

QUANTITATIVE STUDIES ON T CELL DIVERSITY

I. Determination of the Precursor Frequencies for Two Types of Streptococcus A-specific Helper Cells in Nonimmune, Polyclonally Activated Splenic T Cells

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The elucidation of the molecular construction of the antigen receptors of T cells is a fundamental prerequisite for our understanding of the function of the immune system. Short of sufficient material for the analysis of structure, most of the present information on T cell receptors stems from serological, genetic, and functional studies (1-3). Such studies from a number of laboratories have suggested that the immunoglobulin heavy chain variable region participates in antigen recognition by T cells, but other structural elements, although clearly inferred in some experiments, have not been unequivocally identified (3-6).

One critical property that characterizes an immunological receptor system is the degree of diversity of its combining sites. For antibodies, a variety of different approaches to measure diversity has provided fairly clear estimates (7-9). For T cell receptors—because of the absence of well-characterized, soluble antigen-binding molecules—the only feasible approach is the precursor frequency analysis of T cells reactive to a given antigen in limiting dilution (10-12).

The analysis of the combining-site diversity of T cells is complicated by the existence of functionally and physically distinct T cell subsets that contain the precursors for the regulatory and effector T cells (13). For the understanding of the relative role of a functional T cell subset in an immune response, it is important to determine its quantitative representation within the population of lymphocytes reactive to the antigen to which the immune response occurs.

We have therefore designed a method to determine the frequencies of antigen-specific precursor T lymphocytes for various T cell regulator and effector functions. The method is based on the recent observation by a number of laboratories that specifically or polyclonally activated T cell blasts can be induced to grow in vitro in media that contain a factor (T cell growth factor [TCGF])¹ released from spleen cells stimulated with concanavalin A (Con A) (14-16). This opens the possibility to perform limiting-dilution experiments in which large numbers of progeny from limiting numbers of T cells of nonimmunized animals are tested for antigen-specific effector

¹ *Abbreviations used in this paper:* A-CHO, group A streptococcal carbohydrate; Con A, concanavalin A; CTL, cytotoxic T lymphocyte(s); FCS, fetal calf serum; IMDM, Iscove's modified Dulbecco's medium; 2-ME, 2-mercaptoethanol; PFC, plaque-forming cell(s); SRBC, sheep erythrocyte(s); Strep A, group A streptococci; TCGF, T cell growth factor; T_H, helper T; TNP, trinitrophenyl.

functions. Without expansion of T cells before their activation by antigen, limiting-dilution experiments with T cells were successfully done only with antigen-primed populations (12) or for reactivity to alloantigens (10, 11).

In the present paper we introduce the method and describe experiments that identify two different sets of antigen-specific helper cell precursors in nonimmune, splenic T cells. One set is relatively frequent (from 1/1,000 to 1/6,000 T cells), is sensitive to suppression by other T cells, and has the phenotype Lyt-123. The other set is relatively rare (from 1/10,000 to 1/70,000 T cells), is insensitive to suppression by other T cells, and its phenotype is Lyt-1. The results are discussed in relation to other experimental systems that show involvement of two sets of helper cells in antibody production (17-21).

Materials and Methods

Mice. Mice of strains A/J and A.SW were produced in our own animal breeding unit. Some A/J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. All mice were vaccinated with ectromelia vaccine at the age of 4 wk and were used, if not otherwise stated, for experiments at 6-8 wk. Sprague-Dawley rats were obtained from our own animal breeding unit.

Antigens. Group A streptococci (Strep A), strain J17A4, were prepared as described (22) and conjugated with trinitrophenyl (TNP) according to the method of Rittenberg and Pratt (23). Sheep erythrocytes (SRBC) were obtained from our own animal facility.

Preparation of TCGF from Rat Spleen Cells. The spleens of 10-40 Sprague-Dawley rats were removed and single-cell suspensions were cultured for 24 h at 5×10^6 /ml in 70-ml portions of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), 10^{-5} M 2-mercaptoethanol (2-ME), 1% penicillin-streptomycin, 0.025 M Hepes, and 5 μ g/ml Con A in a 5% CO₂ atmosphere. The supernate that contained TCGF was collected by centrifugation, aliquoted, and stored at -70°C . The efficiency of each TCGF preparation was determined by its support of the proliferation of various in vitro T cell lines available in our laboratory (P. Krammer, Unpublished results.). Samples of 1×10^4 of such T cells were cultured overnight in 200 μ l of various dilutions of TCGF in IMDM in round-bottom Linbro microtiter plates (Linbro Chemical Co., Hamden, Conn.), labeled with [³H]thymidine for 4-6 h, and ³H incorporation was then determined. Optimal support of T cell growth for most batches of TCGF was at dilutions from 1:20 to 1:5. Most experiments described in this paper were done with a single TCGF preparation, which gave optimal results at a dilution of 1:20.

Limiting-Dilution Analysis. T cells were prepared by double nylon-wool passage of spleen cells, as previously described (24). T cells were then cultured at a density of 3×10^5 /ml for 2 d with 5 μ g/ml Con A in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% FCS, 10^{-5} M 2-ME, 0.025 M Hepes, 1% penicillin-streptomycin, and 0.2 mM glutamine. Thereafter, Con A-activated T cells were purified by centrifugation over Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) as previously described (24). Portions of such T cells were then placed in Linbro flat-bottom microtiter wells (7600305; Linbro Chemical Co.) that had received 1×10^4 3,000-rad-irradiated (⁶⁰Co) syngeneic peritoneal exudate cells/well as filters, 2 d before. The portions of T cells ranged from 2/well to 8×10^4 /well and were cultured in 200 μ l of appropriately diluted TCGF in IMDM with 10% FCS, supplemented as described above. The medium was changed every 3 d by centrifugation of the plates followed by the removal and replacement of the medium with Titertek pipettes (Flow Laboratories, Inc., Rockville, Md.). Routinely, 24 wells were set up for each cell concentration.

The frequency of cultures with growing cells was determined after 1 wk of culture by visual examination with an inverted microscope. Filler cells were largely present as fragments at that time and, therefore, did not interfere with the assessment of cell growth. Helper activity in each culture was determined on day 7 or 8 by adding a fraction of the cells of each culture after transfer into Click's medium to a fresh Linbro flat-bottom microwell that contained antigen and 5×10^5 splenic B cells, purified by treatment with anti-Thy-1.2 and rabbit complement.

Cultures can be split in up to four fractions to assess helper activity for different B cells or for different antigens.

We use a conventional (AKR \bar{a} C3H) anti-Thy-1.2 antiserum, which we find superior to the commercially available monoclonal anti-Thy-1.2 antisera. As antigens, we used either 3×10^5 SRBC/well or 1×10^7 TNP-Strep A/well. Click's medium was used in these cultures in a 2% CO₂ atmosphere, as previously described (25). After 4 d, responses were assessed by assay for direct plaque-forming cells (PFC) for TNP or SRBC, as previously described (25). Positive PFC responses were determined by comparison with PFC responses of cultures that contained only B cells and antigen (Results). Further processing of data was done by Poisson's distribution (26).

Establishment of Short-Term T Cell Lines. 10^3 - 10^4 purified splenic T cells were placed into Linbro microtiter wells in 200 μ l of RPMI, 10% FCS and 5 μ g/ml Con A, supplemented as described above. After 2 d, the medium was exchanged against appropriately diluted TCGF. Numbers of viable cells in each well were assessed by trypan blue exclusion, counting every 2-3 d. When cell densities reached 5×10^5 /ml, cultures were transferred to Linbro semimicrowells in 2 ml of TCGF and, thereafter, to tissue culture flasks in 10 ml of TCGF. At various times during the culture period, the cells were tested for helper function by using 1×10^5 cultured T cells together with 5×10^5 B cells and antigen as described above. Cells were also tested for suppressor function by adding 1×10^5 cultured T cells to 9×10^5 spleen cells from mice previously primed with Strep A (25), cultured together with TNP-Strep A as described above. Responses of such cultures were assessed by assaying for direct PFC to TNP, as previously described (25).

Treatment of T Cells with Anti-Lyt Antisera. Con A-activated T cells were treated with anti-Lyt-1 and anti-Lyt-23 antisera and selected rabbit complement as previously described (27).

Results

In Vitro Expansion of Con A-activated T Cells. The aim of precursor frequency analysis is to determine that number of cells that contains a single precursor cell. This is done by disseminating graded numbers of cells that are subsequently activated and tested for the function that is attributed to the effector cell whose precursor is to be determined. Therefore, the assay to test for the function must either be sufficiently sensitive to detect a single precursor cell, or, alternatively, the disseminated cells must be sufficiently expanded before the test to allow detection of the progeny of a single precursor cell.

Because most T cell assays do not detect a single cell, we decided to use TCGF to expand small numbers of disseminated T cells. As shown by others, TCGF acts only on activated T cells (28). Con A is perhaps the least selective of all known T cell activators, and, therefore, we analyzed the growth characteristics of Con A-activated T cells cultured in TCGF. Fig. 1 shows the results of cultures initially containing 1,000 cells, and reveals an $\sim 10^4$ -fold increase in cell numbers over a 2-wk period. Thereafter, cell lines behave unpredictably and, for the purpose of the present study, are not further analyzed. The growth observed during the initial 2-wk period suggests a rate of division slightly $>1/d$. We conclude from these experiments that at day 7 or 8 after the initiation of culture, we obtain between 500 and 1,000 progeny of each dividing cell. This number is similar to that observed in limiting-dilution assays for alloreactive T cells (11), whereas in limiting-dilution assays for helper T cells to conventional antigens, clone sizes of 8-16 were estimated (12). Thus, although the conditions of our limiting-dilution assay (see below) are not entirely the same as those used to analyze the growth of T cells in TCGF, the observed clone sizes suggest that the sensitivity of our assay is not significantly below that of assays for alloreactive T cells.

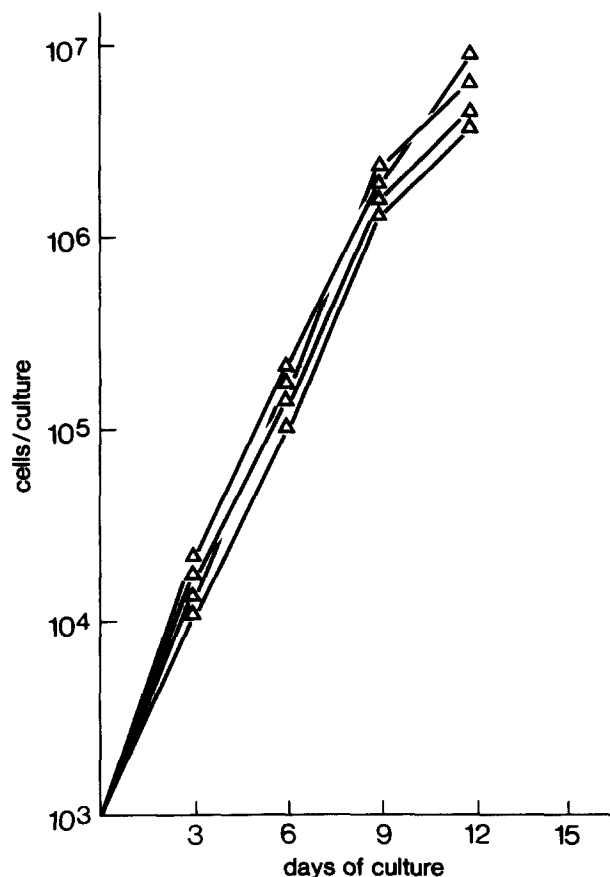


FIG. 1. Growth of cells in four different cultures that were started with 10^3 splenic T cells from strain A/J and 5 $\mu\text{g}/\text{ml}$ Con A in 100 μl of medium on day 2. On day 0, the medium was removed and replaced by 100 μl of appropriately diluted TCGF. On day 5, the cells were transferred to semimicrowells that contained 2 ml, and on day 9 to tissue culture flasks that contained 10 ml. The cells were counted on the indicated days.

Do Con A-activated T Cells Represent the Normal T Cell Pool? When Con A-activated T cells are used as the starting population for precursor frequency analysis, conclusions as to the quantitative representation of precursors in normal T cells can be drawn only if Con A activation were completely random and nonselective. Complete lack of selectivity cannot be conclusively established in a quantitative fashion, but there is good qualitative evidence that Con A activates Lyt-1 and Lyt-23 cells, as well as nonfractionated T cells, to perform helper, suppressor, and cytotoxic effector functions, respectively (29).

As an extension of these studies to T cells after TCGF-induced proliferation, we have analyzed various effector T functions in short-term T cell lines obtained by growing aliquots of 10^4 splenic T cells after 2 d of Con A activation for 10 d in TCGF. As will be shown below, 10^4 T cells are within the range of T cell numbers at which Strep A-specific helper cells show clonal distribution. Helper function and suppressor function in these lines were assayed in cultures of B cells or spleen cells, respectively,

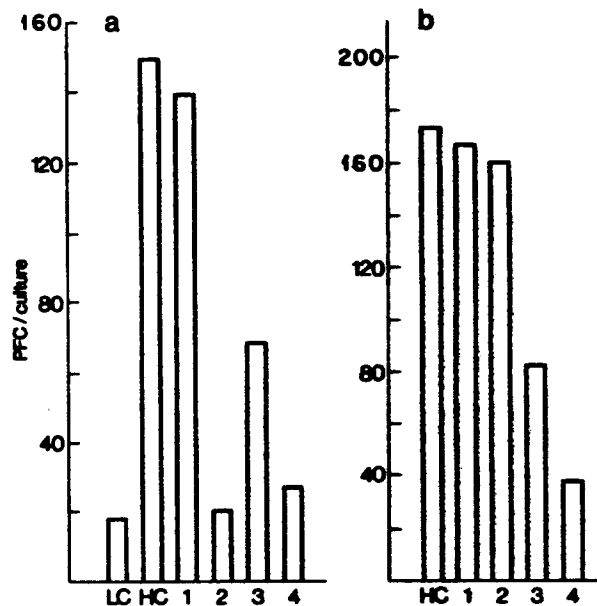


FIG. 2. Analyses of helper activity (a) and of suppressor activity (b) of four different T cell lines initially started with 10^4 splenic T cells of strain A/J that were kept in $5 \mu\text{g/ml}$ Con A for 2 d and thereafter grown for 10 d in TCGF. For helper activity (a), 1×10^5 T cells were added to 5×10^5 B cells and 1×10^7 TNP-Strep A particles, and TNP-PFC were determined on day 4. LC, TNP-PFC of B cells alone. HC, TNP-PFC of B cells supplemented with 5×10^5 splenic T cells from mice immunized with 2×10^9 Strep A particles 3 wk before. For suppressor activity (b) 1×10^5 T cells were added to 1×10^7 TNP-Strep A and 9×10^5 spleen cells of mice previously immunized with Strep A. TNP-PFC responses were determined on day 4. HC, TNP-PFC response of 9×10^5 spleen cells primed with Strep A. The results are arithmetic means of six identical cultures from which the mean PFC response of two cultures without antigen was subtracted.

responding to TNP-Strep A. Out of 14 such lines tested, 2 had exclusive helper activity, 4 had exclusive suppressor activity, 5 had activity in both types of assay, and 3 had no detectable activity. Examples for each of the four types are given in Fig. 2. We have also, as have others (30–32), found cytotoxic activity in such T cell lines (J. Goronzy, K. Eichmann, and M. M. Simon. Manuscript in preparation.).

Thus, although we have no information on the precise quantitative representation of normal T cell subsets in our *in vitro* expanded T cell populations, we have no evidence to conclude that our expansion method selects strongly against or for any of the known functional T cell populations. However, when precursor frequencies are determined in these expanded populations, they apply, strictly speaking, to T cells that are sensitive to Con A activation and growth in TCGF.

Frequency of Con A-activated T Cells That Grow in TCGF. The proportion of Con A-activated T cells that are sensitive to the growth-inducing capacity of TCGF is an important factor for the correction of the precursor frequencies determined with TCGF-expanded populations. This proportion was determined in cultures initially seeded with small numbers of Con A-activated and Ficoll-purified T cells. After 1 wk of culture, wells with growing cells were identified by microscopic inspection. As shown in Fig. 3, the results are compatible with straight lines that cross 1.0 on the ordinate in the Poisson plot, thus suggesting that induction of growth in TCGF is a

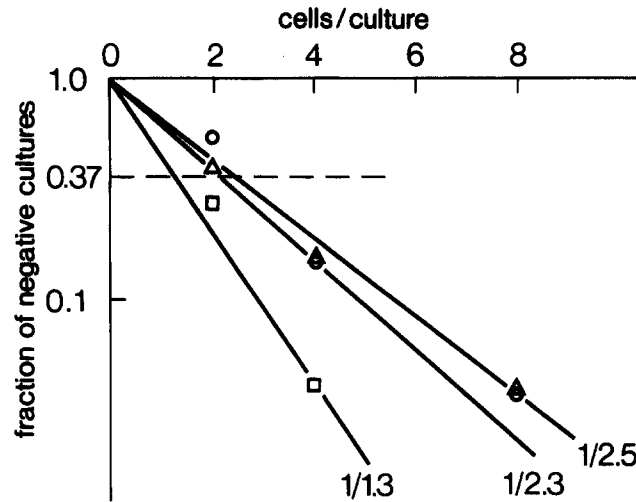


FIG. 3. Precursor frequency analysis of Con A-activated T cells that grow in TCGF. For each cell concentration given on the abscissa, 24 wells were set up. Wells with growing cells were identified by microscopic inspection after 1 wk of culture. Lines were drawn manually through 1.0 on the ordinate to accommodate the maximal number of experimental points. Frequencies were read from the intercept with a line crossing 37% negative cultures on the ordinate. (□) strain A.S.W., (○) strain A/J (experiment 1), (△) strain A/J (experiment 2).

single-cell phenomenon. The frequencies for growing cells in the three experiments range from 1/1.3 to 1/2.5. Thus, we conclude that one out of approximately two Con A-activated T cells is sensitive to the growth-inducing capacity of TCGF. In some experiments, the frequencies of growing cells were considerably lower (1/10–1/100), particularly when Con A-activated spleen cells were used and when Con A activation was done for 3 instead of 2 d. Whereas results from such experiments can be used after proper correction, the present paper contains only uncorrected results from experiments in which frequencies of growing cells were close to 1/2. The frequencies determined below for helper T cell precursors represent, therefore, approximately one-half of the real frequencies.

Frequencies of Helper T Cell Precursors. Fig. 4 shows a histogram of the direct PFC responses obtained when Con A-activated T cells from strain A/J are disseminated in limiting dilution, expanded in TCGF, and, thereafter, tested for helper activity toward B cells responding to TNP-Strep A. With increasing numbers of T cells per well, the number of cultures with positive TNP-PFC responses increases. The difference between responding and nonresponding cultures is readily apparent from the comparison with the PFC responses of purified B cells to TNP-Strep A. In most of our experiments, we find the borderline that separates positive and negative cultures to be 2–3 SD above the arithmetic mean of the PFC responses of cultures without T cells. Accordingly, we routinely use the arithmetic mean + 3 SD as a borderline for distinguishing positive and negative cultures.

The analysis of data such as those in Fig. 4 by Poisson's distribution yields the results shown in Fig. 5. The values have a diphasic distribution such that two linear lines, both crossing 1.0 on the ordinate, can be drawn through two sets of values. One of the lines, which connects values obtained with 2×10^4 to 8×10^4 cells, suggests a

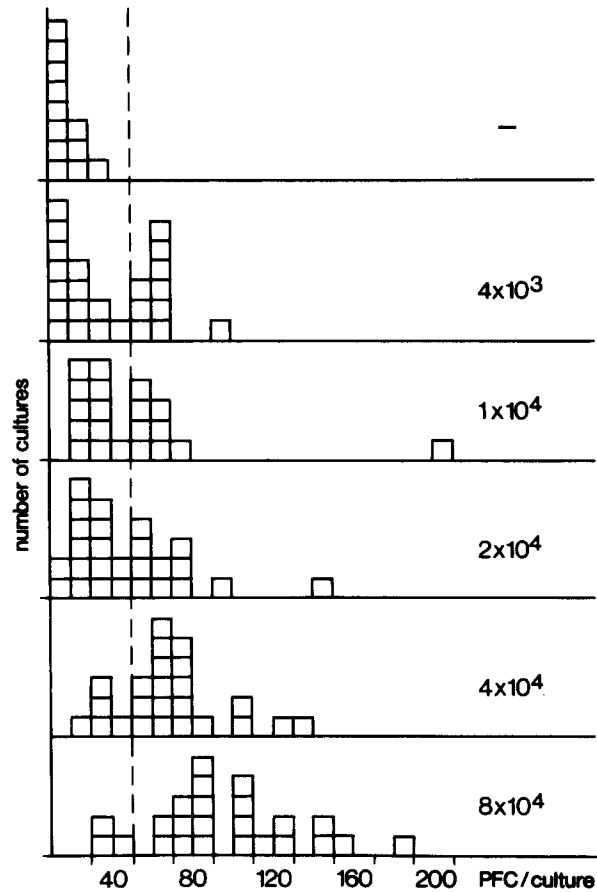


FIG. 4. Histogram of the PFC responses to TNP of cultures containing 5×10^5 B cells and 1×10^7 TNP-Strep A particles to which either no T cells were added (upper panel) or to which one-half of the T cells were added that were recovered from individual cultures initially started with the indicated numbers of Con A-activated T cells and subsequently grown for 1 wk in TCGF. Each box represents the result on one culture. All cells were from strain A/J. The vertical dashed line represents the arithmetic mean + 3 SD of the responses of B cells to TNP-Strep A alone (upper panel).

helper cell frequency of 1/15,000 for A.SW and 1/30,000 for A/J. The other line, which connects values obtained with 1×10^3 to 4×10^3 cells, suggests a helper cell frequency of 1/1,500 for A.SW and 1/3,000 for A/J. In a greater number of experiments—with A/J, A.SW, and (A/J \times A.SW) F_1 mice—we have found the frequencies for the frequent helpers between 1/1,000 and 1/6,000, mostly close to 1/3,000. The frequencies of rare helpers were found between 1/10,000 and 1/70,000, mostly close to 1/30,000. At the intermediate cell concentrations, the proportion of positive cultures decreases with increasing cell numbers, thus suggesting the influence of a suppressing cell population. Thus, we interpret these data to suggest the existence of two different sets of helper cells. One set is relatively frequent, but is sensitive to suppression by other T cells. The other set is less frequent, and insensitive to suppression by other T cells within the cell concentrations tested.

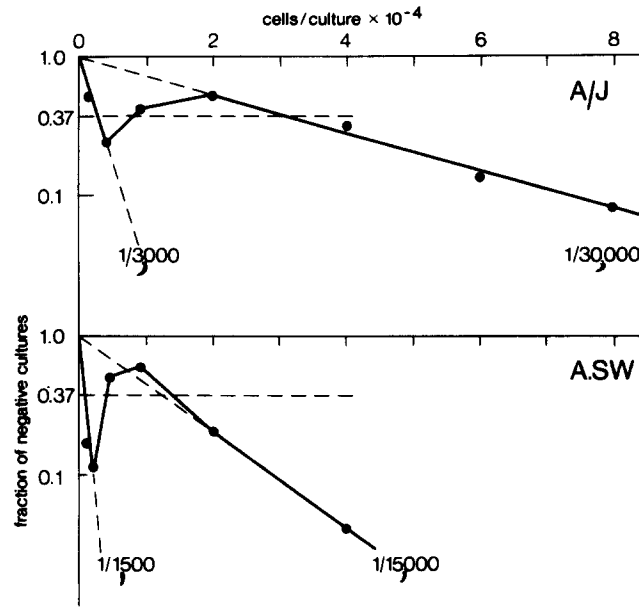


FIG. 5. Precursor frequency analyses of T_H cells for B cells responding to TNP-Strep A. 24 wells were set up with the numbers of Con A-activated T cells indicated on the abscissa. After 1 wk of growth in TCGF, one-half of the T cells of each culture were tested for T_H activity in a new culture of 5×10^6 B cells and 1×10^7 TNP-Strep A particles. Responding cultures were identified by comparing the TNP-PFC response on day 4 with that of 5×10^6 B cells to TNP-Strep A. Cultures with a PFC response greater than the arithmetic mean +3 SD of the B cell control were taken as positive. Lines were drawn manually through 1.0 on the ordinate to accommodate a maximal number of experimental points. Precursor frequencies were read as in Fig. 3.

TABLE I
Independent Assortment of Helper T Cells for Responses to SRBC and to TNP-Strep A

T cells/ well	Threshold*	Number of re- sponses to SRBC	p‡	Number of re- sponses to TNP- Strep A	q‡	p × q‡	Number of re- sponses to SRBC and TNP-Strep A	
							Ex- pected‡	Ob- served
1×10^3 §	Control + 1 SD	6/24	0.25	14/24	0.58	0.15	3.6	5
1×10^3 §	Control + 2 SD	5/24	0.21	10/24	0.42	0.09	2.2	2
1×10^3 §	Control + 3 SD	5/24	0.21	10/24	0.42	0.09	2.2	2
1×10^3 §	Control + 4 SD	5/24	0.21	6/24	0.25	0.05	1.2	1
2×10^4 §	Control + 1 SD	10/24	0.42	18/24	0.75	0.32	7.7	7
2×10^4 §	Control + 2 SD	8/24	0.33	16/24	0.67	0.22	5.3	5
2×10^4 §	Control + 3 SD	8/24	0.33	16/24	0.67	0.22	5.3	5
2×10^4 §	Control + 4 SD	5/24	0.21	14/24	0.63	0.13	3.1	4

* Because of our limited experience with responses to SRBC, data were analyzed with a series of thresholds to distinguish positive and negative responses.

‡ Fraction of responding cultures; the fraction of double responders is the product of the fractions of single responders, if cells distribute independently of one another.

§ At 1×10^3 T cells/well, only the frequent T_H is active, whereas at 2×10^4 T cells/well, the frequent T_H is suppressed and the rare T_H is active.

Specificity of Helper T Cells. The antigen specificity of the helper cells detected in this assay was investigated by testing the same cultures of A/J T cells in limiting dilution for help in responses to TNP-Strep A and to SRBC. Helper cells to SRBC appear to have a lesser frequency (1/70,000–1/140,000) than those to TNP-Strep A, and no clear indication for two sets of helpers to SRBC could be obtained (data not shown). Our data on SRBC-specific helpers are limited and do not, as yet, allow general conclusions. The expected degree of coincidence of help for SRBC and for TNP-Strep A was calculated from the frequencies of cultures positive for help with each of the two antigens (Table I). The comparison with the observed coincidence at cell concentrations at which only the rare helper (2×10^4 cells/well) and only the frequent helper (1×10^3 cells/well) are clonally distributed reveals the completely independent assortment of both specificities at both cell concentrations (Table I). This suggests that both sets of helper cells are antigen specific or idio type specific. As a remote possibility, both types of helper cells may be nonspecific, but their helper effect may be suppressed by different antigen-specific suppressor cells in responses to different antigens. This latter possibility, although not formally excluded, is unlikely because of the single-hit results obtained for each of the two types of helper cells.

Lyt Phenotypes of Helper T Cells. Previous work has shown that functional helper T cells possess the Lyt-1 phenotype (13, 29). For precursor cell analysis, we decided to select Lyt-1 and Lyt-23 cells at the stage after Con A activation, to exclude distortions of the repertoire that may have been caused by separate Con A activation of the selected T cell subsets.

The results on limiting-dilution analysis of helper cells in unselected T cells, Lyt-1, and Lyt-23 T cells of strains A/J and A.SW are shown in Fig. 6. Lyt-23 cells of both strains appear to be largely depleted of helper cell precursors. Unselected T cells of strain A/J, in this particular experiment, contained frequent helpers at 1/3,000 and rare helpers at 1/70,000, whereas unselected T cells of strain A.SW contained frequent helpers at 1/1,000 and rare helpers at 1/11,000. Lyt-1 cells appear to be enriched with respect to the precursors of rare helpers by a factor of 2–4, which is close to the enrichment expected from the proportion of 30% Lyt-1 T cells in the normal population. Precursors of the frequent helper set, however, do not appear to be contained in Lyt-1 T cells, which suggests that its precursor phenotype is Lyt-123.

Frequency of Helper Cells in Old Mice. Strep A are ubiquitous antigens, and, therefore, it is difficult to prove that our helper cell frequencies indeed reflect a nonimmune state. In previous work we have shown that spleen cells of young mice in a clean colony produce marginal PFC responses to TNP-Strep A in vitro, whereas spleen cells of old mice and mice primed with Strep A produce high PFC responses (1, 4, 20, 25) (K. Eichmann. Unpublished data.). Fig. 7 shows a precursor frequency determination of helper cells to TNP-Strep A in retired breeders older than 8 mo. Only one type of helper can be identified with certainty at a frequency of 1/3,500. It is not clear whether it corresponds to the frequent or to the rare helper of young mice. Although these data do not prove the nonimmune state of our young mice, the results indicate that natural immunization by environmental streptococci modifies the quantitative and qualitative representation of helper cells. Thus, helper cell representation, as revealed by limiting dilution of T cells from young mice, may be reflective of little or no previous exposure to environmental streptococci.

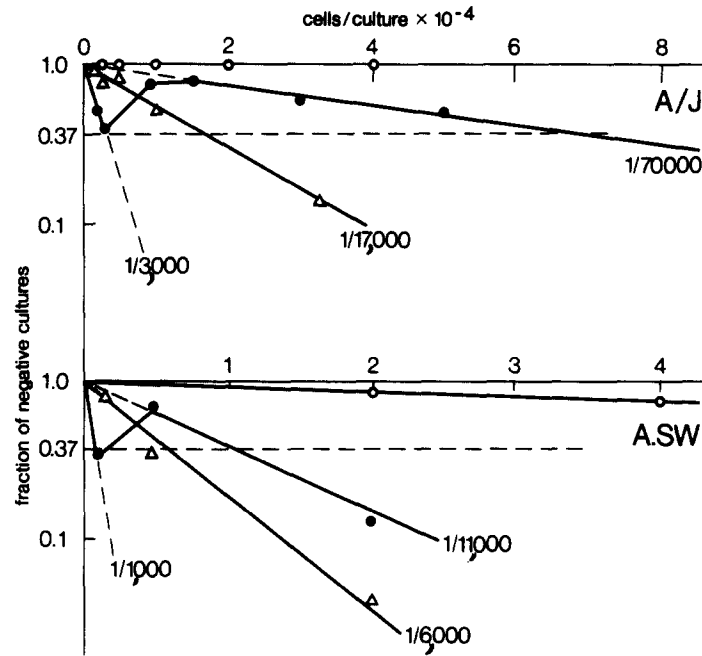


FIG. 6. Precursor frequency analysis of T_H cells for B cells responding to TNP-Strep A, with T cells that were activated for 2 d in Con A, then treated with either rabbit complement (C') alone (\bullet), with anti-Lyt-2.2 antiserum + C' (Δ), or with anti-Lyt-1.2 antiserum + C' (\circ). Cell recoveries were as follows: A/J: C' , 64%; anti-Lyt-1.2, 7%; anti-Lyt-2.2, 7%; A.S.W: C' , 68%; anti-Lyt-1.2, 36%; anti-Lyt-2.2, 40%. Thereafter, cells were grown for 1 wk in 24 wells for each cell concentration and T_H activity was determined as in Fig. 5.

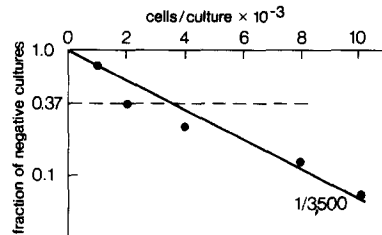


FIG. 7. Precursor frequency analysis of T_H cells for B cells responding to TNP-Strep A, with T cells from ~ 8 -mo-old A/J females that had been used for breeding multiple times. The experiment was performed as described in the legend to Fig. 5.

Discussion

The method for analyzing T cell diversity presented in this paper opens the possibility of studying the representation and function of antigen-specific subsets of T cells in a quantitative fashion and on the level of single clones. The major advantage as compared with previous limiting-dilution systems is the use of TCGF to expand T cell numbers before testing for T cell function. Because we obtain clonal expansion of the analyzed precursor cells by a factor of 500–1,000, we need not worry about limitations of the method set by its sensitivity. Indeed, we can split cultures into several parts, each of which can be tested for another T cell function. It was shown that helper cell frequencies were the same when 1/4, 1/2, and all of each culture was

tested (K. Eichmann. Unpublished results.), which suggests that, after clonal expansion in TCGF, T cell numbers far exceed those that would limit their detectability. A disadvantage of the system appears to be that it is not certain as to what extent we work with nonrandomly selected T cell populations. In addition to Con A activation and TCGF-induced proliferation, our cells have experienced fractionation over nylon wool and over Ficoll. As was discussed above, we have qualitative evidence that each of the T cell functions that we can determine in our laboratory is represented in our TCGF-expanded, Con A-activated T cells. Furthermore, under our culture conditions, one out of two Con A-activated T cells proliferates in TCGF. In spite of this, our expanded T cell populations may provide a biased view of the normal T cell pool.

On the other hand, the possibility to preselect T cells by activation before their expansion offers a number of interesting opportunities for the study of the frequencies of various precursor cells upon different preselection procedures such as activation by a series of different mitogens or antigens. For example, the question of whether alloreactive T cells possess overlapping specificity repertoires with T cells reactive to conventional antigens (33), can definitively be answered in our limiting-dilution system.

The present series of experiments was devoted to the study of Strep A-reactive helper cells in polyclonally activated, nonimmune T cells. We could demonstrate two sets of different frequencies, a rare set with frequencies between 1/10,000 and 1/70,000 and a frequent set with frequencies of from 1/1,000 to 1/6,000. The difference between the frequencies of the two sets of helpers in various experiments was between 5- and 20-fold, in most experiments close to 10-fold.

Both sets of helpers possess antigen specificity to an extent that T cell clones that help in responses to TNP-Strep A are distinct from those that help in responses to SRBC. We could not find any evidence for nonspecific helpers (34-36) that helped in responses to both antigens, although our data do not exclude their existence at low frequency or their function at different experimental conditions. In preliminary experiments, we could also show that both sets of helpers are restricted to the I region of the B cells used for the detection of helper function.

We have previously reported the precursor frequencies of idiotypically defined B cells with specificity for Strep A carbohydrate (A-CHO) to be on the order of 1/2,000 (37). It should be stressed, in this context, that our determination of B cell frequencies included only those B cells that are reactive to A-CHO, which is but one of multiple antigens on Strep A presumably accessible to T cells in a helper assay. Indeed, in previous work, we have shown that <30% of the Strep A-reactive helper cells are specific for A-CHO (38). Thus, the frequencies determined for Strep A-specific helper T and B cells are difficult to compare. Nevertheless, the frequencies of frequent helpers and of B cells in nonimmune spleen cells seem to be similar, whereas rare helpers seem to be an order of magnitude less frequent. This may also be the case for SRBC specificity, the B cell frequency for which was reported to be on the order of 1/1,000 (8). In general, it should be stressed that sets of T helper (T_H) cells can only be clearly distinguished in limiting-dilution assays when they dilute at sufficiently different cell concentrations so that single hit results can be obtained over a series of cell concentrations. It is, therefore, also possible that for SRBC two different helper cells exist, the more frequent of which remained undetectable because of a suppressor cell of equal or slightly greater frequency.

A comparison of the frequencies reported for alloreactive T cells (10, 11) with that of our frequent Strep A-specific T_H reveals both to be similar within one order of magnitude. Thus, our data extend the question about the high frequency of alloreactive T cells to T cells specific for conventional antigens. In a previous report we have made the suggestion that the T cell repertoire for alloreactivity may be highly cross-reactive, and that the apparent high degree of specificity of T cells is mainly the result of regulatory mechanisms (39). In view of the present evidence, we may extend this argument to the T cell repertoire in general.

By using serologically selected T cells, we find that Lyt-23 cells are largely depleted of all T_H cell precursors, whereas Lyt-1 cells are enriched for the rare T_H precursor but depleted of the frequent T_H precursor. Whereas this result was expected for Lyt-23 cells, it was surprising for Lyt-1 cells because previous work had suggested all T_H and their precursors to be Lyt-1 (13). In contrast, we find 90% of all T_H precursors to be Lyt-123. This situation is reminiscent of previous reports from our laboratory that show that most primary cytotoxic T lymphocyte (CTL) precursors are Lyt-123, whereas most Lyt-23 CTL precursors are memory cells (27, 39). These results are also in line with a recent report by Shen et al. (40), which revealed primary T_H cells to arise primarily from the Lyt-123 T cell pool. Thus, we are presently testing the hypothesis that our frequent T_H are primary T_H cell precursors, whereas the rare T_H are secondary T_H cell precursors or memory cells. This would be in line with their Lyt-123 and Lyt-1 phenotypes, respectively, if we assumed that, upon activation with antigen, Lyt-123 precursors mature into T_H effector and T_H memory cells, both of Lyt-1 type. The lesser frequency of Lyt-1 precursors would have to be interpreted as a consequence of selection by previous exposure to antigens other than Strep A, some of which possess cross-reactivity with Strep A.

Upon testing Strep A-specific T_H in old mice we found evidence for only a single T_H type at a frequency of 1/3,500. We suspect that the difference to young mice is the result of natural immunization through exposure to environmental streptococci. Thus, as expected, immunization appears to modify precursor frequencies of antigen-specific T cell subsets. We are presently investigating the effect of various deliberate immunization protocols on the quantitative representation of the two different sets of T_H defined by limiting-dilution analysis and have obtained preliminary evidence that both types of T_H cells increase in frequency upon immunization with anti-idiotypic antibody. Precursor frequency determination of antigen-primed T cells from other laboratories have mostly given monophasic distributions such that only a single T_H could be identified (12), i.e., at a frequency of 1/4,000 for SRBC (41). We plan to study the details of the relationships between the nonimmune and the immune status of T_H cell frequencies.

A number of reports in the literature have described the participation of two different T_H in antibody production. Tada et al. (17) have shown that by nylon column fractionation two types of helpers can be separated, one of which adheres to nylon wool. It is unlikely, but not impossible, that our results reflect these two T_H , particularly because our T cells are preselected by nylon column passage.

In another series of experiments, Bottomly et al. (18) have demonstrated a synergistic effect between two types of antigen-specific T_H cells, one of which requires the presence of immunoglobulin or of B cells for its induction. In our experiments, we

have not, as yet, studied Ig requirements or putative synergy between the two types of T_H because, in normal T cells, they dilute at different cell concentrations.

In four different idiotypic systems, investigated independently in our own and three other laboratories, antibody responses of B cells expressing major idiotypes have been shown to depend, in addition to their dependence upon T_H cells recognizing the antigen, upon T_H cells recognizing the idiootype of the B cells (18–21). In our own studies, the idiootype-recognizing T_H were shown to be independent of antigen and could help B cells without antigen-specific T_H cells present (42). These observations have given support to the notion that the elements of the immune system are interconnected by idiotypic interactions and that these interactions play a major immunoregulatory role (4, 43).

Superficially, the limiting-dilution experiments presented here and by other authors (12, 41) suggest that one clone of T cells can cause a culture of B cells and antigen to produce antibody. However, in our own experiments, the role of a second T_H may have been overcome by TCGF. In other experiments in which primed B cells were used for the detection of help, the second helper signal may have been delivered during the priming that occurred *in vivo* in the presence of T cells (12). Thus, none of the limiting-dilution experiments performed to date formally excludes the participation of two types of T_H cells in antibody responses. We plan to investigate the function and frequency of idiootype-specific T_H (and T suppressor cells) in limiting dilution and hope to be able to define their role in more detail. It should be mentioned that the antigen specificity of our two sets of helpers does not exclude the possibility that one set (or even both) is indeed idiootype specific, because in this case its reactivity would also be restricted to one of the two antigens tested. Indeed, we have obtained preliminary evidence that some of the frequent T_H may be antigen independent and thus behave like idiootype-specific T_H cells (42).

It is interesting to note the difference in susceptibility to suppression between the two sets of T_H . The frequent T_H , as cell numbers per culture are increased, become totally inactivated. We interpret this as suppression by other T cells that exist at lower frequency but that are either more potent on a per cell basis, or that proliferate more rapidly in TCGF. Our data do not as yet allow conclusions as to the mechanism of suppression and as to how many suppressive T cell types are involved. In contrast to the frequent T_H , the rare T_H is totally insensitive to this suppressive mechanism, and we cannot find a suppressor cell for our rare T_H within the cell concentrations tested. This situation also has an analogy to our previous finding on the regulation of CTL responses in which it was shown that Lyt-123 CTL precursors and Lyt-23 CTL precursors are sensitive to different sets of suppressor cells (27, 39). Taken together, with the possibility that the frequent and rare T_H correspond to primary and secondary T_H cells, respectively, these data suggest that upon activation with antigen, T cells move to a memory pool that is distinct from primary T cells by its lack of sensitivity to suppression. Such a mechanism would be ideal for providing a highly active memory pool for acquired immunity to environmental pathogens.

Taken together, we feel that the limiting-dilution method described in this paper has great potential for elucidating the structural and functional details of the T cell network. Limits as to which T cell subsets can be studied are set only by the methods used for the detection of T cell functions. In principle, any T cell function that can be detected in bulk cultures can also be analyzed in our limiting-dilution system. The

definition of T cell subsets by their frequency, particularly in combination with serological or other cell fractionation procedures, and the possibility to analyze large numbers of T cell clones, should provide much more quantitative information on T cell diversity than the study of bulk cultures.

Summary

A limiting-dilution system is described that makes use of T cell growth factor for T cell expansion and allows the determination of precursor frequencies for various regulatory and effector T cells in nonimmune, polyclonally, or specifically activated T cell populations. Two different sets, a frequent and a rare set, of T helper cell precursors with specificity for trinitrophenyl-group A streptococcal vaccine, could be identified: the frequent set is of the Lyt-123 phenotype, and is present at frequencies of from 1/1,000 to 1/6,000 splenic T cells. It is only active at low cell numbers, whereas it is completely inactivated at greater cell numbers, presumably by suppressor T cells of lower frequency but greater potency. The rare set is of the Lyt-1 phenotype, is present at frequencies of from 1/10,000 to 1/70,000, and is not sensitive to suppressor cells present within the tested cell numbers. We suggest that the frequent set contains primary helper cell precursors, whereas the rare set contains helper T memory cells preselected by previous exposure to other antigens. The results are discussed with respect to other reports on the involvement of more than one set of helper cells in antibody production.

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