussion

The present report describes for the first time the simultaneous estimation of precursor frequencies for T lymphocytes secreting IL-2, MAF, and GM-CSF in normal C57BL/6 spleen cells responsive to the combined H-2 plus Mls allogeneic stimulus of DBA/2 spleen cells. By the combination of flow cytometry, a microculture system for the activation of unprimed lymphocytes at limiting dilution, and sensitive microassays for cytolytic and lymphokine activities, it has been possible to compare directly in each series of microcultures the frequencies of lymphokine-secreting cells and cytolytic T lymphocytes in populations separated according to Lyt-2 phenotype. The Lyt-1 phenotype was not taken into consideration in this study in view of recent demonstrations by several laboratories that this marker is expressed by essentially all T lymphocytes (24–26).

The three lymphokines assayed in these experiments were chosen because they have been shown to be functionally and biochemically separable and to act on different target populations. Thus, analysis of the soluble products of T cell clones (11) indicated that IL-2, MAF, and GM-CSF could be produced independently by different clones, whereas biochemical approaches have clearly demonstrated that IL-2 and GM-CSF are separate molecular species (27). IL-2 appears to act exclusively as a growth factor for T lymphocytes (28), whereas GM-CSF is a differentiative and proliferative stimulus for committed progenitor cells of the myeloid lineage (29), and MAF appears to be a nonproliferative, activating stimulus for differentiated cells of the monocyte/macrophage series (20, 30). Although the target populations of MAF

and GM-CSF may overlap and these factors may have additional as yet undefined effects, comparison of supernatants from various T cell clones and tumor lines (11) has shown that each of the assays used here is specific for the activity being measured and is not influenced by the presence of the other lymphokines.

Primary activation of alloreactive lymphocytes was performed in limiting dilution microcultures by stimulation with allogeneic cells in the presence of exogenous IL-2, under conditions previously defined for the generation of alloreactive cytolytic and noncytolytic T lymphocytes (13). This system was modified in the present study by the addition of a 24-h restimulation step, with allogeneic cells in the absence of growth factors, before harvest of culture supernatants for lymphokine assays. It was shown that restimulation increased the apparent precursor frequency of MAF-secreting cells by approximately sixfold (Fig. 1) and that 24 h was optimal for the release of maximal IL-2, MAF, and GM-CSF activities in positive microcultures (Fig. 2 and Table I). Sensitive conditions for the detection of lymphokines in culture supernatants had previously been defined in studies with long-term T cell clones (10, 11). These studies showed that MAF and IL-2 release was antigen specific, occurred within several hours of restimulation, and could not be detected in supernatants of irradiated T cell-depleted stimulator spleen cells cultured alone. GM-CSF, on the other hand, is known to be released by many cell types in addition to T lymphocytes (29). However, as this constitutive release by non-T cells is much lower than production by antigen-stimulated T cells, parameters of the GM-CSF assay could be defined so that background activity released by stimulator cells alone was not detected. Under these conditions, the thresholds of detection of the three lymphokine assays were similar (Fig. 2). It should be noted that the three lymphokine assays required only 100 μl of the 150 μl of supernatant harvested from each limiting dilution microculture. It would clearly be feasible to test other lymphokine activities in the same supernatants, provided sensitive microassays suitable for "spot-testing" are available.

The relationship between Lyt-2 phenotype and the precursor frequencies of cytolytic and lymphokine-secreting cells was examined both by negative selection with anti-Lyt-2 antibody and complement and by positive selection using a flow cytometer to separate Lyt-2^+ and Lyt-2^- subpopulations. Equivalent results were obtained by both approaches.

First, essentially all precursors of CTL were found in the Lyt-2^+ population (57-fold more on average than in the Lyt-2^- fraction). This result is in agreement with many previous reports that both precursor and effector cytolytic T lymphocytes bear the Lyt-2 marker (26, 31, 32). In addition, the CTL precursor frequencies obtained here (1 in 300–400 for unfractionated spleen cells, 1 in 100–200 for Lyt-2^+ cells) are comparable with those recorded in earlier studies for the C57BL/6 anti-DBA/2 combination (12) and thus provide a positive control that efficient conditions for the activation of Lyt-2^+ precursors had been achieved. It could not be determined here whether the occasional cytolytically active microculture established with FACS-separated Lyt-2^- responders was derived from Lyt-2^- precursors or contaminating Lyt-2^+ cells. However, the low frequency of such cultures suggests that contamination of the Lyt-2^- fraction with Lyt-2^+ cells was $<2\%$.

The second finding, that the precursors of IL-2-secreting cells were present at much higher (average of 42-fold) frequency in the Lyt-2^- than the Lyt-2^+ population, is more controversial. Three groups (33–35) have reported that, at least in bulk MLC,

Lyt-2$^+$ cells responding to H-2K/D region stimulation produce comparable quantities of IL-2 as Lyt-2$^-$ cells responding to H-2I or Mls stimulation. In contrast, similar analyses of bulk cultures in this (A. L. Glasebrook and H. R. MacDonald, unpublished observations) and other laboratories (36) have detected little or no soluble or cell-mediated "helper" activity in the absence of H-2I or Mls antigenic disparity, even when purified Lyt-2$^+$ responder populations were used. Although there is evidence that a subpopulation of Lyt-2$^+$ H-2K/D-reactive lymphocytes, including CTL, can release IL-2 (A. L. Glasebrook, manuscript in preparation), the present study as well as those by Lutz et al. (37), Miller and Stutman (38), and Krönke et al. (39) suggest that the frequency of such cells is much lower than the frequency of IL-2-secreting cells in Lyt-2$^-$ H-2I- or Mls-reactive populations. Thus, Lutz et al. found that although H-2K or D region-reactive helper T cells could be detected in primary limiting dilution microcultures, the precursor frequencies of helper cells responding to H-2I and non-H-2 (putatively Mls) determinants were respectively 6- and 20-fold higher. Similarly, Miller and Stutman (38) and Krönke et al. (39) observed much higher frequencies of IL-2-secreting cells in Lyt-2$^-$ populations compared with Lyt-2$^+$ cells and in Mls-reactive compared with H-2-reactive unfractionated populations.

In considering this apparent dissociation, it is important to note that T lymphocytes, and particularly Lyt-2$^+$ cells, have specific membrane receptors for IL-2 (40). Thus, it cannot be excluded that the low apparent frequencies of Lyt-2$^+$ precursors of IL-2-secreting cells observed in this and other studies may result, at least in part, from IL-2 absorption during the culture period. Despite these potential limitations to the quantitation of IL-2-secreting cells, it seems likely that the observation of a much higher precursor frequency in separated Lyt-2$^-$ than Lyt-2$^+$ populations is qualitatively valid. In this case, these results and those of Lutz et al., Miller and Stutman, and Krönke et al., are consistent with the view that most of the IL-2 produced in response to alloantigenic stimulation is released by Lyt-2$^-$ cells that respond preferentially to H-2I or Mls antigens.

The third finding described here is that, in contrast to IL-2, precursors of cells secreting MAF and GM-CSF were present in both the Lyt-2$^+$ and Lyt-2$^-$ lymphocyte populations. Precursor frequency determinations for cells releasing these two factors have not been reported before; however, the observation by Krammer et al. (41) that a high proportion of both cytolytic and noncytolytic cells in concanavalin A-activated populations release interferon-γ may be related in view of the correlation between production of this factor and MAF by long-term T cell clones (11). The finding of the present study that the MAF (and GM-CSF) assay detected the majority of responding Lyt-2$^-$ cells but only about one-third of responding Lyt-2$^+$ cells was unexpected in view of our previous analysis of MAF release by a large group of long-term T cell clones (11; unpublished observations). Of the alloreactive clones studied, 15 out of 17 cytolytic clones directed against H-2K and H-2D antigens and 21 out of 24 H-2I- or Mls-reactive noncytolytic clones were MAF secretors. There was, therefore, no indication that the incidence of MAF-secreting cells was higher among Lyt-2$^-$ H-2I or Mls antigen-reactive than Lyt-2$^+$ H-2K/D-reactive clones. This apparent discrepancy between short-term and long-term clones could reflect (*a*) selection of MAF-producing Lyt-2$^+$ clones with continued in vitro culture, (*b*) suboptimal conditions for the detection of lymphokine-secreting Lyt-2$^+$ cells at limiting dilution, or (*c*) weaker (subthreshold) factor production by Lyt-2$^+$ than Lyt-2$^-$ cells.

No information is yet available about the first of these alternatives. However, the latter two possibilities were addressed directly for the limiting dilution protocol by comparing the amounts of MAF, GM-CSF, and IL-2 present in pooled microwell supernatants from unselected, Lyt-2^+ and Lyt-2^- responder cells at various intervals after restimulation (Table I). For both MAF and GM-CSF, approximately equivalent activities were detected in supernatants collected after 24, 48, and 72 h from all three responder populations. This result paralleled previous studies (9, 11) where lymphokine activities in supernatants from cytolytic and noncytolytic long-term clones were found to be maximal by 24 h and, for all factors except IL-2, to remain at the same level for a week thereafter. There was, thus, no evidence that harvesting supernatants 24 h after restimulation favored the detection of lymphokine production by Lyt-2^- cells over Lyt-2^+ cells. This experiment also indicated that the average MAF and GM-CSF activities released by Lyt-2^+ cells were 10-fold lower than those from Lyt-2^- cells. This weaker total production by Lyt-2^+ cells can be attributed in part to the threefold lower precursor frequency of cells producing these factors among Lyt-2^+ than Lyt-2^- cells, but may point further to lower average production by those Lyt-2^+ cells that do secrete MAF and GM-CSF. Some support for this possibility has been obtained with long-term alloreactive clones, where the average titers of MAF released by cytolytic H-2K/D-reactive clones were found to be 2- to 3-fold lower than average production by noncytolytic H-2I- or Mls-reactive clones (unpublished observations).

The limiting dilution approach used here has the important advantage over work with expanded clones that the frequency of responsive lymphocytes with different functions can be determined in unprimed populations with a minimum of selection by in vitro culture. Experiments are now in progress to compare the precursor frequencies of lymphokine-producing cells among T lymphocytes responding to defined regions of the H-2 complex or the M locus, where gross differences in the frequencies of CTL-P and helper or IL-2-secreting T lymphocyte precursors have already been noted (37–39; H. R. MacDonald and J.-C. Cerottini, unpublished observations).

The main new finding reported here, that most Lyt-2^- and about one-third of Lyt-2^+ alloreactive T lymphocytes are precursors of MAF- and GM-CSF-secreting cells, may have important implications for our understanding of in vivo alloreactivity. Thus, in addition to the well-established in vitro functions of Lyt-2^+ cells in cytolysis and of Lyt-2^- cells in IL-2 production, both these populations may amplify the immune response nonspecifically by release of factors that induce differentiation, proliferation, and activation of cells of the granulocyte and monocyte/macrophage lineage.

Summary

The frequencies of precursors of C57BL/6 T lymphocytes that respond to DBA/2 alloantigens by secreting the lymphokines interleukin 2 (IL-2), macrophage-activating factor (MAF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been directly compared with cytolytic T lymphocyte precursor (CTL-P) frequencies in limiting dilution microcultures established from spleen cells positively or negatively selected on the basis of Lyt-2 phenotype. A clear dichotomy was observed between CTL-P, which were contained in the Lyt-2^+ fraction, and precursors of IL-2-secreting cells, which were detected almost exclusively in the Lyt-2^- population. In

contrast, precursors of cells secreting MAF and GM-CSF were found in both populations: almost all responding cells from the Lyt-2^- fraction produced both these factors, whereas the precursor frequency of MAF-secreting and GM-CSF-secreting cells was three- to fourfold lower in the Lyt-2^+ population. These frequency data were consistent with quantitative differences observed in the average production of these lymphokines by Lyt-2^+ and Lyt-2^- populations.

We thank Dr. F. W. Fitch, Dr. J. A. Ledbetter, Dr. J. J. Farrar, and Dr. M. Nabholz for their gifts of reagents, Anne-Lise Peitrequin and Chantal Frey for excellent technical assistance, and Pierre Zaech for operation of the FACS.

Received for publication 13 July 1982.

References

1. Biology of the Lymphokines. 1979. S. Cohen, E. Pick, and J. J. Oppenheim, editors. Academic Press, Inc., New York.
2. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T-cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120:**2027.
3. Schimpl, A., and E. Wecker. 1972. Replacement of T cell function by a T-cell product. *Nat. New Biol.* **237:**15.
4. Lohmann-Matthes, M.-L., F. G. Ziegler, and H. Fischer. 1973. Macrophage cytotoxicity factor. A product of *in vitro* sensitized thymus-dependent cells. *Eur. J. Immunol.* **3:**56.
5. Parker, J. W., and D. Metcalf. 1974. Production of colony-stimulating factor in mitogen-stimulated lymphocyte cultures. *J. Immunol.* **112:**502.
6. Kirchner, H., R. Zawatzky, H. Engler, V. Schirrmacher, H. Becker, and P. von Wussow. 1979. Production of interferon in the murine mixed lymphocyte culture. II. Interferon production is a T cell-dependent function, independent of proliferation. *Eur. J. Immunol.* **9:**824.
7. Nabel, G., J. S. Greenberger, M. A. Sakakeeny, and H. Cantor. 1981. Multiple biologic activities of a cloned inducer T-cell population. *Proc. Natl. Acad. Sci. U. S. A.* **78:**1157.
8. Marcucci, F., M. Waller, H. Kirchner, and P. Krammer. 1981. Production of immune interferon by murine T-cell clones from long-term cultures. *Nature (Lond.).* **291:**79.
9. Ely, J. M., M. B. Prystowsky, L. Eisenberg, J. Quintans, E. Goldwasser, A. L. Glasebrook, and F. W. Fitch. 1981. Alloreactive cloned T cell lines. V. Differential kinetics of IL-2, CSF, and BCSF release by a cloned T amplifier cell and its variant. *J. Immunol.* **127:**2345.
10. Glasebrook, A. L., A. Kelso, R. H. Zubler, J. M. Ely, M. B. Prystowsky, and F. W. Fitch. 1982. Lymphokine production by cytolytic and non-cytolytic alloreactive T cell clones. *In* Isolation, Characterization, and Utilization of T Lymphocyte Clones. C. G. Fathman and F. W. Fitch, editors. Academic Press, Inc., New York. 341.
11. Kelso, A., A. L. Glasebrook, O. Kanagawa, and K. T. Brunner. 1982. Production of macrophage-activating factor by T lymphocyte clones and correlation with other lymphokine activities. *J. Immunol.* **129:**550.
12. MacDonald, H. R., J.-C. Cerottini, J.-E. Ryser, J. L. Maryanski, C. Taswell, M. B. Widmer, and K. T. Brunner. 1980. Quantitation and cloning of cytolytic T lymphocytes and their precursors. *Immunol. Rev.* **51:**93.
13. Ryser, J.-E., and H. R. MacDonald. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. I. Comparison of precursor frequencies for proliferative and cytolytic responses. *J. Immunol.* **122:**1691.
14. Cerottini, J.-C., H. D. Engers, H. R. MacDonald, and K. T. Brunner. 1974. Generation of cytotoxic T lymphocytes *in vitro*. I. Response of normal and immune mouse spleen cells in

mixed leukocyte cultures. *J. Exp. Med.* **140:**703.
15. Maryanski, J. L., H. R. MacDonald, B. Sordat, and J.-C. Cerottini. 1981. Cell surface phenotype of cytolytic T lymphocyte precursors in aged nude mice. *Eur. J. Immunol.* **11:**968.
16. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3:**645.
17. Farrar, J. J., J. Fuller-Farrar, P. L. Simon, M. L. Hilfiker, B. M. Stadler and W. L. Farrar. 1980. Thymoma production of T cell growth factor (Interleukin 2). *J. Immunol.* **125:**2555.
18. Glasebrook, A. L., and F. W. Fitch. 1979. T-cell lines which cooperate in generation of specific cytolytic activity. *Nature (Lond.).* **278:**171.
19. Ceredig, Rh., A. L. Glasebrook, and H. R. MacDonald. 1982. Phenotypic and functional properties of murine thymocytes. I. Precursors of cytolytic T lymphocytes and Interleukin 2-producing cells are all contained within a subpopulation of "mature" thymocytes as analyzed by monoclonal antibodies and flow microfluorometry. *J. Exp. Med.* **155:**358.
20. Meerpohl, H.-G., M.-L. Lohmann-Matthes, and H. Fischer. 1976. Studies on the activation of mouse bone marrow-derived macrophages by the macrophage cytotoxicity factor (MCF). *Eur. J. Immunol.* **6:**213.
21. Ryser, J.-E., J.-C. Cerottini, and K. T. Brunner. 1978. Generation of cytolytic T lymphocytes *in vitro.* IX. Induction of secondary CTL responses in primary long-term MLC by supernatants from secondary MLC. *J. Immunol.* **120:**370.
22. Marchal, G., and G. Milon. 1980. Enumeration of haemopoietic progenitors (CFU-C and BFU-E) in liquid microculture using a limiting dilution analysis. *Ann. Immunol.* (Inst. Pasteur) **131D:**111.
23. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* **126:**1614.
24. Mathieson, B. J., S. O. Sharrow, P. S. Campbell, and R. Asofsky. 1979. A Lyt differentiated thymocyte subpopulation detected by flow microfluorometry. *Nature (Lond.).* **277:**478.
25. Ledbetter, J. A., R. V. Rouse, H. S. Micklem, and L. A. Herzenberg. 1980. T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. Two parameter immunofluorescence and cytotoxicity analysis with monoclonal antibodies modifies current views. *J. Exp. Med.* **152:**280.
26. Cerottini, J.-C., and H. R. MacDonald. 1981. Limiting dilution analysis of alloantigen-reactive T lymphocytes. V. Lyt phenotype of cytolytic T lymphocyte precursors reactive against normal and mutant H-2 alloantigens. *J. Immunol.* **126:**490.
27. Hilfiker, M. L., R. N. Moore, and J. Farrar. 1981. Biologic properties of chromatographically separated murine thymoma-derived interleukin 2 and colony-stimulating factor. *J. Immunol.* **125:**1983.
28. Smith, K. A. 1980. T-cell growth factor. *Immunol. Rev.* **51:**337.
29. Burgess, A. W., and D. Metcalf. 1980. The nature and action of granulocyte-macrophage colony stimulating factors. *Blood.* **56:**947.
30. Ruco, L. P., and M. S. Meltzer. 1978. Macrophage activation for tumor cytotoxicity: increased lymphokine responsiveness of peritoneal macrophages during acute inflammation. *J. Immunol.* **120:**1054.
31. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly^+ cells in the generation of killer activity. *J. Exp. Med.* **141:**1390.
32. Teh, H.-S., and S.-J. Teh. 1980. The use of a clonal assay for cytotoxic T lymphocytes to determine the Ly phenotypes of cytotoxic precursor and effector cells to alloantigens and trinitrophenyl-modified self antigens. *J. Immunol.* **125:**1977.
33. Swain, S. L. 1981. Significance of Lyt phenotypes: Lyt2 antibodies block activities of T cells that recognize class I major histocompatibility complex antigens regardless of their function. *Proc. Natl. Acad. Sci. U. S. A.* **78:**7101.

34. Kern, D. E., S. Gillis, M. Okada, and C. S. Henney. 1981. The role of interleukin-2 (IL-2) in the differentiation of cytotoxic T cells: the effect of monoclonal anti-IL-2 antibody and absorption with IL-2 dependent T cell lines. *J. Immunol.* **127:**1323.
35. Andrus, L., S. J. Prowse, and K. J. Lafferty. 1981. Interleukin 2 production by both Lyt-2^+ and Lyt-2^- T-cell subsets. *Scand. J. Immunol.* **13:**297.
36. Miller, R. A., and O. Stutman. 1982. Use of positively selected Lyt-2^+ mouse splenocytes to examine interleukin-2 secretion in responses to alloantigens and to TNP-modified syngeneic cells. *Cell. Immunol.* **68:**114.
37. Lutz, C. T., A. L. Glasebrook, and F. W. Fitch. 1981. Enumeration of alloreactive helper T lymphocytes which cooperate with cytolytic T lymphocytes. *Eur. J. Immunol.* **11:**726.
38. Miller, R. A., and O. Stutman. 1982. Enumeration of IL-2-secreting helper T cells by limiting dilution analysis, and demonstration of unexpectedly high levels of IL-2 production per responding cell. *J. Immunol.* **128:**2258.
39. Krönke, M., P. Scheurich, K. Pfizenmaier, M. Röllinghoff, and H. Wagner. 1982. T-T cell interactions during *in vitro* cytotoxic T lymphocyte (CTL) responses. V. Precursor frequencies and specificity of alloreactive helper T cells. *J. Exp. Med.* **156:**41.
40. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factor receptors. Quantitation, specificity, and biological relevance. *J. Exp. Med.* **154:**1455.
41. Krammer, P. H., F. Marcucci, M. Waller, and H. Kirchner. 1982. Heterogeneity of soluble T cell products. I. Precursor frequency and correlation analysis of cytotoxic and immune interferon (IFN-γ)-producing spleen cells in the mouse. *Eur. J. Immunol.* **12:**200.