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Clonal deletion and clonal anergy in allogeneic bone marrow chimeras prepared with TBI or TLI

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Abstract The evolution of Vβ6− expressing C_3H (H_2^k, Thy 1.2, Mls a−) lymphocytes was investigated in C_3H recipients mice pretreated with total body irradiation (TBI) or total lymphoid irradiation (TLI) and infusion of AKR (H_2^k, Thy 1.1, Mls a+) cells. After TBI (9.5 Gy) all Vβ6+ Thy 1.2 (C_3H) cells, which are capable of reacting against the Mls a antigen that like expressed by AKR mice, were deleted in the thymus and the periphery in stable bone marrow (BM) chimeras obtained by infusion of 5×10^6 T-cell-depleted (TCD) AKR BM cells. When, in the opposite combination, 30×10^6 C_3H spleen cells were infused into TBI-treated AKR cells, all animals developed graft-versus-host disease (GVHD) with no clonal deletion and in contrast, showed an increase in Vβ6+ C_3H cells. After injection of 30×10^6 AKR BM cells into TLI-treated C_3H mice no C_3H cells were detected in the thymus and only a small percentage in the periphery. Within these C_3H cells Vβ6+ cells were only partially deleted and anergized as they did not respond in vitro after stimulation with Mls a+ AKR cells or anti-Vβ6 mAb. Cells suppressing anti-Mls a-reacting C_3H cells were not found. After injection of 15×10^6 AKR cells more C_3H cells were found in the thymus, but only a minority of Vβ6+ cells persisted in the periphery of these animals. In conclusion in TBI-prepared chimeras only clonal deletion occurred, whereas in TLI-prepared chimeras both clonal deletion and anergy occurred in maintaining tolerance.

Key words Bone marrow chimeras
Total body irradiation
Total lymphoid irradiation
Clonal deletion · Clonal anergy

Introduction

Transplantation tolerance is now generally believed to be based on two major mechanisms: clonal deletion or clonal anergy. Because of the availability of monoclonal antibodies that recognize specific T cell receptors, these two mechanisms can now be differentiated from each other. The present study was undertaken to investigate which mechanism was underlying immune tolerance after bone marrow transplantation. Two different preparative regimens, both leading to stable bone marrow chimerism were compared. In the first model, mice were given T cell depleted allogeneic bone marrow cells after lethal total body irradiation. The second model involved infusion of

untreated allogeneic bone marrow into recipient mice that were given fractionated total lymphoid irradiation. In both models, host type lymphocytes expressing T cell receptors directed against donor antigens were looked for in peripheral blood and lymphoid organs in order to see whether clonal deletion had occurred.

Methods

Mice

C_3H (H_2^k, Thy 1.2, Mls 1a−) and AKR (H_2^k, Thy 1.1, Mls 1a+) mice were kept in conventional housing during the whole experiment.

Irradiation

One 9.5 Gy dose of total body irradiation (TBI) or 14 Gy of total lymphoid irradiation (TLI) given in daily doses of 2 Gy were administered as described previously [1].

Bone marrow transplantation

TBI-treated animals received 5×10^6 T-cell-depleted (TCD) (using Thy 1.2 monoclonal antibody (mAb) and complement bone marrow (BM) cells or 30×10^6 untreated spleen cells. TLI-treated animals were given 15×10^6 or 30×10^6 unmanipulated BM cells.

Scoring of chimerism and Vβ6+ cells

At various times after BM transplantation peripheral blood, thymus, spleen and lymph nodes were analysed for lymphoid chimerism using Thy 1.2 (= C_3H) or Thy 1.1 (= AKR) mAbs and FACS analysis. By double colour FACS analysis the number of Vβ6+ T cells in Thy 1.2-positive C_3H cells was determined as described previously [3].

Functional assays

The reactivity of remaining Vβ6+ Thy 1.2 cells was investigated in some chimeras using an anti-Vβ6 induced in vitro proliferation assay as described elsewhere [6] or by measuring their proliferation after stimulation with Mls a+ control AKR spleen cells. Putative suppressor cells within the chimeras were sought by adding them to a control $C_3H \times$ AKR mixed lymphocyte reaction (MLR) reaction.

Results

Survival and chimerism after various transplantation regimens

Figure 1 shows the survival after BM or spleen transplantation. All C_3H mice were healthy and showed 100% survival after both TBI and infusion of 5×10^5 Thy 1.1-depleted AKR BM cells or TLI and infusion of 15 or 30×10^6 unmanipulated AKR cells. In contrast, all TBI-treated AKR mice showed clinical signs of graft-versus-host disease (GVHD) (weight loss, diarrhoea, hunched back), and 50% of them died, after infusion of 30×10^6 C_3H cells. Table 1 shows the percentage of C_3H (= Thy 1.2 cells) remaining in the chimeras after the various transplantation schedules.

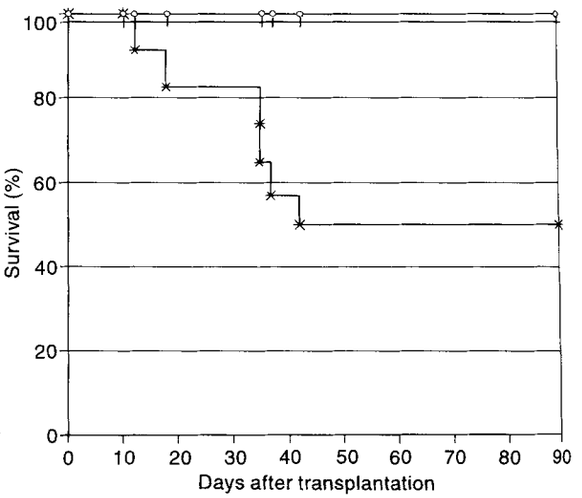

Fig. 1 Actual survival of TLI-treated C_3H mice ($n = 12$) injected with unmanipulated AKR BM cells (□), TBI-treated C_3H mice ($n \pm 10$) injected with T-cell depleted AKR BM cells (+) and TBI-treated AKR mice ($n = 12$) injected with C_3H splenocytes (*)

After 9.5 Gy of TBI and injection of 5×10^6 TCD AKR BM cells all mice became mixed chimeras (63% and 49% of C_3H cells were detected in the peripheral blood and thymus, respectively, at 2–4 months after transplantation). As would be expected, the majority of cells found in mice developing GVHD after spleen infusion were of donor origin both in the periphery (92%) and thymus (86%). After TLI, the level of chimerism depended on the number of BM cells infused. When 15×10^6 AKR were injected, host-type C_3H cells were allowed to grow out sufficiently leading to 44% and 28% of C_3H cells in peripheral blood and thymus, respectively, at 1–4 months after transplantation. However, when the number of donor AKR cells infused was increased to 30×10^6 the majority of cells found in the chimeras were of donor origin with only 7% of C_3H T cells in the periphery and no significant numbers of C_3H thymocytes in the thymus.

Table 1 Percentage of chimerism and persisting Vβ6-positive C_3H cells after allogeneic BM transplantation using various regimens

Treatment group	Inoculum	Percent Thy 1.2 (C_3H) cells in the peripheral blood [a]	Percent Thy 1.2 (C_3H) cells in the thymus [a]	Percent of Vβ6+ cells in the Thy 1.2 population
1. TBI ($n=10$)	5×10^6 TCD BM cells	63 (±11)	49 (±46)	0
2. TBI ($n=8$)	30×10^6 spleen cells	92 (±15)	86 (±28)	16 (±7)
3. TLI ($n=5$)	15×10^6 non-TCD BM cells	44 (±23)	28 (±38)	1 (±0.8)
4. TLI ($n=6$)	30×20^6 non-TCD BM cells	7 (±3)	0	5 (±0.5)

[a] In relation to the total number of T cells (Thy 1.2 + Thy 1.1)

Table 2 Suppressor assay using control of chimeric C_3H cells as regulators in a $C_3H\times$AKR MLR

Responder cells	Stimulator cells [a]	Regulator cells [b]	cpm [c]	Percent suppression [d]
C_3H control spleen cells	AKR	–	$315\pm12\times10^3$	–
C_3H control spleen cells	AKR	Control C_3H	$349\pm24\times10^3$	0
C_3H control spleen cells	AKR	Purified chimeric C_3H (exp 1)	$327\pm10\times10^3$	0
C_3H control spleen cells	AKR	Purified chimeric C_3H (exp 2)	$288\pm26\times10^3$	9

[a] Mitomycin-treated to prevent proliferation but allowing Mls a presentation
[b] In vitro irradiated with 15 Gy to prevent proliferation
[c] Counts per minute after thymidine incorporation after a 3-day culture
[d] Expressed as $\frac{(\text{cpm with control cells} - \text{cpm with experimental cells})}{\text{cpm with control cells}} \times 100$

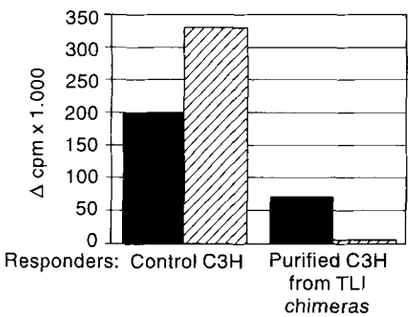

Fig. 2 Proliferation of control C_3H spleen cells and C_3H cells isolated from TLI-treated AKR B C_3H chimeras after in vitro stimulation for 3 days with BALB/C (H_2^d) (■) or AKR (Mls a+) (▨) mitomycin-treated spleen cells. The proliferation is expressed as the increase in thymidine incorporation (cmp) as compared with the background stimulation provoked by syngeneic (C_3H) mitomycin-treated spleen cells

Clonal deletion and clonal anergy after various transplantation regimens

As can also be seen in Table 1 TBI-prepared stable chimeras (group 1) developed complete clonal deletion of lymphocytes with anti-donor reactivity (0% of Vβ6+ cells were found in the C_3H T lymphocytes). In contrast, in group 2 mice which developed GVHD an increase (16%) in Vβ6+ Thy 1.2 cells was found (normal control C_3H mice had 9–12% Vβ6+ T cells).

In TLI-treated recipients clonal deletion of Vβ6+ Thy 1.2 cells was observed only to a limited extent depending on the number of donor cells infused. When a relatively low number of donor cells was transplanted (group 3), host-type C_3H cells grew out, even in the thymus, and most Vβ6+ cells were deleted (only 1% were detected in the periphery). In contrast when a high number of donor cells was infused, only a low number of C_3H T cells were found in the periphery, and they seemed to originate only

from peripheral proliferation as no C_3H T cells were found in the thymus. These remaining C_3H T cells were not totally deleted as 5% Vβ6+ cells were left. To investigate whether these cells were anergized functional assays were performed (Fig. 2). C_3H lymphocytes were purified from the spleen of TLI chimeras by treating them with Thy 1.1 (= anti-AKR) mAb and complement. These cells were then stimulated with mitomycin-treated AKR spleen cells (to investigate Mls-a-directed reactivity) or allogeneic (BALB/C) mitomycin-treated splenocytes. In contrast to control C_3H cells which strongly reacted against AKR cells (even more strongly than against allogeneic BALB/C cells), C_3H cells from TLI chimeras were anergic for AKR as they showed no proliferation. The somewhat lower response of TLI-C_3H cells as compared with control C_3H cells towards BALB/C is not surprising as long-term MLR hyporesponsiveness after TLI is well documented [7]. Also, when these C_3H cells were stimulated with anti-Vβ6 or anti-Vβ8 mAb as described elsewhere [6], they did not respond to anti-Vβ6 mAb or generally to anti-Vβ8 mAb (data not shown). In order to determine whether this anergy was mediated by suppression a suppressor assay was performed (Table 2) using purified C_3H cells obtained from TLI-treated chimeras. As can be seen from Table 2, in two separate experiments no significant suppression was observed when chimeric C_3H from TLI mice were added to a control $C_3H \times AKR$ MLR as compared with control C_3H cells. Suppressor cells could thus be excluded as the reason for the anergy of the chimeric C_3 cells.

Discussion

The present study was undertaken to investigate the mechanisms underlying immune tolerance in TLI-treated allogeneic BM chimeras. For this purpose we used AKR and C_3H mice as the latter are Mls a− and their T lymphocytes express Vβ6 receptors which confer reactivity against Mls a [5]. This enabled us to determine whether Vβ6+ cells were deleted and, if persisting, whether they were anergic for Mls a. At the same time we compared the results of TLI with those obtained in mice developing either stable chimeras or GVHD after TBI. Stable chimeras obtained with TBI and infusion of TCD BM cells developed complete clonal deletion of Vβ6+ cells. This deletion occurred in the thymus as no bright-staining single positive CD4− Vβ6+ thymocytes were observed (data not shown). These results are in agreement with those of previous studies [4]. In contrast, in the GVHD situation clonal expansion of Vβ6+ cells was observed with no clonal deletion illustrating the lack of tolerance between donor and host in this situation.

In TLI-treated animals both clonal deletion and anergy was found. When only a low number of C_3H cells remained in the TLI chimeras, Vβ6+ C_3H cells were not totally deleted (5% persisted). We hypothesize that these Vβ6+ cells are cells that remain in the periphery after TLI, and are peripherally anergized by the injected AKR cells. Peripheral anergy of Vβ6+ C_3H cells after injected of AKR cells has also been reported previously [5]. In another strain combination Field and Steinmuller [2] also found clonal anergy as a major mechanism underlying tolerance in TLI-treated chimeras. In accordance with their results, we found that the non-deleted Vβ6+ cells were anergized because they could not be stimulated in vitro with Vβ6 mAb (data not shown), nor did they proliferate significantly after stimulation with AKR splenocytes (Fig. 2). The fact that suppressor cells were not involved in anergy was apparent from the results of the experiments shown in Table 2. In their study Field and Steinmuller [2] did not investigate whether the remaining anergized cells originated from post-TLI T cells persisting in the peripheral compartment, or whether they arose from thymic emigrants. The former hypothesis seems more likely from the results of our experiments comparing TLI C_3H mice injected with low (15×10^6) or high (30×10^6) numbers of AKR cells. When high numbers of AKR cells were injected, all thymocytes were of AKR origin (Table 1, group 4) and hence the peripheral C_3H T cells were probably peripheral T cells which had survived the TLI regimen. However, when a low number of AKR cells were injected after TLI, many more C_3H cells were allowed to grow out even in the thymus (28%; Table 1, group 3) and thus the peripheral C_3H T cell compartment comprised mainly thymic emigrants. As their Vβ6+ thymocytes had undergone deletion in the thymus (no Vβ6 single positive CD4 thymocytes were detected, data not shown), these emigrating Vβ6− C_3H cells diluted the number of peripherally anergized C_3H cells (Table 1, group 3).

The importance of the persistence or absence of T cells with T-cell receptors directed against donor or host antigens is not known yet, but they may play a role in the stability of chimeras. We are presently investigating this by challenging TLI chimeras with host-type spleen cells.

In conclusion, whereas TBI-prepared stable chimeras used clonal, deletion as a tolerizing mechanism, TLI-treated chimeras used both clonal deletion when T cells orginated from the thymus and clonal anergy for T cells persisting peripherally after TLI.

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