The immunosuppressive effect of fresh allogeneic bone graft in mice

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Summery. *We investigated the immunosuppressive effect of fresh bone allografts after donor specific spleen cell transfusion in mice. Allografts from major histocompatibility complex incompatible mice were performed one week after the transfusion. Cellular and humoral immune responses were recorded by measuring alloreactive cytotoxic T-lymphocyte activity and antibody activity. Survival in days of a second set skin graft was measured for determining clinical immunity. Alloreactive cytotoxic T-lymphocyte activity was stimulated at 17 days (10 days after bone grafting) and then suppressed at 31 days (24 days after bone grafting). The survival of the second set skin graft was not diminished at this time. This outcome indicates that the spleen cell stimulated cellular immune response was suppressed after fresh bone allografts, suggesting that these allografts have an immunosuppressive effect and induce immunosuppressive actions.*

Résumé. *Nous avons fait des recherches sur l'effet immunosuppresseur des greffes d'os allogéniques fraîches après la transfusion de cellules spléniques spécifiques de donneurs chez les souris. Des greffes d'os allogéniques provenant de souris à complexes tissulaires incompatibles ont été effectuées 1 semaine après la transfusion de cellules spléniques spécifiques de donneurs. Les réponses immunes aussi bien cellulaires que humorales ont été enregistrées en mesurant l'activité de lymphocyte T cytotoxique alloréactive et l'activité d'anticorps.*

La survivance (en jours) d'une greffe de peau de deuxième application a été mesurée pour déterminer l'immunité clinique. L'activité de lymphocyte T cytotoxique alloréactive a été stimulée une fois à 17 jours (10 jours après la greffe d'os), puis supprimée à 31 jours (24 jours après la greffe d'os). La survivance de la greffe de peau de deuxième application n'avait pas diminuée à 31 jours (24 jours après la greffe d'os). Ce résultat indique que la réponse immune cellulaire stimulée des cellules de rate a été supprimée après la greffes d'os allogéniques fraîche. Ceci suggère que les greffes d'os allogéniques présentent un effet immunosuppresseur et induisent des actions d'immunosuppresseur retardées.

Introduction

Fresh bone allografts cause a cellular and humoral immune response [1, 5–7, 13, 17], and bone marrow is more immunogenic than bone itself [3, 6, 7, 10]. Although previous studies confirm that bone is immunogenic, fresh bone allograft is not always rejected after transplantation. Burchardt et al. reported that 20% of fresh allografts showed normal successful repair in a canine fibular model [2]. Decreased immune responses after bone allografts have also been reported [3, 11, 16].

We studied the possible immunosuppressive effect of a fresh allogeneic bone graft in an experimental model in which mice were primed by receiving H-2 incompatible donor specific spleen cell transfusion before bone grafting.

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Table 1. Mice groups and experiment days

Groups	Day 1 Spleen cell transfusion	Day 7 Bone graft
I	Yes	No
II	Yes	Yes
III	No	Yes

Materials and methods

Mice

C57BL/10(H-2^bK^bD^b) and DBA/2(H-2^bK^bD^b) mice purchased from the Shizuoka Laboratory Animal Centre (Shizuoka, Japan) were bred in the Institute for Laboratory Animal Research, Nagoya University School of Medicine. H-2 complex is major histocompatibility complex (MHC) of mouse C57BL/10 and has H-2^b haplotype; DBA/2 has H-2^d haplotype. Thus there is a MHC incompatibility between these 2 types of mice.

C57BL/10 mice, 8 weeks old, were used as recipients and DBA/2 mice of the same age were donors in all the experiments. There were 3 experimental groups (Table 1); group I mice received only a spleen cell transfusion; group II, a spleen cell transfusion and a bone graft; group III, a bone graft only. Groups I and II received donor specific spleen cell transfusions on day 1. Groups II and III received a bone allograft on day 7. Unprimed mice did not have a graft or transfusion. The experiment days were numbered with day 1 as the day on which spleen cell transfusion was performed.

Donor specific spleen cell transfusion

This was used to stimulate an alloreactive immune response. The spleen cells were obtained from DBA/2 mice and suspended in Eagle's Minimum Essential Medium (MEM) at a concentration of 1×10^8 ml⁻¹. Each mouse in groups I and II received an intravenous injection of 0.1 ml of the suspension (1×10^7 cells) on day 1.

Bone grafting

A modification of a previously described technique was used [8]. The donor DBA/2 mice were anaesthetised with pentobarbital sodium. An allograft 2 mm long was taken from the

middle third of the tibial shaft and placed in physiological saline. The medullary space was irrigated with normal saline to remove most of the bone marrow. The recipient C57BL/10 mice were operated on in an identical fashion and a segment of bone removed; this was then placed into the recipient tibia using a 27-gauge needle for intramedullary fixation. The procedure was clean, but not sterile.

Alloreactive cytotoxic T-lymphocyte activity (CTL)

Anti-H-2^d CTL activity, which indicates cellular immune response, was measured using a ⁵¹Cr release assay. The cell line P-815, originally established by Dunn et al. [4] from a DBA/2 mouse, was used as target cells after labelling with ⁵¹Cr. These cells are specifically attacked by cytotoxic T-lymphocytes. P-815 cells were incubated with 100 µl of Na₂ ⁵¹Cr in 0.5 ml of Roswell Park Memorial Institute Medium (RPMI) 1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated foetal calf serum for 1 h at 37 °C. These cells were washed twice with Eagle's MEM and resuspended at a concentration of 1×10^6 ml⁻¹ in RPMI 1640 containing 10% heat-inactivated foetal calf serum. Spleen cells obtained from each group of mice at 10, 17 and 31 days were used as effector cells. These cells contain the cytotoxic T-lymphocyte to be measured. The spleen cells were suspended in RPMI 1640 at a concentration of 1×10^7 ml⁻¹ heat inactivated foetal calf serum. The spleen cells were then incubated with irradiated (20 Gy) DBA/2(H-2^d) splenic stimulator cells in 24-well flat bottom microtitre plates in a humidified incubator of 5% CO₂ for 4 days at 37 °C. Data from our preliminary study showed that 4 days of incubation with stimulator cells was the most needed to measure the anti-H-2^d CTL activity. The preincubation with stimulator cells was to amplify allospecific CTL activity. Viable cells were then counted and suspended in fresh RPMI 1640 containing 10% heat-inactivated foetal calf serum. ⁵¹Cr-labelled target cells (1×10^5) were mixed with effector cells at different effector-target cell ratios (E:T ratios) in 200 µl of RPMI 1640 containing 10% heat inactivated foetal calf serum in a 96-well U-shape bottom microplate at 37 °C for 4 h. Radioactivity was determined from 100 µl aliquots of supernatant from each well using a γ-scintillation counter. All assays were carried out twice. The percentage of specific lysis was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. The maximum release was obtained by exposing target cells to 10% Nonidet P-40.

Table 2. Effect of donor specific bone grafting on survival days of allogeneic grafted skin^a

Days of skin grafts	ST ^b	Type of first set grafts	Survival days of skin ^c	n	P ^d
17	+	Bone graft	7.5 ± 0.7	7	<0.01
	+	–	6.9 ± 2.9	8	<0.05
	–	Bone graft	7.8 ± 2.0	6	NS
	–	Skin graft	6.9 ± 1.5	8	<0.01
31	+	Bone graft	10.3 ± 1.6	7	NS
	+	–	7.9 ± 1.1	7	<0.05
	–	Bone graft	7.7 ± 2.4	6	<0.05
	–	Skin graft	6.4 ± 0.5	7	<0.01
First set skin graft			9.9 ± 2.1	15	

^a C57BL/10 mice were grafted with DBA/2 skin after donor specific spleen cell transfusions or donor specific bone graft or both

^b donor specific spleen cell transfusion on Day 1

^c mean survival days ± SD (days)

^d These values are for the survival days of the first set skin graft and those of each of the other groups, using a 2-tailed Student's *t*-test. NS (*P* > 0.05)

Fig. 1. Comparison of the in vivo alloreactive CTL activities of unprimed mice with mice which were grafted with allogeneic bone. Although the CTL activity of group III mice was higher than that of unprimed mice on any observed day, a significant difference first appeared on day 31, demonstrating the CTL priming effect of the allogeneic bone graft. Effector – target cell ratio = 10

Alloreactive antibody activity

The anti-H-2^d activity in the serum indicates humoral immune response. Serum samples were obtained from each group of mice at 10, 17 and 31 days and stored at –20 °C until assayed. They were then serially diluted and tested for antibodies to a strain of DBA/2(H-2^d) thymocytes using an indirect immunofluorescence assay. DBA/2 cells (1×10⁶) were assayed with serum serially diluted with phosphate buffered saline in a range of 1:1 to 1:1024 at 4 °C for 2 h. Cells were washed with and then reactivated with fluorescein isothiocyanate conjugated goat anti-mouse Immunoglobulin G (Tago Inc, Burlingame, CA) at 4 °C for 30 min. After washing the cells were fixed with 5% paraformaldehyde phosphate buffered saline and analysed on a cell sorter (EPICS profile, Coulter Corporation, Hialeah, FL) for immunofluorescence.

Skin grafting

The survival test of the second set skin graft is a measurement of clinical immunity [12, 14]. On days 17 and 31, the graft from a DBA/2 mouse was carried out. Some mice had allogeneic skin grafts on day 7 instead of bone grafts. The combinations are shown in Table 2. The donor mice were anaesthetised with pentobarbital sodium and placed in a prone position. The skin of the upper back was cleaned with alcohol and a full thickness circular skin graft, 8 mm in diameter, was taken. It was then defatted and left in physiological saline. The recipient mice were operated on in the same way to produce a

Fig. 2. There was no significant difference in the anti-H-2^d CTL activities of group I and II mice on day 17. The anti-H-2^d activities of these mice were significantly higher than those of group III mice. On day 31, the anti-H-2^d CTL activity of group II mice was significantly less than group I. There was also no significant difference between the anti-H-2^d CTL activity of group II and that of group III. By day 31, fresh bone allografting had reduced the spleen cell stimulated immune response. Each group comprised 4 mice. Effector – target cell ratio = 10

skin defect into which the graft was sutured. At 24 hours, all failures were omitted and classed as technical complications. The remainder were observed daily for rejection.

Statistics

Analysis was by a 2-tailed Student's *t*-test and Mann-Whitney's nonparametric test.

Results

To determine a cellular immune response, the in vitro alloreactive CTL activities of unprimed mice were compared with mice which were grafted with allogeneic bone (Fig. 1). Although the activities of the group III mice were higher than those of un-

Fig. 3. No unprimed C57BL/10 mice produced any anti-H-2^d antibodies. Although group III produced a lower level than group I, these antibodies were detected showing the humoral immunogenic capacity of allogeneic bone. The anti-H-2^d antibody titre of group I was first detected on day 10, and the level was not changed by days 17 and 31. The anti-H-2^d titre of group II was maintained at days 10 and 17, but had a tendency to decrease at day 31. Each black dot (●) indicates one mouse

primed mice, significance first appeared at 31 days. No immunosuppressive effect was seen after fresh bone allograft, although this type of graft evoked a cellular immune response.

The anti-H-2^d CTL activities in groups I and II were significantly higher than that of group III mice at 17 days (Fig. 2). At 31 days, this activity in group II was significantly less than in group I, but not different to group III, demonstrating a cellular response in group II which once elicited by a spleen cell transfusion was suppressed to a low level, as in group III after a fresh bone graft.

The anti-H-2^d antibody titre of group I was detected at day 10, and its level was not changed on days 17 and 31 (Fig. 3). At 31 days, the number of group II mice producing a lesser amount of anti-H-2^d increased. There was no significant difference between groups I and II at 31 days by the Mann-Whitney's test ($P = 0.1$). Group III mice had a lower, yet detectable, level of antibodies, and

unprimed C57BL/10 mice had no anti-H-2^d, demonstrating the humoral immunogenicity of allogeneic bone.

The mean survival days of the second set skin graft in group II were shortened at day 17, but prolonged at day 31 (Table 2). No such effect was seen in group I, indicating that fresh bone allografts suppressed clinical immunity that had been elicited by spleen cell transfusion. The mean survival days of second set skin graft in group III was also reduced slightly demonstrating the immunogenicity of allogeneic bone.

Discussion

We have observed cellular and humoral immune responses and estimated the clinical effects after fresh bone allografting. When such a graft was implanted, alloreactive CTL activity was stimulated, alloreactive antibodies were produced and the survival days of grafted skin were shorter, indicating the immunogenicity of fresh allogeneic bone. When the allograft was carried out after donor specific spleen cell transfusion, the alloreactive CTL activity was suppressed and the survival of grafted skin was not shortened at 31 days. These results demonstrate that allogeneic bone graft caused the immunosuppression of cellular immune response.

A donor specific spleen cell transfusion was used for priming recipient mice in this study. Such a donor transfusion will reduce the recipient's CTL response [9, 15]. This beneficial effect was only seen when H-2 compatibility existed between donor and recipient [15]. We chose H-2 incompatible mice for donor and recipient, and a donor specific spleen cell transfusion stimulated an immune response. Thus the specific transfusion does not explain the immunosuppressive effect. There are, however, two possibilities. Bone allografts may induce late immunosuppressive actions, also group I mice had a peak response at 17 days which had subsided at 31 days, probably due to a double challenge to an allogeneic antigen. This implies a distinctive character of bone grafts compared to other tissues which generally cause more immune response, as with skin grafts. The mechanism of immunosuppression remains unexplained.

Our results also demonstrate that the suppressive effect could not eliminate all of the alloreactive response, and the immune response evoked by a spleen cell transfusion was suppressed to the same level after a fresh bone graft as if the graft itself was immunogenic. We only studied one

combination of H-2 incompatibility. Burchardt et al. reported successful repair in only 20% of bone allografts [2] which suggests that the immunosuppressive effect may only occur in certain combinations of MHC incompatibilities. Variations of the effect of different combinations of H-2 need further investigation.

Our results suggest that better clinical outcomes may be expected by choosing certain combination of human lymphocytic antigen in cases of allogeneic bone grafting.

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