QUANTITATIVE STUDIES ON T CELL DIVERSITY

III. Limiting Dilution Analysis of Precursor Cells for T Helper Cells Reactive to Xenogeneic Erythrocytes

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The identification of T cell growth factors (TCGF)[1] (1–3) has brought major progress to the study of T lymphocytes, allowing long-term tissue culture of selected T cells. Such lines can be cloned in vitro and kept functionally active as killer (2, 4–7) or helper cells (7–10), or cells showing antigen-specific proliferation (11, 12). The major drawback of the use of long-term culture lines, however, is that it is uncertain to what extent they represent the normal T cell pool or a selection of rare unusual cells. For the study of the T cell repertoire it is thus important to perform clonal assays under conditions that allow the analysis of a large proportion of all T cells.

We have therefore developed a method in which polyclonal activation, differentiation, and growth of normal splenic T lymphocytes occurs in vitro under limiting dilution conditions (13–15). The method was designed to determine the relative frequencies of the various precursors of regulatory and effector T cells, as well as to analyze T cell specificity at a clonal level, thus allowing an evaluation of T cell receptor diversity.

In the present paper we confirm and extend our previous experiments. First, we confirm our earlier results on streptococcus A-reactive helper T cell precursors, using various kinds of xenogeneic erythrocytes as antigens. Again we find with increasing concentrations of T cells alternate increases and decreases in the numbers of positive cultures. We refer to these increases and decreases as "peaks" and characterize them by the T cell concentrations that induce the highest number of positive cultures in each peak. Second, by testing a broader range of T cell concentrations (0.5–80,000 cells/well), we were able to detect an additional third "peak" of precursor helper T (pre-T_H) cells, at rather low T cell concentrations (40–200 cells/well).

We have suggested earlier (13–15) that in our experiments each peak of T_H activity reflects an independent population of pre-T_H cells that is suppressed at higher cell doses. Furthermore, we have previously used the peaks to estimate the frequencies of these various precursor populations by Poisson analysis. However, as the single-hit Poisson distribution does not always fit our data, such an interpretation of the results certainly is oversimplified. Nevertheless, a common sense argument can be made that the frequency of the most frequent pre-T_H cells is on the order of 1/10–1/100. For

[1] *Abbreviations used in this paper:* Con A, concanavalin A; Con A sup, supernatant of Con A-induced rat spleen cells; FCS, fetal calf serum; LD, limiting dilution; PEC, peritonael exudate cells; PFC, plaque-forming cells; RBC, erythrocytes; CRBC, HRBC, SRBC, chicken, horse, and sheep erythrocytes, respectively; TCGF, T cell growth factor; T_H, helper T cell, pre-T_H, precursor helper T cell.

more precise frequency determinations we are currently developing a mathematical system that is appropriate for estimating accurate frequencies from multi-hit limiting dilution results and for testing parameters of regulation.[2]

Because of the controversial nature of our results and because we feel that the "multi-hittedness" of these results reflects fundamentally important regulatory mechanisms of the immune system, we decided to present in this paper a detailed description of the experimental design used to generate the data, as well as experiments that show that we are indeed investigating a cellular phenomenon that is evoked by T_H cells.

Materials and Methods

Mice and Antigens. Male and female C57Bl/6 mice were obtained from Zentralinstitut für Versuchstierzucht, Hannover, Federal Republic of Germany. They were vaccinated with ectromelia vaccine at the age of 4 wk and used for experiments at 6–10 wk. Sheep erythrocytes (SRBC) and chicken erythrocytes (CRBC) were obtained from our own animal facility. Horse erythrocytes (HRBC) were kindly provided by Dr. Y. Rosenberg, National Institute for Medical Research, Mill Hill, London, England.

Preparation of Concanavalin A Supernatant (Con A Sup) from Rat Spleen Cells. Spleens of Sprague-Dawley rats were used for the preparation of Con A sup as described earlier (13).

Cell Culture Conditions. Splenic T cells were prepared by nylon-wool passage of spleen cells (16). For the preparation of Con A-activated T cells, purified T cells or spleen cells were cultured for 2 d at a density of $1–2 \times 10^6$/ml with 5 μg/ml Con A (Serva Feinbiochemica, Heidelberg, Federal Republic of Germany) in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 10^{-5} M 2-mercaptoethanol, 0.025 M Hepes, 1% penicillin-streptomycin, and 0.2 mM glutamine (Con A bulk cultures). After 2 d in Con A bulk cultures, limiting dilution (LD) cultures were set up: cells were washed three times, and then placed in Linbro flat-bottomed microtiter wells (7600305; Linbro Chemical Co., Hamden, CT), together with 1×10^4 peritoneal exudate cells (PEC)/well, irradiated with 3,000 rad (^{60}Co). Cell concentrations used ranged from 0.5 cells/well to 1×10^6 cells/well and are indicated in individual experiments. Either 24, 48 or 96 wells were set up for each group. Culture medium (200 μl/well) was Iscove's modified Dulbecco's medium supplemented with 10% FCS, 1% penicillin-streptomycin, 10^{-5} M 2-mercapthoethanol, and 10–20% Con A sup. Cells were kept at 5% CO_2 and fed two to three times a week by replacing 150 μl medium. Cells were held in LD cultures for 7–8 d as indicated. In some experiments, splenic T cells were directly activated in LD cultures. In this case they were seeded into microtiter wells together with 1×10^4 irradiated PEC, fed for 2 d with medium containing Con A (as described for Con A bulk cultures), and then with medium containing Con A sup (as described for LD cultures). At the end of LD cultures, plates were centrifuged and all medium was removed. The cells were then carefully washed three times with PBS containing 5% FCS, then mixed with B cells (3×10^5/well) and RBC (1×10^5/well) in 100 μl Click's medium (17) in Linbro flat-bottomed microtiter wells and kept at 2% CO_2 (test cultures). B cells were prepared as previously described (13), or by treating spleen cells twice with a monoclonal anti-Thy-1.2 (F7D5 Olac, Blackthorn, England) at a final dilution of 1:2,000, using 5×10^7 spleen cells/ml and rabbit or guinea pig complement. The response was assayed in a Jerne plaque assay. Numbers of direct plaque-forming cells (PFC) were determined 4 d after beginning the test cultures. Control groups contained irradiated PEC, B cells, and RBC only. Data were analyzed as described below.

Results

Titration of In Vitro Differentiated SRBC-reactive Helper Cell Precursors. Most experiments to determine the frequencies of SRBC-reactive pre-T_H cells within the normal T cell pool were performed exactly as described for the analysis of the precursor

[2] Fey, K., I. Melchers, and K. Eichmann. Manuscript in preparation.

frequencies of helper cells reactive to streptococcus A (13). Nylon-wool-purified splenic T cells of C57Bl/6 mice were activated for 2 d in Con A bulk culture and thereafter seeded in graded numbers into microtiter wells containing 10^4 irradiated PEC, and kept for 1 wk in medium containing 10% Con A sup. After this period, growth frequencies were determined by visual examination and analysis according to Poisson statistics (18). Usually 1 out of 2–10 cells seeded grew under these conditions. After 7 d of LD culture, T cells were cultured together with B cells and SRBC in 4-d test cultures. The responses were assayed by counting the number of direct PFC/well.

Several ways of analyzing the results of such an experiment are depicted in Fig. 1. Fig. 1 a shows a plot of the total PFC/group. With increasing numbers of T cells placed into LD cultures, we find alternate increases and decreases of the numbers of total PFC/group. In most experiments we find three such "peaks," e.g., in Fig. 1 a there is one peak at a concentration of 20,000 T cells/well, another at 625 T cells/well, and a third at 20 T cells/well. Plotting the fraction of positive cultures for each T cell concentration (Fig. 1 b) yields results that very much resemble Fig. 1 a, with peaks at 20,000, 625, and 40 T cells/well, respectively. The differences in the numbers of PFC/group are therefore mainly due to differences in the numbers of responding wells rather than to variations in the means of all cultures per group. We can therefore use the fraction of negative cultures to construct plots conventionally used for limiting dilution results (19). Such plots clearly show the decreases and increases in the fraction of negative cultures in relation to the T cell concentration (Fig. 1 c).

In Fig. 2 the results of two other experiments are shown. Table I summarizes the results of eight additional experiments, in which sheep, horse, or chicken RBC were used as antigens. Each peak is characterized by the T cell concentration(s) giving the highest number of positive cultures. As can be seen by comparing the data of Table I and Figs. 1 and 2, our results are highly reproducible. In more than 20 experiments, we found three definite peaks of response. As a general rule, we observe peak I at 40–190 T cells/well, peak II at 600–3,000 T cells/well, and peak III at 20,000–100,000 T cells/well.

Properties of the Detection System for Help. To investigate whether our detection system is suitable for the determination of help in LD experiments, we asked the following questions: (*a*) Does the detection system contain sufficient numbers of B precursor cells for the SRBC response? (*b*) What are meaningful criteria to distinguish positive from negative cultures? (*c*) Is the PFC assay sufficiently reproducible to make reliable distinctions between positive and negative cultures? (*d*) Is the help detected indeed a function of the cultured T cells themselves, or is it due to factors present either in Con A sup or in the supernatants of the T cell cultures? (*e*) Is it indeed a T cell that is activated in LD cultures and induces positive responses in the test culture?

Frequency of B Cell Precursors. The classic assay system to detect helper cell function in vitro is the Mishell-Dutton culture system (20). We use xenogeneic RBC as T-dependent antigens and measure "help" as the response of a B cell population in terms of direct PFC, found in the presence and absence of T cells after 4 d of culture. In our experiments only T_H cells should be limiting. Values given in the literature for SRBC-specific B cell precursors vary between 1/3,000 and 1/100,000 C57Bl/6 spleen cells (21–23). To prove that our cultures offer sufficient numbers of antigen-presenting cells and especially of B cell precursors that can be antigen specifically induced to proliferation and differentiation by T_H cells, we determined the SRBC-reactive B cell

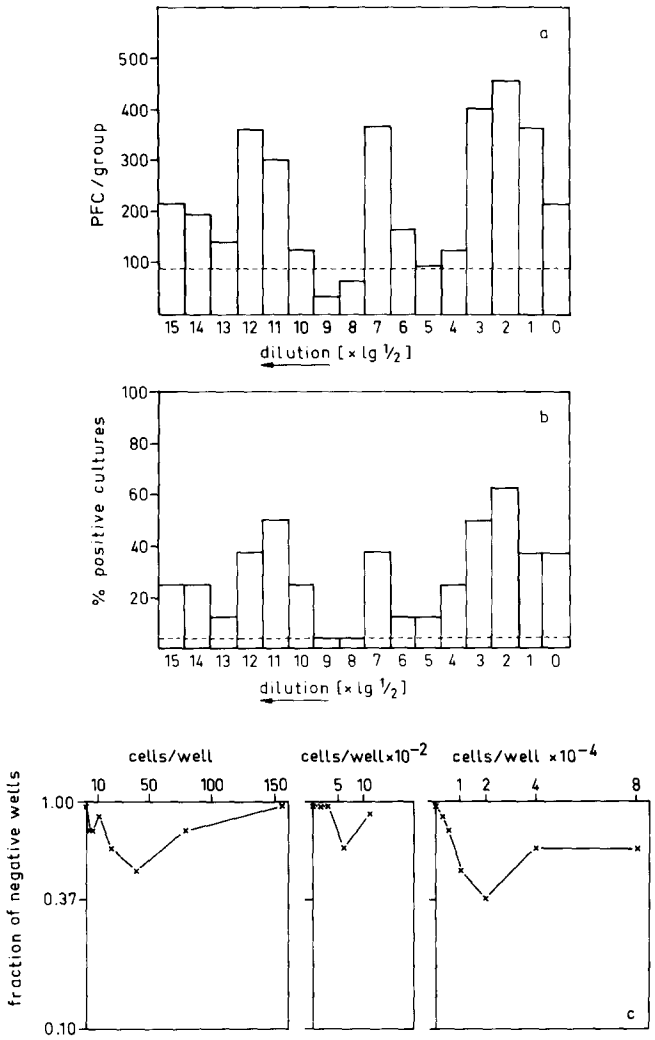

FIG. 1. Titration of in vitro differentiated SRBC-reactive pre-T_H. T cells were activated in Con A bulk culture for 2 d and then seeded in LD culture (24 wells/group). The highest concentration used was 80,000 T cells/well; the lowest was 2.5 T cells/well. Cells were fed with LD medium for 7 d and then washed, and B cells and SRBC were added. After 4 d direct PFC/well were measured. The data obtained are given in three different ways: as total number of PFC/group (a), as percent positive cultures/group (b), and as fraction of negative cultures/group (c). Broken lines indicate background values. Controls were 95.8% negative, $\bar{x} = 2.8$, $\sigma = 4.2$, $t = 15$.

precursor frequency under our culture conditions using limiting numbers of B cells. Although we never met 100% saturating conditions for T_H cells, we can be sure from our data (e.g., Fig. 3) that the frequency of B cell precursors is $>1.54 \times 10^{-5}$ (1/65,000), i.e., above the value at which, in a Poisson distribution, 99.0% of all wells will contain ≥ 1 specific B cell precursor if a total of 3×10^5 B cells/well is used. (At 3×10^5 B cells/well the mean number of SRBC specific precursors then is ~5/well).

Distinction between Positive and Negative Cultures. A second essential requirement of LD analysis is to be able to distinguish between a negative and a positive response,

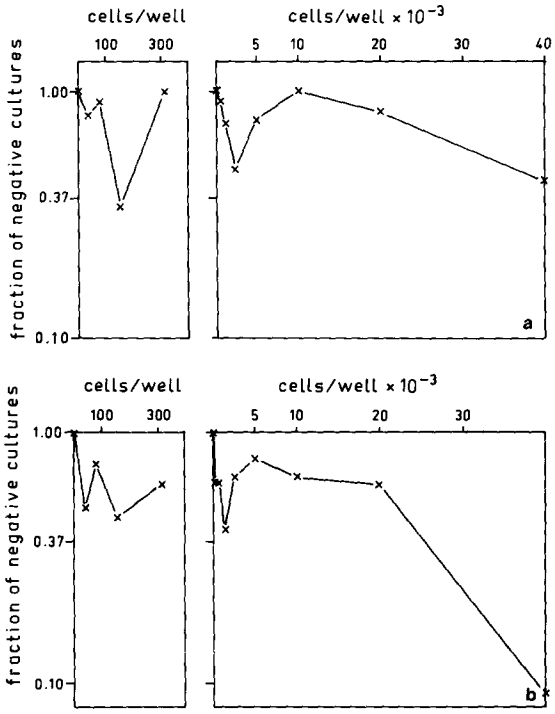

FIG. 2. Titration of in vitro differentiated pre-T_H. The experiments were performed exactly as described in the legend of Fig. 1. SRBC (a) or CRBC (b) were used as antigens. All cultures (24 wells/group) responded at the highest concentration of T cells (8×10^4/well; not shown here). The lowest concentration used was 40 T cells/well. Control values were 95.8% negative, $\bar{x} = 3.9$, $\sigma = 3.9$, $t = 16$ (a), and 100% negative, $\bar{x} = 3.2$, $\sigma = 4.0$, and $t = 16$ (b).

TABLE I

T Cell Concentration-dependent Peaks of the PFC Responses to Xenogeneic Erythrocytes

Experiment*	Antigen	Peaks in the fraction of positives			Range of titration (T cells/well)	N§
		Peak I (T cells/well)‡	Peak II (T cells/well)	Peak III (T cells/well)		
1	SRBC	40	625	20,000	2.5–80,000	16
2	SRBC	40–80	625	40,000	40–80,000	16
3	SRBC	190	3,000	25,000–100,000	190–100,000	12
4	SRBC	40	2,500	20,000–80,000	40–80,000	16
5	CRBC	150	1,250	40,000–80,000	40–80,000	12
6	CRBC	40	625	80,00	40–80,000	12
7	HRBC	200	3,000	25,000–100,000	200–100,000	10
8	HRBC	40	625	20,000	2.5–80,000	16

* Only a representative sample of many experiments is given. Experiments shown in the figures are not included.

‡ All T cells were activated in Con A bulk cultures for 2 d, and then exposed in LD cultures to Con A sup containing medium for 7 d, before combining them with 3×10^5 syngeneic B cells and 1×10^5 RBC in test cultures for 4 d. The responses were analyzed as described in the legend of Fig. 1. 24 wells/group were tested, in all cases.

§ Number of different concentrations tested in each experiment.

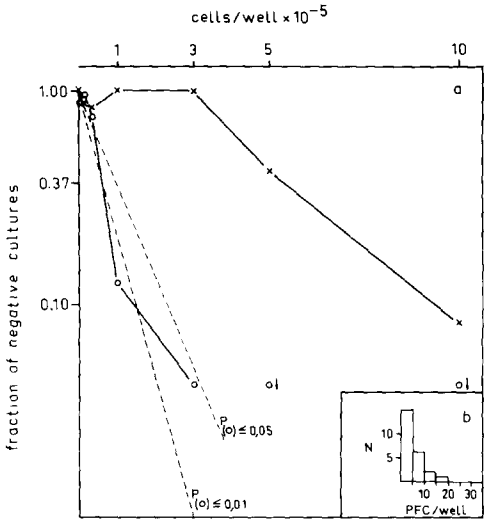

FIG. 3. Titration of B cells specific for SRBC. B cells were prepared as described in Material and Methods. They were titrated from 1×10^6 to 4×10^3 cells/well, holding the total number of cells/well at a minimum of 3×10^5 by adding appropriate numbers of irradiated (1,500 rad) B cells (×). A second set of groups (○) also received T cells, which had been activated in Con A bulk culture for 2 d, then seeded at 4×10^4 cells/well into microtiter plates together with 10^4 irradiated (3,000 rad) PEC, and kept in LD cultures for 8 d. All groups consisted of 24 wells and received 1×10^5 SRBC/well. Direct PFC responses were assayed on day 4. The responses of the group containing only irradiated B cells and SRBC (b) served to define the threshold (= t) between positive and negative wells (compare with Fig. 4). All controls were negative, with $\bar{x} = 4.0$, $\sigma = 4.6$, and $t = 17.8$. Arrows indicate groups in which all cultures responded (<4.2% negative). Broken lines are theoretical regression lines constructed for $f_0 \leq 0.01$ and ≤ 0.05, respectively, at 3×10^5 cells/well.

i.e., in our case, between no B cells and one B cell responding. From the data shown in Fig. 3 we can calculate the "burst size" of a single, activated B cell by dividing the total number of PFC per group by the frequency of precursors. In accordance with other observations (24), we find in repeated experiments a burst size of ~30 PFC after 4 d of culture. In theory, one should therefore find at the end of a 4-d test culture positive wells with ~30 (or more) PFC, and negative wells with close to zero PFC. In reality, we observe the results illustrated in Fig. 4. If the distribution of control values is plotted in a histogram, we often find it to be a single distribution (Fig. 4a), of which the mean ($\bar{x}$) and standard deviation (σ) can be calculated. Means of such "all-negative" controls are usually found to be below 10 PFC/well. We then use the value of $\bar{x} + 3\sigma$ as threshold (= t) below which a culture is counted "negative." By this procedure, 99.8% of all negative wells will be found within a negative population that shows a Gauss distribution. As an example for the results thus obtained, Fig. 4a and b shows the histograms of a control and an experimental group containing 1.5×10^5 SRBC-immunized T cells in addition to B cells and antigen. By our criteria we here find 100 and 16.7% negatives, respectively.

In some experiments, particularly when large numbers of control wells are tested, we do not observe a single distribution but two or three populations instead (Fig. 4c). Probably the populations with higher PFC numbers/well represent wells where one or more B cells respond specifically to SRBC in a primary response due to residual T cells in the B cell population. Fig. 3 shows that 3×10^5 B cells/well is close to a

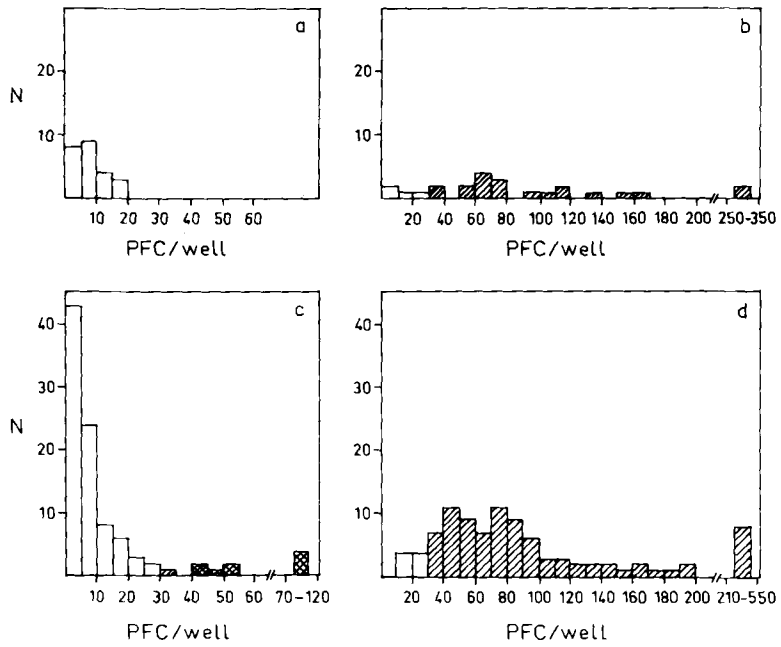

FIG. 4. Definition of "positive" and "negative" cultures. PFC responses of selected groups are shown as histograms, where N equals the number of cultures with a certain number of PFC/well. In experiment 1 (a and b), groups consisted of 24 wells each. The controls (a) are distributed as one population. They are all considered negative and used for the calculation of $\bar{x}(= 9)$, $\sigma(= 5.6)$, and $t = \bar{x} + 3\,\sigma(= 26)$. The group of experiment 1 depicted here (b) contained in addition to B cells and SRBC 1.5×10^5 T cells, primed in vivo with SRBC. In experiment 2 (c and d) groups consisted of 96 wells. The controls (c) are distributed as one negative and probably two positive populations (▩). Only the 87 negative cultures are used to calculate $\bar{x}(= 7.8)$, $\sigma(= 7.2)$, and $t(= 29)$. The experimental group shown here (d) contained 2,000 T cells/well, taken from a 2-d Con A culture, and fed with LD medium for 7 d before the addition of B cells and SRBC. Positive cultures are indicated (▨).

concentration at which a certain number of wells respond. In addition, we find in titration experiments of normal T cells, T_H cells at a frequency of about $\leq 1/300{,}000$ (data not shown here). In this paper we only present data from LD experiments in which >89% of the control wells belong to a single, negative population. Only these are then used to calculate $\bar{x}$, σ, and t as described above. The t value thus obtained is used to define the fraction of negative cultures ($= f_0$) in control as well as experimental groups. As an example for this type of experimental data, Fig. 4c and d shows histograms of a control and an experimental group, where we calculated 89.6 and 8.3% negatives, respectively. If necessary, such data then can be normalized by dividing the observed f_0 of any experimental group by the f_0 of the control group.

Frequently, t is found to be close to 20–30 PFC/well, i.e., close to the value expected to represent the positive response of one B cell. As becomes clear from looking at Fig. 4b–d, negative and positive populations overlap, and the "negative" population determined by $t = \bar{x} + 3\sigma$ may contain quite a number of positives. Therefore, the values obtained for f_0 are most likely overestimated, leading to minimal estimates in the frequency determination.

Reliability of the Plaque Assay. Because the decision about a well being positive or

negative is crucial for the result of any experiment, we also tested the reliability of our plaquing technique. Fig. 5 demonstrates in two examples that we can split test cultures just before the plaque assay with reasonable accuracy into identical halves. P values, based on Fisher's exact test, ranged from 0.001 to 0.05, thus rejecting the null hypothesis of independent assortment at the 5% level of significance.

Are "Factors" Found in Con A Sup Responsible for the B Cell Response Observed? Our Con A sup presumably contains a variety of factors (Con A, interleukin 1, interleukin 2, T cell-replacing factor, etc.) which by themselves might be able to induce B cells, either directly or via some other contaminating cell in the B cell preparation. We thus had to check whether the helper effect observed in our cultures is not simply due to Con A or factors transmitted from the LD cultures to the test cultures.

When we titrated a preparation of Con A sup directly into cultures containing B cells and SRBC, we indeed found that at 10% Con A sup, the number of positive wells increased from 14 to 53%, with the distribution shifting to more cultures with higher numbers of PFC (Fig. 6). At 1% Con A sup, however, we found even fewer positive wells (5%) than in the controls without Con A sup, with the distribution

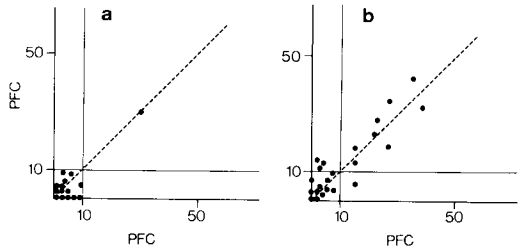

FIG. 5. Splitting of individual wells containing PFC clones. 10^4 irradiated (3,000 rad) PEC were placed into microtiter wells either alone (a) or together with 400 T cells/well taken from a 2-d Con A bulk culture (b). All wells were fed with LD medium for 7 d and washed, and 3×10^5 B cells and 1×10^5 SRBC added. After 4 d of culture, wells were split into equal volumes just before the plaque assay. PFC numbers obtained in the first half of individual wells are plotted against PFC numbers obtained in the second half. Lines indicate the threshold between positive and negative responses (———). The hypothesis of independent distribution is rejected at a rejection zone of $P \leq 0.05$, because the probability to obtain the present results, based on Fisher's exact test (25), is $P = 0.042$ (a) and $P = 0.002$ (b).

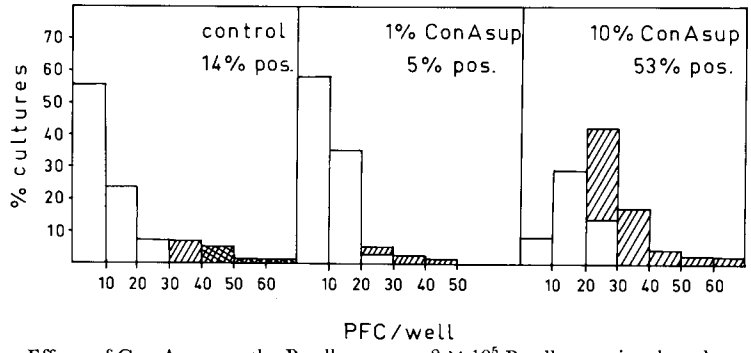

FIG. 6. Effects of Con A sup on the B cell response. 3×10^5 B cells were incubated together with 1×10^5 SRBC in media containing no, 1%, or 10% of rat Con A sup. 4 d later, the numbers of PFC were determined. 96 wells/group were tested. In the control group six wells with >40 PFC were counted positive (▩), and only the remaining wells (= 94%) were used to determine t(= 24). Positive cultures are indicated (▨).

shifting to more cultures with fewer PFC. When we set up test cultures, we estimate that we do not transfer more than 10% of LD culture medium, equivalent to 1% of the original Con A Sup. Therefore, we should not find a response induced by Con A sup.

Our T cell cultures are fed with 10% Con A sup, and the medium is regularly changed for the last time 2 d before setting up test cultures. During this time factors may have been released or activated by the cultured T cells. We therefore checked whether we can induce B cells by transferring supernatants from these cultures. Fig. 7 a and b shows that there is some activity in these supernatants when used at 10%, corresponding to 1% of the original Con A sup (18.7% positive wells compared with 10.4% positive wells in the controls). However, if carefully washed T cells are used instead, there are more positive cultures (92.6%), and the whole population has shifted to more cultures with higher PFC numbers (Fig. 7 c). If T cells are used together with 10% of their own supernatant, fewer cultures are found positive (49.5%) and the population is shifted back to more cultures with lower PFC numbers (Fig. 7 d). We interpret these results as showing that (*a*) there is some "helping" activity in these culture supernatants, (*b*) most of the activity is transferred by cells, and (*c*) there is also some "suppressive" activity in the culture supernatants which negatively interacts with the helping activity of the cells. This suppressive activity, however, can be washed away quite efficiently (washing three times with PBS/FCS followed by 10 min of equilibration and centrifugation).

As a sideline, we investigated whether the helping activity of supernatants of LD cultures (Fig. 7 b) is due to factors released by the T_H cells that are active in the test cultures. We therefore checked for a correlation between "help" transferred by cells and by the corresponding supernatants, but so far we could not find any (data not shown). We therefore conclude that the two helper effects probably represent different mechanisms. In summary, our protocol ensures that although Con A sup itself, as well as the culture supernatants, has helping and also suppressing activities, the helper activity determined in our experiments is transferred by cells.

Are "Positive Responses" Induced by T Cells Activated in LD Cultures? By definition, a

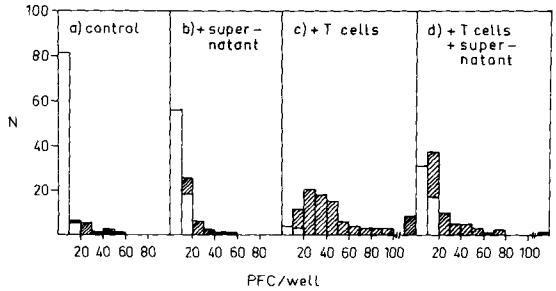

FIG. 7. Effects of in vitro activated T cells and their supernatants on the B cell response. 3×10^5 B cells were incubated with 1×10^5 SRBC and (a) 10% LD medium, taken from wells containing 10^4 irradiated PEC (= control), (b) 10% LD medium, taken from wells containing 10^4 irradiated PEC and in vitro activated T cells, (c) in vitro activated T cells, washed three times with PBS/5% FCS, each wash followed by 10 min equilibration and centrifugation, or (d) in vitro activated T cells, not washed, together with 10% supernatant from the same well. All T cells were activated in Con A bulk cultures for 2 d, then placed in LD culture at 2,000 T cells/well for 7 d. Media were changed for the last time 2 d before setting up test cultures. 96 wells/group were tested. Controls were 89.6% negative with $\bar{x} = 3.9$, $\sigma = 3.6$, $t = 15$. Positive cultures are indicated (▨).

T_H cell is a T lymphocyte that helps a B cell to respond to a T-dependent antigen. Although we regularly use nylon-wool-purified T lymphocytes in our cultures, it appeared necessary to determine whether T cells are indeed responsible for the "peaks" observed. We therefore prepared T cell-enriched and T cell-depleted populations of spleen cells, placed them into microtiter wells on irradiated PECs, and induced them *in situ* with Con A and Con A sup in the usual way. Before setting up test cultures, cells were irradiated with 1,500 rad, which does not change the helper effect (data not shown). The results are illustrated in Fig. 8. T cells, activated *in situ*, induce the usual response pattern with peaks at 80,000, 2,500, and 40 T cells/well. With B cells, we also find three peaks, but at higher concentrations (800,000, 100,000, and 200 B cells/well). If the helper effect were due to T cells, and if there were a randomly selected 5% T cell contamination within the B cell population, one would expect the maxima to shift to 20-fold-higher concentrations. Therefore, the result appears reasonable, although not all maxima are shifted to higher concentrations in a parallel fashion. (The mean of the differences, however, is 18-fold.) In summary, we conclude that help detected by our test cultures is indeed the effect of T cells originally placed into LD cultures.

Titration of T Cells at Very Low Concentrations. In most experiments we titrated T cells between 80,000 and 40–100 cells/well to test for helper activity. The most

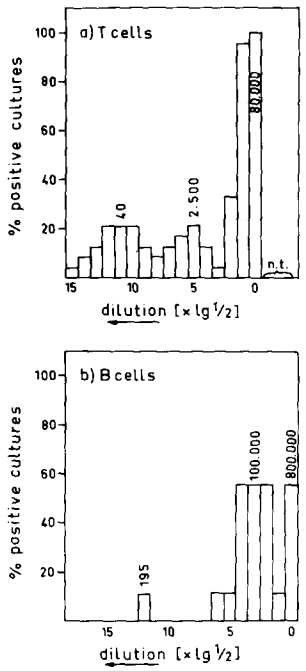

FIG. 8. Titrations of in vitro differentated pre-T_H cells present in T cell-enriched and T cell-depleted populations. T and B cells were prepared as described in Materials and Methods, and placed directly into microtiter wells (24 wells/group), together with 10^4 irradiated PEC. Cells were fed with Con A (2 d) and Con A sup (7 d) containing media, then irradiated (1,500 rad) and washed. Freshly prepared B cells and SRBC were added, and the number of PFC/well determined 4 d later. The concentrations tested ranged from 80,000 to 2.5 cells/well (T cells) and 800,000 to 3 cells/well (B cells). nt, not tested. Controls (48 wells) were 100% negative, with $\bar{x} = 8.4$, $\sigma = 4.5$, $t = 22$.

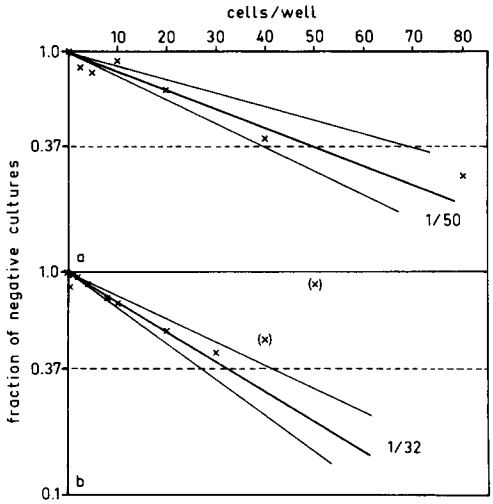

FIG. 9. Titrations of in vitro differentiated pre-T_H cells at low cell concentrations. T cells were activated in Con A bulk culture, then seeded in LD culture. 48 wells/group were set up. Test cultures contained B cells and SRBC, and were assayed after 4 d. Controls were 93.8% negative with $\bar{x} = 4.5$, $\sigma = 3.8$, $t = 16$ (a), and 100% negative with $\bar{x} = 6$, $\sigma = 3.2$, $t = 16$ (b). Data were analyzed as shown in Table II. Regression lines shown were calculated with the "weighted means" method (26), their 95% confidence intervals are also indicated. Data points shown in brackets were not used for regression analysis.

TABLE II

Frequency Estimates of Pre-T_H Cells at Low Cell Concentrations

Experiment*	Antigen	Wells/ group	Range‡	Frequency§	95% confidence interval	Closeness of fit‖
1 (Fig. 9 a)	SRBC	48	0–40 (6)	1/39	1/32–1/49	0.005
2 (Fig. 9 b)	SRBC	48	0–30 (8)	1/33	1/27–1/41	0.81
3 (Fig. 1)	SRBC	24	0–40 (6)	1/39	1/30–1/53	0.001
4 (Fig. 8 a)	SRBC	24	0–20 (5)	1/67	1/43–1/149	0.9
5	HRBC	24	0–40 (5)	1/45	1/33–1/68	0.1–0.3

* Experiments 1–4 are described in the legends of the figures. Experiment 5 was done exactly as experiment 1, with HRBC as the antigen.

‡ The range of the titration (cells/well) and the number of points (in parentheses) are given that were used for the calculations.

§ Frequencies and 95% confidence intervals were determined by using the minimal χ^2 fitting method, as described by Taswell (26).

‖ The closeness of fit was tested with χ^2 testing. P values are given.

surprising result of these experiments was that we found many wells responding at low numbers of T cells originally placed into culture, which suggests that cultures of <100 T cells frequently contain at least one SRBC-reactive pre-T_H cell (compare Figs. 1, 2, 8, and Table I). We therefore looked into this range in more detail, testing more concentrations between 0 and 100 cells/well, with 48 wells/group. The result of two experiments are illustrated in Fig. 9. We find increasing numbers of positive wells up to 40 cells/well (a) or 80 cells/well (b). At higher cell concentrations, the curves bend backwards to more negative wells and reach nearly complete suppression at 50 cells/well (b) and 160 cells/well (a, data not shown), respectively.

One might expect that at such low cell concentrations only one cell type may be

limiting for help. In this case, the initial portion of the curve would be linear and suitable to estimate precursor frequencies according to the zero term of the Poisson distribution (18, 19). We therefore constructed linear regression lines by several different methods, as described by Taswell (26) and tested with χ^2 testing the closeness of the fit of a straight line to our data points.

Table II shows the results of such analyses, giving frequencies obtained with the minimal χ^2 fitting method (26) between 1/23 and 1/67. However, when we tested the goodness of fit with χ^2 testing, we found a broad range of P values, indicating that at least not in all cases a single-hit curve fits the data. This result is not totally unexpected if one considers all data generated in the whole titration range. Nevertheless, as we always find many positive wells at 10–50 cells/well and sometimes can construct excellent linear regression lines (experiments 2, 4, and 5), we have to assume that there exist pre-T_H cells with frequencies of $\geq 1/100$.

Specificity of the Response. Frequencies of pre-T_H cells of $>1/100$ T cells are difficult to reconcile with a high degree of specificity of T_H cells. To define the antigen specificity of T_H cells as differentiated under our in vitro conditions, we performed experiments in which Con A-activated T cells were combined after 7 d of LD culture with B cells and two non-cross-reactive antigens, SRBC and CRBC, in the same culture. After the test culture, individual wells were split and PFC were determined on both types of RBC. We proved in separate experiments that (*a*) the presence of CRBC in the test system does not alter the SRBC response, and (*b*) there is no cross-reaction between SRBC and CRBC on the PFC level (data not shown).

The results of two experiments and the analysis of the data for independent or linked assortment of SRBC- and CRBC-specific responses is shown in Table III. With both antigens we detect three peaks of response, at very similar cell concentrations, for example at 40,000 (both antigens), 2,500 and 1,250, and 160 and 80 T cells/well for CRBC and SRBC, respectively. The observed distribution of positive wells fits the

TABLE III
Specificity of T_H Cells

T cells/well*		20,000		10,000		5,000		2,500		1,250		625		313		156		78		39	
Wells tested		22		24		24		24		23		24		24		24		24		24	
Percent negative on SRBC		52.3		76.2		66.6		38.1		69.5		38.1		67.7 (70.8)‡		71.4 (58.3)		47.7 (45.8)		47.7 (33.3)	
Percent negative on CRBC		62.5		66.7		79.2		66.7		41.7		62.5		62.5 (62.5)		45.8 (70.8)		75.0 (54.2)		50.0 (37.5)	
Response pattern		Obs.	Exp.§																		
SRBC	CRBC																				
+	+	6	5.5	4	2.7	1	2.1	4	5.3	4	5.1	6	6.0	4	3.7	6	4.9	6	3.5	7	7.0
+	−	9	9.5	4	5.3	9	7.9	12	10.7	5	3.9	10	10.0	6	6.2	3	4.1	8	10.5	7	7.0
−	+	2	2.5	4	5.3	4	2.9	4	2.7	9	7.9	3	3.0	5	5.3	7	8.1	0	2.5	5	5.0
−	−	5	4.5	12	10.7	10	11.1	4	5.3	5	6.1	5	5.0	9	8.8	8	6.9	10	7.5	5	5.0
P‖		0.49		0.22		0.28		0.22		0.31		0.67		0.58 (0.22)		0.30 (0.31)		0.02 (0.30)		0.66 (0.28)	

* Splenic T cells were activated in Con A bulk culture for 2 d and then seeded into LD cultures in the concentrations indicated. After 7 d test cultures were set up containing 3×10^5 B cells, 1×10^5 SRBC, and 1×10^5 CRBC. 4 d later test cultures were assayed with SRBC and CRBC. Control values were $\bar{x} = 6.9$, $\sigma = 3.4$, $t = 20$ (SRBC), and $\bar{x} = 3.2$, $\sigma = 4.0$, $t = 4.0$, $t = 16$ (CRBC) (experiment 1), $\bar{x} = 5.2$, $\sigma = 4.0$, $t = 17$ (SRBC), and $\bar{x} = 2.8$, $\sigma = 3.4$, $t = 13$ (CRBC) (experiment 2), respectively.

‡ Data from a second experiment are given in parentheses.

§ Expected values are calculated for an independent distribution of positive cultures, i.e., T_H cells (= Null hypothesis). Obs., observed; Exp., expected.

‖ *P* value is based on Fisher's exact test (25).

hypothesis of independent assortment over the whole range of concentrations tested, down to as few as 39 cells/well seeded. Such a pattern is compatible with the idea that all T_H cells detected in our experiments indeed are antigen specific, and, in addition, need hapten carrier linkage and presumably direct cell contact in order to help. This observation makes it unlikely that the titrated T cells function as nonspecific inducers for pre-T_H cells contaminating the B cell population.

Discussion

Con A activation of splenic T cells and subsequent expansion in diluted supernatant of Con A-stimulated rat spleen cells result in growth of a large proportion (~50%) of T cell blasts, and in differentiation of precursor T cells into functional effector T cells of various types (13–15). We have shown before that pre-T_H cells differentiate under these conditions into functional T_H cells, which cooperate with B cells in response to group A streptococci (13). By thus activating T_H cells in limiting dilution, we observed that with decreasing concentrations of T cells, the expected decrease in the fraction of positive cultures was followed by an unexpected second increase and decrease. We have suggested that this bimodal LD curve reflects separate precursor populations with different frequencies (a high- and a low-frequency population), and a separate suppressor population that specifically suppresses the more frequent effector cell population (13). We now present data that add another peak of responsiveness to the two previously described, when the concentration of T cells is further decreased.

In contrast to our observations, many previous LD experiments have revealed single-hit curves for a variety of T cell functions (27–30). We feel that with polyclonal activation we determine not only the effector precursor T cells under study but also those T cells that can modify their generation and function. These regulatory T cells are most probably neglected by other LD protocols. Indeed, we feel that our ability to determine effector T cells as well as regulator T cells in the same LD experiment opens a new quantitative approach for studying immune regulation. We therefore decided to study our experimental system in more detail to exclude the possibility that our results are influenced by tissue culture artifacts.

The activation protocol used by us is well known from bulk culture experiments (31, 32): splenic T cells of normal mice are polyclonally activated in vitro such that effector cell functions appear that cannot be detected without activation. Activation can be achieved under LD conditions, down to rather low cell concentrations, and the function of the activated cells is subject to some form of regulation. Activation and regulation occur with the same titration curve independent of whether the T cells are placed into LD cultures after Con A activation in bulk culture (most experiments) or before Con A activation, as normal T cells (Fig. 8).

To exclude possible artifacts, we investigated whether "help" as observed in our system is classical T_H cell-mediated help by several criteria: (*a*) reconstitution of the PFC response of a spleen cell population depleted for T cells; (*b*) mediation of help by the cells titrated, and not by factors present in the Con A sup, used to convert normal T cells into T_H cells; (*c*) T cell nature of the helper cell; (*d*) antigen specificity and requirement of direct cell contact (hapten carrier linkage). To measure help as the limiting factor, we found it adequate to prepare a B cell population by treating normal spleen cells twice with an antiserum to Thy-1 and complement C′. It is clear that this leaves a certain contamination of the B cells with T cells (Figs. 3 and 8),

which, however, do not provide sufficient help for a primary response, if the total number of "B cells" added per well is kept at 3×10^5 (Fig. 3).

What is the origin of help in our system? This question is complicated by the fact that we use normal T cells that have been exposed to Con A and a variety of factors present in rat Con A sup. These molecules themselves help if their concentration is high enough (10% Con A sup), but not at a concentration (1% Con A sup) that corresponds to the estimated volume transferred by nonwashed T cells (10% of LD medium) (Fig. 6). Indeed, T cells mixed with 10% of LD medium (1% Con A sup) show less help than carefully washed T cells which by dilution should only transfer an equivalent of ~0.001% of Con A sup (Fig. 7). Virtually all help detected in our system therefore is of cellular origin.

Help from the titrated cells is largely due to T cells, because the peaks of responses are shifted to a lower total cell concentration if a population depleted for T cells is compared with one enriched for T cells (Fig. 8). Although we cannot formally prove that the three peaks found in each of the two populations actually correspond, the shifts are in the range expected, if there is a 20-fold difference in the total T cell numbers.

The T_H cells we find seem to be antigen specific and probably work by direct cell contact, as we do not observe a bystander effect (Table III). This is true over a large range of concentrations, including three peaks, and for two classically non-cross-reacting antigens. We have repeated this experiment twice with essentially the same result. Of course, one would like to test a broader range of antigens, including soluble proteins, to look for possible cross-reactions, especially at very low T cell concentrations. Also the possibility has to be formally excluded that antigen specificity is not directly a function of the titrated T cells, but is introduced into the system by some other mechanism, e.g., a limiting cell in the detection system. However, it appears to us that the easiest interpretation of the results is that pre-T_H cells differentiate into mature T_H cells that function in an antigen-specific way via direct cell contact.

What is the interpretation of the limiting-dilution curve? A large number of experiments done under similar conditions have given the same result: we find three peaks at ~40–200, 600–3,000, and 20,000–40,000 T cells/well. We find these using several different antigens (SRBC, CRBC, HRBC, and streptococcus A) in systems testing T_H cell activity, and we similarly find three peaks in systems testing cytotoxic T lymphocytes with specificity for trinitrophenol (33). Thus, the limiting-dilution curve is reproducible in a variety of systems testing different T effector functions. The final interpretation of these curves will not be possible until mathematical models have been established that permit the analysis of multi-hit results (see footnote 2). Preliminary results suggest that our initial interpretation of multi-phase limiting-dilution curves are correct to the extent that a mathematical analysis based on this interpretation yields curves very similar to those observed experimentally. The mathematical analysis is based on the assumption that we look at the result of cellular interactions and that each peak of responsiveness reflects a different pre-T_H population, which becomes suppressed at increasing cell concentrations. We hope that our experiments can provide information about how these interactions work and what the exact frequencies of each of these populations may be. Using our published data (13), Hoffmann was able to construct similar curves making assumptions similar to ours (34). We therefore think that models will be identified that fit the data, allow the

exact estimation of frequencies, and also make predictions about regulation that can be tested in further experiments.

In addition to questions concerning regulation, the second important message of our experiments concerns the frequency estimates of pre-T_H cells. Because of missing mathematical models we can only do some not quite appropriate estimates (Table II). Nevertheless, our experiments show that whatever the mechanism is in detail, something like one T cell out of 20–100 can produce what we call "antigen-specific help." Therefore, frequencies of "specific" pre-T_H cells have to be in that order of magnitude. At present it appears unclear how this surprisingly high frequency can be reconciled with the apparent antigen specificity. So far our results add further constraints to estimates about the size of the T cell repertoire and to theories on the molecular mechanism of antigen recognition by T cells.

Summary

Splenic T cells exposed to concanavalin A (Con A), and subsequently to factors produced by rat spleen cells in response to Con A (Con A sup), aquire the ability to function as helper T (T_H) cells in response to xenogeneic erythrocytes (RBC). Help is measured as the reconstitution of the plaque-forming cell response of a spleen cell population depleted of T cells by treatment with anti-Thy-1 serum and complement. We propose that precursor T_H cells differentiate during the in vitro treatment into mature T_H cells. As differentiation occurs under limiting dilution conditions, an estimation of the precursor frequency should in principle be possible. However, a single-hit Poisson distribution does not fit our data. Instead, we observe, dependent on the T cell concentration, three separate "peaks" of response. In many experiments, using sheep, horse, and chicken RBC as antigens, we reproducibly find these "peaks" at 40–190, 600–3,000, and 20,000–100,000 T cells, placed into limiting dilution cultures, respectively.

By various experiments we can show that the helper activity is not due to passively transferred rat factors, but to the titrated cells themselves. The active cell is a T cell that appears to function in an antigen-specific way and to require direct cell contact to do so. It thus resembles the classical helper T cell.

As we find precursor T_H cells already at very low concentrations of T cells, we titrated the range between 0 and 100 T cells/well carefully. The bent shape of the titration curves does not always allow a statistically satisfying regression analysis, and we therefore cannot estimate precise precursor frequencies from every experiment. However, a common sense argument can be made that these frequencies must be on the order of 1/10–1/100 T cells.

We propose that the limiting dilution curves obtained in this system most likely reflect fundamentally important cellular interactions that regulate immunological effector functions. We favor a concept of independently interacting sets of helper and suppressor T cells of various frequencies, but other models are possible.

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References

1. Paetkau, V., G. Mills, S. Gerhart, and V. Monticone. 1976. Proliferation of murine thymic lymphocytes in vitro is mediated by the Con A-induced release of a lymphokine (co-stimulator). *J. Immunol.* **117:**1320.
2. Gillis, S., and K. A. Smith. 1977. Long term culture of tumor specific cytotoxic T cells. *Nature (Lond.).* **268:**154.
3. Andersson, J., K. O. Grönvik, E. L. Larsson, and A. Coutinho. 1979. Studies on T lymphocyte activation. I. Requirement for the mitogen-dependent production of T cell growth factors. *Eur. J. Immunol.* **9:**581.
4. Nabholz, M., H. D. Engers, W. Collaro, and M. North. 1978. Cloned T cell lines with specific cytolytic activity. *Curr. Top. Microbiol. Immunol.* **81:**176.
5. Baker, P. E., S. Gillis, and K. A. Smith. 1979. Monoclonal cytolytic T cell lines. *J. Exp. Med.* **149:**273.
6. v. Boehmer, H., H. Hengartner, M. Nabholz, W. Lernhardt, M. H. Schreier, and W. Haas. 1979. Fine specificity of a continuously growing killer cell clone specific for H-Y antigen. *Eur. J. Immunol.* **9:**592.
7. Glasebrook, A. L., M. Sarmiento, M. R. Loken, D. P. Dialynas, J. Quintans, L. Eisenberg, C. T. Lutz, D. Wilde, and F. W. Fitch. 1981. Murine T lymphocyte clones with distinct immunological functions. *Immunol. Rev.* **54:**225.
8. Watson, J. 1979. Continuous proliferation of murine antigen specific helper T lymphocytes in culture. *J. Exp. Med.* **150:**1510.
9. Glasebrook, A. L., and F. W. Fitch. 1979. T cell lines which cooperate in generation of specific cytolytic activity. *Nature (Lond.).* **278:**171.
10. Schreier, M. H., N. N. Iscove, R. Tees, L. Aarden, and H. v. Boehmer. 1980. Clones of killer and helper T cells: growth requirements, specificity and retention of function in long term culture. *Immunol. Rev.* **51:**315.
11. Fathman, C. G., and H. Hengartner. 1978. Clones of alloreactive T cells. *Nature (Lond.).* **272:**617.
12. Sredni, B., H. Y. Tse, and R. H. Schwartz. 1980. Direct cloning and extended culture of antigen-specific MHC-restricted, proliferating T lymphocytes. *Nature (Lond.).* **283:**581.
13. Eichmann, K., I. Falk, I. Melchers, and M. M. Simon. 1980. Quantitative studies on T cell diversity. I. Determination of the precursor frequencies for two types of streptococcus A-specific helper cells in non-immune, polyclonally activated splenic T cells. *J. Exp. Med.* **152:**477.
14. Goronzy, J., U. Schaefer, K. Eichmann, and M. M. Simon. 1981. 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 cell. *J. Exp. Med.* **153:**857.
15. Eichmann, K., J. Goronzy, U. Hamann, P. H. Krammer, R. C. Kuppers, I. Melchers, M. M. Simon, and G. Zahn. 1981. Clonal analyses of helper and cytotoxic T cells: multiple, independently regulated precursor sets of frequencies suggesting a limited repertoire. *In* Isolation, Characterization, and Utilization of T Lymphocytes. C. G. Fathman and F. W. Fitch, editors. Academic Press, Inc., New York. 233.
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. Click, R. E., L. Benck, and B. S. Alter. 1972. Immune responses in vitro. I. Culture conditions for antibody synthesis. *Cell. Immunol.* **3:**264.
18. Ostle, B. 1963. Statistics in Research. The Iowa State University Press, Ames, Iowa. 75.
19. Lefkovits, I., and H. Waldmann. 1979. Limiting Dilution Analysis of Cells in the Immune System. Cambridge University Press, Cambridge, England. 1–257.
20. Mishell, R. J., and R. W. Dutton. 1966. Immunization of normal mouse spleen cell

suspensions in vitro. *Science (Wash. D. C.).* **153:**1004.
21. Quintans, J., and I. Lefkovits. 1973. Precursor cells specific to sheep red cells in nude mice. Estimation of frequency in the microculture system. *Eur. J. Immunol.* **3:**392.
22. Watson, J., and C. Whitlock. 1978. Effect of a synthetic adjuvant on the induction of primary immune responses in T cell depleted spleen cultures. *J. Immunol.* **21:**383.
23. Schreier, M. H. 1978. B cell precursors specific to sheep erythrocytes. Estimation of frequency in a specific helper assay. *J. Exp. Med.* **148:**1612.
24. Tannenberg, W. J. K., and A. N. Malaviya. 1968. The life cycle of antibody-forming cells. I. The generation time of 19S hemolytic plaque-forming cells during the primary and secondary responses. *J. Exp. Med.* **128:**895.
25. Ostle, B. 1963. Statistics in Research. The Iowa State University Press. Ames, Iowa. 132.
26. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* **126:**1614.
27. Waldmann, H., I. Lefkovits, and J. Quintans. 1975. Limiting dilution analysis of helper T cell functions. *Immunology.* **28:**1135.
28. Skinner, M. A., and J. Marbrook. 1976. An estimation of the frequency of precursor cells which generate cytotoxic lymphocytes. *J. Exp. Med.* **143:**1562.
29. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatibility antigen activated cytotoxic T lymphocytes. II. Estimation of the frequency and specificity of precursors. *J. Exp. Med.* **145:**508.
30. Swain, S. L., P. R. Panfili, R. W. Dutton, and I. Lefkovits. 1979. Frequency of allogeneic helper T cells responding to whole H-2 differences and to an H-2K difference alone. *J. Immunol.* **123:**1062.
31. Dutton, R. W. 1973. Inhibitory and stimulatory effects of concanavalin A on the response of mouse spleen cell suspensions to antigen. II. Evidence for separate stimulatory and inhibitory cells. *J. Exp. Med.* **138:**1496.
32. Tse, H., and R. W. Dutton. 1976. Separation of helper and suppressor T lymphocytes on a Ficoll velocity sedimentation gradient. *J. Exp. Med.* **143:**1199.
33. Hamann, U., K. Eichmann, and P. H. Krammer. 1982. Frequencies and regulation of trinitrophenyl-specific cytotoxic T precursor cells: immunization results in release from suppression. *J. Immunol.* In press.
34. Hoffmann, W. G. 1981. Nature or nurture? On the difference between helper and suppressor T cells in the framework of the symmetrical network theory. *J. Supramol. Struct. Cell. Biochem.* **5**(Suppl.):84.