EFFECTS OF T CELL DEPLETION IN RADIATION BONE MARROW CHIMERAS

II. Requirement for Allogeneic T Cells in the Reconstituting Bone Marrow Inoculum for Subsequent Resistance to Breaking of Tolerance

By MEGAN SYKES, MICHAEL A. SHEARD, AND DAVID H. SACHS

From the Transplantation Biology Section, Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

Recent clinical and experimental protocols for allogeneic bone marrow transplantation (BMT)[1] have demonstrated that T cell depletion of bone marrow can dramatically reduce the incidence and severity of graft-vs.-host disease (1). One undesirable effect of such depletion, however, has been an increased incidence of failure of alloengraftment, both early and in the late post-BMT period (1, 2). These problems present a major obstacle to the application of clinical BMT across complete MHC barriers. We have previously reported that the ability of non-T cell-depleted (non-TCD) allogeneic BM inocula to increase levels of chimerism in murine radiation bone marrow chimeras was at least partly independent of a genetic potential for GVH reactivity (2a). The present studies address possible mechanisms whereby depletion of T cells from allogeneic BM may result in delayed failure of previously engrafted BM.

Previous studies have demonstrated that lethally irradiated mice reconstituted with a mixture of TCD syngeneic and TCD allogeneic BM develop stable mixed lymphohematopoietic chimerism, with specific and permanent tolerance to donor and recipient alloantigens (3–5). In addition, if only the syngeneic components of mixed BM inocula are TCD, the animals repopulate as 100% allogeneic chimeras and do not develop clinical GVHD (6, 2a). The latter result appears to be due to a protective effect against GVHD provided by the TCD syngeneic BM component (6–8). Since chimeras produced in this manner appear healthy and survive long-term, regardless of whether or not the allogeneic BM component is TCD, they provide a useful model for examination of the effects of T cells in allogeneic BM inocula on long-term tolerance. Using this model, we have tested the ability of tolerance in long term chimeras to withstand challenge with an intravenous inoculum containing normal, nontolerant recipient strain splenocytes. The results indicate that tolerance is surprisingly fragile in long-term mixed allogeneic chimeras prepared using TCD allogeneic BM. This was not due to the mixed chimerism per se, since tolerance in allogeneic chimeras initially reconstituted with TCD allogeneic BM alone was equally

Address correspondence to Dr. David H. Sachs, NIH, Bldg. 10, Room 4B17, Bethesda, MD 20892.

[1] *Abbreviations used in this paper:* A, allogeneic chimera; BM, bone marrow; BMT, BM transplantation; CA, completely allogeneic chimera; FCM, flow cytometry; HVG, host-vs.-graft; M, mixed chimera; non-TCD, non-T cell-depleted; RAMB, rabbit anti-mouse brain; TCD, T cell-depleted; WBC, white blood cells.

susceptible to the effects of such a challenge. In striking contrast to these results, recipients of mixed marrow inocula in which the reconstituting allogeneic BM inoculum was non-TCD were completely resistant to the tolerance-breaking effects of the same recipient strain splenocytes. Data are also presented concerning the dependence of this phenomenon on potential GVH alloreactivity within the allogeneic T cell population.

Materials and Methods

Animals. Male C57BL/10SnJ (B10), B10.D2nSn (B10.D2), and (C57BL/10Sn × B10.A)F_1 [(B10 × B10.A)F_1] mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Male (B10.S/Sx × C57BL/10Sn)F_1 mice were bred in our own colony. Bone marrow chimeras were maintained in a specific pathogen-free facility for at least 8 wk after BMT.

Chimera Preparation. Chimeras were prepared as previously described (5). Briefly, recipient B10 mice, aged 12-16 wk, were lethally irradiated (1,025 rad, 8.6 min, ^{137}Cs source) and reconstituted on the same day with bone marrow obtained from the femora and tibiae of B10 and B10.D2 or (B10 × B10.A)F_1 donors aged 6-12 wk. T cell depletion was performed using rabbit anti-mouse brain (RAMB) serum and guinea pig complement (C') (Gibco Laboratories, Grand Island, NY) as described (5). Animals were reconstituted with B10 and B10.D2 marrow in the following combinations: Mixed allogeneic (M) chimeras received 5 × 10^6 TCD B10 plus 15 × 10^6 TCD B10.D2 bone marrow cells (BMC); allogeneic (A) chimeras received 15 × 10^6 TCD B10.D2 BMC; completely allogeneic (CA) chimeras, so named because of the 100% allogeneic lymphohematopoietic repopulation (2a) received 5 × 10^6 TCD B10 plus 15 × 10^6 non-TCD B10.D2 BMC. The designations used for chimeras prepared according to these regimens are summarized in Table I.

Monoclonal Antibodies (mAb). FITC-conjugated 5F1 anti-K^b mAb (9) was used for detecting cells of B10 origin. Biotinylated mAb 34-2-12 (10) with specificity for D^d was used for detection of cells of B10.D2 or (B10 × B10.A)F_1 origin. FITC-conjugated Leu-4 (Becton Dickinson & Co., Mountain View, CA) was used as an irrelevant antibody to test for background staining.

Flow Cytometry (FCM) Analysis. FCM analysis was performed as described (11) using a modified Becton Dickinson & Co. FACS 2. Data were displayed as cell frequency histograms in which log fluorescence intensity was plotted on the x axis and cell number on the y axis. The percentage of cells considered positive after staining with a mAb was calculated using a cut-off for positivity determined from the fluorescence profiles of negative and positive control populations, which were PBL from normal donor and recipient strain mice.

Staining of Splenocytes and Peripheral Blood Lymphocytes (PBL) of Chimeras. Initial PBL phenotyping was performed 6-10 wk after BMT. Mice were bled and PBL were isolated as described (4). Cells were washed, then incubated with FITC or biotin-conjugated anti-K^b or anti D^d (see above) mAb for 45 min at 4°C, then washed twice. For staining of biotinylated mAb, a second incubation with FITC-avidin (Sigma Chemical Co., St. Louis, MO) for 30 min at 4°C was performed, cells were washed twice, then resuspended and analyzed using FCM. Cell populations from negative and positive strains with respect to MHC haplotype (i.e., PBL from normal donor and recipient strain mice) were used as negative and positive controls, respectively, for staining with each mAb, and the percent of positively staining cells in a chimera relative to the percent staining in the positive control population was calculated using the formula: Relative percent staining = 100 × [(Experimental percent staining − negative control percent staining)/ × 100% (positive control percent staining − negative control percent staining)]. For those experiments in which increased nonspecific staining of experimental cell populations was observed, staining of the experimental cell population with an irrelevant antibody (Leu-4-biotin) was substituted for staining of a cell population from a negative strain with the test reagent.

Host-vs-Graft (HVG) Inocula. Sterile B10 spleen cell suspensions were prepared as described (7). 2-50 × 10^6 cells were injected in a volume of 0.5 ml into the tail veins of chimeras 8-13 wk after BMT.

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

Skin Grafting. 6 d after administration of HVG inocula, full-thickness tail skin grafts were placed on the lateral thorax of chimeras using the method of Billingham (12). Rejection was considered complete when no viable skin was visible. Grafts that showed persistent signs of inflammation and/or atrophy were considered to be undergoing chronic rejection.

White Blood Cell (WBC) Counts. Small samples of blood were obtained by tail bleeding into a microfuge tube containing 2 U heparin sulfate (2 μl). Red blood cell lysis was achieved by incubation of a 10 μl aliquot of blood for 5 min in 250 μl hypotonic ammonium chloride buffer, and WBC were counted on a standard hemocytometer.

Results

Allogeneic T Cells in the Reconstituting Marrow Inoculum Affect Stability of Long-Term Tolerance. The levels of allogeneic chimerism resulting in animals reconstituted with various combinations of allogeneic TCD or non-TCD BM plus TCD syngeneic BM are summarized in Table II. Appropriate levels of allogeneic chimerism after reconstitution with each BM combination were documented by PBL phenotyping of all long-term chimeras before use in the studies reported here. Previous studies have established that recipients of mixed marrow inocula containing TCD syngeneic plus TCD or non-TCD allogeneic BM are specifically and permanently tolerant to donor alloantigen in the strain combination used here, and that allogeneic chimerism is permanent (3–6). To determine whether there were differences in the nature of tolerance observed in these types of chimeras, we challenged long-term stable chimeras with normal (nontolerant) splenocytes syngeneic to the recipients. Tolerance was then assessed by following the survival of donor-specific skin grafts placed 6 d after the HVG inoculum was administered. In addition, the level of chimerism in the PBL of these animals was assessed before, and at several time points after inoculation. Unmanipulated long-term chimeras from the same experimental groups were used as controls. B10 chimeric recipients originally reconstituted with TCD B10 plus TCD B10.D2 BM (M chimeras) were compared with B10 chimeric recipients originally reconstituted with TCD B10 plus non-TCD B10.D2 BM (CA chimeras). An additional group, long-term B10 chimeric recipients originally reconstituted with TCD B10.D2 marrow alone (A chimeras), was included, since the level of chimerism (~98%) before HVG challenge of these animals more closely approximated that of the CA recipients (100%) than did that of the M recipients (see Table II). Animals in all three groups were inoculated intravenously with 0, 2 × 10^6, or 20 × 10^6 spleen cells from normal B10 donors.

The effects of these inocula on chimerism are shown in Fig. 1. The upper and middle panels indicate that, throughout the 107-d follow-up period, the level of chimerism remained stable in CA, A, and M chimeras receiving 0 or 2 × 10^6 B10

TABLE I

Designations for Radiation Bone Marrow Chimeras

Chimera	Abbreviation	Reconstitution*
Mixed	M	(B10+B10.D2)(TCD)→B10
Allogeneic	A	B10.D2-TCD→B10
Completely allogeneic	CA	B10-TCD+B10.D2-non-TCD→B10

* Donor bone marrow strains are indicated before the arrow; recipient is indicated after the arrow.

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

TABLE II
Donor Cell Chimerism In Radiation Chimeras Reconstituted with Various Bone Marrow Inocula

Reconstitution	Levels of donor cell chimerism*			
	Donor TCD		Donor Non-TCD	
	Donor BM only	Donor BM plus TCD host-strain BM	Donor BM only	Donor BM plus TCD host-strain BM
		%		%
B10.D2→B10	94-99 (A)[b]	30-60 (M)	100	100 (CA)
(B10 × B10.A)F1→B10	92-96	60-65	96-98	90-94

* Chimerism determined using mAb staining and FCM analysis. Numbers shown represent range for means obtained from at least three experiments.
‡ Letters in parentheses indicate designations used for B10.D2→B10 chimeras in this paper (see Table I).

spleen cells intravenously. However, the injection of 20 × 10^6 B10 spleen cells into long-term M chimeras resulted in a marked decline in the level of chimerism in all animals, with complete syngeneic repopulation in two of three animals. A similar result was obtained in A chimeras (Fig. 1, *lower panel*). In contrast, the level of chimerism was not altered in any of the three CA chimeras receiving 20 × 10^6 B10 spleen cells. Thus, the receipt of non-TCD allogeneic marrow at the time of BMT led to increased stability of chimerism as indicated by increased resistance to the effects of an HVG inoculum.

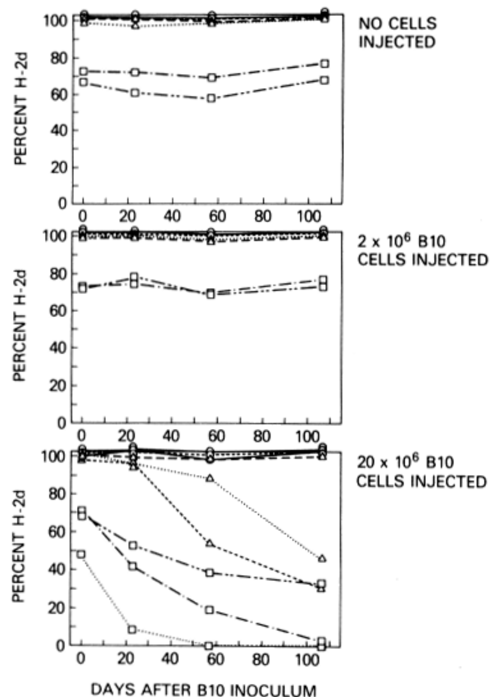

FIGURE 1. Effects on chimerism of inoculation of long-term chimeras with varying numbers of fresh normal B10 splenocytes. Long-term established mixed B10-TCD + B10.D2-TCD → B10 (□), allogeneic B10.D2-TCD → B10 (Δ), and completely allogeneic B10-TCD + B10.D2-non-TCD → B10 (O) chimeras received 0, 2 × 10^6, or 20 × 10^6 fresh B10 splenocytes intravenously on day 0. The level of B10.D2 chimerism (percent H-2^d) was determined at the time points indicated by staining of PBL with fluorescence labeled mAb and FCM analysis. Each curve represents data from an individual chimera.

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

TABLE III
Donor Skin Graft Survival in Mixed, Completely Allogenic and Allogeneic Chimeras After Inoculation With B10 Splenocytes

Chimera	B10.D2 skin graft survival after B10 spleen cells were injected	
	Exp. 1 (2 × 10^7)*	Exp. 2 (3 × 10^7)
Mixed B10-TCD+B10.D2-TCD→B10	116‡	
	CR§	
	70	
Completely allogeneic B10-TCD+B10.D2-non-TCD→B10	>137‖	>100
	>137	>100
	>137	>100
Allogeneic B10.D2-TCD→B10	CR	8
	22	29
	>137	15
		12
		15

* Number of B10 spleen cells injected.
‡ Rejected on day indicated.
§ Chronic rejection ongoing on last day of observation, day 137.
‖ No evidence of rejection by last day of observation indicated.

The survival times of donor strain skin grafted 6 d after administration of B10 spleen cells were also followed. All CA, M, and A chimeras receiving 0 or 2 × 10^6 B10 spleen cells retained B10.D2 skin grafts without evidence of rejection for the duration of the follow-up period (data not shown). Skin graft survival times of recipients of 20 × 10^6 B10 spleen cells are shown in Table III (Exp. 1). In this group, all skin grafts on CA chimeras remained perfectly intact throughout the observation period, while three of three M chimeras and two of three A chimeras rejected their grafts. One A chimera showed no evidence of rejection by termination of follow-up at day 137, and this animal was also the only A recipient of 20 × 10^6 B10 splenocytes that did not demonstrate a reduction in the level of allogeneic chimerism by day 107 (Fig. 1, *lower panel*).

These data indicated that CA chimeras were relatively resistant, compared with M or A chimeras, to the tolerance-breaking effects of normal recipient strain splenocytes. Although loss of chimerism correlated generally with rejection of donor-specific skin grafts, the rejection of skin grafts and loss of chimerism in M and A chimeras was slow and sometimes incomplete, and did not occur in every animal. We therefore repeated the experiment using a somewhat larger B10 splenocyte inoculum, consisting of 30 × 10^6 cells. We also wished to address the possibility that the loss of chimerism in A and M but not in CA chimeras reflected the presence (in A and M) or absence (in CA) of syngeneic stem cells in the chimeras capable of repopulating the animals after allogeneic hematopoietic elements were eliminated by the HVG inoculum, rather than reflecting actual resistance to the tolerance-breaking properties of such an inoculum. In this case, the injection of B10 splenocytes would result in a more severe and prolonged pancytopenia in CA chimeras than in M or A, since

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

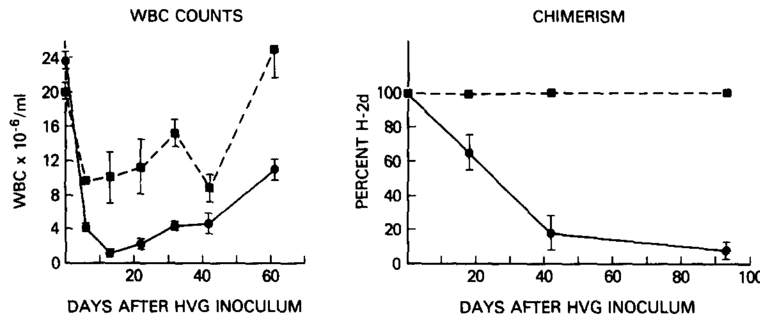

FIGURE 2. Effects on chimerism and peripheral blood WBC counts of inoculation of 30 × 10^6 fresh B10 splenocytes into long-term A [B10.D2-TCD → B10] (—●—) and CA [B10-TCD + B10.D2-non-TCD → B10] (. . ■. .) chimeras. B10 splenocytes were administered on day 0, and WBC counts (*left panel*) and levels of chimerism (*right panel*) were determined at the time points indicated, as described in Materials and Methods. Mean ± SE for three to five mice are shown at each time point.

CA chimeras would lack stem cells capable of replacing the eliminated allogeneic elements. We therefore followed peripheral blood WBC counts after administration of an HVG inoculum to CA and A chimeras. Since both groups began with fairly similar levels of allochimerism, reduced WBC counts after this inoculation might reflect destruction of chimeric lymphohematopoietic elements.

The results shown in Fig. 2 indicate that WBC counts fell in both groups after injection of B10 splenocytes, reaching a nadir at 6–13 d. However, this drop was much more profound in A recipients (nadir 1,150 ± 400 [SEM] WBC/μl), which experienced severe leukopenia, than in CA recipients (nadir 9,600 ± 400 [SEM] WBC/μl), in which WBC counts did not differ significantly from those observed in unmanipulated chimeras (13,400 ± 900 [SEM] WBC/μl). All A chimeras, and none of the CA recipients, demonstrated a partial or complete loss of chimerism. In addition, A chimeras rejected B10.D2 skin grafts acutely, with rejection times ranging from 8 to 29 d (Table II, Exp. 2). In contrast, all CA chimeras retained B10.D2 skin grafts permanently, with no sign of rejection for the duration of the 100-d follow-up period. All animals appeared healthy throughout the experiment.

In another experiment, three of three M chimeras given 30 × 10^6 B10 spleen cells also lost chimerism rapidly, with complete or nearly complete repopulation by B10 lymphopoietic cells by 48 d after inoculation (data not shown). These results demonstrated that T cells in reconstituting BM inocula lead to resistance in long-term chimeras to the tolerance-breaking effects of normal recipient strain lymphocytes.

Effects of HVG Inocula in F_1 into Parent Chimeras. We next attempted to determine whether T cells in donor BM inocula would lead to resistance to breaking of tolerance in long-term chimeras if the potential for T cells in the donor marrow inoculum to react against the host (i.e., GVH reactivity) were minimized. This was done using an F_1 donor into parental recipient (F_1 → P) strain combination. As shown in Table II, chimeras prepared by infusing either TCD or non-TCD (B10 × B10.A)F_1 BM alone into lethally irradiated B10 recipients repopulated as almost completely allogeneic chimeras, with slightly greater levels of chimerism in recipients of non-TCD BM than in recipients of TCD F_1 BM, as previously reported (2a). The magnitude

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

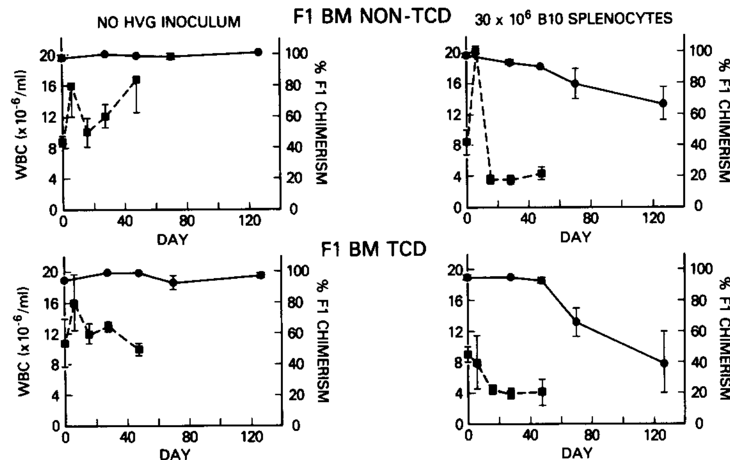

FIGURE 3. Effects of HVG inocula on chimerism and WBC counts in long-term $F_1 \rightarrow P$ chimeras. Long-term (B10 × B10.A)F_1-non-TCD → B10 (*upper panel*) and (B10 × B10.A)F1-TCD → B10 (*lower panel*) either underwent no additional manipulations (*left panels*) or received an intravenous inoculum containing 30 × 10^6 B10 splenocytes on day 0 (11 wk after BMT) (*right panels*). Total peripheral WBC counts (. . ■. .) and percent F_1 chimerism (—●—), determined as described in Materials and Methods, are shown at various time points after inoculation with B10 splenocytes. Each point represents mean ± SE of results from three or four animals.

and direction of the difference in levels of chimerism between the former and the latter group were similar to those between the CA and A chimeras discussed above (see Table II). 11 wk after BMT, F_1-non-TCD → P and F_1-TCD → P chimeras were either inoculated with 30 × 10^6 B10 spleen cells (test group) or left unmanipulated (control group). WBC counts, chimerism, and skin graft survival were followed. As shown in Fig. 3, a significant drop in WBC counts in $F_1 \rightarrow P$ chimeras inoculated with B10 splenocytes was observed in injected chimeras and not in unmanipulated controls, regardless of whether or not the initial F_1 bone marrow inoculum had been T cell depleted. In addition, levels of F_1 chimerism declined gradually in both F_1-TCD and F_1-non-TCD-reconstituted mice inoculated with B10 splenocytes. This decline was slightly more pronounced in F_1-TCD than in F_1-non-TCD chimeras, and one animal in the former group lost its chimerism completely. Uninjected control animals in both groups maintained their levels of F_1 chimerism throughout the follow-up period.

Donor strain F_1 skin grafts were partially or completely rejected by injected animals in both groups, regardless of whether or not the original F_1 BM inoculum had been TCD (Table IV). In contrast, uninjected control animals in both groups retained donor F_1 skin grafts in perfect condition permanently (Table IV). Injected and uninjected animals in both groups rejected third-party (B10. S × B10)F_1 skin grafts briskly and with equal efficiency (data not shown), indicating that chimeras were immunocompetent and, if left unmanipulated, were specifically tolerant of donor (B10 × B10.A)F_1 alloantigen.

Overall, these results indicated that animals reconstituted with non-TCD semiallogeneic BM were not substantially more resistant to the tolerance-breaking effects

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

TABLE IV

Effects of HVG Inocula on Donor Skin Graft Survival in $F_1 \rightarrow P$ Chimeras

Chimera	Skin graft survival: Uninjected controls	Skin graft survival: Recipients of B10 splenocytes*
	d	
(B10 × B10.A)F_1-TCD→B10	>148, >148, >148‡	91§, CR‖148, 52
(B10 × B10.A)F_1-non-TCD→B10	>148, >148, >148, >142¶	129, 133, 21

* Animals received 3×10^7 fresh B10 splenocytes on day −6.
‡ Grafts in perfect condition at termination of follow-up period, 148 d.
§ Grafts rejected on day indicated.
‖ Chronic rejection ongoing at termination of follow-up period, 148 d.
¶ Animal died on day indicated with skin graft intact.

of recipient strain lymphocytes than were animals reconstituted with TCD BM. However, loss of chimerism, skin graft rejection, and decline in WBC counts after administration of 30 × 10^6 B10 spleen cells were less dramatic in F_1-TCD → B10 than was observed in B10.D2-TCD → B10 chimeras (Fig. 2; Table III, Exp. 2). The result in F_1 → B10 chimeras was therefore confirmed in a repeat experiment in which larger numbers of B10 spleen cells were administered. More rapid rejection of skin grafts and loss of chimerism were observed in five F_1-TCD → B10 and five F_1-non-TCD → B10 chimeras inoculated with 50 × 10^6 B10 spleen cells. There were no apparent differences in rates of graft rejection and loss of chimerism between animals originally reconstituted with TCD versus those reconstituted with non-TCD F_1 marrow (data not shown). Five control chimeras of each type which received saline injections retained skin grafts and chimerism permanently (data not shown). In contrast to results obtained in P1 → P2 chimeras, therefore, T cells in reconstituting marrow inocula did not confer resistance to breaking of tolerance in F_1 → P chimeras.

Discussion

The model described in this paper was designed to determine the effects of T cells present in reconstituting allogeneic BM inocula on the stability of tolerance to donor antigen in long-term radiation bone marrow chimeras. We have previously observed that coadministration of TCD syngeneic BM along with non-TCD allogeneic BM to lethally irradiated recipients provides protection from GVHD while still allowing 100% allochimerism to develop (6, 8). These apparently healthy chimeras were therefore suitable for long-term studies. The effects of HVG inocula in such chimeras were compared with those in long-term chimeras reconstituted with TCD allogeneic BM, with or without TCD syngeneic BM. Surprisingly, tolerance was quite easily broken in recipients in which the original allogeneic marrow inoculum had been TCD. 20 × 10^6 recipient strain splenocytes were sufficient to break tolerance in most M and A chimeras, as evidenced by a reduction in or complete loss of chimerism, and slow, partial, or complete rejection of donor-specific skin grafts. CA chimeras, which had received the same reconstituting mixed marrow inoculum as the M group, except that the allogeneic marrow was non-TCD, were completely resistant to these tolerance-breaking effects.

Further evidence for the relative sensitivity of A compared with CA chimeras to

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

the alloaggressive effects of HVG inocula was the marked leukopenia in the former and not the latter group in the early period after inoculation with 30 × 10^6 B10 splenocytes (Fig. 2). The higher WBC counts in CA chimeras do not reflect proliferation of administered host strain splenocytes, since PBL of CA chimeras typed in this early period were solely of donor phenotype (Fig. 2). Recovery of WBC counts after their dramatic decline in A chimeras was associated with repopulation by cells of recipient H-2 phenotype, while CA chimeras remained completely allogeneic throughout the experiment. This repopulation of A chimeras may have been derived from stem cells already present in the chimera at the time of HVG inoculation, or from stem cells present in the spleen cell HVG inoculum itself. In this experiment, donor skin grafts were rapidly rejected by A chimeras and permanently accepted by CA chimeras. All three parameters (leukopenia, loss of chimerism, and rejection of donor skin grafts) therefore confirm the greater sensitivity of A compared with CA chimeras to the alloaggressive effects of HVG inocula.

These results demonstrate that : (*a*) Tolerance to donor alloantigen, which is permanent in otherwise unmanipulated whole body irradiation bone marrow chimeras prepared with TCD allogeneic BM, can be broken by inoculation with relatively small numbers of nontolerant recipient strain lymphocytes. Loss of tolerance is accompanied by a loss of donor chimerism regardless of whether or not the initial level of donor chimerism was almost complete (A chimeras) or was only partial (M chimeras); and (*b*) one or more cell populations in donor marrow, removed during T cell depletion, confer resistance in long-term chimeras to such tolerance-breaking effects of recipient strain lymphocytes.

There are several possible explanations for the ability of allogeneic T cells in reconstituting marrow inocula to confer resistance to breaking of tolerance. Suppressor cells in the original reconstituting marrow inoculum, possibly similar to those implicated in several rodent models of experimentally induced tolerance (13–15), might play a role in resisting breaking of tolerance. Resistance to breaking of tolerance in CA chimeras might also be due to anti–host T cell reactivity of allogeneic T cells administered in the original reconstituting marrow inoculum. Such T cells, after being primed in vivo by B10 host antigen, might eliminate inoculated B10 splenocytes before the latter population could effect breaking of tolerance. Indeed, anti–host CTL activity can be detected in splenocyte populations from some long-term CA chimeras (6). This possibility is strongly supported by findings in F_1 → P chimeras, in which tolerance is broken with equal facility in recipients of non-TCD vs. TCD F_1 marrow (Fig. 3, Table IV), since in this strain combination the potential for GVH T cell reactivity is greatly diminished.

In studies involving a P1 → P2 strain combination, we used chimeras reconstituted with non-TCD allogeneic plus TCD syngeneic BM, since animals reconstituted with non-TCD allogeneic BM alone tend to show increased early mortality (6) and are therefore not suitable for long-term studies. However, the presence of TCD syngeneic BM in the original reconstituting inoculum did not appear to enhance the resistance to HVG inocula, since animals reconstituted with TCD syngeneic BM in addition to TCD allogeneic BM were equally or more susceptible to breaking of tolerance than animals reconstituted with TCD allogeneic marrow alone. When studying an F_1 into parent combination, we elected to compare animals reconstituted with F_1 (TCD vs. non-TCD) BM alone, rather than a mixed F_1/syngeneic BM combination,

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

since the relative levels of chimerism in the former groups most closely paralelled those of CA vs. A chimeras (Table II).

CA chimeras are clinically free of GVHD (6), and in vitro studies (Sykes, M., et al., unpublished data) have provided no evidence for GVH-related immunodeficiency (16, 17). Thus, GVH reactivity required to prevent the alloaggressive effects of injected recipient strain lymphocytes may be quantitatively or qualitatively insufficient to produce either clinical GVHD or in vitro evidence for GVH-related immunodeficiency.

It is possible that the resistance to breaking of tolerance attributed to T cells in allogeneic reconstituting BM inocula is mediated by a cell population other than T lymphocytes, since the RAMB serum used for T cell depletion may eliminate additional non-T cell BM subpopulations. Studies performed to date suggest that stem cells are not selectively depleted but that NK cells in bone marrow are eliminated by RAMB/C treatment (2a). Since similar protection from HVG inocula by RAMB/C-sensitive cells in donor BM inocula was not observed in the $F_1 \rightarrow P$ combination, the effects in P1 → P2 chimeras are most likely to be related to potential GVH reactivity, which is an established property of T cells (18, 19).

Attempts to break tolerance in murine whole body irradiation bone marrow chimeras have not, to our knowledge, been previously reported. The ability to break experimentally induced tolerance in neonatally tolerized mice, however, was first demonstrated by Billingham et al. (20) and has subsequently been demonstrated in neonatally tolerized rats (21, 22). The numbers of lymphoid cells required to break neonatally induced tolerance in mice (23, 24) were much greater than those required in our irradiation TCD-BM chimeras. In these earlier reports, skin grafting was performed long before recipient strain lymphocytes were administered, and it is possible that changes in the grafts themselves might render them less susceptible to rejection by the time an HVG inoculum is adminstered (25). Since we grafted donor-specific skin 6 d after administration of recipient strain splenocytes, prior adaptation of the graft could not have played a role. The strikingly different susceptibilities of neonatally tolerized mice and radiation bone marrow chimeras to breaking of tolerance might also reflect inherent differences in the mechanisms maintaining tolerance in the two models.

The small number of normal recipient strain lymphocytes (one-fifth to one-third spleen equivalent) required to break tolerance in radiation chimeras reconstituted with TCD allogeneic BM suggests that if suppression plays any role in maintaining tolerance to donor antigen, it is not sufficiently potent to suppress the activity of a relatively small number of additional lymphocytes reactive to donor antigen. Thus, clonal deletion may be the dominant mechanism maintaining tolerance to donor alloantigen in radiation bone marrow chimeras prepared with TCD allogeneic marrow.

We have recently demonstrated that much of the ability of allogeneic T cells in allogeneic or mixed allogeneic bone marrow inocula to increase the level of allochimerism is independent of their potential GVH reactivity (2a). The observation that resistance to breaking of tolerance is associated with T cells potentially capable of producing GVHD therefore implies that these two effects may be mediated by different mechanisms, and/or different cell subpopulations. Both phenomena might be important clinically, since failure of engraftment after reconstitution with TCD allogeneic bone marrow has been observed long after initial engraftment had been achieved

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

(1). Our results indicate that the mechanisms maintaining tolerance to donor antigen in radiation chimeras constructed with TCD allogeneic BM are fragile and cannot resist the addition of relatively small numbers of nontolerant lymphocytes. In the clinical situation, small numbers of host T cells might escape the ablative regimen used for preparation for BMT (26), and could subsequently expand to cause delayed rejection of a donor marrow allograft. This might be prevented if the original BM inoculum contained anti–host-reactive T cells capable of eliminating residual host lymphocytes. Regardless of the mechanisms determining marrow allograft rejection at different times, our results indicate that a small number of T cells in allogeneic BM inocula might be sufficient to prevent both early and late marrow rejection. The use of a mixed syngeneic (TCD) and allogeneic (non-TCD or partially TCD) marrow inoculum might permit achievement of this goal since syngeneic marrow appears to provide protection from GVHD (6–8) while permitting development of complete allochimerism in association with stable specific tolerance to donor antigen.

Summary

The ability of normal recipient-type lymphocytes to break tolerance in long-term allogeneic radiation chimeras has been investigated. Reconstitution of lethally irradiated mice with a mixture of syngeneic and allogeneic T cell–depleted (TCD) bone marrow (BM) has previously been shown to lead to mixed chimerism and permanent, specific tolerance to donor and host alloantigen (3–5). If allogeneic T cells are not depleted from the reconstituting inoculum, complete allogeneic chimerism results; however, no clinical evidence for GVHD is observed, presumably due to the protective effect provided by syngeneic TCD BM. This model has now been used to study the effects of allogeneic T cells administered in reconstituting BM inocula on stability of long-term tolerance. We have attempted to break tolerance in long-term chimeras originally reconstituted with TCD or non-TCD BM by challenging them with inocula containing normal, nontolerant recipient strain lymphocytes. Tolerance was broken with remarkable ease in recipients of mixed marrow inocula in which both original BM components were TCD. In contrast, tolerance in chimeras originally reconstituted with non-TCD allogeneic BM was not affected by such inocula. Susceptibility to loss of chimerism and tolerance was not related to initial levels of chimerism per se, but rather to T cell depletion of allogeneic BM, since chimeras reconstituted with TCD allogeneic BM alone (mean level of allogeneic chimerism 98%) were as susceptible as mixed chimeras to the tolerance-breaking effects of such inocula. The possible contribution of GVH reactivity to this resistance was investigated using an F_1 into parent strain combination. In these animals, the use of non-TCD F_1 BM inocula for reconstitution did not lead to resistance to the tolerance-breaking effects of recipient strain splenocytes. These results suggest that the ability of T cells in allogeneic BM inocula to confer resistance to late graft failure may be related to their graft-vs.-host reactivity, even in situations in which they do not cause clinical GVHD. These findings may have relevance to the mechanism whereby T cell depletion of allogeneic BM leads to an increased incidence of late graft failure in clinical BM transplantation situations.

We wish to thank Drs. Susan McCarthy and John Wunderlich for helpful review of the manuscript, Ms. Judith Jaworek for expert secretarial assistance, Mr. Donald Holt and his staff

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

for excellent animal care, and Ms. Susan Sharrow and her staff for expert microfluorometry analyses.

Received for publication 29 February 1988 and in revised form 21 April 1988.

References

1. Martin, P. J., J. A. Hansen, C. D. Buckner, et al. 1985. Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts. *Blood.* 66:664.
2. Soderling, C. C. B., C. W. Song, B. R. Blazar, and D. A. Vallera. 1985. A correlation between conditioning and engraftment in recipients of MHC-mismatched T cell-depleted murine bone marrow transplants. *J. Immunol.* 135:941.

2a. Sykes, M., M. Sheard, and D. H. Sachs. Effects of T-cell depletion in radiation bone marrow chimeras: 1) evidence for a donor cell population which promotes alloengraftment without the potential to produce GVHD. *J. Immunol.* In press.

3. Singer, A., K. S. Hathcock, and R. J. Hodes. 1981. Self recognition in allogeneic radiation bone marrow chimeras: a radiation-resistant host element dictates the self specificity and immune response gene phenotype of T-helper cells. *J. Exp. Med.* 153:1286.
4. Ildstad, S. T., and D. H. Sachs. 1984. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature (Lond.).* 307:168.
5. Ildstad, S. T., S. M. Wren, S. A. Barbieri, and D. H. Sachs. 1985. Characterization of mixed allogeneic chimeras. Immunocompetence, in vitro reactivity, and genetic specificity of tolerance. *J. Exp. Med.* 162:231.
6. Ildstad, S. T., S. M. Wren, J. A. Bluestone, et al. 1986. Effect of selective depletion of host and/or donor bone marrow on lymphopoietic repopulation, tolerance, and graft-vs-host disease in mixed allogeneic chimeras (B10+B10.D2 → B10). *J. Immunol.* 136:28.
7. Sykes, M., A. Eisenthal, and D. H. Sachs. 1988. Mechanism of protection from graft-vs-host disease in murine mixed allogeneic chimeras. I. Development of a null cell population suppressive of CML responses and derived from the syngeneic bone marrow component. *J. Immunol.* 140:2903.
8. Sykes, M., C. H. Chester, and D. H. Sachs. 1988. Protection from GVHD in fully allogeneic chimeras by prior administration of T-cell-depleted syngeneic bone marrow. *Transplantation (Baltimore).* In press.
9. Sherman, L. A., and C. P. Randolph. 1981. Monoclonal anti-H-$2K^b$ antibodies detect serological differences between H-$2K^b$ mutants. *Immunogenetics.* 12:183.
10. Ozato, K., Mayer, N. M., and D. H. Sachs. 1982. Monoclonal antibodies to mouse major histocompatibility complex antigens. IV. A series of hybridoma clones producing anti-H-2^d antibodies and an examination of expression of H-2^d antigens on the surface of these cells. *Transplantation (Baltimore).* 34:113.
11. Segal, D. M., S. O. Sharrow, J. F. Jones, and R. P. Sirigaanian. 1981. Fc(IgG) receptors on rat basophilic leukemia cells. *J. Immunol.* 126:138.
12. Billingham, R. E. 1961. Free skin grafting in mammals. *In* Transplantation of Tissues and Cells. R. E. Billingham and W. K. Silvers, editors. The Wistar Institute Press, Philadelphia, 1–26.
13. Dorsch, S., and B. Roser. 1982. Suppressor cells in transplantation tolerance. 1. Analysis of the suppressor status of neonatally and adoptively tolerized rats. *Transplantation (Baltimore).* 33:518.
14. Dorsch, S., and B. Roser. 1982. Suppressor cells in transplantation tolerance. II. Identification and probable mode of action of chimeric suppressor cells. *Transplantation (Baltimore).* 33:525.

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023

15. Lancaster, F., Y. L. Chui, and J. R. Batchelor. 1985. Anti-idiotypic T cells suppress rejection of renal allografts in rats. *Nature (Lond.).* 315:336.
16. Moser, M., T. Iwasaki, and G. M. Shearer. 1985. Cellular interactions in graft-versus-host-induced T cell immune deficiency. *Immunol. Rev.* 88:135.
17. Via, C. S., S. O. Sharrow, and G. M. Shearer. 1987. Role of cytotoxic T lymphocytes in the prevention of lupus-like disease occurring in a murine model of graft-vs-host disease. *J. Immunol.* 139:1840.
18. Dicke, K. A., J. van Hooft, and D. W. van Bekkum. 1968. The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures. *Transplantation (Baltimore).* 6:562.
19. Yunis, E. J., R. A. Good, J. Smith, and O. Stutman. 1974. Protection of lethally irradiated mice by spleen cells from neonatally thymectomized mice. *Proc. Natl. Acad. Sci. USA.* 17:2544.
20. Billingham, R. E., L. Brent, and P. B. Medawar. 1956. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 239:357.
21. Gowans, J. L., and D. D. McGregor. 1965. The immunological activities of lymphocytes. *Prog. Allergy.* 9:1.
22. Elkins, W. L., J. S. Adams, D. H. Fox, D. B. Wilson, and F. P. Stuart. 1973. Partial tolerance and immunity after adoptive abrogation of transplantation tolerance in the rat. *Cell. Immunol.* 9:412.
23. Ramseier, H. 1973. Immunization against abolition of transplantation tolerance. *Eur. J. Immunol.* 3:156.
24. Silvers, W. K. 1980. Studies on the apparent serial passage of transplantation immunity in tolerant mice. *Transplantation (Baltimore).* 10:538.
25. Gruchalla, R. S., P. G. Strome, and J. W. Streilein. 1983. Analysis of neonatally induced tolerance of H-2 alloantigens. III. Ease of abolition of tolerance of class I, but not class II, antigens with infusions of syngeneic, immunocompetent cells. *Transplantation (Baltimore).* 36:318.
26. Bunjes, D., W. Heit, R. Arnold, T. Schmeiser, M. Weisneth, F. Carbonell, F. Porzsolt, A. Raghavachar, and H. Heimpel. 1987. Evidence for the involvement of host-derived OKT8-positive T cells in the rejection of T-depleted, HLA-identical bone marrow grafts. *Transplantation (Baltimore).* 43:501.

Downloaded from http://rup.silverchair.com/jem/article-pdf/168/2/661/1098356/661.pdf by guest on 01 April 2023