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Factors involved in peripheral T cell tolerance: the extent of clonal deletion or clonal anergy depends on the age of the tolerized lymphocytes

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Abstract. After injection of SEB (staphylococcus enterotoxin B), normal adult mice, or thymectomized irradiated mice (TX irr.) reconstituted with lymphocytes taken from normal adult mice became specifically tolerant of SEB. At the same time the percentage of Vβ8 positive CD4 lymphocytes known to be responsive to SEB was almost 50% decreased, indicating that a high level of clonal deletion was realized. In contrast, mice with an exclusively old T cell compartment (old thymectomized mice, TX irr. mice reconstituted several months previously) became tolerant of SEB without deleting their Vβ8 + CD4 + cells, indicating that clonal anergy was the major mechanism in play in the induction of tolerance. Finally, TX irr. mice reconstituted with single positive thymocytes known to become recent thymic emigrants developed tolerance for SEB together with a high level (70%) of clonal deletion. Altogether these results indicated that the mechanism involved in peripheral tolerance depended on the age of the lymphocyte: very young lymphocytes underwent mainly clonal deletion whereas long lived lymphocytes underwent predominantly clonal anergy.

Key words: T cell tolerance – Clonal deletion – Clonal anergy

The mechanisms by which T lymphocytes become tolerant for self or foreign antigens have been extensively investigated in the recent years. Whereas the concept of active suppression is still controversial, the mechanisms of clonal deletion or clonal anergy are generally accepted as the major ones in T cell tolerance. The advent of superantigens, which are able to stimulate large fractions of T cells [2, 4, 5, 14], and the availability of monoclonal antibodies identifying the T cell receptors capable of reacting against these antigens [16, 20, 23] has made the discrimination between clonal deletion and clonal anergy very reliable. It is generally accepted that immature T cells are tolerized within the thymus. This process is called central tolerance and is realized mainly by clonal deletion [7, 10, 11, 15].

After their arrival in the periphery T cells have recently been shown to be susceptible to tolerance induction as well (called peripheral tolerance) [3, 6, 8, 19, 22]. However, the mechanisms involved in peripheral tolerance are still unclear and controversial. Some studies indicate that clonal anergy is the predominant type of peripheral T cell tolerance [6, 8, 18, 19], whereas other experiments – sometimes involving the same models – unequivocally show that clonal deletion can be achieved to a large extent in the periphery as well [22]. The present studies were undertaken to know more about the factors which determine whether a peripheral T cell undergoes tolerance induction through clonal deletion as apposed to clonal anergy. It was our hypothesis that recent thymic emigrants would still be susceptible to clonal deletion within the first days after their arrival in the periphery, whereas older (memory?) T cells would mainly undergo tolerogenic signals leading to clonal anergy.

To investigate this we created experimental models in which the peripheral T cell pool could be expected to be made up predominantly of old mature T cells or alternatively of a high proportion of recent thymic emigrants. Here we showed that in the former situation, tolerance was almost exclusively realized by clonal anergy, whereas a high level of clonal deletion was observed in the case of a T cell compartment consisting of a relatively important fraction of recent thymic emigrants.

Materials and methods

Animals. In all experiments BLAB/C (H-2^d) mice were used.

Neonatal thymectomy. Newborn mice, 1–3 days old were anesthetized by cooling, and thymectomy was performed through a sternotomy between the second and third ribs by vacuum suctioning of the thymus with a pipette.

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Table 1. Effect of SEB priming on Vβ8 and Vβ6 receptor antigen expression and on the in vitro proliferative response of T lymphocytes

Groups	Percentage[d] of CD4+ lymphocytes expressing		Stimulation index:[e] after in vitro blastogenesis by	
	Vβ8+	Vβ6+	SEB	SEA
A. 2–4 month BALB/C ($N=5$)	24 (15–29)	9[f]	284 (196–405)	308 (172–444)
B. 2–4 month BALB/C ($N=3$)[a] primed with SEB	14 (9–17)	14[f]	11 (4–29)	206 (147–267)
C. LN reconstituted BALB/C[b] ($N=2$)	21 (20–22)	ND	27 (3–52)	24 (2–47)
D. LN reconstituted BALB/C[c] primed SEB ($N=2$)	12 (9–15)	ND	2 (2–3)	11 (3–20)

[a] SEB (33 µg) was injected 3 times within 1 week
[b] Lethally irradiated, thymectomized BALB/C mice, reconstituted with T cell depleted BM and lymph node cells from 2–4 month old BALB/C donors
[c] SEB (3 × 33 µg) was injected within 4 weeks after reconstitution
[d] Mean (range) are shown
[e] See materials and methods, means (range) are given
[f] Only one sample in group A and group B was examined on Vβ6 expression
ND = Not determined

Total body irradiation. Mice were exposed to irradiation using gamma rays from a 60-cobalt source (0.35 Gy per min) at a focus-skin-distance of 100 cm. A total dose of 8.5 Gy (850 Rad) was given.

Lymphohaematopoietic reconstitution after irradiation. Bone marrow (BM) cells were obtained from the femur of adult BALB/C mice. Subsequently, T cells were depleted from the BM by adding 50 µl of Thy 1.2 (Becton Dickinson, Mountain Vieuw, CA) for 30 min at 4°C. Thereafter, the cells were washed twice and rabbit complement diluted 1/9 was added and incubated for another 45 min at 37°C. Within one day of TBI, irradiated thymectomized mice received 20×10^6 T-depleted bone marrow cells together with either 50×10^6 single positive thymocytes obtained from 3–4 week old cortisone treated BALB/C mice in the first group, or in the second group, 60×10^6 peripheral T cells obtained from lymphnodes of 2–4 month old BALB/C mice

SEB injection: Staphylococcal enterotoxin B (SEB) was purchased from Sigma Chemical Company (St. Louis, Mo., USA). It was diluted and administered IP at a concentration of 33 to 100 µgr in 0.2 ml saline solution (phosphate-buffered saline). All in vitro tests were done 6–14 days after SEB injection.

Flow cytometry: One million splenocytes (passed through a nylon wool column) were stained for the expression of Vβ8 or Vβ6 positive T cell receptors using 20 µl mAbs obtained respectively from undiluted hybridoma culture supernatant from clones F23.1 [20] and F44-22-1 [16], followed by 50 µl fluoresceinated anti-mouse IgG (Fab) portion (Serotec, Oxford, England) for Vβ8 (F23.1) binding and 50 µl of rabbit anti-rat IgG (Fab) portion (Serotec, Oxford, England) for Vβ6 (F44-22-1) binding.

Double staining was done with an anti-mouse CD4 phycoerythrin conjugate (anti-L3T4, Becton Dickinson, Mountain View, Calif.) to measure the expression of Vβ receptors on CD4 positive cells only. Analysis was done using FACSTAR-PLUS (Becton Dickinson, Mountain View, Calif.) interfaced with a Hewlett Packard 300 computer. Cells were gated to exclude non viable ones.

Data are presented as percentage of positive cells after substraction of the number of cells stained with the fluorescent conjugate and irrelevant antibody.

Functional anergy to SEB. The responsiveness of lymphocytes and splenocytes in vitro was analyzed by measuring the stimulation index (SI) of lymphocytes after adding SEB (10 µg/ml), or staphylococcal enterotoxin A (SEA, 10 µg/ml, SERVA, Heidelberg, Germany) or phytoheamaglutinin (PHA, 72 µg/ml, Wellcome, England) into flat bottom micro-culture wells containing 5×10^5 cells in 0.2 ml RPMI 1640 medium supplemented with 20% human AB serum. After 72 h of incubation, cells were pulsed with 1 µCi of ^{3}H-thymidine collected to a glass filter and counted 18 h later.

$$\text{SI: } \frac{\text{cpm (cells + SEB)}}{\text{cpm (cells + medium)}}$$

The SI was calculated using the mean of quadriplucate counts per minute (cpm) samples measured by a scintillation counter:

Results

Injection of SEB into mice with an adult peripheral T cell compartment (2–4 month old) resulted in tolerance with a high level of clonal deletion (Table 1). BALB/C control (group A) mice, 2–4 months old, had a mean of 24% of Vβ8-CD4 positive T lymphocytes in their spleen and reacted vigourously in vitro on stimulation with SEB (mean SI = 284). When a similar group of mice (group B) was injected with 100 µg of SEB IV one week before testing their lymphocytes became hyporeactive against SEB (SI = 11), and at the same time they deleted an important fraction of their Vβ8+ CD4 cells known to be reactive against SEB (decrease from 24% to 14%). These results were identical to those previously published by other groups [8, 9, 23]. The tolerance achieved after IV injection of SEB was specific for SEB as the reactivity against another stimulus (SEA) was not significantly decreased (SI of 206 as compared to 308 in uninjected mice). The somewhat decreased reactivity of SEB injected mice against SEA could be a consequence of the lymphokine release syndrome known to occur after super antigen injection [13, 14]. The deletion of lymphocytes after SEB injection was limited to those expressing a Vβ8 TCR known to be reactive against SEB, as the percentage of Vβ6 positive CD4 cells (which do not react to SEB) was not decreased by SEB injection (14% in a SEB injected mouse as compared to 9% in an uninjected animal).

Also BALB/C mice (group C) which were thymectomized, lethally irradiated, and reconstituted with T depleted BM cells and peripheral lymphocytes (spleen cells and lymphnode cells) obtained from 2–4 months old donors became specifically tolerant of SEB after IV SEB injection 1 week before. Indeed, the in vitro reaction to SEB was reduced to about 10% of that observed in uninjected reconstituted mice (SI decreased from 27 to 2), whereas the reactivity against SEA remained considerable (SI decreased only from 24 to 11). Once again, SEB injection led to a significant deletion of Vβ8+ CD4 cells (decrease from 21 to 12%).

Thus, in both situations – 2–4 month old unmanipulated mice, or irradiated, thymectomized mice recon-

Table 3. Effect of SEB priming on Vβ8 and Vβ6 receptor antigen expression and on proliferative response of T lymphocytes in "young" mice

Groups	Percentage[d] of CD4 + lymphocytes expressing		Stimulation index:[c] after in vitro blastogenesis by		
	Vβ8 +	Vβ6 +	SEB	SEA	PHA
A. 3 week old BALB/C ($N = 2$)	23 (21–26)	9 (9–10)	50 (23–78)	ND	288 (25–551)
B. 3 week old BALB/C primed with SEB ($N = 2$)	11 (10–12)	13 (12–15)	5 (2–7)	ND	64 (45–83)
C. 3 week old thymect BALB/C ($N = 2$)	15 (15–17)	6	4	ND	13
D. 3 week old thym. BALB/C primed with SEB ($N = 3$)	9 (8–14)	10	7 (3–14)	ND	26 (13–39)
E. thymocyte reconstituted[a] BALB/C ($N = 3$)	22 (19–25)	ND	59 (55–64)	48 (58–81)	ND
F. thymocytes reconstituted[b] primed with SEB ($N = 2$)	7 (5–10)	ND	4 (4–5)	36 (7–66)	ND

[a] Lethally irradiated, thymectomized BALB/C mice, reconstituted with T cell depleted BM and thymocytes from 3–4 month old BALB/C cortisone treated donors
[b] SEB (3 × 33 μg) was injected within 1 week after reconstitution
[c] See materials and methods
[d] Mean (range) was shown
ND = Not determined

Table 2. Effect of SEB priming on Vβ8 and Vβ6 receptor antigen expression and on proliferative response of T lymphocytes in "old" mice

Groups	Percentage[d] of CD4 + lymphocytes expressing		Stimulation index:[e] after in vitro blastogenesis by		
	Vβ8 +	Vβ6 +	SEB	SEA	PHA
A. 6–7 month BALB/C ($N = 2$)	28 (28–29)	10 (10–11)	552 (273–831)	ND	148 (84–212)
B. 6–7 month BALB/C ($N = 2$)[a] primed with SEB	24 (24–25)	9 (9–10)	6 (6–7)	ND	327 (133–522)
C. 6 month old thymect[b] ($N = 2$)	25 (24–25)	11	10 (51–152)	137	722
D. 6 month old thymect primed with SEB ($N = 2$)	25 (24–25)	6	2 (1–5)	35	895
E. TX-reconstituted with thymocytes primed with SEB[c] ($N = 1$)	30	ND	5	58	ND

[a] SEB (3 × 33 μg) was injected 10 days before the test
[b] Thymectomy was done within 2 days of birth
[c] Lethally irradiated, thymectomized BALB/C mouse reconstituted with thymocytes 2 months before SEB injection
[d] mean (range) are shown
[e] See materials and methods
ND = not determined

stituted with peripheral T cells from 2–4 month old mice – SEB injection induced specific tolerance, which seemed to be mediated to a significant extent by clonal deletion.

Injection of SEB into mice with an old peripheral T cell compartment resulted in specific tolerance but without significant clonal deletion (Table 2). In the next series of experiments we used various groups of mice whose peripheral T cell compartment would be expected to be composed mainly of long-lived T cells. The first groups (A and B) consisted of 6 months old mice. At that age it has been shown that the thymic export of T cells becomes extremely low [21] hence peripheral T cells consist mainly of old T cells. However, to exclude any thymic influence, old thymectomised mice (group C and D) were investigated as well. Finally, a thymectomized, lethally irradiated BM reconstituted mouse (E) was injected with single positive thymocytes, but was used in the experiments only 2 months after reconstitution and therefore could be considered as having a peripheral T cell pool of cells of at least 2 month old. As can be seen on Table 2, in all groups of mice SEB injection resulted in functional tolerance of SEB (signifiant decrease of the SI of SEB induced blastogenesis to very low levels), but this time without any significant decrease of the number of Vβ8 + CD4 cells. Tolerance was specific, as the blastogenesis to another stimulus (PHA or SEA) was not consistently decreased. Also the number of irrelevant Vβ6 + CD4 cells was not significantly altered by the SEB injected (see group B and D). The overall conclusion of these experiments was, therefore, that in mice having a predominantly old T cell compartment SEB injection did lead to tolerance but without apparent clonal deletion.

Injection of SEB in mice with a predominantly young T cell compartment resulted in specific tolerance and clonal deletion of a high percentage of Vβ8 + CD4 cells. Three experimental situations were investigated where the peripheral T cell compartment could be considered as being composed mainly by young T cells. The first groups (A and B) consisted of 1 week old BALB/C mice. The following groups (C and D) were made up of 1 week old neonatally thymectomized mice to exclude thymic influence, and finally, in groups (E and F), lethally irradiated, thy-

mectomized animals were reconstituted with single positive thymocytes (obtained from thymuses of cortisone treated animals) and injected 1 week later with SEB.

As shown on Table 3, in all situations SEB injection led to a high level of clonal deletion of Vβ8 + CD4 cells and this was most pronounced in group F (a decrease of Vβ8 + CD4 cells of about 70%). In this latter group the group of reconstituted mice could be expected to have the highest percentage of very young T cells, as they were reconstituted with single positive thymocytes, known to be the cells which migrate to the periphery. Once again, injection of SEB resulted in specific tolerance as the PHA or SEA stimulation indexes were not considerably influenced. Again the decrease of CD4 cells was not seen in the subpopulation of T cells bearing the irrelevant Vβ6 positive TCR.

Discussion

Recently, various experiments have indicated that peripheral T cell tolerance can be achieved. As far as the mechanisms are concerned, some authors find that clonal anergy is the major mechanism underlying peripheral T cell tolerance although they admit that the results can vary from animal to animal [18]. Other investigators [22] have shown that clonal deletion can also take place in the periphery. The present experiments were undertaken to solve this controversy. It was our hypothesis that T lymphocytes that only recently emigrated from the thymus would still be very susceptible to clonal deletion in the periphery, whereas those which persisted for a longer period would become resistant to deletion and would be tolerized without deletion, thus becoming anergic. We showed that in cases where the peripheral T cell pool would be expected to be composed of a high fraction of young lymphocytes, tolerance after SEB injection was specific and associated with a high level of clonal deletion (a decrease of about 50%) of the Vβ8 + CD4 cells known to be reactive against SEB. In contrast, in all three situations where the peripheral T cells could be expected to consist almost exclusively of long lived lymphocytes, tolerance was achieved without significant clonal deletion, and hence was due to clonal anergy.

We asked ourselves why young peripheral T cells are still susceptible to clonal deletion, and old T cells resistant to it. It is possible that young peripheral T cells contain a majority of virgin T cells, whereas old T cells should be mainly memory cells. Indeed, T cells probably die rapidly in the periphery if they do not see an appropriate antigen [12]. Memory T cells express more adhesion molecules, and therefore their contact with the antigen presenting cell (APC) must be different from that of virgin cells. The intensity of contact with the APC is known to be an important determinant in the type of tolerance (clonal deletion versus anergy) which is induced after a tolerogenic signal [1, 17]. Memory cells are also known to have lymphokine secretion profile other than that of virgin cells [1]. Interference with the lymphokine secretion profile of cells was has recently been shown to be an important factor in the induction of clonal deletion [1]. Therefore, the differences in the mechanisms underlying tolerance in virgin as opposed to memory T cells could be related to their constitutive lymphokine secretion profiles. We are presently trying to clarify these issues using mice reconstituted with T cells with either virgin or memory characteristics or by interfering with their specific lymphokine secretion patterns.

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