

## QUANTITATIVE STUDIES ON T CELL DIVERSITY

### II. Determination of the Frequencies and Lyt Phenotypes of Two Types of Precursor Cells for Alloreactive Cytotoxic T Cells in Polyclonally and Specifically Activated Splenic T Cells

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In the first paper of this series (1), we described a limiting dilution assay designed for the determination of any precursor T cell whose effector function can be determined *in vitro*. For this assay, normal T cells are used that are polyclonally activated in bulk culture with concanavalin A (Con A)<sup>1</sup> and thereafter expanded by limiting dilution in the presence of T cell growth factor (TCGF). The expansion step ensures a clone size of 500–1,000 so that T cell functions can be easily determined. The use of this assay for the determination of antigen-specific T helper cells provided for the first time information on the precursor frequencies for helper cells in nonimmune T cells. Furthermore, two types of helper cell precursors could be identified, the more frequent one being sensitive to and the less frequent one being insensitive to suppressor T cells.

In the present paper, we apply the same method to the determination of precursor frequencies for cytotoxic T cells (CTL). Although, in contrast to helper T cell precursors, a host of information on CTL precursor frequencies is available (2–10), we decided to undertake this study to compare the frequencies determined in polyclonally activated T cells with those determined in alloantigen-activated T cells. We found that CTL precursor frequencies determined in both assays were similar. However, in addition to the single type of CTL precursor found by us and others in alloantigen-activated T cells, polyclonally activated T cells show an additional CTL precursor of lesser frequency. As in the case of frequent and rare T helper cells, frequent CTL precursors are sensitive to suppression, whereas rare CTL precursors are not.

The determination of CTL precursor frequencies in selected Lyt-1 and Lyt-23 T cells showed an absolute depletion of all CTL precursors in Lyt-1 and a slight depletion of frequent CTL precursors in Lyt-23 T cells. This was the case for specifically and polyclonally activated cells, and it confirms previous data from our laboratory showing that the majority of CTL precursors are of Lyt-123 phenotype (11). Rare CTL precursors are enriched in the Lyt-23 population, a finding that is

<sup>1</sup> *Abbreviations used in this paper:* C', complement; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; CTL-P, CTL precursor; FCS, fetal calf serum; MLR, mixed lymphocyte reaction; PEC, peritoneal exudate cells; TCGF, T cell growth factor; T<sub>H</sub>, T helper cells.

discussed with respect to the possible function of the two types of CTL precursors as primary and secondary cells.

### Materials and Methods

*Mice.* C57BL/6 and DBA/2 mice were purchased from the Institut für Versuchstierforschung, Hannover, Federal Republic of Germany. All mice were vaccinated with ectromelia vaccine at the age of 4 wk and were used for experiments at 6–10 wk. Sprague-Dawley rats were obtained from our own animal breeding unit.

*Preparation of TCGF.* Preparation of TCGF from rat spleen cells has been described in detail previously (1). Briefly, TCGF was prepared by stimulating spleen cells from Sprague-Dawley rats with Con A ( $5 \mu\text{g/ml}$ ) at  $5 \times 10^6/\text{ml}$  in complete Iscove's modified Dulbecco's medium. Supernatant fluids were collected after 24 h of incubation. The efficiency of each TCGF preparation was determined by its support of the proliferation of various in vitro T cell lines and in a costimulator assay according to the protocol described by Paetkau et al. (12). All experiments described in this paper were done with TCGF preparations giving optimal support in both detector systems at dilutions of 1:5–1:20.

*Enrichment of T Cells.* T cells were prepared by nylon wool passage of spleen cells, as described previously (13).

*Preparation of Antisera and Selection for Lyt T Cell Subsets.* Conventional anti-Lyt-1.2 and anti-Lyt-3.2 antisera were prepared according to Shen et al. (14), as described in detail elsewhere (11). Monoclonal anti-Lyt-2.2 antibodies, kindly provided by Dr. U. Hämmerling (Memorial Sloan-Kettering Cancer Center, New York), were prepared using the cell fusion techniques as described (15).

For selection of Lyt T cell subsets, spleen cells and splenic T cells, as well as Con A-activated spleen cells and splenic T cells, were treated with anti-Lyt-1.2 and anti-Lyt-2.2, plus anti-Lyt-3.2 antisera, respectively, and selected rabbit complement, as described (11).

*Limiting Dilution Analysis.* Two limiting dilution protocols were employed for determination of CTL precursor (CTL-P) frequencies. In the first protocol, unselected or selected C57BL/6 spleen cells or splenic T cells were cultured in limiting numbers (32–48 microcultures/group) with  $10^6$  irradiated (2,200 rad) DBA/2 spleen cells for 7 d in Linbro flat-bottomed microtiter wells (7600305; Linbro Chemical Co., Hamden, Conn.) in a volume of 200  $\mu\text{l}$  RPMI-1640 supplemented with 10% fetal calf serum (FCS),  $10^{-5}$  M, 2-mercaptoethanol, 0.025 M HEPES, 1% Pen-Strep, 0.2 mM glutamine, and the appropriate concentration of TCGF (5–20%). In the second protocol, unselected or selected C57BL/6 spleen cells or splenic T cells were polyclonally activated at a density of  $2 \times 10^6/\text{ml}$  for 2 d with  $2.5 \mu\text{g}$  Con A/ml in RPMI-1640 supplemented as described above (no TCGF). Recovered cells were purified by centrifugation over Ficoll as described previously (1) and plated in limiting numbers in Linbro flat-bottomed microtiter wells in the presence of TCGF and  $1 \times 10^4$  irradiated (3,000 rad;  $\text{Co}^{60}$ ) syngeneic peritoneal exudate cells (PEC) per well as fillers, and incubated for 7–9 d. Medium was exchanged every 3 d. Frequencies of growing cells were determined after 1 wk of culture by visual examination using an inverted microscope and analyzed according to Poisson's distribution (1). In all experiments described, growth frequencies were similar for unselected lymphocytes and for selected Lyt T cell subsets. Results from only those experiments in which the frequencies of growing cells were  $>1$  in 5 are considered.

*Assay for Cytolytic Activity.* After incubation for the indicated time, microcultures were assayed for cytolytic activity in a 4-h  $^{51}\text{Cr}$  release assay on the appropriate target cells. For this purpose, aliquots of the individual cultures were transferred to round-bottomed microtiter plates (Linbro IS-MRC 96; Linbro Chemical Co.), washed once with medium, and thereafter mixed with  $2 \times 10^3$   $^{51}\text{Cr}$ -labeled P815 tumor target cells in a final volume of 0.2 ml. In the case of polyclonal activation, individual microcultures were split and tested for specific CTL activity on  $^{51}\text{Cr}$ -labeled P815 targets and for total cytolytic activity on P815 tumor cells in the presence of Con A ( $5 \mu\text{g/ml}$ ). Spontaneous release was determined by transferring aliquots from control microcultures containing no responder cells on the appropriate target cells. An aliquot of the target cells was frozen and thawed four times so that maximum  $^{51}\text{Cr}$  release could be determined.

*Calculations of Cytolytic Activity and of CTL-P Frequencies.* Microcultures were scored as positive when  $^{51}\text{Cr}$  release values exceeded the arithmetic mean of spontaneous release by  $>3$  SD.

The relationship between numbers of responder cells added per microculture and the fraction of nonresponding cultures per group were fit by the least-squares method to the zero-order term of the Poisson equation. In case of diphasic distributions, only those values of the curve were taken into the calculation that appeared to be linear in the semilogarithmic plot. Estimates of precursor frequencies are given by the slope of the regression line or, practically, by the number of cells corresponding to 37% negative cultures.

## Results

*Comparison of Specific CTL-P Frequencies in Polyclonally Activated T Cells and in Normal T Cells Activated by H-2 Alloantigen.* Spleen cells or purified T cells from normal C57BL/6 mice were assayed for their precursor frequencies for CTL against DBA/2 target cells by two different limiting dilution systems. In the first method, spleen cells or T cells were placed in microwells together with DBA/2 stimulator cells and TCGF and, after 1 wk of culture, each well was assayed for CTL activity against DBA/2 target cells. In the second method, spleen cells or T cells were activated in bulk culture with Con A for 2 d, and then placed in microwells together with TCGF and syngeneic peritoneal macrophages but without stimulator cells. After a period of 7–9 d, each microwell was assayed for CTL activity against P815 tumor target cells. The data obtained were analyzed according to Poisson's distribution, and the results are depicted in Fig. 1 (first method) and in Fig. 2A and B (second method).

It is evident from Fig. 1 that for both spleen cells and T cells, the number of cells per well is in linear relationship to the logarithm of the fraction of cultures negative for CTL activity. The lines cross 1.0 on the ordinate, which suggests that a single cell type is limiting. For this experiment, the frequencies, read from the 0.37 intercept of

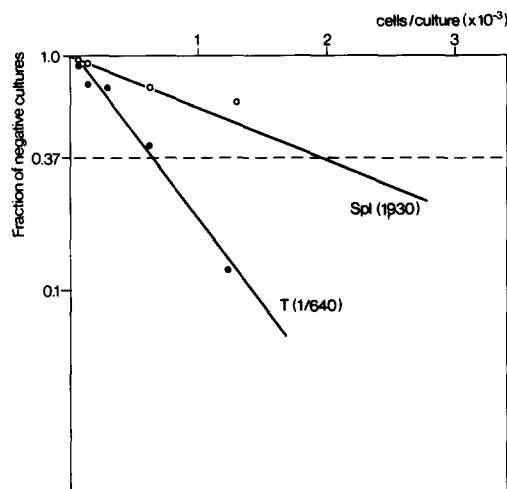


FIG. 1. Comparison of the frequencies of CTL-P specific for H-2<sup>d</sup> in alloantigen-activated C57BL/6 spleen cells (○) and splenic T cells (●). Groups of 32 microcultures containing 75–1,200 responder cells were sensitized to  $1 \times 10^6$  irradiated DBA/2 spleen cells for 7 d in the presence of TCGF. Aliquots of each well were subsequently tested for cytolytic activity on  $^{51}\text{Cr}$ -labeled P815 tumor targets in a 4-h chromium-release assay. Cultures were scored positive when chromium release exceeded the average spontaneous  $^{51}\text{Cr}$  release by at least 3 SD. Data are plotted according to Poisson statistics and evaluated as described in Material and Methods.

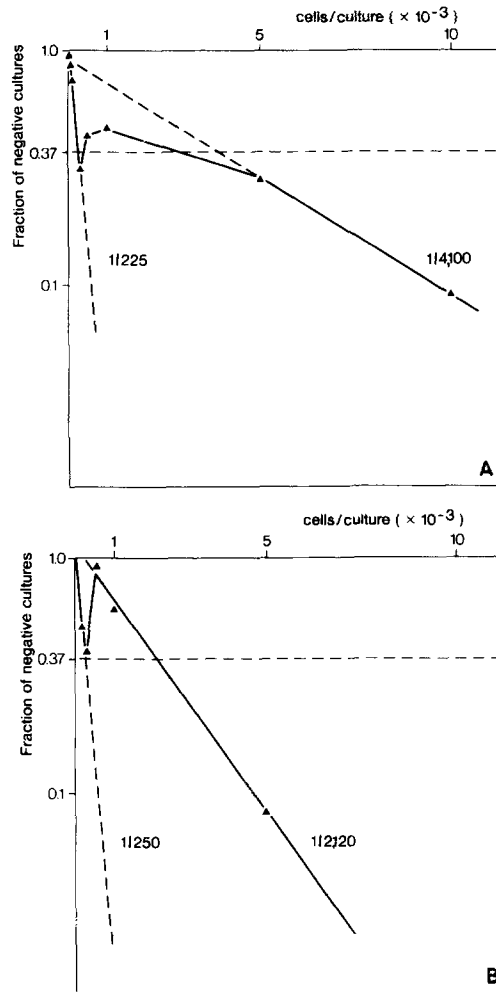


FIG. 2. Comparison of frequencies of CTL-P specific for H-2<sup>d</sup> in C57BL/6 splenic T cells (A) and spleen cells (B) polyclonally activated by Con A. After 2 d of stimulation, groups of 32 microcultures containing 5–20,000 responding cells were expanded on PEC for 8 d in the presence of TCGF. Determination of CTL activity and evaluation of data was as described in Fig. 1.

each line, suggest a CTL-P frequency of 1/1,930 for spleen cells and 1/640 for T cells. These numbers agree with the enrichment expected from a proportion of ~30% T cells in the spleen. Furthermore, they agree with the frequencies determined for alloantigen-specific CTL-P as reported by a number of other laboratories (4, 5, 8).

In Fig. 2 A and B, the results with T cells and spleen cells, respectively, obtained by the second method, are depicted. It is evident that the relationships between the numbers of cells per well and the fraction of negative wells show a diphasic distribution consisting of two linear portions. The 0.37 intercepts of the first linear area suggest a CTL-P frequency of 1/225 for T cells and 1/250 for spleen cells. The 0.37 intercepts of the second linear area suggests a CTL-P frequency of 1/4,100 for T cells and 1/2,120 for spleen cells. Both linear areas cross close to 1.0 on the ordinate, suggesting that each represents the distribution of a single cell type. At the intermediate cell

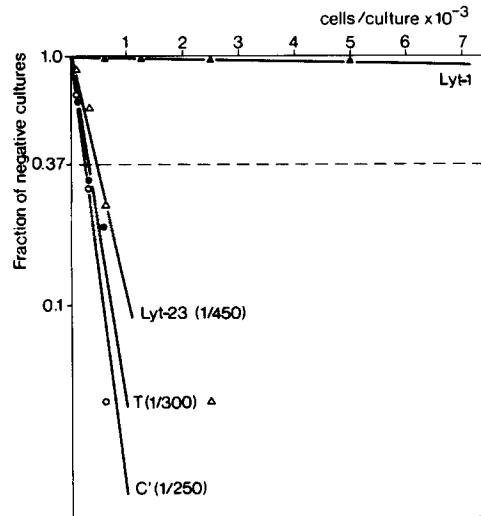


FIG. 3. Comparison of frequencies of CTL-P specific for H-2<sup>d</sup> in alloantigen activated unselected C57BL/6 splenic T cells and selected Lyt T cell subsets. Before sensitization with alloantigen and TCGF under limiting dilution conditions, responder cells were either left untreated (●) or were treated with C' alone (○), with anti-Lyt-1.2 + C (Lyt-2, 3 cells; Δ) or with anti-Lyt-2.2 plus anti-Lyt-3.2 + C (Lyt-1 cells; ▲) and subsequently adjusted to the appropriate cell numbers. Culture conditions, determination of CTL activity, and evaluation of data were as described in Fig. 1.

concentration, the number of positive wells decreases with increasing cell numbers, suggesting the existence of a suppressive mechanism which inhibits the frequent CTL-P but not the infrequent one. The frequencies observed are slightly but perhaps insignificantly greater than that observed by stimulator cell activation (Fig. 1). The enrichment expected for CTL-P cells in purified T cells is not seen in this type of experiment, because cell numbers per well refer to those recovered after 2 d of Con A activation, when presumably most of the recovered cells are T cells, even when spleen cells are initially used.

The data obtained by this method are essentially similar to those previously reported for antigen-specific helper cells (1). As for T helper cells, two types of CTL-P can be identified. The more frequent CTL-P occurred with a frequency of 1/100–1/300 in different experiments, whereas the frequency of the rare CTL-P was found between 1/2,000 and 1/8,000. At higher cell concentrations, the frequent CTL-P are suppressed, presumably by a suppressor T cell. Rare CTL-P appear to be insensitive to this suppressive mechanism. Even at very high cell concentrations we could not demonstrate inhibition of the rare CTL-P (data not shown). The latter finding also rules out the possibility that culture conditions such as crowding are responsible for suppression of CTL activity within the range of cell concentrations tested.

In further experiments, we have analyzed the effect of the time period for which the cells are kept in TCGF on the precursor frequencies. We found that frequent CTL-P tend to diminish, whereas rare CTL-P appear to increase with an increased period of incubation in TCGF (data not shown). This indicates that both cell types may have different growth kinetics in TCGF. Furthermore, it became clear that upon analyzing frequencies for both CTL-P at a single point in time, we may miss the

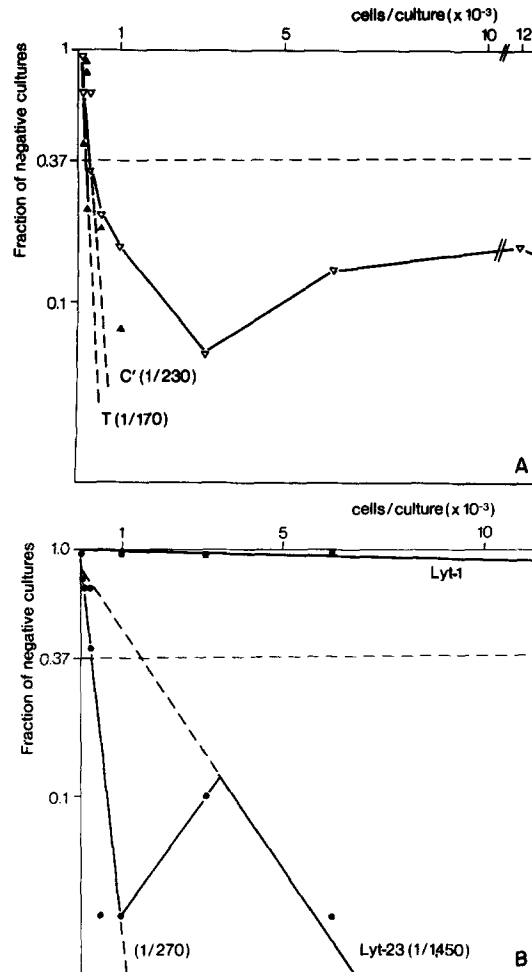


FIG. 4. Comparison of frequencies of CTL-P specific for H-2<sup>d</sup> in C57BL/6 splenic T cells and Lyt T cell subsets polyclonally activated by Con A. Before Con A stimulation in bulk cultures, splenic T cells were either left untreated (A; ▲) or were treated with C' alone (A; ∇), with anti-Lyt-1.2 + C' (Lyt-2,3 cells; B ●) or with anti-Lyt-2.2 plus anti-Lyt-3.2 + C' (Lyt-1 cells; B ■). After activation with Con A for 2 d, responder cells were expanded under limiting dilution conditions for 8 d on PEC in the presence of TCGF and subsequently tested on <sup>51</sup>Cr-labeled P815 tumor targets. Determination of CTL activity and evaluation of data were as described in Fig. 1.

optimal point for one or both precursors. For this and other reasons, our frequencies clearly represent minimal estimates.

*Lyt Phenotypes of Specific CTL-P Cells.* As for nonselected T cells, we analyzed CTL-P frequencies in T cells selected for their Lyt phenotypes, and compared the results obtained by the two different types of limiting dilution methods described above. B6 T cells were treated with anti-Lyt-1, and anti-Lyt-2 plus anti-Lyt-3, respectively, and selected rabbit complement, or with complement alone, and the residual cell populations were subjected to limiting dilution analysis using TCGF and stimulator cell activation (Fig. 3). Alternatively, the residual cell populations were activated for 2 d with Con A in bulk cultures and then placed in limiting dilution together with TCGF (Fig. 4A and B).

As shown by the experiments in Fig. 3, alloantigen-activated T cells reveal a single CTL-P whose frequency depends on the Lyt phenotype of the T cell population originally placed in limiting dilution. Lyt-1 T cells appear to be completely devoid of CTL-P, whereas Lyt-23 T cells appear to be slightly depleted of CTL-P when compared with T cells that were untreated or treated with complement alone. Because the population remaining after treatment with anti-Lyt-1 is <10% of the unselected splenic T cells, these data show that most CTL-P are eliminated by anti-Lyt-1.

The data obtained with polyclonal activation after anti-Lyt treatment of T cells are represented in Fig. 4A and B. In this particular experiment, we could not quantitatively determine the infrequent CTL-P in unselected complement-treated T cells, presumably because the generation of suppression was less efficient than in a typical experiment. However, the frequency of the rare CTL-P could not have been >1/5,000. Again, as in the previous experiment, Lyt-1 cells were largely depleted of CTL-P cells (Fig. 4B). In Lyt-23 cells, we find two populations whose frequencies are similar, as in unselected T cells. In contrast to unselected T cells, the rare precursor is now readily detectable with a frequency of 1/1,450, suggesting that it has been enriched by treatment with anti-Lyt-1 and complement (C'). We cannot make exact statements about the degree of enrichment because the frequency in the unselected population could not be measured. Similar results have been obtained with spleen cells instead of T cells (data not shown), but also in this experiment, no exact measurement on the enrichment of rare CTL in Lyt-23 cells was obtained.

Together with the results on alloantigen-activated T cells, these results suggest that most CTL-P cells are of Lyt-123 phenotype. These cells appear mostly within the frequent set of CTL-P. The minority of CTL-P are of Lyt-23 phenotype and appear to be preferentially within the infrequent set of CTL-P. It is clear, however, that there is no absolute association between Lyt phenotypes and frequent and rare CTL-P cells.

In further experiments, we analyzed the Lyt phenotypes of CTL-P cells after 2 d of Con A activation *in vitro*, by performing the treatment with anti-Lyt antisera and C' after the Con A activation step and before the limiting dilution in TCGF. At this point, treatment with anti-Lyt-1 and complement eliminates only 60–70% of the cells, in contrast to >90% of normal T cells, indicating a three- to fourfold increase in the Lyt-23 population. Interestingly, and in contrast to anti-Lyt-1 treatment before Con A activation, we find a two- to fourfold increase not only in the rare, but also in the frequent, CTL-P. This is shown by the experiments depicted in Fig. 5A for T cells and Fig. 5B for spleen cells, the frequencies of which have to be compared with those in Fig. 2A and B, respectively. In conjunction with previous work on the Lyt phenotypes of primary and secondary CTL-P cells (11), and in analogy to our previous observations on frequent and rare helper T cell precursor cells (1), these data suggest that upon activation to mature cytotoxic effector cells, Lyt-123 cells lose their Lyt-1 antigen and move from the frequent to the rare set of CTL-P. The loss of Lyt-1 appears to precede the appearance of the rare CTL-P type so that, shortly after activation, an Lyt-23 CTL-P of the frequent CTL phenotype is enriched. In our opinion, this transient phenotype is then replaced by the Lyt-23 positive, rare type of CTL-P, which represents a memory cell. Limiting dilution experiments using T cells activated for various lengths of time will be needed to clarify this point.

*Frequencies and Lyt Phenotypes of the Precursors for All T Cells with Cytotoxic Potential.* To determine the proportion of alloantigen-specific CTL-P within all CTL-P cells,

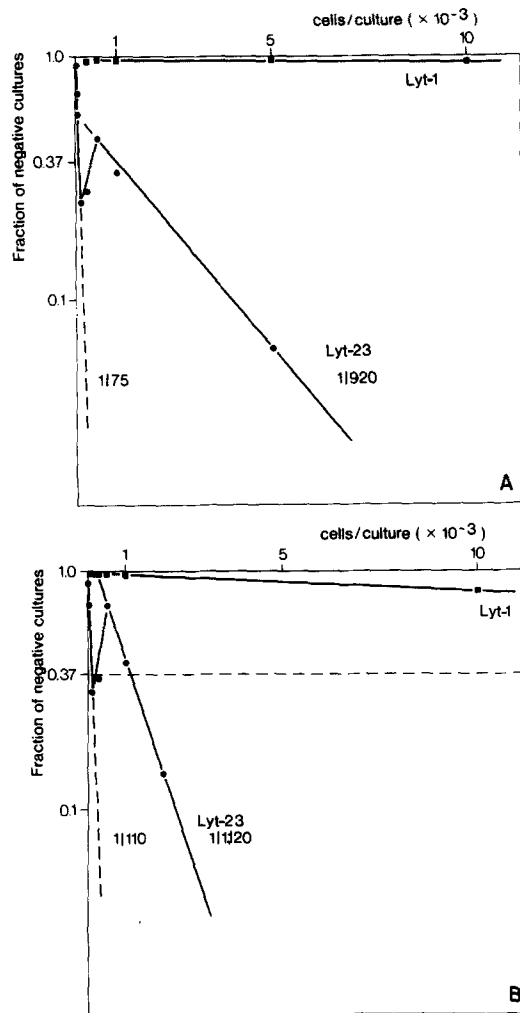


FIG. 5. Comparison of frequencies of CTL-P specific for H-2<sup>d</sup> in Lyt T cell subsets selected after Con A activation of C57BL/6 splenic T cells (A) and splenic cells (B). After 2 d of Con A stimulation in bulk cultures, C57BL/6 spleen cells or splenic T cells were selected for either Lyt-1 cells (■) or Lyt-2,3 cells (●) as described in Fig. 4. After adjustment to the appropriate cell numbers, each population was subsequently expanded under limiting dilution conditions for 8 d on PEC in the presence of TCGF. Determination of CTL-activity on <sup>51</sup>Cr-labeled P815 tumor targets and evaluation were as described in Fig. 1.

limiting dilution experiments were performed in which each well was assayed for cytolytic activity on target cells in the presence of Con A, thus presumably determining every cell with cytolytic potential independent of antigen specificity. For limiting dilution we used our protocol in which T cells activated for 2 d in Con A are assayed. The data in Fig. 6A and B give the results of a representative experiment whose frequencies have to be compared with those of T cells specific for one haplotype depicted in Fig. 4A and B. Again an ~100-fold depletion of CTL-P in Lyt-1 cells and a twofold depletion in Lyt-23 cells is shown (Fig. 6B). Also in these experiments we observed a zone of suppression that was only revealed when the actual amount of



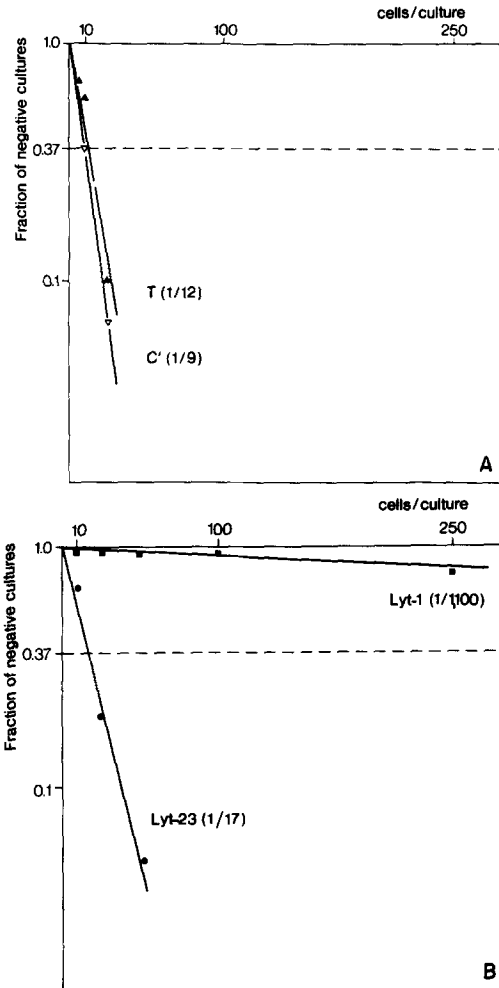


FIG. 6. Comparison of frequencies of non-antigen-specific CTL-P in C57BL/6 splenic T cells and Lyt T cell subsets. Before stimulation with Con A, C57BL/6 responder T cells were either left untreated (A; ▲) or were treated with C' alone (A; ▽), with anti-Lyt-1.2 + C' (Lyt-2,3 cells; B; ●) or with anti-Lyt-2.2 plus anti-Lyt-3.2 + C' (Lyt-1 cells; B; ■). Each subset was readjusted to the appropriate cell numbers and subsequently expanded under limiting dilution conditions for 8 d on PEC in the presence of TCGF. After the culture period aliquots of each well were tested for cytolytic activity on <sup>51</sup>Cr labeled P815 tumor targets in the presence of Con A. Determination of CTL activity and evaluation of data were as described in Fig. 1.

cytotoxic activity in each culture was considered (data not shown). Because suppression was incomplete, this assay did not allow the definition of a second or rare CTL-P. The data, when compared with Fig. 4 A and B, show that the proportion of H-2 alloantigen-specific CTL within all CTL-P cells is ~5%, which agrees with measurements in other limiting dilution systems (3, 5). Moreover, as in the analysis of specific CTL-P, Lyt-123 is the predominant phenotype of precursors for all T cells with cytotoxic potential.

### Discussion

In this paper, we compare the results of precursor frequency analyses of cytotoxic T cells obtained in two different limiting dilution systems. The first system uses normal T cells or spleen cells that are activated in limiting dilution with allogeneic stimulator cells in the presence of TCGF. This system is therefore limited to the determination of the precursor frequencies of T cells reactive to H-2 or other cell surface determinants. In the second system, T cells activated polyclonally with Con A in bulk culture are subjected to limiting dilution in the presence of TCGF but without any specific activation step. This second system is therefore not restricted to T cells specific for cell surface determinants. Rather, it can be applied to the determination of the precursor frequencies for T cells with any antigenic specificity whose function can be measured by an *in vitro* assay.

It is advantageous to use such a widely applicable assay in studies aimed at comparing the frequencies of various different types of precursor T cells, such as, for example, a comparison of the frequencies of alloantigen-specific T cells with T cells reactive to conventional antigens, or a comparison of the frequencies of helper, suppressor, or killer T cells specific for any antigen.

In contrast to these advantages, interpretations of results obtained with polyclonally activated T cells suffer from the uncertainty about the degree of selectivity of polyclonal activation. Part of the purpose of this study was therefore a comparison with an assay in which normal spleen cells are placed in limiting dilution without any previous selection step. The frequencies of alloreactive CTL-P can be most readily determined in this way and, therefore, the present comparison was performed.

We find that nylon wool fractionation of T cells does not significantly alter the CTL-P frequencies when spleen cells and T cells are compared in both types of assay. Most important, we determined similar precursor frequencies for CTL in both types of assay, which suggests that polyclonal activation with Con A selects neither for nor against cytotoxic T precursor cells. In a previous report, we made the qualitative observation that Con A-activated T cell blasts contain antigen-specific helper and suppressor cells as well as cytotoxic T cells (1). Now we show quantitatively that Con A-activated T cell blasts contain CTL-P cells in frequencies similar to those of normal T cells.

On the basis of our comparison of the two different limiting dilution methods, we cannot draw conclusions about a possible selectivity of the expansion of precursor cells by TCGF. We have, however, shown previously and confirmed here that the frequencies of Con A blasts growing in TCGF approaches 1/2 in many experiments, although sometimes lower frequencies are observed (1). In this paper, we consider results from only those experiments in which the frequency of growing cells was  $>1/5$ . These growth frequencies, determined at cell concentrations between 1 and 20 per well, reflect low estimates of the frequencies of growing cells, at high cell concentrations of which the functional assays are performed. Thus, we think that precursor frequencies determined in these assays deviate from those in the normal T cell pool by perhaps no more than a factor of three.

As we could previously show for T helper cells (1), Con A-activated T cells also contain two different types of CTL-P cells that can be distinguished because the more frequent one is suppressed at higher cell numbers. In some exceptional experiments using alloantigen-activated T cells, indications for two types of CTL-P could also be

obtained, but in most typical experiments using C57BL/6 lymphocytes responding to H-2D alloantigens (DBA), only a single CTL was found whose frequency was similar to the more frequent one found in Con A blasts. The difference between these two results can be explained in several ways. The most plausible explanation is that in the C57BL/6 anti-DBA combination, stimulator cell activation does not favor the sensitization of suppressor cells, so that the infrequent CTL-P cannot be demonstrated. Con A stimulation, in contrast, may sensitize regulatory and effector cells more or less equally, or may activate suppressor T cells preferentially. Regulatory interactions between the suppressors and CTL-P during the limiting dilution period may then lead to inhibition of the frequent CTL-P type above a certain cell concentration. Therefore, the rare CTL-P, which is insensitive to this suppression can become apparent (see below).

Other explanations for the detection of two CTL-P in Con A-activated T cells are possible, but have to invoke the argument that more than one cell type is limiting for CTL formation at the cell concentrations that give rise to the linear areas of our frequency distribution lines. Because we find both linear areas in agreement with the zero order term of the Poisson equation, and because the *Y*-axis intercepts determined by the least-square method were always close to 1.0, with coefficients of determination exceeding 0.95, we are convinced that these linear portions represent measurements of single cell types.

The frequent and rare CTL-P are characterized by their sensitivity and apparent insensitivity, respectively, to suppressor T cells in the analyzed T cell population. These suppressor cells appear to be of lower frequency than the frequent CTL-P. The rare CTL-P is apparently insensitive to this suppressor cell but may be sensitive to another suppressor cell whose frequency is too low for detection in our analyzed cell numbers. In recent work from others (16-23) and from this laboratory (24), it has been shown that in the course of an *in vitro* primary anti-H-2 cytotoxic response, T suppressor cells are generated that are able to prevent the development of CTL from precursors in fresh primary mixed lymphocyte reactions (MLR) but not in the ongoing MLR in their own cultures. In contrast, the present data show a suppression of CTL-P by concomitantly sensitized suppressor cells in polyclonally activated cultures. Are the suppressive mechanisms in both systems different or the same? The similarity of the frequencies of the suppressor-sensitive CTL-P population after Con A activation to that of CTL-P in antigen-activated cultures suggests that both CTL-P are the same, and that both represent targets for suppression. We know very little about the characteristics of the suppressor T cell detected in Con A-activated T cells and, therefore, it remains to be determined whether the T cell is identical to that demonstrated in bulk culture. We should like to stress, however, that suppression detected in both assays may indeed be mediated by one type of suppressor cell. The failure to demonstrate this suppressor cell in limiting dilution experiments using alloantigen-activation strongly suggests that Con A stimulation results in an early stimulation, whereas alloantigen stimulation results in a late sensitization of suppressor cells. A comparison of parameters such as antigen specificity or Lyt phenotype may provide an answer to the question of the relationships between the suppressive mechanisms observed in bulk culture and those seen in limiting dilution.

What is the nature and function of two CTL-P cells? In our previous work on T helper cell ( $T_H$ ) frequencies, we suggested that frequent  $T_H$  and rare  $T_H$  represent

primary and secondary T helper cells, respectively (1). This suggestion has since been supported by frequency determination of  $T_H$  precursors in T cells immunized *in vitro*, which leads to a diminution of frequent  $T_H$  and an increase of rare  $T_H$  (K. Eichmann, unpublished observations). Furthermore, the Lyt-123 phenotype of frequent  $T_H$  and the Lyt-1 phenotype of rare  $T_H$  point in that direction.

Are our data on frequent and rare CTL-P compatible with their roles as primary and secondary precursor cells? In previous work from this laboratory, it was shown that in primary bulk culture MLR, CTL derive only from the Lyt-123 cells but not from the Lyt-23 cells, which are suppressed. In secondary bulk culture MLR, CTL derive mainly from the Lyt-23 cells but not from Lyt-123 cells (11).

These previous results are in agreement with our present findings that most CTL-P determined in limiting dilution assays that use unprimed T cells are Lyt-123 cells. However, we find that unprimed Lyt-23 cells contain both types, frequent and rare, of CTL-P. This is not in agreement with our previous findings if we assume that the loss of the Lyt-1 antigen and the acquisition of the differentiation state of the rare precursor cell type are simultaneous events in the transition of primary to secondary CTL-P cells. We could show, however, that after 2 d of Con A activation, both frequent and rare CTL-P are equally enriched in the Lyt-23 population, which suggests that loss of Lyt-1 is an early event in the CTL maturation pathway. We therefore think that the frequent CTL-P with Lyt-23 phenotype is a transient stage after activation, which is supported by its lack of enrichment in Lyt-23 cells prepared from nonactivated T cells. Furthermore, we think that the acquisition of the rare precursor phenotype is a late event in CTL maturation, but this remains to be determined experimentally. Taken together, with respect to the Lyt phenotype of CTL-P, the data obtained in limiting dilution are in agreement with those reported by other groups (25; J. Langhorne, personal communication), and with those obtained previously in bulk cultures (11).

With respect to the absolute frequencies of specific CTL-P, total CTL-P, and the proportion of specific precursors within the total pool, the data obtained with our two limiting dilution systems agree with one another and with those reported in the literature (4-6, 8, 25). In addition, we can now compare for the first time the frequencies of alloreactive T cells with those of T cells reactive to a conventional antigen, determined by the same assay. Streptococcus A-specific  $T_H$  were found at frequencies of 1/1,000-1/6,000 for the frequent and 1/10,000-1/70,000 for the rare precursor type. Alloantigen-specific CTL were found at frequencies of 1/100-1/600 for the frequent and 1/2,000-1/8,000 for the rare precursor type. We hope that this type of comparison, when more data are available on T cells with the same rather than with different effector function, will allow a deeper insight in the diversity of the T cell receptor repertoire.

### Summary

Two different limiting dilution systems have been applied to compare precursor frequencies of alloreactive cytotoxic T cells (CTL-P) in polyclonally and specifically activated lymphocyte populations and in selected Lyt T cell subsets. Both systems make use of T cell growth factor for T cell expansion but differ with respect to the activation step in that lymphocytes are either activated directly with allogeneic

stimulator cells or are sensitized polyclonally with concanavalin A (Con A) in bulk culture before their expansion under limiting dilution conditions.

In polyclonally activated C57BL/6 lymphocyte populations, two types of CTL-P specific for H-2<sup>d</sup> alloantigens could be identified: a frequent set with a frequency of 1/100–1/300, and a rare set with a frequency of 1/2,000–1/8,000. In contrast, only a single CTL-P set was found in specifically activated populations with a frequency similar to that of the frequent CTL-P found in Con A blasts. In Con A blasts, the frequent CTL-P, which only became apparent at low cell concentrations, were inhibited at higher cell concentrations by suppressor T cells, whereas rare CTL-P were insensitive to this suppressive mechanism. Whereas in specifically activated T cells, the predominant CTL-P phenotype was Lyt-123, the predominant Lyt phenotypes for the frequent and the rare CTL-P found in Con A blasts were Lyt-123 and Lyt-23, respectively, which suggests that they represent primary and secondary CTL-P, respectively. The results are discussed with respect to previous reports on the involvement of Lyt T cell subsets in the generation of cytotoxic responses and their regulation by T suppressor cells.

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