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Induction of tolerance towards TNP entails down-regulation of an autoimmune attack

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SUMMARY

In order to follow the process of induction and maintainance of tolerance, BALB/c mice were tolerized by free hapten, and effector and regulatory cell interactions were analysed by limiting-dilution (LD) cultures. Injection of trinitrobenzenesulphonic acid (TNBS) resulted, predominantly, in the activation and expansion of self-reactive cytotoxic T cells (CTL), which were observed transiently at frequencies comparable to allo-specific CTL. In addition, self-reactive helper T cells (Th) were activated and expanded in tolerized mice. TNP-specific reactivity was difficult to evaluate, since cytotoxic activity against haptenized self followed the pattern of self-reactivity throughout the test period. But in LD cultures determining proliferation, two populations of Th responding to TNP-self were observed, while only one Th population could be detected in response to self. Expansion/activation of Th and CTL pecursors (CTLp) was followed by activation of suppressor T cells (Ts). The suppressor population could be divided into two subpopulations, one interfering with Th, the second interacting directly with CTL (veto cells). The results indicate that during the induction of tolerance, animals pass through an autoimmune attack, with expansion and activation of self-reactive clones (CTL, Th). The final status of non-responsiveness towards TNP is not due to the deletion of effector or regulatory cells, but results from the establishment of a steady state of dominance of self-reactive and TNP-self-reactive suppression.

INTRODUCTION

Despite intense efforts, the mechanisms by which the immune system generates and maintains a status of tolerance towards self are not understood. Two main hypothesis are discussed (reviewed by Nossal, 1983; Scott, 1984). One is Burnet's (1957) hypothesis of clonal deletion of self-reactive lymphocytes. This includes clonal abortion (failure to resynthesize surface receptors), or clonal anergy (inability to respond). The other proposes active suppression of self-reactive clones. The latter theory is supported by the demonstration of autoreactive clones (Zauderer *et al.*, 1984; Gatenby *et al.*, 1982) and of suppressor cells in many models of tolerance. Clonal analysis should have solved the problem, yet the debate is still going on, with evidence for both mechanisms being presented even at the clonal level. Examples of deletion mechanisms are Gibson *et al.* (1985), Good & Nossal (1983), Wood, Strome & Streilein (1984), Oki & Sercarz (1985) and Feldman, Zanders & Lamb (1985), and of regulatory mechanisms, Stockinger (1984) and Cooper & Eichmann (1985).

Limiting-dilution (LD) analysis not only makes it possible to quantify effector and regulatory cell populations, but also yields information about qualitative interactions between effector and regulatory cells. For example, it can be shown, via LD analysis, that responsiveness is due to release from suppression rather than clonal expansion (Zöller & Andrighetto, 1985; Zöller *et al.*, 1985; Eichmann *et al.*, 1983). Since the immune system of untreated animals thus seems to maintain a status of suppression, it was interesting to analyse the status of tolerance, to see whether or not there may exist differences in the quantitative and qualitative interactions of effector and regulatory cells. When tolerance was induced by i.v. injection of a reactive hapten (TNBS) we could demonstrate activation firstly of auto-aggressive CTL and secondly of cells that directly inhibit the latter (veto cells; Miller, 1986). Thus tolerance appears to be a sequel to an autoimmune attack. It is suggested that the resulting stable state of tolerance is due to a shift in the mode by which regulatory and effector cells interact. Since the TNP-substituted self components persist for a long period (mice stay yellowish), these data may have fundamental bearing on the question of self tolerance.

Abbreviations: Con A, concanavalin A; CTL, cytotoxic T cells; CTLp, CTL precursors; Fo/+, fraction of non-responding cultures; IL-2, interleukin-2; LD, limiting dilution; RPMI-s, RPMI-1640, supplemented with antibiotics, L-glutamine, 10^{-3} M HEPES buffer, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum; SC, spleen cells; Th, helper T cells; TNBS, trinitrobenzenesulphonic acid; TNP, trinitrophenyl; TNP^{10}-cells/TNP^{1}-cells, lymphocytes haptenized wtih 10 mM/1 mM TNBS.

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MATERIALS AND METHODS

Mice

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

Coupling of TNP to lymphocytes

Lymphocytes were haptenized with TNP by incubating 10^8 cells in 1 ml Hanks' balanced salt solution (BSS) with 4 ml of 10 mM trinitrobenzenesulphonic acid (TNBS; Sigma) in cacodylate buffer, pH 6·8, for 15 min at 37° (Zöller & Andrighetto, 1984).

Induction of tolerance

Mice received five to six injections of 5 mg TNBS in 0·2 ml of PBS within a period of 3 weeks.

Effector cells

Spleens and thymuses were pressed through fine gauze and washed with BSS. Where indicated L3T4$^+$ and L3T4$^-$ (Dialynas *et al.*, 1983) populations were prepared from splenic T cells. T cells were obtained by panning on anti-mouse Ig-coated plates (Wysocki & Sato, 1978). The non-adherent population was panned again on GK1·5-coated plates, collecting the non-adherent as well as adherent fraction, as described elsewhere (Zöller & Andrighetto, 1984).

Cultures

Concanavalin A (Con A; Pharmacia) blasts were obtained by culturing 3×10^6 spleen cells (SC) in 2 ml RPMI-1640, supplemented with antibiotics, glutamin, 10^{-3}M HEPES buffer, 5×10^{-5}M 2-mercaptoethanol and 10% fetal calf serum (RPMI-s), containing 10 μg/ml Con A, for 48-72 hr at 37°, in 5% CO_2 in a humidified atmosphere.

For re-stimulation of CTL in bulk cultures, 2×10^6/ml SC were cultured with 2×10^6/ml 3000 rads gamma-irradiated syngeneic thymocytes or syngeneic TNP-haptenized thymocytes as stimulator cells in RPMI-s, plus 10% IL-2 containing medium for 5–6 days. As a source of IL-2, Con A supernatant of rat SC was used, Con A being blocked with 20 mM α-methylmannoside.

Limiting-dilution (LD) cultures

BALB/c SC were diluted from 6400 to 100 cells/well in RPMI-s with or without 10% of IL-2-containing medium. Where indicated 1×10^4 L3T4$^+$ cells were added to dilutions of L3T4$^-$ splenic T cells. No IL-2-containing medium was added to these cultures. Cells were stimulated with 3×10^5 irradiated CBA, BALB/c or TNP^{10}-BALB/c thymocytes. For every dilution 60 replicates were set up. Cells were cultured for 3 to 7 days.

Proliferation

Proliferation was determined in LD cultures after 3 days by adding 1 μCi of [^{3}H]thymidine during the last 4 hr of culture. Thymidine incorporation exceeding the mean from 24 control wells (no stimulator cells) by a factor of 5 was considered as indicating proliferation.

Cytotoxicity assay

CBA, BALB/c or BALB/c-TNP^1 (haptenized with 1 mM TNBS) Con A blasts were used as target cells. Freshly harvested SC were added to ^{51}Cr-labelled target cells at ratios from 100 to 12·5:1. In LD cultures, target cells were added after 7 days of culture. ^{51}Cr release was determined after 6 hr. Cytotoxicity was calculated as percentage cytotoxicity:

$$100 \times \frac{\text{counts in test wells} - \text{counts in medium control wells}}{\text{maximum release} - \text{counts in medium control wells}}.$$

In LD cultures values exceeding the mean ± 3 SD of 48 medium control wells were considered to indicate cytotoxicity.

Limiting-dilution calculation

The frequency of CTL or Th was calculated according to the formula $u = \ln Fo$, where u is the mean number of CTL or Th per well, and Fo is the frequency of *non-responding* wells. When $\ln Fo$ is plotted against number of SC per well, as in the diagrams which follow, then, initially, a straight line is obtained at low numbers of cells per well, which cuts the 37% ordinate at $u = 1$ Th or CTL per well. As the numbers of SC are increased, then this line may change direction abruptly, indicating that another type of cell (Ts) begins to be present in significant numbers, which can counteract, in a dominant fashion, the effect of the first type. LD assays for Ts were similar, except that every well contained excess of the cells required for the response. The frequency of Ts was then obtained from $u = \ln F+$, where F+ is the frequency of *responding* wells (Lefkovits & Waldmann, 1979).

RESULTS

Evaluation of the status of tolerance

Tolerance was induced in adult BALB/c mice by five to six i.v. injections of 5 mg TNBS. Cytotoxic activity of SC from tolerized mice was tested in bulk cultures 1–10 weeks after tolerization, and compared with the cytotoxic potential of SC from control animals. SC of untreated mice displayed no cytotoxic potential against self or TNP-self. After *in vitro* stimulation with syngeneic thymocytes, a low level of cytotoxic activity against self and TNP-self was observed; after *in vitro* stimulation with haptenized self, SC from control mice preferentially lysed haptenized target cells. On the other hand, SC of tolerized mice restimulated either with self or TNP-self did not lyse either type of target cell throughout the test period. SC of tolerized mice which had not been restimulated, however, were reactive against self when tested 1 week after tolerization. Reactivity against self disappeared within 3 weeks after tolerization and could not be recovered after *in vitro* restimulation with either syngeneic thymocytes or haptenized thymocytes (Table 1).

These data indicate that tolerization initiates a sequence of events, starting with activation of self-reactive CTL and being followed by activation of self- and/or TNP-self-specific suppression. To test this assumption, the frequencies of self-reactive and TNP-self-reactive CTLp, as well as of regulatory cells, were evaluated in LD cultures.

Apparent frequencies of CTLp in TNBS-tolerized mice

In a first set of experiments, we tested whether the unreactivity of bulk cultures towards TNP was due to deletion of TNP-specific CTLp (Fig. 1a). Four days after the last injection of TNBS, TNP-specific CTLp were detected at high frequency. By

Table 1. TNP-self- and self-specific cytotoxic activity after TNBS-induced tolerance: time-course

SC donor	Weeks after tolerization	*In vitro* restimulation*	% cytotoxicity BALB/c (T:E) 1:100	50	25	12	BALB/c-TNP[1] (T:E) 100	50	25	12
Untreated		—	1	0	0	0	1	1	1	0
TNBS-tolerized	1	—	17	9	6	3	17	12	4	1
	3	—	0	1	0	0	0	0	3	0
	6	—	0	0	0	0	0	0	3	0
	10	—	0	0	1	0	0	0	0	1
Untreated		Thymocytes		12	7	5		14	7	4
TNBS-tolerized	1	Thymocytes		4	1	1		1	1	0
	3	Thymocytes		6	2	1		4	2	0
	6	Thymocytes		6	5	1	5	4	1	
	10	Thymocytes		9	1	1		3	1	0
Untreated		TNP[10]-thymocytes		13	6	2		39	24	14
TNBS-tolerized	1	TNP[10]-thymocytes		4	3	1		1	1	0
	3	TNP[10]-thymocytes		4	1	1		1	1	0
	6	TNP[10]-thymocytes		4	0	0		4	2	1
	10	TNP[10]-thymocytes		2	5	3		7	4	0

* Responder:stimulator = 1:1.

1 week after TNBS injection, the frequency of CTLp declined. Moreover, the maturation of CTLp declined at higher numbers of cells per well being 'counter-regulated' by suppressor cells. This is consistent with the ratio-dominance model of suppression (Lefkovits, Aarden & Corley, 1980), i.e. suppressor cells must be present at a lower frequency than the cells they suppress, and yet their influence must be dominant. Two to four weeks after tolerization, hardly any cytotoxic clones could be detected. After 6 and 10 weeks there appeared to be two populations of CTLp/CTL, one population with a frequency of 1/5000, which was counter-regulated, and a second population of lower frequency (1/20000–1/25000) which did not appear to be suppressible. The same pattern of reactivity was seen when LD cultures stimulated with TNP[10]-thymocytes were tested against non-haptenized targets (Fig. 1b), but they did not lyse allogeneic (CBA) Con A blasts (data not included). Thus, the most impressive feature was the transient activation of self-reactive CTLp. But it was not possible to determine whether TNBS-tolerized mice also contained (in addition to self-reactive CTL) TNP-specific CTL.

To support the hypothesis that tolerization via TNBS is initiated by an autoimmune attack, syngeneic and, for comparison, allogeneic lymphocytes were used as stimulators in LD cultures (Fig. 2a). Alloreactive CTLp were detected at comparable frequencies in untreated and TNBS-tolerized mice, thus excluding the possibility that TNBS injection may induce general unresponsiveness. However, 4 days after tolerization, self-reactive CTL were also detected and at comparably high frequencies. To demonstrate the remarkable cytotoxic potential of auto-aggressive CTL, cytotoxicity values are shown in Fig. 2b. Four days after tolerization, not only the frequency but also the cytotoxic potential of auto-aggressive CTL were comparable to alloreactive CTL. The frequencies of self-reactive CTLp/CTL decreased rapidly during the observation period, the reactivity profile changing during the time exactly as described for LD cultures stimulated with TNP[10]-thymocytes. This confirmed that tolerization initially resulted in expansion of self-reactive CTL. The period of autoreactivity was transient and was followed by a status of anergy towards self as well as TNP-self.

Activation of help in TNBS-tolerized mice

Determination of CTLp/CTL in LD cultures supported by exogenous IL-2 does not allow any estimate to be made of intrinsic helper activity. Nevertheless, the observation of expansion of self/TNP-self-reactive CTLp argues for activation/expansion of Th. This was confirmed with the use of LD cultures in which [^{3}H]thymidine incorporation was determined. One week after tolerization clones proliferating in response to self were detected at a high frequency. The frequency of proliferating clones decreased with high numbers of cells per well, indicating that a population of cells suppressing proliferation was present at a lower frequency. In non-tolerized mice the frequency of proliferating clones was significantly lower, and counter-regulation was not obvious in the tested range (Fig. 3a). In LD cultures stimulated with TNP-self, two populations of proliferating clones appeared. In tolerized animals both proliferating populations were counter-regulated, while in control mice only the population with the higher frequency was counter-regulated (Fig. 3b). Thus, the proliferative responses to self and TNP-self were distinct. the frequency of Th began to decline as early as 2 weeks after tolerization, and control levels were reached by 6–10 weeks. The frequencies of Ts interacting with proliferating clones were estimated from F+ of LD cultures

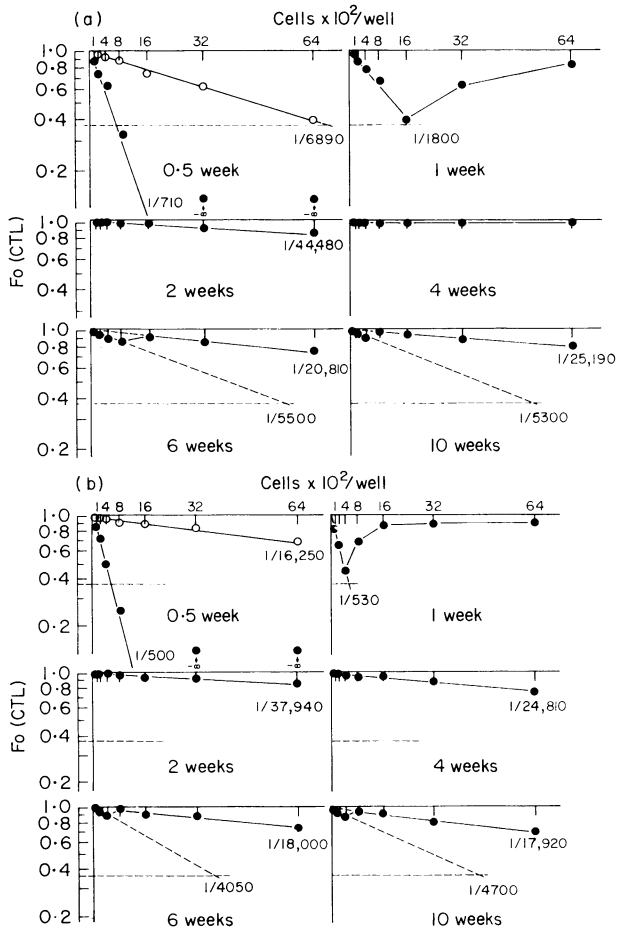

Figure 1. Frequency of cytotoxic T-cell precursors after stimulation by TNP-self. BALB/c SC of an untreated animal (○) and of TNBS-tolerized mice, 4 days, 1 week, 2, 4, 6 and 10 weeks after tolerization (●) were cultured under LD conditions using IL-2-containing medium and irradiated BALB/c TNP^{10}-thymocytes as stimulator. LD cultures were split after 7 days and the fraction of cytotoxic cultures was determined against BALB/c TNP^1-Con A blasts (a) and against BALB/c Con A blasts (b)

determining [^{3}H]thymidine incorporation. Frequencies of Ts remained rather constant for at least 4 weeks, and were still at an elevated level 10 weeks after tolerization (Table 2).

These results show that tolerization leads to the expansion not only of CTLp, but also of both TNP-self- and self-reactive Th and Ts. Furthermore, it can be concluded that the status of anergy, which followed the initial autoimmune attack, need not be due to clonal deletion, but may be maintained by active suppression.

Establishment and maintainance of tolerance via suppression

Further support for the suppression rather than the deletion hypothesis was provided by data obtained using a modification of the LD protocol for determination of CTLp. This comprised of a supply of help using an addition of a constant number of $L3T4^+$ cells from tolerized mice, rather than by IL-2. Using this assay CTLp/CTL were observed throughout the test period at low numbers of cells per well, but these were counter-regulated at high numbers of cells per well. CTLp/CTL were detected in response both to haptenized (Fig. 4a,b) and non-haptenized (Fig. 4c) thymocytes. Again, there was no significant difference between the frequencies of CTL reactive against TNP-self and unmodified self. Nevertheless, by supplying help via cells instead of via factors, it could be shown conclusively that in the tolerant state CTLp are not clonally deleted, but are functionally inactivated by suppression.

Table 3 shows the frequencies of CTLp/CTL after tolerance induction, as detected by the LD protocol described in Fig. 4. (For comparison the frequencies detected in IL-2-supported LD cultures are added in brackets.) Up to 6 weeks after tolerization, TNP-self-reactive CTLp/CTL were detected at an elevated level compared to non-tolerized mice. After 10 weeks comparable frequencies were detected in LD cultures supported by helper cells or helper factors (1/5300) and these were close to the frequency detected in untreated controls (1/7000). Frequencies of self-reactive CTLp/CTL decreased from 1/500 (4 days after tolerization) to 1/9000 (10 weeks after tolerization), thus still exceeding the frequency of self-reactive CTLp in control animals (1/16000).

Besides enabling us to discover CTLp/CTL in the established tolerant state, LD cultures supported by helper cells also provided information that SC of tolerized mice contain a population of Ts that can interact directly with CTLp/CTL. This was concluded from the observation that 2–4 weeks after tolerization CTLp/CTL did not become visible in LD cultures supported by an excess of helper factors. Since the presence of CTLp/CTL was hidden, despite the supply of helper factors, CTLp/CTL must have been the direct target of suppression. The frequencies of these Ts, calculated from F+ of LD cultures supported by L3T4+ cells, are shown in Table 3. Since counter-regulation of CTLp/CTL was already observed at a low fraction of responding cultures, estimations of the frequencies of these Ts may be rather imprecise. But it can be stated that in TNBS-tolerized mice self- and TNP-self-reactive Ts interacting directly with CTL were detected at high frequencies, maximal frequencies being reached 2–4 weeks after tolerization. From then on, the frequencies decreased slowly. Finally, it should be noted that TNP-self-reactive Ts for CTLp/CTL were detected at higher frequencies than self-reactive Ts.

Figure 5 summarizes the characteristics of TNBS-induced tolerance. The frequencies of self-reactive/TNP-self-reactive CTL were greatly increased due to the activation of self- and TNP-self-reactive Th, and reached their maximum 4 days after tolerance induction. The following period of functional inactivity was not due to clonal deletion/anergy. Instead, unreactivity resulted from the activation of TNP-self- and self-reactive Ts. Tolerant animals contained two populations of Ts, one capable of interacting with Th, the other capable of interacting directly with CTL. Ts reached maximal frequencies at 1–2 weeks and remained at an elevated level throughout the observation period.

DISCUSSION

Intravenous injection of TNBS drives the immune system into a state of tolerance, i.e. after challenge with TNP in an immunogenical form neither production of TNP-specific antibodies nor activation of TNP-specific CTL is observed (Good & Nossal, 1983; Fidler & Golub, 1973; Asherson & Ptak, 1979).

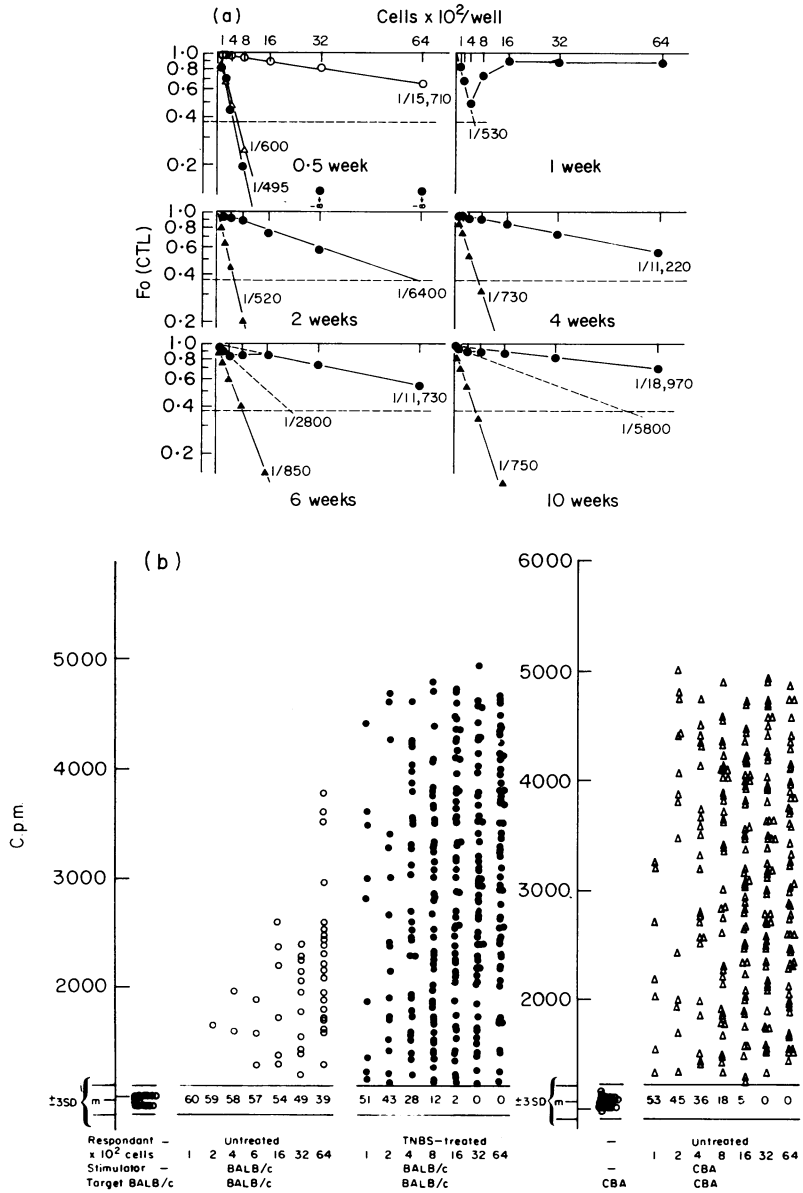

Figure 2. Frequency of cytotoxic T-cell precursors after stimulation by unmodified self. (a) BALB/c SC of an untreated animal (open symbols), and of TNBS-tolerized mice, 4 days, 1 week, 2, 4, 6 and 10 weeks after tolerization (closed symbols) were cultured under LD conditions using IL-2-containing medium and irradiated BALB/c or CBA thymocytes as stimulator. Seven days later the fraction of cytotoxic cultures stimulated with BALB/c thymocytes was determined against BALB/c Con A blasts (○/●) and of cytotoxic cultures stimulated with CBA thymocytes against CBA Con A blasts (△/▲). (b) The individual cytotoxicity values of LD cultures from SC of an untreated and a tolerized (4 days after tolerization) animal after stimulation with irradiated BALB/c or CBA thymocytes are shown. Release ± 3 SD of 24 medium (m) control wells (○); * maximum release. Further symbols (and data) correspond to the curves in the upper lefthand corner of Fig. 2a).

When analysing the unresponsiveness with respect to CTL in bulk cultures, we observed that tolerance was maintained until 10 weeks after TNBS injection, since after *in vitro* restimulation with TNP^{10}-thymocytes no TNP-specific cytotoxicity could be detected. In spite of this, the unrestimulated SC of tolerized mice displayed cytotoxic activity against self shortly after TNBS injection. Hence, TNBS injection resulted in long-lasting tolerance towards TNP, but the animals passed through a period of an autoimmune attack. A similar observation, i.e. a short period of hyper-reactivity during the initiation of tolerance, was described by Sterzl (1966). During this period animals passed through a phase of miserable conditions: they were lethargic, anaemic, had rough fur, and histological examination revealed mononuclear infiltrates in the liver and depletion of the red pulp in the spleen (data not shown). Autoreactivity was confirmed by analysis at the single-cell level. As summarized in Fig. 5,

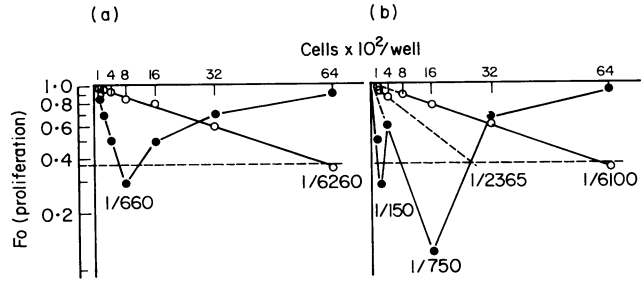

Figure 3. Frequencies of proliferating clones in TNBS-tolerized mice. LD cultures of BALB/c SC of an untreated animal (○), and of a TNBS-tolerized mouse (4 weeks after tolerization) (●) were set up using irradiated thymocytes (a) or irradiated TNP^{10}-thymocytes (b) as stimulators. Proliferation was determined after 3 days by [^{3}H]thymidine incorporation.

Table 2. Frequencies of proliferating clones and of clones suppressing proliferation in response to self and TNP-self

SC donor	Weeks after tolerization	Frequency^{-1} of proliferating clones		Frequency^{-1} of suppressor clones	
		TNP-self reactive	Self-reactive	TNP-self reactive	Self-reactive
Untreated		2365+6100	6260	2400+ND*	ND
TNBS-tolerized	1	150+750	660	550+3300	2040
	2	590+2300	955	500+3500	2510
	4	1090+4020	1466	600+3500	2480
	6	1655+6000	2665	1130+5000	3900
	10	3240+5900	5400	1260+5120	4780

* ND, not detectable.

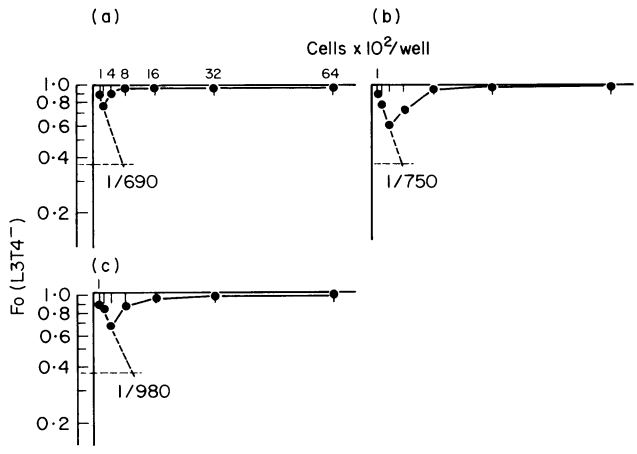

Figure 4. Determination of the frequencies of TNP-self- and self-reactive cytotoxic T-cell precursors and suppressor T cells in TNBS-tolerized mice in the presence of an excess of $L3T4^+$ cells from tolerized mice. $L3T4^-$ splenic T cells of a TNBS-tolerized BALB/c mouse (4 weeks after tolerization) were cultured under LD conditions adding excess of $L3T4^+$ T cells of tolerized mice and irradiated BALB/c TNP^{10}-thymocytes (a,b) or irradiated BALB/c thymocytes (c) as stimulators. The fraction of cytotoxic cultures was determined 7 days later against BALB/c TNP^1-Con A blasts (a) and BALB/c Con A blasts (b,c).

autotoxic CTL were detected 4 days after TNBS injection at a 30-fold plus frequency, compared to untreated controls. Correspondingly, autoreactive Th were highly increased in frequency. Subsequently, autoreactive Ts, which were not detectable in untreated animals, were found at high frequencies too. While frequencies of autoreactive Th and CTLp declined rapidly, Ts frequencies persisted at the same level until 4–6 weeks after tolerization, thus creating a status of dominance of suppression. Essentially the same pattern of reactivity was observed with respect to TNP-self-reactive clones. However, it should be mentioned that it was not possible to dissect self- and TNP- self-reactive CTLp. Analysis of proliferating clones revealed two populations, one being detected in response to self and TNP-self, the other only in response to TNP-self. We therefore suggest that SC of TNBS-tolerized mice may contain populations both of self as well as of TNP-self-reactive CTLp, which probably could not be detected as separate clones due to proximity in frequencies.

Two features of TNBS-induced tolerance deserve discussion, namely the induction of tolerance as a highly dynamic process and the maintenance of tolerance via suppression. It was pointed out before (Cooper & Eichmann, 1985) that tolerance induced by reactive haptenic groups appears to be particularly suited for comparison to self-tolerance. Since TNBS binds

Table 3. Frequencies of TNP-self and self-reactive cytotoxic T-cell precursors and of suppressor T cells interfering with cytotoxic T cells as detected in limiting-dilution cultures supported by helper cells

SC donor	* Weeks after tolerization	Frequency^{-1} of CTLp/CTL†‡		Frequency^{-1} of Ts†	
		TNP-self reactive	Self-reactive	TNP-self reactive	Self-reactive
Untreated		7200 (6890)	16450 (15710)	ND	ND§
TNBS-tolerized	0·5	740 (710)	520 (495)	ND	ND
	1	1020 (1800)	683 (530)	250	1150
	2	1518 (44480)	2155 (6400)	286	1364
	4	1509 (ND)	2219 (11220)	352	1364
	6	1824 (5500)	2915 (2800)	792	2090
	10	5290 (5300)	8756 (5800)	858	4070

* L3T4$^-$ splenic T-cells were titrated into L3T4$^+$ splenic T-cells of tolerized mice.
† Calculated from the L3T4$^-$ SC population and corrected for the frequency in unseparated SC.
‡ In brackets: frequencies as detected in LD cultures supported by IL-2.
§ ND, not detectable.

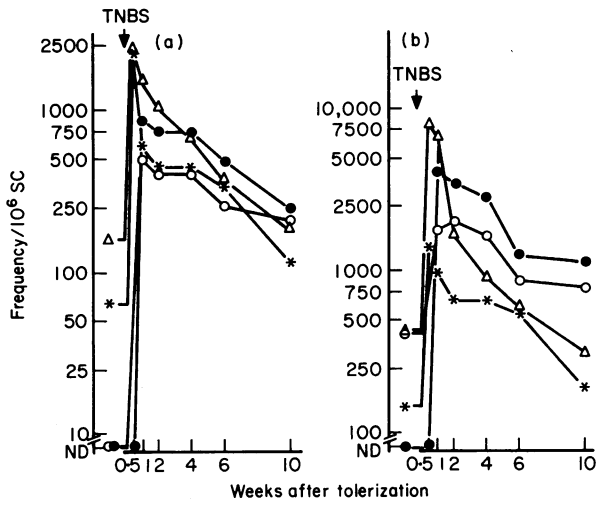

Figure 5. Synoptic presentation of activation/expansion of TNP-self and self-reactive clones after TNBS treatment. Time-course of frequencies of self- (a) and TNP-self- (b) reactive effector and regulatory cells after TNBS injection: CTLp/CTL (derived from Fo of LD cultures supported by L3T4$^+$ cells) (*); Th (derived from Fo of LD cultures determining [^{3}H]thymidine incorporation) (△); Ts interacting with Th (derived from F+ of LD cultures determining [^{3}H]thymidine incorporation) (○); Ts interacting with CTL (derived from F+ of LD cultures supported by L3T4$^+$ cells) (●). ND, not detectable.

readily to cells and proteins, and TNP persists in the organism for several weeks (mice stay yellowish), the hapten may be considered to mimick a self component, i.e. the immune system is forced to repeat the process of learning to tolerate self. Although it is still unknown how self-tolerance is established during ontogeny, the frequent finding of self-reactive clones (Ts, Th, B) (Hansen, 1986; Julius & Heusser, 1986; Bretscher, 1986; de Talance *et al.*, 1986; Portnoi *et al.*, 1986; Kieber-Emmons & Köhler, 1986) strongly supports the idea that self-tolerance is an active process. Our data show that initiation of tolerance in the adult is accompanied by an autoimmune attack. Although we cannot explain why the immune system has to repeat the process of learning self, i.e. does not recognize self anymore as self, when it presents itself ubiquitously as TNP-self, the appearance of autoreactivity convincingly demonstrates the permanent presence of self-reactive clones, and further argues against the possibility that self tolerance is based on clonal deletion/abortion.

Furthermore, what can be clearly derived from the model are the processes of regulation of autoreactivity and establishment of tolerance. Both self and TNP-self-reactive clones are functionally inactivated by suppressor cells, the tolerant state being characterized by the presence of two types of suppressor cells, antigen-specific Ts, which interact with Th (Andrighetto & Zöller, 1985; Duncan, Steplowski & Bitter-Suermann, 1986) and a second population of Ts, undetectable in non-tolerized mice, which can interact directly with CTL. The presence of this second Ts population, and its target, were inferred from the observation that CTLp/CTL were counter-regulated in LD cultures supported by an excess of helper factors—under which culture conditions the presence of antigen-specific Ts interacting with Th will be hidden. It should be mentioned that 2 and 4 weeks after tolerization, CTLp/CTL could only be detected in LD cultures supported by L3T4+ cells but not in those supported by IL-2. This may be explained by assuming that L3T4+ cells can compete with CTL as targets for the second Ts population, which would imply that the second Ts population can interact with both CTL and activated Th. Irrespective of the potential targets, the fact that the second Ts population can interact directly with CTL makes it likely to be identical with veto cells (Miller, 1986). Thus, the status of tolerance would be maintained by both Ts interacting with Th, and veto cells, which implies that tolerance is controlled both at the effector and the Th level.

Finally, we would like to argue that veto cells are an essential prerequisite for the maintenance of tolerance. Both the naive and the tolerant state are defined by suppression, but in the naive animal antigenic stimulation leads to a response due to the

release from antigen-specific suppression of Th, which then provides help for effector cells to proliferate and mature. In TNBS-induced tolerance, on the other hand, auto-aggressive CTL become activated by introducing an omnipresent neodeterminant, which then initiates the activity of veto cells. Thus, as long as the neodeterminant persists in the organism, further challenge with TNBS will not yield a response because CTL recognizing the 'neo-self' will be vetoed.

Finally, TNBS-induced tolerance appears to be comparable to self-tolerance in that the 'tolerogen' is omnipresent in the organism. (Conversely, this implies that TNBS-induced tolerance may not be comparable to some other antigen-specific tolerance models.) If so, then these data strongly support the view put forward by many authors that self-tolerance is a highly dynamic (and reversible) process (e.g. Zauderer *et al.*, 1984; Gibson *et al.*, 1985; Ramensee & Bevan, 1984). Such tolerance is compatible with the life-long persistence of anti-self clones because these are under continuous control by regulatory cells.

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