Immunology 1985 **55** 703

Limiting dilution analysis of the frequencies of helper and suppressor T cells in untreated and TNP-treated BALB/c mice: response as a consequence of perturbation of a stable steady state

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Accepted for publication 15 March 1985

Summary. B-cell response and suppression of response were analysed with respect to changes in frequency or composition of regulatory elements in comparison to untreated BALB/c mice. As antigenic stimulus the hapten TNP was used. Suppression was induced by intravenous (i.v.) injection of TNP-haptenized syngeneic lymphocytes; for inducing response, mice received TNP-horse red blood cells (HRBC). As the read-out system, plaque-forming cells (PFC) were determined after either the addition of naive B cells only [determination of the frequency of helper T-cells (T_H)], or the addition of naive B cells plus saturating doses of TNP-specific T_H [determination of the frequency of suppressor T-cells (T_S)].

The data indicate that, in untreated animals, a network of help and suppression is pre-existing, wherein T_H are more frequent (1/1471) than T_S (1/4413), but T_S are dominant, i.e. when the fraction of non-responding cultures (F_0) (determination of help) was plotted, an inversion of the curve was seen at high numbers of cells per well; however, the fractions of responding cultures (F_+) (determination of suppression) could be plotted on a straight line.

Application of antigen in suppressogenic or immunogenic form resulted in a two- to four-fold increase in the corresponding regulatory population but, concomitantly, a minor increase in the frequency of the mutual counteracting population was observed. Irrespective of any immunization schedule, T_H were more frequent than T_S.

The overall shape of the 'helper' and 'suppressor' curves with spleen cells (SC) from suppressed mice resembled—besides changes in the frequencies—those obtained with SC of untreated animals. This corresponds to the maintenance of a state of unresponsiveness as in untreated animals. However, when SC from primed or suppressed plus primed mice were analysed, a different type of curve was obtained. Suppressor curves no longer followed a ratio dominance model; instead, at high numbers of cells per well, the frequency of wells with suppressive activity decreased. Correspondingly, in helper curves, a second slope of increasing numbers of wells with helper activity was seen at a high input of cells. Hence, response cannot be explained solely by expansion/activation of T_H, but obviously a third regulatory population is involved which could not be detected in untreated animals. This third regulatory population could either be non-sup-

Abbreviations: BSS, Hanks' balanced salt solution; Con A, concanavalin A; F_0, fraction of non-responding cultures; F_+, fraction of responding cultures; HRBC, horse RBC; IL-2: interleukin-2; i.v., intravenous; PFC, plaque-forming cells; RPMI-s, RPMI-1640, supplemented with antibiotics, L-glutamine, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum; OVA, ovalbumin; RBC, red blood cells; SC, spleen cells; SRBC, sheep RBC; TNBS, trinitrobenzenesulphonic acid; TNP, trinitrophenyl; T_H; helper T cells; T_S, suppressor T cells.

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pressible T_H or—more likely, as will be discussed—contrasuppressor cells.

INTRODUCTION

The two prerequisites of the immune system are stability and rapid adaptation to external stimli (reviewed by Bell, Perelson & Pimbley, 1978; De Lisi & Hiernaux, 1982). How this apparently contradictory behaviour is organized is still a matter of debate. Two main lines of thinking exist: one hypothesis describes the immune system as resting in a stable state which needs strong signals to move into a responding state (Cantor & Gershon, 1978; Germain & Benacerraf, 1980; Herzenberg, Black & Herzenberg, 1980; Okumura & Tada, 1980). The other describes the stable state as the result of a continuous interaction of lymphocytes via recognition of self antigens (idiotypic determinants) which respond to external antigens only if the latter mimic the former (Eichmann, 1978; Jerne, 1955, 1974a, b; Richter, 1978; Rowley, Köhler & Cowan, 1980; Hiernaux & Bona, 1981; Hoffmann, 1975). We supposed that limiting dilution anaysis of T_H and T_S in untreated *vs* primed or suppressed animals might give some indications about the mechanisms of inducible response, supporting the likelihood of one of the two alternative hypotheses mentioned above, i.e. the first hypothesis would be supported by the pre-existence of only small clone sizes of T_H and T_S, and the exclusive expansion of either T_H or T_S after immunogenic or suppressogenic stimulation. Instead, the experimental data indicate that the immune system continuously maintains a balance between T_H and T_S. Movements towards response as initiated by external stimuli do not depend exclusively on expansion of T_H cells but are due to the appearance of one or more additional population(s) of regulatory cells. This assumption will be discussed briefly.

MATERIALS AND METHODS

Mice

Male BALB/c mice were obtained from the Zentralinstitut für Versuchstierkunde, Hannover, FRG. Mice were kept under specific pathogen-free conditions and were used for experiments at the age of 8–12 weeks.

Coupling of 2,4,6-trinitrophenyl (TNP) to lymphocytes, erythrocytes and protein

Thymus cells were haptenized with TNP by incubating 10^8 cells in 1 ml Hank's balanced salt solution (BSS) with 4 ml of 10 mM trinitrobenzenesulphonic acid (TNBS) in cacodylate buffer, pH 6·8, for 15 min at 37°. Thereafter, cells were washed four times in BSS. Sheep RBC (SRBC) and HRBC were haptenized according to Rittenberg & Pratt (1969) using 20 mg TNBS/1 ml packed RBC. TNP was coupled to ovalbumin (OVA) in phosphate-buffered saline, incubating a solution of 20 mg/ml OVA with a solution of 40 mM TNBS, vol:vol, overnight at 37°. TNP-OVA was exhaustively dialysed against the starting buffer.

In vivo *immunization*

For induction of T_S, mice received a single injection of 5×10^7 thymus cells haptenized with 10 mM TNBS (TNP^{10}-thymus cells) i.v., 7 days before priming or *in vitro* culturing (Pierres *et al.*, 1980). Mice were primed by i.v. injection of 5×10^8 TNP-HRBC, suppressed plus primed mice received the combined treatment, i.e. 5×10^7 TNP^{10}-thymus cells, i.v. on Day -12 plus 5×10^8 TNP-HRBC on day -5. TNP-specific T_H were gained from para-aortic draining lymph nodes after intratail injection of 5×10^7 TNP^{10}-SC in complete Freund's adjuvant. T_H were further selectively increased by culturing draining lymph nodes cells with irradiated TNP^{10}-SC (Martinez-Alonso *et al.*, 1980) in RPMI-1640, supplemented with antibiotics, L-glutamine, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum (RPMI-s) for one week at 37°, 5% CO_2 in air in a humidified atmosphere.

Preparation of concanavalin A (Con A) supernatant

SC from BDX rats were cultured in RPMI-s with 10 μg/ml Con A for 48 hr at 37°. Supernatants were harvested and Con A was blocked by the addition of 20 mM α-methyl mannoside. Interleukin-2 (IL-2) production was tested by [^{3}H]thymidine incorporation into an IL-2-dependent T-cell line.

Effector cells

Spleens, lymph nodes and thymuses were harvested, meshed and washed in BSS. For LD experiments, SC were used exclusively. SC were enriched for T cells by eliminating B cells via 'panning' on anti-mouse Ig-coated plates (Wysocki & Sato, 1978). SC adhering to anti-mouse Ig-coated plates were used as the source of B cells. *In vitro* expanded TNP-specific T_H were freed from dead cells and debris by a Ficoll–Hypaque gradient centrifugation (Bøyum, 1968).

Limiting dilution cultures

SC from untreated, primed, suppressed or suppressed plus primed mice were enriched for T cells, suspended in RPMI-s, supplemented with 10% IL-2-containing supernatants and 20 μg/ml TNP-OVA. Cells were diluted and 100 μl were placed in U-bottomed microtitre wells, together with 3×10^5 TNP^{10}-thymus cells which were irradiated with 3000 rads. Cell concentrations ranged from 100 to 6400 cells/well. Sixty replicates were set up for each dilution. Cells were cultured for 7 days in humidified air containing 5% CO_2 at 37°.

Assay system

This was carried out according to the method of Jerne & Nordin (1963). To a 100 μl aliquot of limiting dilution cultures, 100 μl of a suspension of 3×10^5 naive B cells plus 20 μg/ml TNP-OVA or 100 μl of a mixture of 3×10^5 naive B cells plus 5×10^4 T_H^{TNP} plus 20 μg/ml TNP-OVA in RPMI-s were added. Control wells received only naive B cells plus antigen or a mixture of naive B cells plus T_H^{TNP} plus antigen. After a 4-day culture period, plates were centrifuged, supernatants discarded and replaced by 100 μl BSS, 25 μl TNP-SRBC (1:4 dilution) and 25 μl of guinea-pig serum (1:5 dilution). Individual wells were transferred to 2-ml tubes, mixed with 300 μl 0·5% agar containing 0·75 mg/ml DEAE dextran (at 45°), poured into petri-dishes and incubated for 3 hr at 37°. Plaques were counted immediately thereafter. Plates with a number of PFC out of the range of ± 3 SD around the mean of control wells were considered as indicating suppression or help, respectively. Mean values of PFC in the presence of naive B cells were in the range of 5–15 plaques in individual assays, mean values of naive B cells together with T_H^{TNP} ranged between 120 and 250 PFC/well.

Calculations

The frequency of T_H and T_S was calculated according to the formulae:

$$F_0 = e^{-uT_H} \text{ or } F_+ = e^{-uT_S}$$

F_0 being the fraction of non-responding cultures, F_+ being the fraction of responding cultures and $u =$ number of c cells distributed in w wells (Lefkovits & Waldmann, 1979). Linear regression analysis was performed only at the initial portions of the experimental curves, i.e. for T_H in the range of 100–800 cells/well and for T_S in the range of 400–1600 cells/well. Points with a correlation index of 0·9 were accepted as linear.

RESULTS

Experimental protocol

Limiting dilution analysis of hapten (TNP)-specific T_H and T_S was performed in SC of untreated, primed, suppressed and suppressed plus primed BALB/c. All limiting dilutions were performed in the range of 100–6400 T cells per well, setting up 60 replicates at seven dilutions. Culture medium containing IL-2 was supplemented with irradiated TNP^{10}-thymocytes and TNP-OVA. Soluble as well as cell-bound antigen was added in order to avoid the possibility of preferentially expanding/activating any single cell compartment. For determination of T_H, naive B cells were added and T_S were determined after the addition of naive B cells plus saturating doses of T_H.

Determination of the frequency of T_H in untreated and TNP-treated mice

In the first set of experiments, the frequency of T_H in untreated animals was enumerated by adding naive B cells to limiting dilution cultures. Four representative experiments are shown in Fig. 1a. In all animals tested, the frequency of wells displaying helper activity increased from 100 to 800 cells per well. From the first slope, the frequency was determined in a total of 10 experiments and was found to be $1/1471 \pm 726$. Increasing the number of SC from untreated mice per well, this first slope was followed by a continuous decrease in the number of wells showing helper activity. At the starting point of the inversion, the frequency of non-responding cultures was around 0·54, corresponding to 28 out of 60 wells displaying helper activity. The inversion of the curves indicates the recruitment of regulatory cells (T_S) (Lefkovits & Waldmann, 1979).

A pattern very similar to SC of untreated BALB/c was obtained with SC from suppressed animals (Fig. 1b). Again, there was an increase in the frequency of cultures displaying helper activity in wells with 200 or 400, as compared to 100 cells per well. The frequency of T_H calculated out of 10 experiments was in the range of $1/11041 \pm 316$. The following three points should be mentioned:

(i) the paradoxic effect of an increase in T_H in suppressed animals, defined not only by the higher frequency (1/1471 *vs* 1/1041), but also the more narrow range of spreading (as expressed by standard deviations);

(ii) the inversion at a lower number of cells per

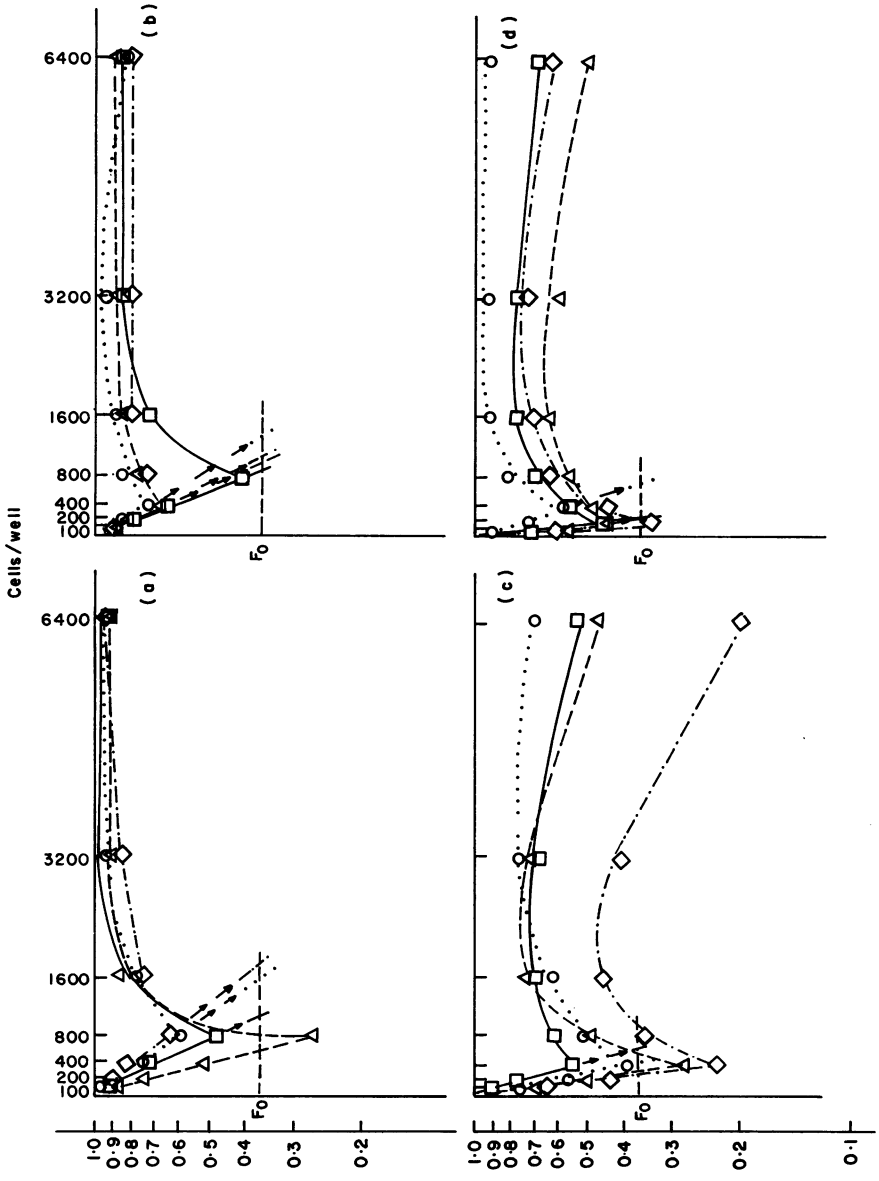


Figure 1. Frequency analysis of TNP-specific T_H in SC from untreated and TNP-treated BALB/c mice. SC of (a) untreated, (b) suppressed (5×10^7 TNP^{10}-thymocytes, i.v., Day −7), (c) primed (5×10^8 TNP-HRBC, i.v., Day −5) and (d) suppressed plus primed (combined treatment on Days −12 and −5) were seeded in limiting dilution cultures (60 wells/group) at seven concentrations ranging from 100 to 6400 cells/well. After 7 days of culture, 3×10^5 B cells and TNP-OVA were added. Direct anti-TNP PFC/well were measured 4 days later. Data are presented as fraction of negative cultures, i.e. within the range of mean ± 3 SD of controls (24 wells). Frequencies of T_H were determined from the 37% intercept of *only* the first positive slope. (a)–(d) each show four independent, representative experiments from a total of at least 10 experiments.

well as compared to untreated mice, with a mean of only 22 responding cultures out of 60;

(iii) the types of curves obtained at high numbers of cells per well. In most experiments, a continuous decrease in wells with helper activity was obtained. Rather seldom, the first inversion of the curve was followed by a second slope of increasing numbers of wells with helper activity at high numbers of cells per well (10/60 responding cultures at 6400 cells/well as compared to 0/60 at 3200 cells/well).

In primed mice, the limiting dilution curves were shifted to the left (Fig. 1c), i.e. a higher frequency of helperized wells was already observed at 100 cells per well. This corresponded to a frequency of T_H of $1/496 \pm 314$. Hence, there was a more than two-fold increase as compared to naive animals. In essence, the first part of the curve in primed mice resembles that of naive and suppressed mice but, opposite to suppressed and naive animals, more than one inversion was seen with SC from primed mice. In all animals tested, there was a tendency towards an increased number of wells with helper activity at the highest number of cells per well (6400) tested.

In suppressed plus primed animals, i.e. in mice having received TNP^{10}-thymus cells at Day -12 followed by injection of TNP-HRBC at Day -5, limiting dilution analysis revealed curves which combined the elements of suppressed and primed mice (Fig. 1d). As in primed mice, the curves were shifted to the left, corresponding to a frequency of T_H of $1/401 \pm 307$. At high numbers of cells per well the frequency of wells with helper activity increased, although the second helper slope was less steep than in primed mice.

Determination of the frequency of T_S in untreated and TNP-treated mice

For the determination of T_S, naive B cells and saturating doses of TNP-specific T_H were added to the limiting dilution cultures. The frequency of T_S was determined by plotting the fraction of responding cultures (F_+) in the range of 400–1600 cells per well (in which range a linear increase in wells with suppressive activity was observed consistently). The fraction of non-responding cultures was drawn out to obtain some information on the general behaviour of the system.

The determination of T_S in untreated mice showed a frequency of T_S of $1/4413 \pm 1207$. The behaviour was quite uniform and compatible with a ratio dominant model (Lefkovits & Waldmann, 1979) (Fig. 2a). In suppressed mice, the frequency of T_S was $1/2200 \pm 784$, but, plotting F_0, the curves are not as uniform as in untreated mice. At high numbers of cells per well, a tendency towards an inversion was observed in about 30% of experiments (Fig. 2b). The frequency of T_S in primed mice was calculated as $1/3683 \pm 1026$. Hence, it was slightly higher than in untreated BALB/c. At low numbers of cells per well, a rather homogeneous, linear behaviour was seen when plotting F_0, but, opposite to untreated mice, an inversion of the curves was regularily observed at high numbers of cells per well (Fig. 2c). As in suppressed mice even the curves determining T_S in suppressed plus primed mice were variable (F_S: $1/2193 \pm 964$). Plotting F_0, two types of curves were principally found. The first type (30% of experiments) resembled the behaviour of untreated animals, whereas 70% of the curves were similar to those seen in primed mice, although the inversed part of the slope was less steep (Fig. 2d).

Evaluation of limiting dilution curves

If one compares the frequencies of T_H and T_S in naive and TNP-treated mice, it is obvious that activation of the system results in expansion of T_H and T_S. Irrespective of any treatment, T_H are more frequently observed than T_S (Table 1). From theory, it is known that under limiting dilution conditions of two populations (i.e. T_H and T_S), an inversion of the limiting dilution curve of the population with the higher frequency can only be observed if the population with the lower frequency is dominant (Lefkovits & Waldmann, 1979). Since T_H are more frequent than T_S, the inversion of the slope of T_H curves indicates that T_S are dominant with respect to T_H. The dominance of T_S thus explains the inversion of the slope of 'helper' curves as seen in limiting dilution cultures of SC from untreated mice. The dominance of T_s does not explain why in limiting dilution cultures of TNP-treated animals a second inversion of the curves determining the frequency of T_H is seen, nor does it give an explanation for the inversion of the curves measuring the frequency of T_S. However, the second inversion of 'helper' curves and the inversion of 'suppressor' curves has to be expected when TNP-treatment activates a third population of regulatory cells which follows a ratio dominance model with respect to T_S. Thus, a third regulatory population, which is less frequent than T_S, but dominant with respect to T_S, has to appear in limiting

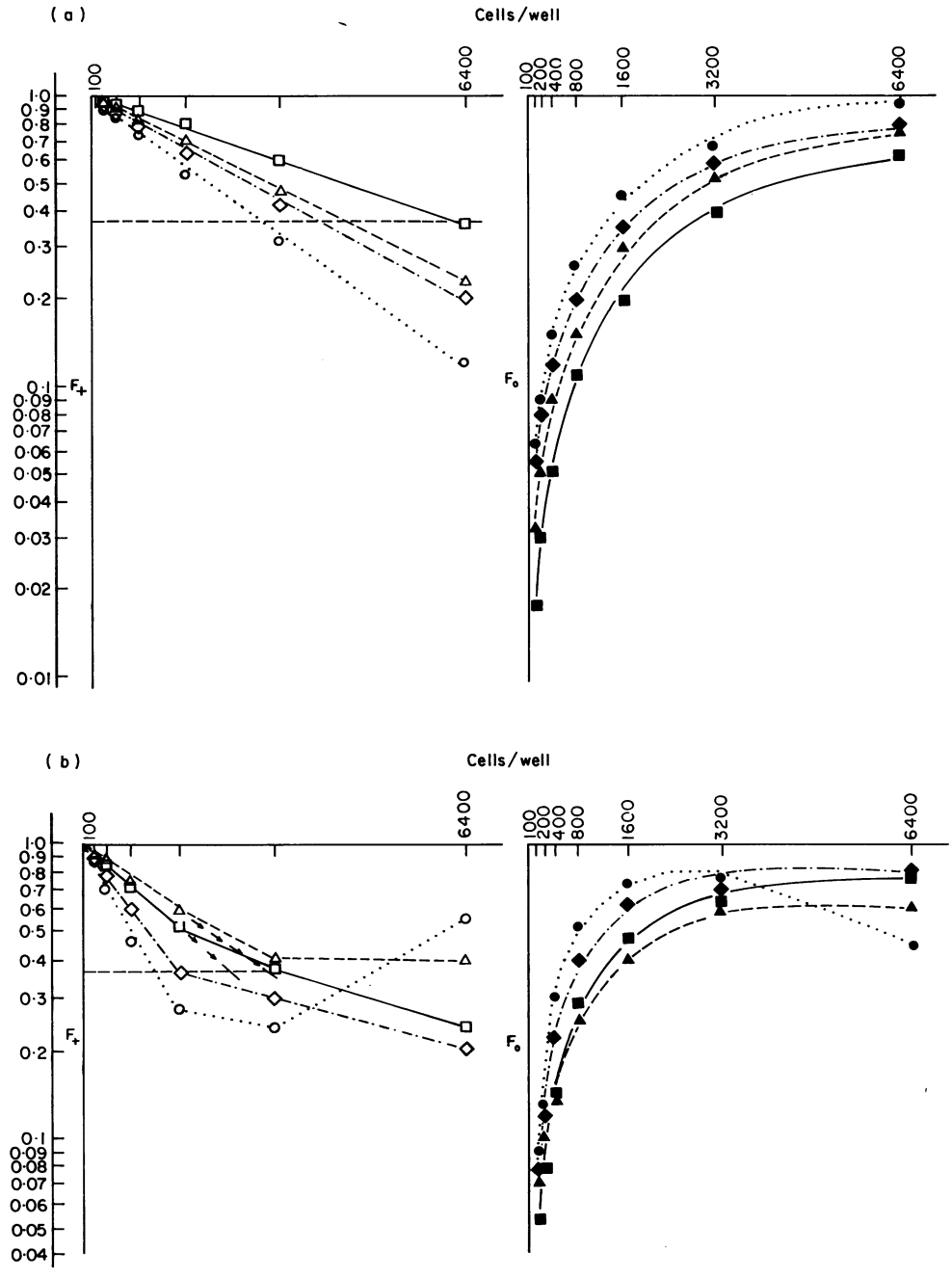
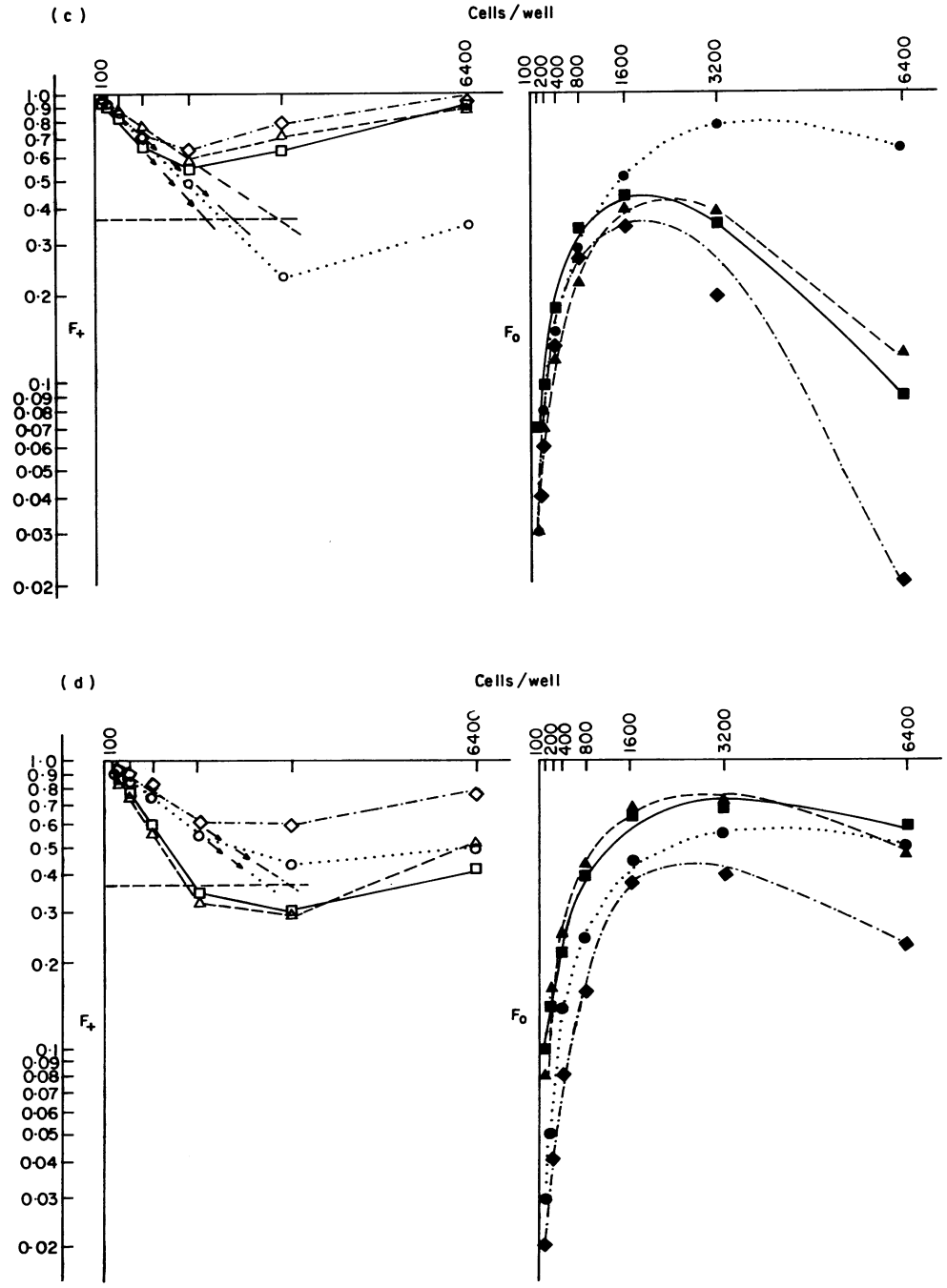


Figure 2. Frequency analysis of TNP-specific T_S in CS from untreated and TNP-treated BALB/c mice. LD cultures were set up exactly as described in Fig. 1, including the *in vivo* pretreatment of (b)–(d). After 7 days of culture, 3×10^5 B cells, 5×10^4 TNP-specific T_H and TNP-OVA were added. Data are presented as fraction of negative cultures, which corresponds with respect to suppression to cultures out of the range of mean $\pm$ 3 SD of controls (24 wells). Frequencies of T_S were determined by plotting



the fraction of responding cultures (37% intercept of the first positive slope). The four experiments from groups (a)–(d) correspond to experiments of Fig. 1, i.e. the same symbols were used for each individual SC population, where T_H and T_S were determined simultaneously.

Table 1. Ratio of T_H and T_S in untreated *vs* TNP-treated BALB/c mice

	T_H*		T_S*		Ratio
T-SC: treatment *in vivo*	Frequency^{-1}	Ratio	Frequency^{-1}	Ratio	T_H:T_S
–	1471 (2198–745)	1:	4405 (5618–3215)	1:	3·00:1
5×10^8 TNP-HRBC, i.v.	496 (810–115)	2·96	3676 (4717–2667)	1·20	7·41:1
5×10^7 TNP^{10}-thymocytes, i.v.	1041 (1357–725)	1·41	2198 (2817–1416)	2·00	2·11:1
5×10^7 TNP^{10}-thymocytes + 5×10^8 TNP-HRBC, i.v.	401 (708–94)	3·67	2193 (3155–1229)	2·01	5·47:1

* Mean of 10 experiments.

dilution cultures determining suppression as an inversion of the curve and, consequently, must be seen in 'helper' curves as a second inversion, i.e. as a second slope of decreasing numbers of non-responding cultures, when increasing the number of cells per well. This type of curve was seen in primed and suppressed plus primed animals.

DISCUSSION

Out of the so called 'naive status', the immune system can be moved into two different states: response or suppression. The naive status represents a stable steady state because it needs an external stimulus to move into response or suppression (Grossmann, 1982; Seghers, 1979; Cooper-Willis & Hoffmann, 1983; Lederberg, 1959; Burnet, 1957), and because after being moved out by external energy it moves back in an oscillatory way towards the starting point (Zembala & Asherson, 1973; Scibienski *et al.*, 1974; Romball & Weigle, 1982). We tried to analyse the regulatory elements of the immune system in different states by using limiting dilution analysis with respect to T_H and T_S in untreated *vs* primed *vs* suppressed *vs* suppressed plus primed mice.

In untreated mice, we observed a high frequency of T_H (1/1471) and a somewhat lower frequency of T_S (1/4413) with dominance of T_S. Hence, the 'naive status' is actually the net result of a neutralized response, as has already been suggested (Eichmann *et al.*, 1982). How then is the system moved out of the non-responsive state and how can response be withdrawn by externally induced suppression?

After priming, the frequency of T_H was increased by a factor of three. After suppressor cell induction, the frequency of T_S was increased by a factor of two. However even after priming, a minor increase in the frequency of T_S was observed (1·2-fold increase) and, on the other hand, T_H were slightly augmented in suppressed mice (1·4-fold increase). Hence, any kind of treatment which pushes one of the starting regulatory populations simultaneously, although not to the same degree, affects the second regulatory population too. These data support the concept that the immune system tries to maintain the starting equilibrium of the steady state of untreated animals.

Suppressive treatment strengthens only the non-responding quality of the system, i.e. the system is not effectively pushed out of balance. Priming, the opposite to suppression, profoundly alters the initial stable state. Response is due not only to a shift in the ratio of T_H:T_S from 3·0:1 in untreated to 7·4:1 in primed mice, but rather seems to be accompanied by the appearance of a third regulatory population which, by the method used, could not be detected in untreated animals and only very occasionally appeared in suppressed mice. However, in primed and in suppressed plus primed animals, the appearance of this third population was constantly indicated by the shape of 'suppressor' as well as 'helper' curves.

The experimental protocol does not allow the quantification or qualification of this third population. The occurrence of the inversion (second inversion in helper curves, first inversion in suppressor curves) as well as the steepness of the inverted slope are finally dependent on the mutual ratios as well as the efficiencies of three interacting populations (T_H, T_S, third regulatory population). Nevertheless, the shape of the curves proves its existence beyond doubt.

With respect to the nature of this third population, two main hypotheses can be put forward. Firstly, the

third population represents another type of T_H, differing from T_H responsible for the first slope in 'helper' curves not only with respect to the frequency, but also with respect to quality, as reflected by 'suppressor' curves of primed and suppressed plus primed animals. 'Suppressor' curves which were performed in the presence of saturating doses of hapten-specific T_H clearly show that this third population cannot be suppressed. Sets of T_H and T_S with different frequencies, possibly corresponding to different stages of activation, were also supposed by other groups (Melchers, Fey & Eichmann, 1982; Eichmann *et al.*, 1983; Fey, Melchers & Eichmann, 1983). Furthermore, it was suggested that response may be the result either of shifts between those sets of T_H and T_S, or that after external stimulation T_H may no longer be suppressible (Hamann, Eichmann & Krammer, 1983). The second hypothesis is that the third population represents a type of cell directly counteracting T_S. It is known that activated contrasuppressor cells can act as potent 'helpful' regulatory cells (Ptak *et al.*, 1981). Furthermore, if the immune system is characterized by an internal activity which is generated in the absence of environmental contacts (Coutinho *et al.*, 1984), activation of contrasuppressor cells could well explain the rapid adaptation to external stimuli. Experiments to differentiate between these two possibilities are in progress. Preliminary results indicate that the cell is cyclophosphamide-sensitive, does not behave like 'classical' T_H, and probably may be idiotype-specific.

In summary, the stable steady state of untreated animals is due to an equilibrium between help and suppression. External interference with this steady state leads to an increase in T_H or T_S, where the system tries to keep the equilibrium by a concomitant, although less effective, increase in the mutual counteracting population. This resembles the suppressed status, in which the system maintains the state of unresponsiveness. Perturbation of the system as reflected by response obviously is not exclusively the result of an expansion in preformed regulatory populations (T_H, T_S), but is brought in mainly by the appearance of a third regulatory population which can not be detected in untreated animals.

ACKNOWLEDGMENT

This investigation was supported by the Deutsche Forschungsgemeinschaft, grant Zo 40/2-1 (MZ) and the MPI 40% (GA).

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