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INCREASED INCIDENCE OF MURINE GRAFT-VERSUS-HOST DISEASE AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION BY PREVIOUS INFUSION OF SYNGENEIC BONE MARROW CELLS[1]

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Different groups of BALB/c mice received supralethal total-body irradiation (TBI; 8.5 Gy,[5] day 0). When 30×10^6 allogeneic (C57B1) bone marrow (BM) cells were infused with or without 10×10^6 syngeneic (BALB/c) BM cells on day 1, many animals (60%) died from graft-versus-host disease (GVHD). Typing of peripheral blood leukocytes for donor antigens showed that, respectively, 22/22 and 17/21 of the mice in both groups became chimeric. When syngeneic bone marrow was given on day 1 and allogeneic bone marrow on day 2 after TBI, a similar number of animals (21/23) became chimeric, but GVHD occurred more frequently in this group (25/26 mice, $P<0.01$). When the syngeneic bone marrow cells were replaced by spleen cells, or when the transplantation of allogeneic bone marrow was delayed till days 3 or 6 after TBI, almost all mice rejected the allogeneic BM graft and became long-term survivors. BALB/c mice receiving 30×10^6 C57B1 BM cells after 17 daily fractions of 0.2 Gy of total lymphoid irradiation (TLI), showed a high incidence of chimerism (15/17) and in none of the latter animals was GVHD observed. Despite the high incidence of GVHD in the mice receiving allogeneic BM after TBI and syngeneic BM transplantation, as compared with mice prepared with TLI which do not develop GVHD, suppressor cells were as easily induced after TBI and syngeneic BM transplantation as after TLI.

In an interesting study Rapaport et al. (*1*) showed that after hemopoietic reconstitution of supralethally irradiated adult dogs with their own stored bone marrow (BM)[5], long-term unresponsiveness of DLA-identical kidney allografts could be obtained, with no need for any additional immunosuppression. However, the time of organ transplantation (within 12–36 hr after BM transplantation) was critical. These authors suggest that the stress of replacing hemopoietic stem cells into an irradiated adult host may trigger a phase of cell replication (mainly suppressor cells) that temporarily recapitulates the events of immunological ontogeny. We were interested to see whether this model could be extended to that of allogeneic BM transplantation in mice. Therefore mice were injected with syngeneic and allogeneic bone marrow at various times after supralethal TBI. At the same time we wondered if this model could teach us more about the role of the autologous bone marrow in the mechanisms underlying graft versus host disease (GVHD). Indeed, several studies point to the role of autologous haematopoietic cells, surviving the conditioning regimen, as effectors (*2, 3*) or as targets (*4–6*) in GVHD after allogeneic BM transplantation.

Another reason for planning these experiments was to learn more about the in vivo role of suppressor cells in the prevention of GVHD. When 17 fractions of 2 Gy of total-lymphoid irradiation (TLI) are given to mice, the experiments of Slavin et al. (*7*) and Strober et al. (*8*)—as well as own experiments (*9*)—showed that these mice become stable BM chimeras without GVHD. It is suggested that the predominance of suppressor cells created after TLI (*10*) could be an important explanation for this phenomenon (*11*). The experiments of Rapaport suggest that syngeneic BM transplantation after total body irradiation (TBI) might also induce a suppressor cell predominance. Hence the interest of looking at the induction of suppressor cells in the mouse model after syngeneic BM transplantation and at the incidence of GVHD in the latter situation when allogeneic BM is infused concomitantly.

MATERIALS AND METHODS

Animals. Inbred BALB/c (H-2-$^{d/d}$) and C57BL/ka (H-$2^{b/b}$) male mice, 2–4 months old were used, respectively, as recipients and donors in all the experiments. The animals were kept in conventional housing in the Animalium of the University of Leuven.

Irradiation. Irradiation was carried out using gamma rays from a 60-cobalt source at a focus skin distance of 80 cm at a dose rate of 0.3 Gy/min. A dose of 8.5 Gy of TBI was given in a single fraction. BALB/c mice receiving 17 daily fractions of 2 Gy of TLI were anesthetized and irradiated as described elsewhere (*9*).

Collection of peripheral blood lymphocytes (PBL). This step was done as described elsewhere (*9*).

Infusion of BM or spleen cells into mice after TLI. BM cells were obtained by flushing minimal essential medium (MEM) through the shafts of the femurs and tibias of donor mice. Spleen cells were obtained by gently disrupting BALB/c spleens and flushing them through a nylon mesh. Cells were washed and resuspended in MEM. Aliquots of 0.25 ml medium, con-

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[5] Abbreviations used in this article: BM, bone marrow; CFU-S, colony-forming units-spleen; GVHD, graft-versus-host disease; Gy, Gray (= 100 rads); HE, hematoxylin and eosin, MEM, minimal essential medium; MLR, mixed lymphocyte reaction; PAS, periodic-acid-Schiff; PBL, peripheral blood lymphocytes; TBI, total-body irradiation; TLI, total-lymphoid irradiation.

taining 10×10^6 BALB/c BM or 30×10^6 C57Bl BM or 10×10^6 BALB/c BM plus 30×10^6 C57Bl BM or 5×10^6 C57Bl BM or 2.5×10^6 BALB/c spleen cells were injected into the tail vein of recipient mice.

Assay for chimerism. This assay has been described elsewhere (*9*) and was performed at day 40 and also at day 120 after BM transplantation to exclude late rejections. The results obtained at days 40 and 120 were always concordant. The results shown are those obtained at day 40.

In vivo and in vitro control of the hemopoietic stem cell content of infused BM. To minimize the influence of possible variation of the stem cell content of the different BM suspensions, experiments were always planned in such a way that groups of mice that were to be compared were transplanted on the same day with the same BM batch.

Each day that BM transplantation was performed, two groups of recipient mice, syngeneic with the BM donors, were also transplanted after receiving 8.5 Gy of TBI on the previous day. The first group received 10×10^6 BM cells of the same batch and was followed for survival (in vivo control of the quality of transfused BM). The second group received only 1×10^5 BM cells of the same BM batch and was killed one week later. After formol fixation, the spleens of these animals were scored for hematopoietic colony formation (colony-forming-unit-spleen, CFU-S assay). This was regarded as an in vitro control for the stem cell content of the infused BM. These in vivo and in vitro controls showed no significant variations during the entire series of experiments.

Suppressor cell assay. To check the suppressor cells in the spleens of irradiated animals, the mixed lymphocyte reaction (MLR) suppressor assay was used as described by King and Strober (*12*). In this assay the capacity of spleen cells of treated BALB/c mice to suppress a MLR is assessed. The MLR takes place between responder lymphocytes of untreated BALB/c mice and stimulator lymphocytes of C57Bl mice. The latter lymphocytes are first inactivated by an irradiation dose by 30 Gy, given in vitro. To eliminate proliferation of the putative suppressor cells they receive 10 Gy radiation in vitro.

Statistical analysis. Analysis of survival and of incidence of chimerism (number of chimeric animals) is done with the chi square test. P values of <0.05 are considered significant.

Histology. Autopsy was done immediately after killing the animals by cervical dislocation, or as soon as possible after spontaneous death. All lymphoid organs (thymus, lymph nodes, spleen, and Peyer's patches)—as well as fragments of the liver, kidney, lungs, testes, and heart—were removed. The tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut at 5 μm. The sections were stained with hematoxylin and eosin (HE) and with the periodic-acid-Schiff stain (PAS).

RESULTS

Incidence and level of chimerism. Table 1 shows the incidence (number of chimeric animals) and level (percentage of donor-type leukocytes in PBL) of chimerism in BALB/c mice after 17 fractions of TLI (0.2 Gy/fraction) or after 8.5 Gy of TBI and infusion of various BM inocula at various days after irradiation. When only allogeneic (30×10^6 C57Bl) BM cells were infused on day 1 (group 2) after 8.5 Gy of TBI all animals (10/10) became chimeric with a high level of chimerism (mean 87%). When, on the same day, 30×10^6 allogeneic and 10×10^6 sygeneic (BALB/c) BM cells were injected (group 3) some animals rejected their graft (4/21) but the majority (17/21) became chimeric with a similar level of chimerism (85%), as after infusion of allogeneic BM alone. The difference in incidence of chimerism between groups 2 and 3 is not significant ($P>0.1$). Also, when syngeneic BM was transplanted on day 1 and allogeneic BM on day 2 (group 4) most of the mice (17/19) became chimeric with a high level of chimerism (83%). However, when the syngeneic bone marrow was infused on day 1 but the allogeneic bone marrow transplantation was postponed till day 3 (group 5) or day 6 (group 6) most animals (7/8 and 10/11, respectively) rejected the allogeneic bone marrow, and the two mice that were chimeric displayed only a low level (55 and 34%, respectively) of chimerism. When the syngeneic bone marrow was replaced on day 1 by 10×10^6 syngeneic spleen cells (group 7) or 5×10^6 syngeneic spleen cells (group 8) all the allogeneic bone marrow grafts (transplanted on day 2) were rejected. BALB/c mice receiving TLI displayed a high incidence

TABLE 1. Incidence (number of chimeric animals) of chimerism and level (number of donor-type leukocytes in peripheral blood) of BALB/c mice after 8.5 Gy of TBI and infusion of bone marrow or spleen cells of various origins at different times after TBI

No. of group	Bone marrow inoculum	Day of BM or Sp transplantation	Incidence of chimerism	(%)	Level of chimerism mean (range)
1	10×10^6 BALB/c BM	D_1[a]	0/10		—
2	30×10^6 C57B1 BM	D_1	10/10	(100%)	87 (84-95)
3	10×10^6 BALB/c BM	D_1	17/21	(81%)	85 (81-94)
	30×10^6 C57B1 BM	D_1			
4	10×10^6 BALB/c BM	D_1	17/19	(89%)	83 (80-93)
	30×10^6 C57B1 BM				
5	10×10^6 BALB/c BM	D_1	1/8	(12%)	55
	30×10^6 C57B1 BM	D_3			
6	10×10^6 BALB/c BM	D_1	1/11	(9%)	34
	30×10^6 C57B1 BM	D_6			
7	10×10^6 BALB/c Sp	D_1	0/5	(0%)	—
	30×10^6 C57B1 BM	D_2			
8	5×10^6 BALB/c Sp	D_1	0/5	(0%)	—
	30×10^6 C57B1 BM	D_2			
TLI[b]	30×10^6 C57B1 BM	D_1	15/17	(88%)	74 (45-95)

[a] The day of irradiation is taken as day 0 (= D_0).

[b] These BALB/c mice received 17 daily fractions of 2 Gy of TLI and 30×10^6 C57B1 BM cells on the first day after the last TLI fraction.

(15/17) and level (74%) of chimerism after allogeneic BM transplantation, which is similar to BALB/c mice receiving allogeneic BM on the same day (group 3) or one day (group 4) after syngeneic BM ($P>0.1$).

Survival and incidence of GVHD. Figure 1 shows the survival of different groups of BALB/c mice after transplantation with syngeneic bone marrow or spleen cells or allogeneic bone marrow, or both, at various times after 8.5 Gy of TBI or after 34 Gy of TLI and allogeneic BM transplantation. When no BM was infused (group 0; Fig. 1A) all mice died within 2 weeks after TBI because of aplasia. When 10×10^6 syngeneic bone marrow was given on day 1 (group 1; Fig. 1A) most animals became long-term survivors with a survival incidence similar to unirradiated control BALB/c mice (control group; Fig. 1A). When 30×10^6 allogeneic (C57Bl) BM was infused on day 1 (group 2; Fig. 1A) many animals (>60%) died within the first three months after TBI, with clinical signs of GVHD (diarrhea, weight loss, ruffled fur, and hunched back). This diagnosis was also confirmed after autopsy. All these animals showed histological signs of GVHD including mononuclear infiltration in the periportal zone in the liver, aplasia of the lymph nodes with the absence of germinal centers, and proliferation of lymphoid cells in the paracortical zone. In the spleen the red pulp was normal but the white pulp displayed various degrees of lymphocyte depletion with absence of germinal centers and occasional proliferating large lymphoblasts. All these histological signs have been described in mice undergoing GVHD (*13*).

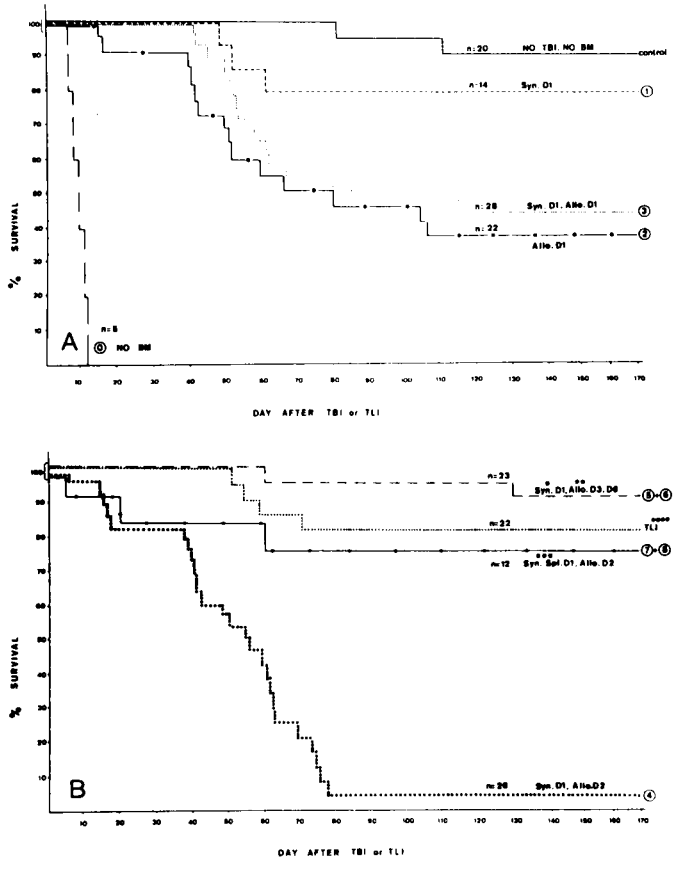

FIGURE 1. (A, B) Survival of various groups of BALB/c mice after transplantation with syngeneic BM or spleen cells, and/or allogeneic BM, at various times after 8.5 Gy of TBI or after 34 Gy of TLI and allogeneic BM transplantation. (•Syn.) = infusion of 10×10^6 syngeneic (BALB/c) BM cells; (••Allo.) = infusion of 30×10^6 allogeneic (C57Bl) BM cells; (•••Syn. spl) = infusion of 10×10^6 or 5×10^6 syngeneic (BALB/c) spleen cells; (••••TLI) = BALB/c mice receiving 17 daily fractions of 2 Gy of TLI and 30×10^6 allogeneic (C57Bl) BM cells on the first day after the last fraction. Day 0 = day of irradiation.

The difference between group 1 receiving only syngeneic BM and group 2 receiving only allogeneic BM is significant ($P<0.025$ at day 170). A similar survival pattern was observed when, at day 1, simultaneously syngeneic and allogeneic bone marrow was infused (group 3; Fig. 1A): most of these animals (>50%) also died with signs of GVHD. When, however, the allogeneic bone marrow was administered one day after the syngeneic bone marrow (group 4; Fig. 1B) almost all animals (25/26) succumbed within 3 months after TBI, with clinical as well as histological signs of GVHD. The difference in survival between this group and the group receiving only allogeneic bone marrow (group 2; Fig. 1A) is significant ($P<0.01$ at day 170). The survival was excellent in groups 5 and 6 (Fig. 1B), in which the allogeneic bone marrow was given, respectively, 2 days and 5 days after the syngeneic bone marrow, and also in groups 7 and 8 (Fig. 1B), in which on day 1 the syngeneic bone marrow was replaced by, respectively, 10×10^6 and 5×10^6 syngeneic splenocytes before allogeneic bone marrow transplantation on day 2. The survival of all these groups (5, 6, 7, and 8) is similar to the survival pattern of group 1, which received only syngeneic bone marrow after TBI. Also BALB/c mice receiving TLI and allogeneic BM transplantation had an excellent survival (more than 80% at day 170; Fig. 1B), and no clinical nor histological signs of GVHD were observed (TLI group).

Induction of suppressor cells in the spleen of BALB/c mice by infusion of syngeneic (10×10^6) BM cells after 8.5 Gy of TBI or by 34 Gy of TLI (without BM transplantation). Fig. 2 shows the induction of suppressor cells of the MLR in the spleen of BALB/c mice by TLI or by syngeneic BM transplantation after 8.5 Gy of TBI. At each time spleen cells of 2 mice of each group were pooled. Each experiment was repeated twice and the data shown are the means of the tests ±SD. As has already been shown by Slavin et al. (*10*), King et al. (*11*), and Waer et al. (*14, 15*) suppressor cells of the MLR are found in the spleen of irradiated mice during the first three weeks after the end of a cumulative dose of 34 Gy of TLI. A similar suppressor cell pattern, however, is also observed in BALB/c mice receiving 8.5 Gy of TBI and syngeneic BM transplantation. The level of suppressor cell activity progressively decreases in both groups, and after three weeks suppressor cell activity is no longer recorded. Table 2 shows the data of a relevant experiment.

DISCUSSION

In this study we looked at the effect of the infusion of syngeneic BM or spleen cells on graft acceptance and on the incidence of GVHD after allogeneic BM transplantation in mice after TBI.

When allogeneic BM was infused on the same day as syngeneic BM, most animals (17/21) became chimeric, which is similar to mice receiving only allogeneic BM ($P>0.1$), but the survival rate of the former animals was not enhanced: more than 50% in both groups died from GVHD. However, when allogeneic BM was given one day after the syngeneic BM, significantly more animals (25/26; $P<0.01$) died within the first months after TBI than when only allogeneic BM was transplanted. This increased mortality was not provoked by aplasia resulting from rejection of the grafts because animals dying from aplasia died much earlier (cf. group 0 receiving no BM;

Fig. 1A). On the contrary, it was shown that most of these animals (17/19; cf. Table 1, group 4) displayed a high level of chimerism, thus proving that they had accepted their graft. The mortality observed was due to GVHD because all mice in this group showed clinical as well as histological signs of GVHD. The possibility that the higher mortality might be caused by intercurrent infections can be ruled out, because all groups of mice were irradiated and transplanted simultaneously, and were kept under the same conditions in the same animal room. Infection by transfused BM batches is also unlikely, because mortality was never seen with the "in vivo controls" of the bone marrow batches used.

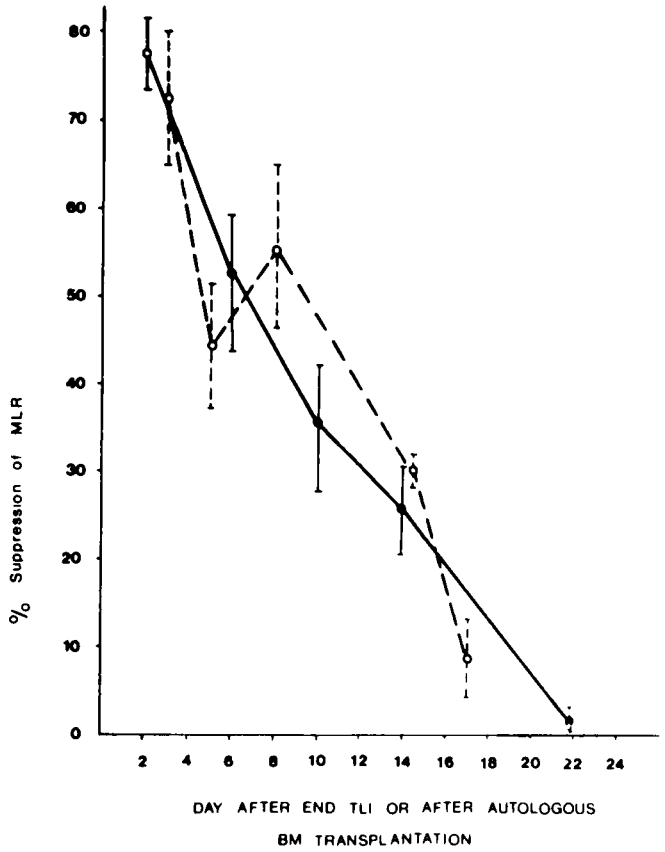

FIGURE 2. Evolution of MLR suppressor cells in the spleen of BALB/c mice after 8.5 Gy of TBI and autologous BM transplantation, or after 34 Gy of TLI (without BM transplantation). (●——●) = TLI; (O- - -O) = TBI and autologous BM transplantation.

There are two possible explanations for the increased incidence of GVHD in the group of mice receiving allogeneic BM one day after grafting syngeneic BM. The first is based upon the observation made by different groups that the main target cells of GVHD are hematolymphopoietic cells of the recipient, and that the other tissues are only attacked as "innocent bystanders." It was shown (e.g., by Steinmuller et al. [4]) that in stable CBA→C3H chimeras that have a CBA hematololymphatic system, GVHD could only be induced by C3H lymphocytes sensitized against CBA, and not by donor lymphocytes sensitized against C3H. A similar observation was made by Streilein (*6*) in supralethally irradiated hamsters in which GVHD in a P (parental) to F_1 (hydrid) model could only be observed when F_1 hematopoietic cells were added to the BM inoculum. It is probably "repopulating" recipient lymphohematopoietic cells that are very important: after sublethal TBI (2 Gy) irradiation of F_1 (hybrid) mice, GVHD induced by P (parental) cells is more severe than without previous irradiation; probably this effect is due to repopulating recipient populations that serve as targets (*16*). After the recipient lymphohematopoietic tissue is attacked, other organs can be destroyed as "innocent bystanders" (*5, 17*). A beautiful illustration of this hypothesis is that intestinal grafts, syngeneic with the BM donor, grafted in the recipient show lesions similar to those of the recipient's own intestine (*17*), or that F_1 hamsters reconstituted with P BM after TBI (thus having a P lymphohematopoietic system) do not show local GVHD lesions after local injection with P lymphocytes in the skin (*2*). It is, therefore, likely that in the mice of group 4 receiving allogeneic BM one day after syngeneic BM, targets for GVHD are more plentiful, due to proliferating syngeneic lymphohematopoietic cells, than when no syngeneic BM is administered or when it is given on the same day as the allogeneic BM graft. To further elucidate whether it was predominantly the hematopoietic or the lymphopoietic cells that served as the main targets, we replaced the syngeneic bone marrow by splenocytes. This experiment, however, was inconclusive because the syngeneic splenocytes led to rejection of the allogeneic BM in all cases.

Another explanation for the high incidence of GVHD in group 4 receiving allogeneic BM one day after syngeneic BM is that the transplanted syngeneic cells contribute to the effector population of GVHD. In the rat model, Elkins (*3*) could show by chromosome identification that after local injection of allogeneic cells, a systemic GVHD can be provoked by the recipient lymphocytes. Also, analysis of the allotype of the antibody responsible for hemolytic anemia in mice (*18*) and hamsters

TABLE 2. The percentage of suppression of the MLR between control responders BALB/c lymph node cells and irradiated (30 Gy) control C57B1 stimulator lymph node cells by irradiated spleen cells (10 Gy) of BALB/c mice one week after receiving 34 Gy of TLI or syngeneic bone marrow transplantation after 8.5 Gy of TBI

Responder cells 0.5×10^6	Stimulator cells 0.5×10^6	Cocultured spleen cells 0.5×10^6	cpm (±SD)	% Suppression
Control BALB/c lymph node cells	Control C57Bl lymph node cells	—	182247 (±9247)	—
Control BALB/c lymph node cells	Control C57Bl lymph node cells	Control BALB/c	192820 (±11322)	0%
Control BALB/c lymph node cells	Control C57Bl lymph node cells	BALB/c after 34 Gy TLI	100677 (±8731)	43%
Control BALB/c lymph node cells	Control C57Bl lymph node cells	BALB/c after 8.5 Gy TBI and autologous BM transplantation	97044 (±7943)	44%

(*19*) developing GVHD indicates the participation of the recipient lymphocytes in GVHD.

The experiments decribed here can be of interest for the explanation of the prevention of GVHD in mice after TLI. It has been shown that after 17 daily fractions of 2 Gy of TLI, GVHD does not occur in mice after H2-incompatible BM transplantation (*7*). It has been suggested by Slavin et al. (*7*) and Strober et al. (*8*)—as well as by our own experiments (*14*)— that suppressor cells proliferating after TLI might play an important role in the prevention of GVHD after TLI. However, in mice receiving 8.5 Gy of TBI and syngeneic BM transplantation we could also detect the presence of suppressor cells during the same period, and to a similar level, as after TLI. Despite this suppressor cell predominance, GVHD is, nevertheless, very frequent in these mice when allogeneic BM is given on the same day as syngeneic BM transplantation, and even more common when allogeneic BM is infused one day afterwards. It is, therefore, likely that, besides suppressor cells, other mechanisms are important in the prevention of GVHD after TLI. It may be that target cells from the lymphoid organs are more completely eliminated after a cumulative fractionated dose of 34 Gy of TLI than after a single dose of 8.5 Gy of TBI. Experiments to verify this hypothesis are in progress.

When allogeneic BM was transplanted 2 days (group 5) or 5 days (group 6) after syngeneic BM grafting, only a minority of the animals (1/8 and 1/11, respectively) became chimeric, which explains the absence of GVHD and good survival in these groups. Two possible explanations can be given for this phenomenon. First, after 3 or 6 days the syngeneic BM may give rise to sufficient immunocompetent cells to reject the allogeneic graft. Secondly, after this time many syngeneic cells can proliferate in the recipient hematopoietic tissues occupying the space known to be essential for the allogeneic stem cells to seed (*20*).

With regard to clinical BM transplantation our data are relevant. It has been demonstrated (*21*) that after prior treatment with cyclophosphamide, which is commonly used in human BM transplantation, hematopoietic stem cell recovery is enhanced after TBI. On the other hand it has also been shown (*22*) that one day after cyclophosphamide injection, F_1 mice show an increased susceptibility to GVHD by P cells. These two experiments are concordant with the hypothesis that after increased syngeneic hematopoiesis (e.g., after syngeneic bone marrow transplantation) GVHD can occur more frequently. This underlines once more the importance of the timing and of an exact conditioning schedule for the prevention of GVHD.

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