

## QUANTITATIVE STUDIES ON T CELL DIVERSITY

### IV. Mathematical analysis of multiple limiting populations of effector and suppressor T cells

BY K. FEY, I. MELCHERS, AND K. EICHMANN

*From the Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.*

Limiting dilution (LD)<sup>1</sup> analysis is one widely used way to analyze properties of lymphocytes at the clonal level (1). Many previous experiments have primarily studied LD systems that provided single hit results and, therefore, could be interpreted using the O-term of the Poisson distribution (reviewed in references 1, 2). The purpose of such experiments was exclusively the determination of precursor frequencies for various effector lymphocytes.

Recently, we described a number of LD systems that suggested more than one limiting population of cells interacting with each other, rather than a single hit mechanism (3). These systems are aimed at studying T cells and are based on *in vitro* differentiation and/or clonal expansion by polyclonal activation rather than by specific antigen (4–7). In a few cases, results from antigen activation protocols have suggested multiple limiting cells as well (8–11, and I. Melchers, unpublished observation). We have previously proposed that in these LD systems not only the effector T cells under study but also regulatory T cells that can modify the generation of effector cells are detected (3–7).

Because we strongly feel that these experimental protocols not only provide information on precursor frequencies but also represent a novel quantitative approach to the study of immune regulation, we have decided to establish the mathematical basis for the correct estimation of the precursor frequencies of effector and regulatory T cells from multi-component limiting dilution results. In this paper we present our mathematical approach and demonstrate that we can reproduce our experimental results by assuming the existence of multiple paired populations of effector and suppressor cells for any given T cell effector function studied. T suppressor cells distribute independently of effector T cells. Within the framework of these assumptions, only in one biological model and with a very restricted choice of parameters can we obtain curves that fit the data satisfactorily. Thus, we think that we can interpret LD results not only with

<sup>1</sup> *Abbreviations used in this paper:*  $\phi$ , frequency;  $a$ , number of  $T_s$  needed for suppression of 1  $T_E$ ;  $A$ , number of  $T_s$  needed for suppression of all  $T_E$  in a culture well; Con A, concanavalin A, CTL, cytotoxic T lymphocytes;  $F_0$ , probability to find a negative culture; HTL, helper T lymphocytes; LD, limiting dilution;  $N$ , cell number;  $P$ , probability to find a given number of cells/culture;  $R$ , ratio  $\phi_s/\phi_E$ ; SRBC, sheep erythrocytes; TCGF, T cell growth factor;  $T_E$ , effector T cell or its precursor;  $T_s$ , suppressor T cell or its precursor;  $u = \langle j \rangle$ , mean multiplicity ( $=\phi_E \cdot N$ ) of  $T_E$ , distributed around the exact number  $j$  of  $T_E$ ;  $v = \langle i \rangle$ , mean multiplicity ( $\phi_s \cdot N$ ) of  $T_s$ , distributed around the exact number  $i$  of  $T_s$ .

respect to effector precursor frequencies but also with respect to suppressor precursor frequencies and with respect to a possible mechanism of immune regulation.

### Rationale of the Approach

We interpret LD experiments in which the progeny of limiting precursor T cell populations produce measurable effects by interacting with nonlimiting indicator cell populations. The limiting T cells may be either the precursors of T effector cells such as helper T cells or cytotoxic T cells, or the precursors of negatively regulating cells such as suppressor T cells. The nonlimiting indicator cells may be either B cells for the determination of helper T cells or target cells for the determination of cytotoxic T cells. To permit maturation of precursor cells into effector cells, a number of other nonlimiting compounds such as (T cell growth factor) TCGF and filler cells must be present in culture.

In this paper we refer to the various effector T cell precursors as  $T_E$ ,  $T_{E'}$ , etc, to the suppressor T cell precursors as  $T_S$ , and to the variable number of all limiting cells as  $N$ . The frequencies of  $T_E$  cells are referred to as  $\phi_E$ ,  $\phi_{E'}$ , etc. so that each  $T_E$  has a mean multiplicity of  $u_E = \phi_E \cdot N$ . The results of the experiments are represented as the number of nonresponding cultures  $F_0$  as a function of  $N$ ,  $F_0(N)$  or, more often,  $\log F_0(N)$ .

In the simplest case,  $\log F_0(N)$  is represented as a straight line and  $\log F_0(0) = 0$ . This result is commonly explained by a single hit model which proposes that only one type of  $T_E$  is limiting the response. In this case,  $F_0(N)$  is the probability that an event with the multiplicity  $u_E = \phi_E \cdot N$  fails to occur in  $N$  trials. According to Poisson, this probability is described by  $F_0(N) = e^{-u_E}$ , or  $\log F_0(N) = -\phi_E \cdot N$ . Therefore, the slope of  $\log F_0(N)$  is equal to  $-\phi_E$ .

While this is generally accepted as a correct interpretation of this type of result, we like to point out that the single hit model is merely *a model* that was identified among other possible ones to provide a sensible and simple interpretation for straight  $\log F_0(N)$ . With this reasoning we now attempt to identify models that provide equally sensible and simple interpretations for the non-straight  $\log F_0(N)$  we observe in our experiments.

### The Typical Result

In this paper we concern ourselves with LD data to which, according to the various regression line analyses described (12, 13), single straight lines cannot be fitted. Such data do not, however, deviate from linearity in a random way or in many different ways, but usually show the distinct characteristic arrangement of data points of the example shown in Fig. 1. As the cell number is increased, a seemingly linear curve with a negative slope is followed by a sharp kink and a positive slope, then again by a second seemingly linear negative slope. In this experiment, but not always, a second kink and a third negative slope follow. This type of result has been observed for a considerable number of different helper T cell and cytotoxic T cell precursors analyzed (summarized in reference 3).

We can demonstrate linearity for the negative slopes by regression line analyses (Table I and reference 6) and justify this mathematically even in a model with several limiting interacting  $T_E$  and  $T_S$  populations (see below). We therefore

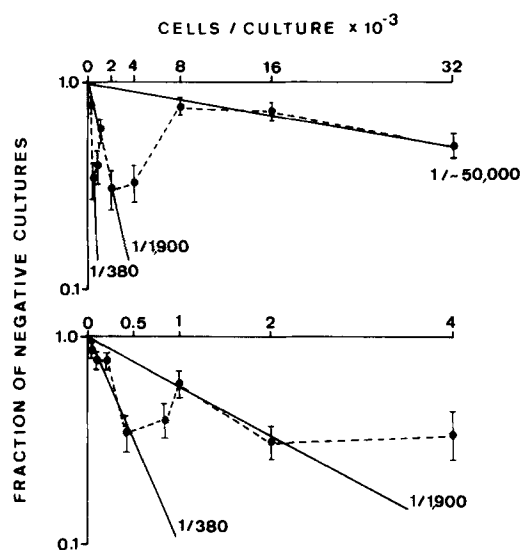


FIGURE 1. Limiting dilution analysis of cytotoxic precursor cells that lyse trinitrophenylated syngeneic target cells. Normal lymph node cells from strain B10.Br were placed in flat bottom microtiter plates in limiting dilution. The medium was supplemented with  $5 \mu\text{g/ml}$  concanavalin A (Con A) for 2 d and with 20% TCGF containing rat spleen cell Con A supernatant for an additional 7 d. Thereafter, each well was assayed for cytotoxic activity on  $^{51}\text{Cr}$ -labeled trinitrophenylated Con A-induced syngeneic spleen cell blasts. For technical details see references 4 and 5. Each data point was determined with 192 cultures; the vertical bars represent the 95% confidence limits for each point. Linear regression analysis according to the minimal  $\chi^2$  method. The lower frame is an extended representation of the cell concentrations up to 4,000 cells/culture; the upper frame shows all cell concentrations tested.

TABLE I  
Linear Regression Analysis of Negative Slopes Determined by LD Experiments on TNP Reactive CTL in Normal Lymph Node Cells\*

| Exp. | Slope 1                                     |               |               | Slope 2                                     |               |               | Slope 3                                     |               |               |
|------|---|---------------|---------------|---|---------------|---------------|---|---------------|---------------|
|      | Precursors <sup>‡</sup><br>$\times 10^{-5}$ | No. of points | $p^{\dagger}$ | Precursors <sup>‡</sup><br>$\times 10^{-4}$ | No. of points | $p^{\dagger}$ | Precursors <sup>‡</sup><br>$\times 10^{-5}$ | No. of points | $p^{\dagger}$ |
| 1    | $2.2 \pm 0.7$                               | 4             | 0.3           | $1.1 \pm 0.27$                              | 4             | 0.04          | $2.6 \pm 1.3$                               | 4             | 0.5           |
| 2    | $2.1 \pm 0.28$                              | 5             | 0.001         | $7.6 \pm 2.8$                               | 3             | 0.2           | $5.1 \pm 1.0$                               | 4             | 0.03          |
| 3    | $5.2 \pm 1.9$                               | 3             | 0.5           | $4.2 \pm 2.1$                               | 3             | 0.9           | $2.2 \pm 0.32$                              | 3             | 0.04          |
| 4    | $5.15 \pm 1.8$                              | 5             | 0.5           | $5.5 \pm 0.79$                              | 4             | 0.4           | —   | —             | —             |
| 5    | $2.12 \pm 1.01$                             | 4             | 0.3           | $3.9 \pm 1.16$                              | 4             | 0.08          | —   | —             | —             |
| 6    | —**   | —             | —             | $13.2 \pm 4.4$                              | 3             | 0.5           | —   | —             | —             |
| 7    | —   | —             | —             | $3.54 \pm 1.58$                             | 5             | 0.7           | —   | —             | —             |

\* Minimum  $\chi^2$  method used (12). For experimental details see Fig. 1 legend and references 3, 5.

<sup>‡</sup> Number of CTL precursors  $\pm$  95% confidence limits.

<sup>§</sup> All points on a negative slope used for analysis, including  $N = 0$ .

<sup>†</sup>  $P$  values  $> 0.05$  signify that slope is acceptable as linear. Thus, 11 out of 15 slopes in this survey are acceptable as linear.

\*\* Not all slopes are observed in all experiments.

define frequencies  $\phi_E$ ,  $\phi_{E'}$  from the negative slopes.

A survey of many experiments revealed that the kinks frequently occur at values in the order of  $F_0 = 0.37$  ( $F_0 = 0.37$  if  $\phi_E \cdot N = 1$ ). This is true for a wide range of  $\phi_E$  values observed ( $1/50$ – $1/10,000$ ). We therefore introduce as a new variable (instead of  $N$ ) the mean multiplicity  $u_E = \phi_E \cdot N$ ,  $u_{E'} = \phi_{E'} \cdot N \dots$  of each  $T_E$  population and conclude that the kinks occur at multiplicities in the order of

$u_E = 1$ . Thus, the position of the kink is dependent on the actual  $T_E$  multiplicities and independent of the cell concentrations in cultures. This argues for a biological mechanism and against tissue culture artefacts introduced by variations in cell densities. We thus assume, as do others (9, 10, 14), that a kink is caused by the influence of suppressor cells.

### Models for Suppression

Because of the observed “universality” of the “typical result” not only with respect to the analyzed T cell functions and specificities but also with respect to their variable frequencies, we attempt to identify models for suppressor cell function whose mathematical analysis allows reproduction of actual experimental data. From our experimental protocol we conclude that suppression must be caused by T cells that are titrated together with the  $T_E$  populations. This conclusion is the basis for all models.

We assume, for the sake of simplicity, that the experimental data are not influenced by unequal proliferation of different cell types. We further assume, for all models, that  $T_S$  and  $T_E$  are distributed independently of each other in a Poissonian fashion. Accordingly, the probability  $P_1$  to find exactly  $j$   $T_E$  that have a mean multiplicity of  $u = \phi_E \cdot N$ , is described by the equation:

$$P_1 = e^{-u} \cdot \frac{u^j}{j!}.$$

Similarly, for  $T_S$  with a mean multiplicity of  $v = \phi_S \cdot N$ , the probability  $P_2$  to find  $i$   $T_S$  is:

$$P_2 = e^{-v} \cdot \frac{v^i}{i!}.$$

If the ratio  $\phi_S/\phi_E$  of the frequencies is denoted by  $R$ ,  $v$  is given by  $v = R \cdot u$ . Initially, we restrict the analysis to one pair of  $T_E$  and  $T_S$  and, accordingly, one kink.

*Model 1: One  $T_S$  or Its Progeny is Sufficient to Suppress a Culture Well Independent of the Number of  $T_E$ .* Such a model has only one variable parameter  $R = \phi_S/\phi_E$ . The value for  $F_0(u)$  is given by the probability  $e^{-u}$  to find no  $T_E$  plus the probability to find any number of  $T_E$  together with at least one  $T_S$ . Thus, the probability  $F_0(u)$  to find a negative well is described by the equation:

$$F_0(u) = e^{-u} + (1 - e^{-u}) \cdot (1 - e^{-v}) \quad (1)$$

As demonstrated before by Hoffmann (15), and as shown in Fig. 2, no variation for  $R$  allows the reproduction of a kink as in Fig. 1. Therefore, all variations of this model as well as all models that assume a  $T_S$  frequency smaller than the  $T_E$  frequency have to be discarded. We like to point out that this model was initially put forward by us to interpret our data intuitively (4).

*Model 2:  $T_S$  or Their Progeny Suppress a Nonlimiting, Constant, Essential Component in LD Cultures.* Nonlimiting, constant and essential components of LD cultures are (a) the B cell indicator population for T helper cells, (b) the target cell indicator population for cytotoxic T cells, and (c) the TCGF or other components

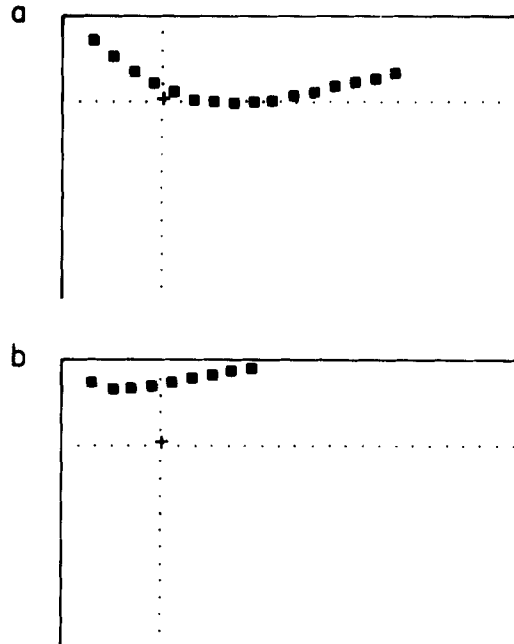


FIGURE 2. Computer printout showing two numerical examples for model 1. Upper frame:  $R = 0.5$ ; lower frame:  $R = 2$ . Horizontal axis:  $N$ , vertical axis:  $\log F_0$ . The vertical dashed line represents  $u = 1$ , the horizontal dashed line is  $\log F_0 = \log 0.37$ . The examples show that for  $R > 1$  one does not reach sufficiently low values for  $\log F_0$ , and for all  $R < 1$  the kink remains too shallow to fit experimental data.

in the culture (i.e. filler cells) necessary for survival, proliferation, and/or maturation of  $T_E$ . Although it is possible that  $T_S$  act on B cells, it is unattractive to assume that  $T_S$  act on target cells for cytotoxic T lymphocytes (CTL). The only common mechanism to explain the similar helper T lymphocyte (HTL) and CTL results in this model would therefore be a neutralization of essential culture constituents such as TCGF by  $T_S$ .

Such a model has two variable parameters:  $R = \phi_S/\phi_E$  and the number  $A$  of  $T_S$  necessary to suppress a culture. As the target component for  $T_S$  is constant,  $A$  is invariant within one experiment. The corresponding formula is derived by adding the probability  $e^{-u}$  to find no  $T_E$  to the probability to find any number of  $T_E$  together with at least  $A T_S$ , that is:

$$F_0(u) = e^{-u} + (1 - e^{-u}) \cdot \left(1 - \sum_{i=0}^{A-1} P_2\right). \quad (2)$$

As can be seen in Fig. 3, such a model can be used to reproduce experimental data, i.e. from an analysis of T helper cells reactive to sheep erythrocytes (SRBC) (6) assuming high numbers for both  $R$  and  $A$ .

Models of this sort are not attractive from a biological viewpoint, however. The difficulty arises in attempts to explain the occurrence of second  $T_E'$  populations that must be insensitive to the  $T_S$  population. If cell numbers are reached that contain sufficient  $T_S$  for inactivation of an essential culture component, no

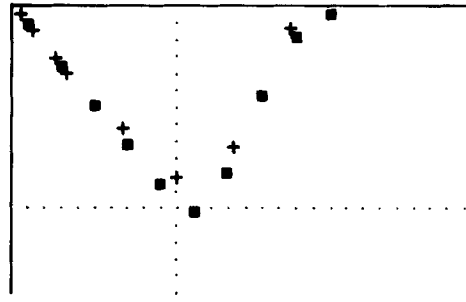


FIGURE 3. Computer printout showing a numerical example for model 2 (■) in comparison to experimental data (+) obtained from a LD experiment on the precursors of helper T cells reactive to SRBC. For technical details see reference 6. The parameters chosen for best fit are  $R = 27$  and  $A = 39$ . Horizontal axis  $N$ , vertical axis:  $\text{Log } F_0$ . The frequency is  $\phi = \frac{1}{2}$ , the vertical dashed line gives  $u = 1$  and the horizontal dashed line represents  $\log F_0 = \log 0.37$ .

response should be possible at further increase of cell numbers. This is not a formal exclusion of model 2, however, because second  $T_E$  populations may require smaller concentrations of such culture components, i.e. TCGF.

*Model 3:  $T_S$  or Their Progeny Suppress  $T_E$  Precursors.* These models assume that the  $T_E$  precursors themselves are the targets of  $T_S$ . Model 1 is the simplest version of this model and has been eliminated. A second version is that a given fixed number  $a$  of  $T_S$  is required to inactivate each  $T_E$ . This model has two variable parameters:  $R = \phi_S/\phi_E$  and  $a$ . In contrast to model 2, the number  $A$  of  $T_S$  required to suppress a culture well is not invariant but increases proportional to the number  $j$  of  $T_E$ :  $A = a \cdot j$ . The probability  $F_0(u)$  of finding a negative well in this model is described by the equation:

$$F_0(u) = \sum_{j=0}^{\infty} P_1 \cdot \left( \sum_{i=a \cdot j}^{\infty} P_2 \right). \quad (3)$$

As shown in Fig. 4, no choice of parameters in this version of model 3 allows satisfactory reproduction of experimental data. This is not immediately obvious when  $a$  is chosen considerably greater than  $R$ . (Fig. 4b). The biphasic curve produced, however, only superficially resembles our experimental data.

Therefore, we have examined a third version of model 3 in which the number  $a$  of  $T_S$  required to inactivate 1  $T_E$  is variable within one experiment and depends on the total number  $j$  of  $T_E$  in a culture well. This model also has two variable parameters:  $R = \phi_S/\phi_E$  and  $A(j)$ , the number of  $T_S$  required to suppress  $j$   $T_E$ . Among the various possibilities for  $A(j)$  only those make biological sense that are monotonously increasing and interpolate between  $A = \text{constant}$  (as in model 2) and  $A(j) = a \cdot j$  (as in model 3, second version). It makes sense to assume such a dependence for  $A(j)$  particularly if the conditions of tissue culture are considered. At low cell concentrations, relevant suppressive interactions between  $T_S$  and  $T_E$  may have a lower probability and may therefore require greater numbers of  $T_S$  than at high cell concentrations.

If  $A(j)$  is the number of  $T_S$  required to suppress  $j$   $T_E$ , the probability  $F_0(u)$  of

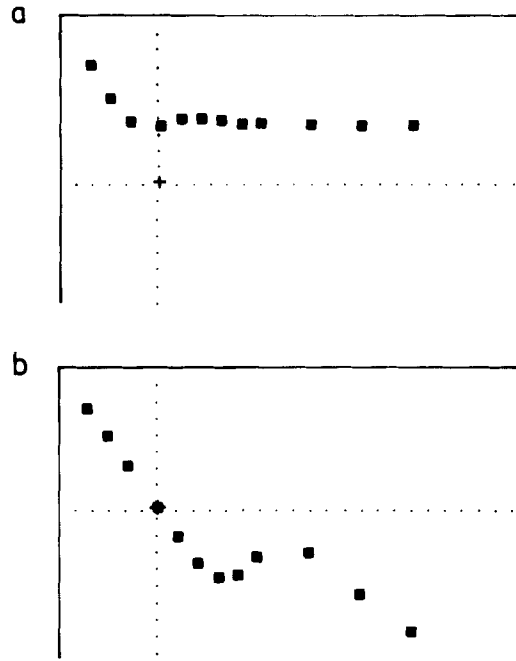


FIGURE 4. Computer printouts of two numerical examples of the second version of model 3. Frame *a*:  $R = 10$ ,  $a = 10$ ; Frame *b*:  $R = 20$ ,  $a = 40$ . Horizontal axis:  $N$ , vertical axis:  $\log F_0$ . The vertical dashed line gives  $u = 1$ , the horizontal dashed line  $\log F_0 = \log 0.37$ . Remarkably, a kink is produced without a second  $T_E$  population when  $a \gg R$  (frame *b*). However, its shape is unlike that observed in most experiments.

finding a negative culture in this version of model 3 is described by the equation:

$$F_0(u) = \sum_{j=0}^{\infty} P_1 \left( \sum_{i=A(j)}^{\infty} P_2 \right), \quad (4)$$

where  $A(0) = 0$ .

This equation is the most universal and can be used to calculate all models described in this paper as well as other models on  $T_E/T_S$  interactions. Thus, Eq. 3 can be derived from Eq. 4 by setting  $A(j) = a \cdot j$ . Eq. 2 can be derived from Eq. 4 by setting  $A(j) = A = \text{constant}$  for  $j > 0$  and  $A(0) = 0$ . Eq. 1 can be derived by setting  $A(j) = 1$ . In general terms, Eq. 4 means that negative wells are produced in all cases in which either the number  $j$  of  $T_E$  is 0, or  $j$  is  $>0$  but the number  $i$  of  $T_S$  is  $\geq A(j)$  and therefore sufficient to suppress  $j$   $T_E$ . In contrast, a positive culture is produced if  $j$  is  $>0$  and  $i < A(j)$ .

Eq. 4 can be derived by a detailed argument that parallels the derivation of the Poisson distribution as the limit of binomial distributions (16).

*Application of Model 3 to Experimental Data.* We now search for choices of the two parameters  $R = \phi_S/\phi_E$  and  $A(j)$  with which experimental curves can be reproduced. Since we have excluded model 1, in which  $A(j) = 1$ , we can also exclude  $R < 1$  for all choices of  $A(j)$ . Thus, the frequency of  $T_S$  must in any case be greater than the frequency of  $T_E$  ( $R > 1$ ).

Since the first kink occurs usually at  $u_E \geq 1$ , we have to postulate that for  $u_E$

$< 1$  the major part of the  $T_S$  distribution must lie at numbers lower than  $A(j)$  and therefore be less than sufficient for suppression. For  $u < 1$  this applies to  $j = 0, j = 1$ . Thus, the probability of finding a suppressed well at cell numbers smaller than  $u$  is low and, therefore,  $F_0$  is influenced primarily by the probability of finding no  $T_E$ . Thus,  $\log F_0$  of the initial slope is linear as in a single hit model (see above). The distribution of the  $T_S$  population at  $u < 1$  is schematically indicated in Fig. 5a, which shows that although the mean multiplicity  $v$  of  $T_S$  is several times greater than the mean multiplicity  $u$  of  $T_E$ , the main part of  $T_S$  is distributed below a given value for  $A(j)$  and therefore does not contribute to suppression.

The kink is usually reached when  $u \geq 1$ . Thus, with increasing cell numbers, the major part of the  $T_S$  distribution must be shifted to numbers  $> A(j)$ , as

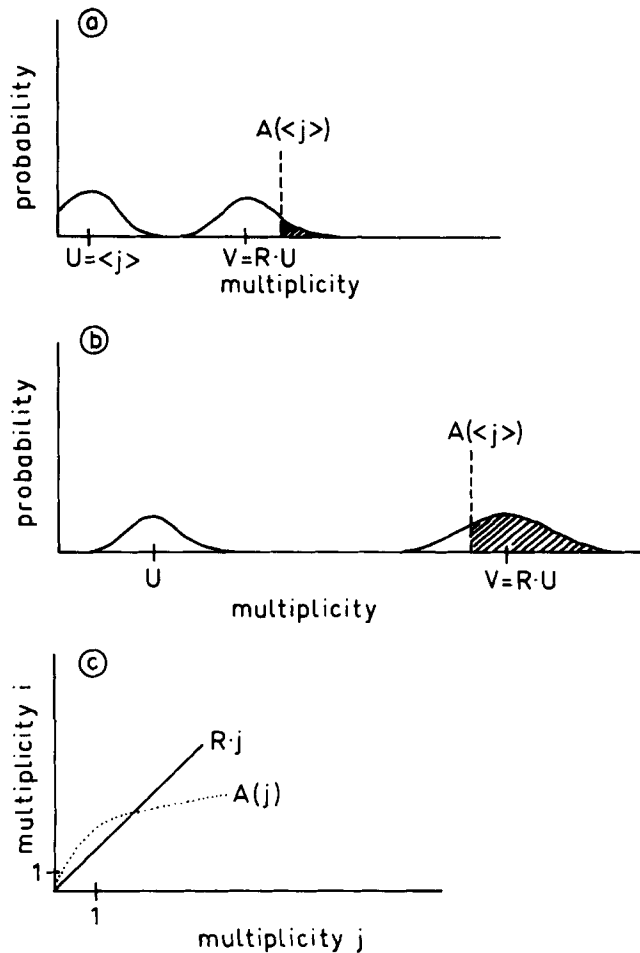


FIGURE 5. Distributions of the  $T_S$  population that contribute to suppression of  $T_E$  (shaded areas).  $T_E$  are distributed around  $u$  and  $T_S$  are distributed around  $v$ . For small  $u$  (frame a), the main part of the  $v$  distribution is below the limit  $A(j)$ . For high  $u$  (frame b), the main part of the  $v$  distribution is above  $A(j)$ . From this follows a corresponding function for  $A(j)$  as shown in frame c.



illustrated in Fig. 5*b*. Hence,  $A(j)$  increases less than proportional to  $N$  and we conclude a general shape for  $A(j)$  as illustrated in Fig. 5*c*.  $R \cdot j$  indicates the increasing numbers of  $T_S$ /culture. The  $j$  value of the intercept between  $A(j)$  and  $R \cdot j$  indicates the point  $u = \langle j \rangle$  at which the number of  $T_S$  becomes sufficient for suppression.

The angle of the intercept between  $A(j)$  and  $R \cdot j$  determines the rate at which  $F_0$  approaches 1 and, therefore, the shape of the positive slope of the kink. To obtain the result in Fig. 6, we therefore examined a number of different functions for  $A(j)$  that provide shapes similar to that in Fig. 5*c*. As can be seen from Fig. 6*a* and *b*, we can find functions for  $A(j)$  that, in combination with the appropriate choice of  $R$ , provide a satisfactory reproduction of experimental data. We like to point out that a value of around 20 for  $R$  is not unique to this experiment but has been found for several T cell types, including cytotoxic T cells reactive to TNP and helper T cells reactive to Streptococcus A and to SRBC. The examples in Fig. 6*c* and *d* show that even small variations of >30% in either  $R$  or  $A(j)$  lead to pronounced changes in the shape of the curves. We have also excluded that variation in one parameter can be compensated by variation of the other. Hence, only one choice for each parameter can reproduce the experimental data.

We like to point out that the complete function  $A(x)$  as a polynomial  $A(x) = \sum_{i=0}^p a_i x^i$ , or as a Taylor expansion cannot be determined by the analysis of  $F_0$  alone, particularly with only a finite number of culture wells per cell number. In the range where  $R \cdot j \gg A(j)$ , i.e. in the range of full suppression,  $F_0$  is equal to 1 regardless of  $A(j)$  being constant or slightly increasing. There clearly exist several possible functions that can reproduce the data in the range of cell concentrations in which  $R \cdot j$  intercepts  $A(j)$ . Exact information on  $A(j)$  could presumably be obtained by the analysis of all  $F$  values, i.e. the fraction of responders with 1, 2, 3, . . .  $n$  number of clones. Since the function for  $A(j)$  is presumably strongly influenced by the conditions of tissue culture, we think that its exact definition is not of outstanding interest.

*Extension of Model 3 to More Than One Pair of  $T_E/T_S$ .* As stated above (Fig. 1), the typical result to be interpreted shows a second negative slope after the kink and, sometimes, a third negative slope after a second kink when cell numbers are further increased. For the second slope, we assume an additional  $T_{E'}$  population that is sensitive to  $T_S$  and that has a mean multiplicity  $u' = \phi_{E'} \cdot N$ . The second slope is then described by the probability  $\phi_{E'} \cdot N$  to find no  $T_{E'}$ . The following equation applies to the extended model:

$$F_0(T_E, T_S, T_{E'}) = F_0(T_E, T_S) \cdot e^{-\phi_{E'} \cdot N}$$

or

$$\log F_0(u, u') = \log F_0(u) - \phi_{E'} \cdot N.$$

Hence,  $-\phi_{E'} \cdot N$  is a straight line that has to be added to the  $T_E/T_S$  term of  $\log F_0$ . This is demonstrated in Fig. 7, which shows a reproduction of the first and second kink of Fig. 1 by a summation of their  $F_0$  values. Above a given  $N$ ,  $\log F_0(T_E/T_S)$  becomes 1 and, therefore, the curve for  $T_{E'}$  and putative  $T_{S'}$ ,  $T_{E''}$ , etc. can be calculated irrespective of and analogous to  $T_E/T_S$ . The difference to model 3 is only that the first slope has to be taken as the sum of the first and all other frequencies  $\phi_E + \phi_{E'} + \phi_{E''} \dots$

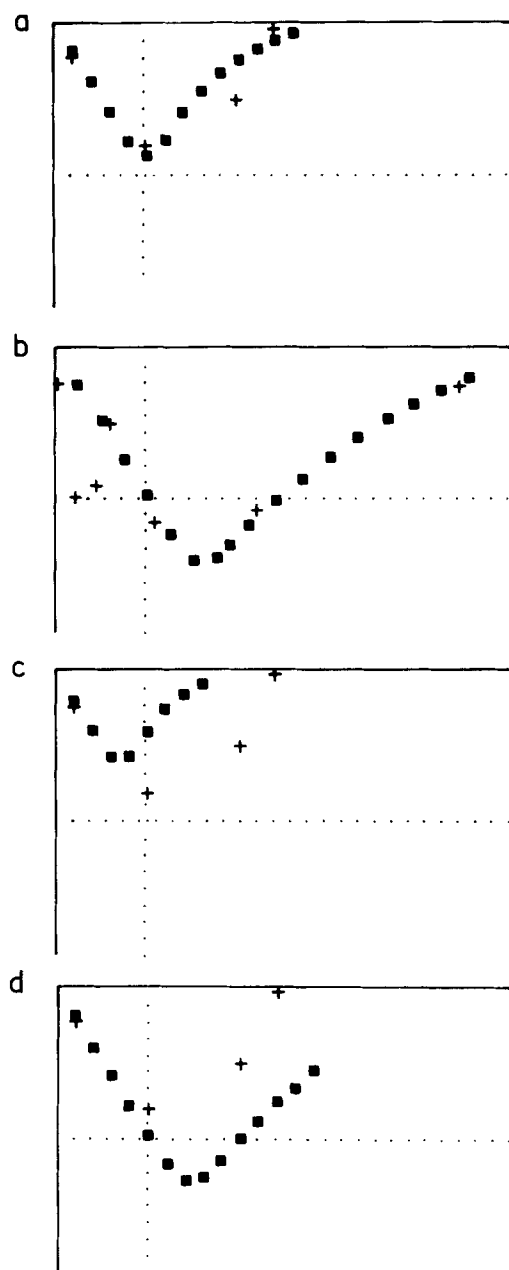


FIGURE 6. Numerical examples of model 3, third version (■) compared with the experimental data shown in Fig. 1 (+). Experimental data are corrected for the contribution of only one population. Horizontal axis:  $N$ , vertical axis:  $\log F_0$ . Vertical dashed line:  $u = 1$ , horizontal dashed line:  $\log F_0 = \log 0.37$ . Frame *a*: first kink ( $\phi = 1/380$ ), parameters are  $R = 22$  and  $A(j) = 8 + 20 \cdot \sqrt{j}$ . Frame *b*: second kink ( $\phi = 1/1,900$ ), parameters are  $R = 20$  and  $A(j) = 8 + 32 \cdot \sqrt{j}$ . Frames *c* and *d* show lack of fit to the first kink if wrong parameters are chosen. Frame *c*:  $R = 15$ ,  $A(j) = 8 + 20 \cdot \sqrt{j}$ . Frame *d*:  $R = 22$ ,  $A(j) = 5 + 15 \cdot \sqrt{j}$ . The examples show that a small variation (<30%) of each parameter leads to total loss of fit. For all functions  $A(j)$  the following convention is adopted:  $A(0) = 0$  and  $A(j)$  is replaced by the next higher integer for noninteger values for  $A(j)$ .

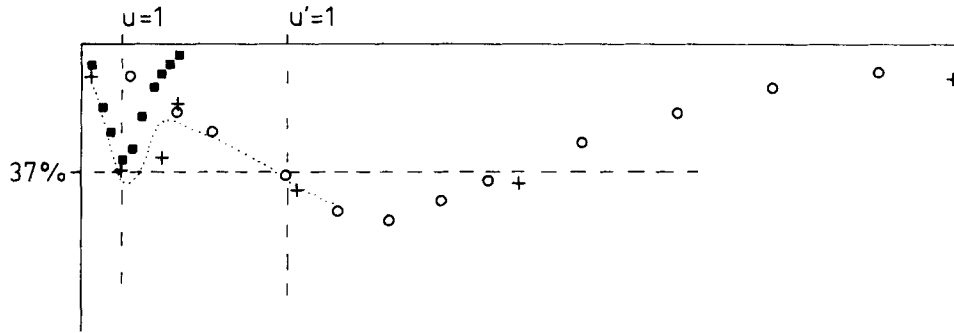


FIGURE 7. Numerical example of model 3, third version, reproducing the first and second kink in Fig. 1 (+). Horizontal axis:  $N$ , vertical axis:  $\log F_0$ ,  $u = \phi_E \cdot N$ ,  $u' = 5u = \phi_E' \cdot N$ . Vertical dashed lines  $u = 1$ ,  $u' = 1$ . Horizontal dashed line:  $\log F_0 = \log 0.37$ . Parameters  $R$  and  $A(j)$  same as in Fig. 6 *a* and *b*. Contribution of first kink: ■, contribution of second kink: ○, sum of both contributions: . . . . .

### Conclusions

In this report we have analyzed results from LD experiments in mathematical terms. We could show that the typical shape of the LD results of polyclonally activated T cells can be reproduced by assuming independent pairs of populations of effector and suppressor cells. Suppressive interactions occur within one pair but not between pairs.

We have tried a number of models for suppressor cell function. Among them, two models can serve to reproduce experimental data. In one of them (model 2), an essential culture component such as TCGF is assumed to be the target of suppression. In the other (model 3, third version), T effector cell precursors are identified as the targets of suppression. We like to point out that neither model makes predictions about the mode of suppression (idiotypic, antigen-specific, factor-mediated, etc.). In our opinion, the latter makes more sense because suppression is restricted to  $T_E$  within one of several paired  $T_E/T_S$  populations. In any case and within both possible models, the parameters that can serve to reproduce experimental data are very similar. We therefore restrict the discussion to model 3, third version.

Within this model, only a narrow choice of parameters can serve to fit experimental data. Thus, suppressor cell frequencies must be  $\sim 20$ -fold greater than effector cell frequencies. In addition, there is a rather large number of  $T_S$  required for suppression of 1  $T_E$ . This number, however, can not be definitively determined because there appear to be variations with cell densities and, presumably, also with the conditions of tissue culture. The lower the T cell density, the larger the number of  $T_S$  required for suppression of 1  $T_E$ . This phenomenon is the reason why the dilution of cells allows the expression of effector functions that are suppressed at high cell densities.

We have previously determined the frequencies of many  $T_E$  to be in the order of  $1/200$  (3), in some cases  $>1/50$  (6). If our model were correct,  $T_S$  frequencies may therefore exceed  $1/10$  to  $1/2.5$ . These results raise the question of T cell specificity even stronger than the high frequencies of many effector T cells.

Moreover, these results may add further restraints to network (17) or circuit (18) theories on immune regulation.

### Summary

Limiting dilution (LD) analyses of polyclonally activated T cells yielded results suggesting the existence of multiple paired populations of effector and suppressor precursors for a number of different T cell functions and specificities analyzed. These populations occur at graded frequencies and suppression occurs within a pair but not between pairs. In this paper, we establish the mathematical basis for the interpretation of these multi-component limiting dilution results. First, we derive equations for a number of mathematical models and identify one model that both makes biological sense and can be used to reproduce experimental data. Second, within this model, we identify parameters such as the frequency of suppressive cells and the number of suppressive cells required for suppression. The results suggest that within each paired population, suppressor precursors are 20 times more frequent than effector precursors. Furthermore, a similar but variable excess of suppressor cells is required for suppression to become effective. Together with the high frequency (1/50–1/500) of most effector T cell precursors previously reported, the results suggest that up to 40% of the T cells can become involved in suppression of an antigen-specific effector T cell population. These studies may provide exact estimates for predictions to be tested in experiments on immune regulation.

The authors are grateful to Drs. M. Simon, R. Kuppers, H. U. Weltzien, and G. Dueck for helpful discussions and suggestions.

*Received for publication 22 November 1982 and in revised form 21 March 1983.*

### References

1. Lefkovits, I., and H. Waldman; editors. 1979. *Limiting dilution analysis of cells in the immune system*. Cambridge University Press, Cambridge, U.K.
2. Miller, R. G. 1982. Analysis of T cells by limiting dilution, an overview. *In* *Isolation, Characterization and Utilization of T Lymphocyte Clones*. C. G. Fathman, and F. W. Fitch, editors. Academic Press, New York. p. 220.
3. Eichmann, K., J. Goronzy, U. Hamann, P. Krammer, R. C. Kuppers, I. Melchers, M. M. Simon, and G. Zahn. 1982. Clonal analysis of helper and cytolytic T cells: multiple independently regulated precursor sets of frequencies suggesting a limited repertoire. *In* *Isolation, Characterization and Utilization of T Lymphocyte Clones*. C. G. Fathman, and F. W. Fitch, editors. Academic Press, New York. p. 233.
4. Eichmann, K., I. Falk, I. Melchers, and M. M. Simon. 1980. Quantitative studies on T cell diversity. I. Determination of the precursor frequencies of two types of streptococcus A-specific helper cells in non-immune, polyclonally activated splenic T cells. *J. Exp. Med.* 152:477.
5. 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 cells. *J. Exp. Med.* 153:857.
6. Melchers, I., K. Fey, and K. Eichmann. 1982. Quantitative studies on T cell diversity. III. Limiting dilution analysis of precursor cells for T helper cells reactive to

- xenographic red blood cells. *J. Exp. Med.* 156:1587.
7. Hamann, U., K. Eichmann, and P. Krammer. 1983. Frequencies and regulation of trinitrophenyl-specific cytotoxic T precursor cells. Immunization results in release from suppression. *J. Immunol.* 130:7.
  8. Simon, M. M., J. Goronzy, U. Schaefer, and K. Eichmann. 1981. Frequency analysis of alloantigen specific T helper cell precursors in unselected T cells and in selected Lyt-1 T cell subpopulations after specific activation. In *Mechanisms of Lymphocyte Activation*. H. Kirchner, and K. Resch, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. p. 270.
  9. Weyand, C., J. Goronzy, and G. Hämmerling. 1981. Recognition of polymorphic H-2 domains by T lymphocytes. I. Functional role of different H-2 domains for the generation of alloreactive cytotoxic T lymphocytes and determination of precursor frequencies. *J. Exp. Med.* 154:1717.
  10. Macphail, S., I. Yron, and O. Stutman. 1982. Primary in vitro cytotoxic T cell response to non-major histocompatibility complex alloantigens in normal mice. *J. Exp. Med.* 156:610.
  11. Miller, R. A., and O. Stutman. 1982. Enumeration of IL-2 secreting helper T cells by limiting dilution analysis and demonstration of unexpectedly high levels of IL-2 production for responding cell. *J. Immunol.* 128:2258.
  12. Taswell, C. 1981. Limiting dilution analysis for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* 126:1614.
  13. Fazecas de St. Groth, S. 1982. The evaluation of limiting dilution assays. *J. Immunol. Methods.* 49:1211.
  14. Pfizenmeier, K., M. Röllinghoff, and H. Wagner. 1982. Mitogen induced clonal expansion of T cells under limiting dilution conditions. A new approach to study the repertoire of CTL-precursors. 5th European Immunology Meeting, Abstract Book. p. 273.
  15. 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. Suppl.* 5:84.
  16. Feller, W. 1968. *An Introduction to Probability Theory and its Application*. Vol. 1, 3rd Ed. John Wiley & Sons, New York. p. 153.
  17. Jerne, N. K. 1974. The immune system, a web of V domains. *Harvey Lect.* 70:93.
  18. Cantor, H., and R. Gershon. 1978. Immunological circuits: cellular composition. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 38:2058.