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Bone Marrow Transplantation Induces Either Clonal Deletion or Infectious Tolerance Depending on the Dose[1]

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The concept of immunologic tolerance arose from bone marrow transplantation in neonatal or irradiated mice, in which the predominant mechanism is clonal deletion of donor-specific T cells by donor hemopoietic cells in the recipient thymus. A short term treatment with nonlytic CD4 and CD8 mAbs can induce tolerance to tissue allografts or reversal of spontaneous autoimmunity. Such tolerance to skin or heart allografts is dependent on "infectious" tolerance mediated by regulatory $CD4^+$ T cells. We show here, for multiple minor Ag differences, that while a large inoculum of donor marrow produces significant deletion of Ag-reactive cells as expected, a low marrow dose generates tolerance with little evidence of clonal deletion. Only this low dose tolerance can be transferred to unmanipulated recipients via $CD4^+$ T cells, can be passed onto naive T cells as if infectious, and can act to suppress rejection of third party Ags when "linked" on F_1 grafts. ***The Journal of Immunology*****, 1998, 160: 2645–2648.**

The demonstration of classical transplantation tolerance induced by allogeneic marrow infusion into the neonatal mouse (1) led to the concept that tolerance was an acquired process and subsequently to the idea that it was dependent on clonal deletion of immature lymphocytes confronting their specific Ags. Streilein and coworkers (2) demonstrated that the mechanisms underlying marrow tolerance in the neonate varied depending on the strain combinations studied. In some cases, tolerance was accompanied by in vitro evidence of lymphocyte nonresponsiveness (consistent with deletion), while in others lymphocyte reactivity still remained. Zamoyska et al. (3) observed clear evidence of dominant tolerance in adult marrow chimeras in which T cells were allowed to develop in one of two implanted, genetically disparate, thymus lobes. More recently, Modigliani and colleagues (4) observed that T cells developing in epithelial thymus grafts also exhibited dominant tolerance, unlike those developing in a normal thymus.

The extent to which dominant tolerance might be exploited therapeutically has been highlighted in studies from our laboratory in which tolerance to skin and heart grafts (across both minor and major histocompatibility barriers) could be induced by nonlytic CD4 and CD8 Ab therapy (5, 6). The subsequent finding that in all cases tolerance was infectious and dependent on $CD4^+$ regulatory T cells (7) led us to reexamine the mechanism by which marrow infusions (under Ab cover) brought about tolerance in the adult. Did this involve dominant tolerance? In 1989, we demonstrated that tolerance could be induced in the adult to bone marrow grafts (incompatible for multiple minor and Mls-1 Ags) using CD4 and CD8 Abs. Tolerance was accompanied by loss of MLR reactivity, although not of $V\beta6^+$ cells from the periphery, suggesting that Mls-1^a-reactive T cells had become anergic (8). This first demonstration of anergy in vivo then seemed a sufficient explanation of the tolerant state and was not pursued further until now. We decided to reinvestigate the mechanisms underlying adult transplantation tolerance by marrow infusion and have concluded that the cell dose of the marrow inoculum influences the way in which tolerance develops.

Materials and Methods

Mice and skin grafting

CBA/Ca, Thy1.1 congenic CBA/Ca, B10.BR, and (AKR × B10.BR) F_1 mice were bred and maintained in conventional conditions at the Sir William Dunn School of Pathology (Oxford, U.K.). AKR/OlaHsd mice were obtained from Harlan Olac (Bicester, U.K.). Recipient mice were between 6 and 8 wk old at the beginning of an experiment. All groups were matched for age and sex. All procedures were performed in accordance with the U.K. Home Office Animals (Scientific Procedures) Act of 1986.

Skin grafts were performed according to a modified method of Billingham et al. (1). In short, full thickness tail skin (0.5 × 0.5 cm) was grafted on the lateral flank. Mice were anesthesized using Hypnodil and Sublimaze i.p. (Janssen, Tilburg, The Netherlands). Skin grafts were considered rejected when no viable donor skin was present. Significance differences in graft survival between groups were estimated by the log rank method (9).

Monoclonal Abs and bone marrow infusions

The following mAbs were produced by culture in hollow-fiber bioreactors in our own laboratory: YTS 177.9 (rat IgG2a mouse CD4 (5)), YTS 3.1 (rat IgG2b mouse CD4 (10)), YTS 191.1 (rat IgG2b mouse CD4 (10)), YTS 156.7 (rat IgG2a mouse CD8β (10)), YTS 169.4 (rat IgG2b mouse CD8α (10)), and 30-H12 (rat IgG2b mouse Thy1.2 (11)). Abs were purified from culture supernatants by 50% ammonium sulfate precipitation and checked for their purity (>50% of total protein) by native PAGE and SDS-PAGE on a PhastGel (Pharmacia, St. Albans, U.K.) system. Mice that were given bone marrow to induce tolerance received three doses each of 0.5 mg YTS 177.9 + 0.25 mg YTS156.7 + 0.25 mg YTS 169.4.

Bone marrow cell donors were T cell depleted using a mixture of 0.5 mg each of YTS 3.1 + YTS191.1.2 + YTS156.7 +YTS 169.4 Abs (10) 3 days before bone marrow cell harvesting. After depletion, bone marrow contained less than 0.5% $CD3^+$ cells. Bone marrow cells were flushed from the femoral and tibial bones using RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 2% FCS, penicillin/streptomycin, and 5 mM HCl. After washing and counting bone marrow cells, cells were resuspended in 200 μl of PBS and injected in the lateral tail vein.

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Table I. *Deletion of Vβ6$^+$CD4$^+$ and Vβ6$^+$CD8$^+$ T cells depends on the dose of AKR bone marrow cell inoculum and correlates inversely with linked suppression of (AKRxB10.BR) F_1 grafts*[a]

Bone Marrow Dose	Thymus CD4$^+$CD8$^+$ % Vβ6$^+$	Peripheral Blood CD4$^+$CD8$^+$ % Vβ6$^+$	Donor Chimerism[b] % Thy1.1$^+$	AKR Skin Graft MST[c] (days)	(AKRxB10.Br) F_1 Skin Graft MST[c] (days)
5×10^7	0.4 ± 0.1/3.2 ± 2.8	0.4 ± 0.22/1.4 ± 0.7	28.9 ± 15.5	>100	27
1×10^7		0.8 ± 0.6/2.4 ± 1.9	10.0 ± 7.8	>100	36
2×10^6		1.3 ± 1.4/3.9 ± 2.0	3.1 ± 1.1	>100	34
4×10^5		1.5 ± 1.1/6.7 ± 1.3	0.2 ± 0.2	>100	>100
1×10^5	8.1 ± 1.2/11.9 ± 0.9	10.1 ± 7.4/14.2 ± 7.5	0.3 ± 0.4	>100	>100
1×10^4				>100	
1×10^3				33	
"100"				51	
None	7.9 ± 0.6/15.6 ± 0.1	12.2 ± 1.5/17 ± 1.2	0.2 ± 0.4	16	18

[a] Different doses of T cell-depleted AKR bone marrow cells were given i.v. to CBA/Ca mice ($n = 10$ in each group) together with a course of nondepleting CD4 and CD8 Abs. AKR tail skin was grafted 6 wk later. Control groups consisting of CBA/Ca infused with syngeneic or no bone marrow cells rejected AKR skin within 20 days. At day 70, the percentage of Vβ6$^+$ cells among single CD4$^+$ and CD8$^+$ cells was determined in the 5×10^7, 1×10^5, and no bone marrow gropus in the thymus ($n = 4$) and in the peripheral blood ($n = 6$). Results are depicted as mean ± SD.

[b] Chimerism was determined by staining the peripheral blood for donor Thy1.1$^+$ among CD4$^+$ cells.

[c] MST, median survival time.

Immunofluorescence analysis and cell sorting

Cell preparations of thymi were washed and resuspended in PBS, 1% w/v BSA, 5% v/v heat-inactivated normal rabbit serum, and 0.1% w/v sodium azide. Cells were incubated for 30 min at 4°C with anti-Vβ6-FITC (clone 44-22-1 (12)), CD4-phycoerythrin (Sigma Chemical Co., cat. no. P-294), and CD8-Quantum Red (Sigma, cat. no. R-3762). Then cells were washed, resuspended in PBS, 1% BSA, and fixed in 1% v/v formaldehyde solution. Tricolor FACScan analysis (Becton Dickinson, Oxford, U.K.) was performed. Staining of peripheral blood lymphocytes was performed in a similar manner but was preceded by a water lysis of the erythrocytes. Chimerism was detected by staining donor lymphocytes with anti-Thy1.1-FITC (Serotec, cat. no. MCA 47F).

To determine the phenotype of APCs required for tolerance induction, T cell-depleted AKR bone marrow was negatively selected using a FACSort (Becton Dickinson) after staining with either anti-B220-Quantum Red (Sigma, cat. no. 4262), anti-mouse Ig-FITC (Sigma, cat. no. F-0257), or anti-CD11b (YMB 6.1.10-FITC (13)). Sorted cells were collected by centrifugation and counted, and 2000 cells were injected into Ab-treated CBA recipients.

Results

High dose marrow infusion generates tolerance by deletion

CBA/Ca (H2^k, Mls-1^b) mice were tolerized to AKR (H2^k, Mls-1^a) using different doses of T cell-depleted bone marrow cells and a short course of nondepleting CD4 (plus depleting CD8) Abs. Transplantation tolerance was tested by grafting donor AKR skin 6 wk later. The percentage of Vβ6$^+$ cells among CD4$^+$ and CD8$^+$ cells in the thymus, peripheral lymphoid organs, and blood was used to reflect the degree of deletion elicited through Mls-1^a recognition (12). Six weeks after transplantation of the test AKR graft, mice were regrafted with skin from (AKR × B10.BR) F_1 donors, and graft survival was again monitored (Table I). Recipients of high dose bone marrow (>4×10^5 cells) showed evidence of central and peripheral deletion of the Vβ6$^+$ cells, sustained donor chimerism, and acceptance of donor skin. Recipients of low dose marrow (≤4×10^5 cells) accepted donor skin, yet had no evidence of donor chimerism nor of deletion of the Vβ6$^+$ population.

Low dose marrow infusion generates tolerance associated with suppression

A second graft expressing the donor Ags and an additional new set of minors ((AKR × B10.BR) F_1) was then used to search for any evidence of linked suppression (14). These grafts were rejected rapidly in the high dose marrow groups but accepted indefinitely in the low dose groups (Table I). Evidence of dominant tolerance was then sought by the transfer of tolerant splenocytes from low or high dose marrow groups into naive CBA/Ca mice simultaneously grafted with AKR skin. Graft rejection was marginally delayed only in the recipients of spleen cells from the low dose bone marrow group. However, when tolerant donor mice were boosted with T cell-depleted AKR bone marrow cells 3 days before splenocyte transfer, graft rejection was significantly delayed in all recipients of low dose-tolerant cells but not in recipients of the high dose group (Fig. 1).

To demonstrate that transferable (dominant) tolerance depended upon CD4$^+$ T cells, we treated low dose-tolerant, boosted mice with either depleting anti-CD4 or anti-CD8 Abs

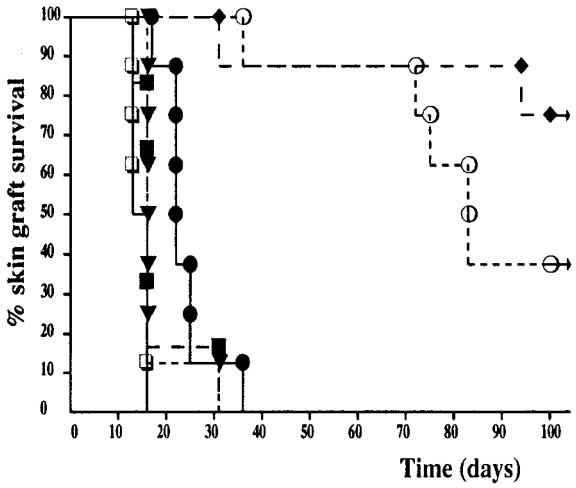

FIGURE 1. Naive CBA/Ca mice were given i.v. 4 to 6×10^7 splenocytes derived from low dose bone marrow-tolerized (nonboosted) donors (●) and equivalent donors boosted with 1×10^7 T cell-depleted AKR marrow cells (○) or from high dose bone marrow-tolerized (nonboosted) donors (■) or their equivalent boosted as above (□), and all were grafted the same day with AKR skin. The median survival times of AKR skin were 22 vs 82 ($p < 0.004$) and 16 vs 13 days (not significant), respectively. Controls consisted of mice treated with 5×10^6 T cell-depleted marrow-plus-AKR skin (median survival time, 14 days) or mice treated with 4 to 6×10^7 splenocytes derived from naive CBA/Ca mice boosted with 1×10^7 T cell-depleted AKR bone marrow cells. When boosted, low dose bone marrow-tolerized mice were injected with either depleting CD4 Abs (▼) or depleting CD8 Abs (◆) 24 h before cell transfer. Median survival times were 16 and 82 days, respectively ($p < 0.013$), demonstrating that the suppressing capacity was within the CD4$^+$ population. All groups consisted of eight mice each.

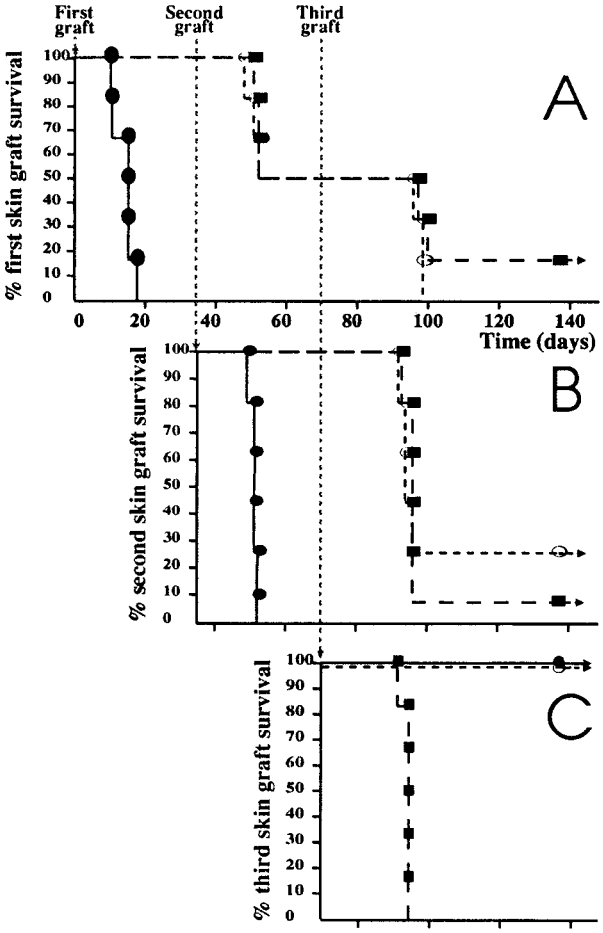

FIGURE 2. Skin graft survival on immunocompetent CBA/Ca mice after adoptive transfer of tolerant splenocytes. Thy-1.2 CBA/Ca mice were tolerized to AKR using 10^4 T cell-depleted AKR bone marrow cells together with a course of nonlytic CD4 plus depleting CD8 Abs. Tolerance was tested by acceptance of an AKR skin graft (not shown). After boosting of these mice with 10^6 T cell-depleted AKR bone marrow cells, tolerant splenocytes (one-half spleen per mouse) were adoptively transferred into Thy-1.1 CBA/Ca recipients. The same day, mice (six in each group) were grafted with AKR skin and received either 2 mg of depleting anti-Thy-1.2 Abs (30-H12; *A*, ●), 2 mg of Campath-1G (an irrelevant isotype matched Ab) (*A*, ○), or no Abs (*A*, ■). Skin grafts in the 30H-12-treated group were rejected within 18 days, whereas graft survival in the other two groups was >35 days ($p < 0.004$). The latter two groups were regrafted on the contralateral side with fresh AKR skin at day 35 after the first skin graft and received either 2 mg of 30H-12 (*B*, ○) or 2 mg of Campath-1G Abs (*B*, ■). Graft survival of this second AKR skin graft was in both groups >35 days, indicative of an infectious suppression of graft rejection when compared with controls ($p < 0.004$) consisting of Thy-1.1 CBA mice grafted with AKR skin that rejected their graft within 18 days (*B*, ●). At day 70 after adoptive transfer, both groups of mice still carrying AKR grafts received a third skin graft from an (AKR × B10.BR) F_1 donor (*C*, ● and ○). These grafts survived beyond 50 days, demonstrating that the infectious suppression could then act on third-party Ags linked through an F_1 graft. Control Thy-1.1 CBA mice rejected their F_1 grafts within 20 days (*C*, ■).

or a combination of both 24 h before transfer into naive CBA/Ca mice (Fig. 1). While those donor populations deprived of $CD8^+$ T cells were still able to delay rejection of AKR grafts by naive host lymphocytes, those deprived of $CD4^+$ T cells failed to do so, confirming that $CD4^+$ T cells were indeed required for the transfer of tolerance.

Low dose marrow tolerance is "infectious"

Adoptive transfer of low dose-tolerized cells ($Thy1.2^+$) into $Thy1.1^+$ CBA/Ca recipients resulted in suppression of graft rejection as before. Depletion of donor lymphocytes immediately after transfer, with a depleting Ab against Thy1.2, abolished suppression (Fig. 2). However, if depletion was delayed until donor cells had resided in the secondary host for 3 wk, this resulted in prolonged survival of a fresh AKR graft and subsequently full acceptance of a graft from (AKR × B10BR) F_1 donors (Fig. 2). This demonstrates for the first time that tolerance to marrow can give rise to infectious tolerance and that the second-generation tolerized T cells also exhibit suppressive properties (linked suppression).

$B220^+$ bone marrow cells are required for low dose tolerance induction

As the injection of as few as 10,000 donor marrow cells was sufficient to induce tolerance, and as few as 100 cells produced a significant delay in graft rejection (Table I), we hypothesized that tolerance might require specific expansion or homing of a viable APC subpopulation from the bone marrow. In favor of this interpretation, neither fixed (1% formaldehyde) nor irradiated (2000 rad) cells (at a dose of 10^5) could induce tolerance, even if the latter were given repeatedly during a 2-wk period (not shown). In addition, removal of $B220^+$ cells (but not surface Ig^+ cells nor myelomonocytic $CD11b^+$ cells) significantly impaired the tolerizing potential of a low dose (2000 sorted cells) marrow inoculum, suggesting that immature B-lineage cells were the most effective population at inducing dominant tolerance in this system.

Discussion

It has generally been thought that bone marrow induces tolerance centrally by clonal deletion in the thymus, and that no peripheral regulation was involved (15). The data in this paper confirm this to be the case when relatively high doses of multiple minor Ag (including Mls-1^a)-mismatched bone marrow are given under cover of CD4 plus CD8 Abs. The demonstration that chimerism, clonal deletion, and tolerance can be achieved without any ablation of $CD4^+$ T cells or myeloid cells and with as few as 2×10^6 marrow cells, is notable but probably a function of the minor Ag differences, as we and others have previously shown that more aggressive protocols are required to achieve the high levels of chimerism necessary to induce deletion across MHC mismatches (16, 17). More surprising is that the infusion of much lower numbers of bone marrow cells (as low as 1000 cells per mouse) also led to tolerance in this Mls-plus-minors Ag difference, as defined by the acceptance of donor-type skin grafts, without any overt deletion of Mls-1^a-reactive $V\beta6^+CD4^+$ cells, and this was then associated with transferable and linked suppression of third-party F_1 skin grafts. It should be noted that the loss of the $V\beta6^+CD4^+$ cells in the high dose marrow groups was found to be gradual (slower in the spleen than in the lymph nodes or blood) and occurred between 6 and 20 wk after transplantation, but it was never observed even at 20 wk in low dose marrow groups. This may explain why anergy rather than deletion was previously reported by Qin et al. 8 wk after high dose AKR marrow infusion to CD4- and CD8-depleted CBA mice (8) and raises the possibility that in these circumstances anergy preceded deletion (18). In the experiments of this paper that used the lower doses of marrow, we were unable to show a reproducible loss of in vitro proliferation to donor Ag, even though the mice were tolerant of donor skin. This is similar to the situation seen with tolerance generated entirely to skin grafts under the cover of CD4 and CD8 Abs (6).

Although there is a general association between deletion and the loss of suppression, this is not absolute, as seen in the group of mice that received an intermediate (4×10^5) dose of bone marrow cells, in which deletion of $CD4^+V\beta6^+$ cells was observed in the peripheral blood and yet (AKR $\times$ B10BR) F_1 skin was still accepted (Table I). This may be because the Mls-1^a Ag is not thought to be a major target for skin graft rejection (19), and this dose of marrow, while deleting the Mls-1^a-reactive T cells, may have been insufficient to delete all the other T cells that recognize Ags on the donor skin that can still act as targets for suppression.

Spleen cells from tolerant mice could suppress the rejection of grafts in otherwise unmanipulated secondary recipients. This suppression was most effective after boosting the tolerant CBA/Ca donor with AKR Ags some 3 to 4 days before transfer and was dependent on $CD4^+$ T cells. This suggests that tolerant $CD4^+$ T cells are not only responsible for initiating suppression, but are also able to respond to fresh Ag challenge in a manner that reinforces tolerance. It has previously been shown by titration that suppression of graft rejection in a secondary recipient represents a quantitative measure of dominant tolerance that is related to the ratio of tolerant to naive $CD4^+$ T cells (20), and it is likely that the Ag boost is increasing the numbers of tolerant, suppressive $CD4^+$ T cells. It is interesting to note that roughly half of the secondary recipient mice rejected their first AKR skin graft soon after the second graft (Fig. 2*A*), even though the latter was not itself rejected until further challenge with a third (AKR $\times$ B10.BR) F_1 graft that was accepted indefinitely. This suggests that even after boosting, insufficient numbers of $CD4^+$-tolerant T cells had been transferred to completely tolerize the secondary recipient.

Although the mechanisms of peripheral tolerance remain unresolved, the APC clearly plays an important role, as evidenced by the suppression of third-party responses only if they are coexpressed on F_1 grafts, both in the primary tolerant mouse as well as in the secondary recipient through infectious tolerance. It was found that $B220^+sIg^-$ cells were required when limiting numbers of bone marrow cells were used to induce tolerance, suggesting a prominent role for an immature B cell. This could be related to the proliferative or Ag-presenting properties of a particular B cell subset (21) or to the restricted expression of the Mls-1^a Ag to B-lineage cells (and $CD8^+$ T cells) but not dendritic or macrophage cells (19). However, it is still possible that long term maintenance of tolerance in these mice became dependent on the AKR skin that was later grafted to test for tolerance.

These findings show that adult transplantation tolerance induced with marrow inocula, under cover of CD4 Abs, can occur either via clonal deletion, and hence passive mechanisms, or dominant tolerance through active suppression. The degree to which either is involved seems dependent on the dose of marrow. These findings may have significant implications in the therapeutic application of tolerance.

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