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Satoshi Chiba
Shigeru Goto
Yoshinori Shimizu
Frank Vari
Roger Lord
Catherine Edwards-Smith
Shigeru Kobayashi
Takenori Ochiai
Kaichi Isono

The characterization of reconstituted passenger leukocytes on the induction of tolerance in rat liver transplantation

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S. Chiba[1] · S. Goto (✉)[2] · F. Vari
R. Lord · C. Edwards-Smith · S. Kobayashi
Transplantation Biology,
Department of Surgery, QIMR,
P.O. Box: Royal brisbane Hospital,
QLD 4029, Brisbane, Australia

Y. Shimizu
The Second Department of Surgery,
Showa University School of Medicine,
Tokyo, Japan

T. Ochiai · K. Isono
The Second Department of Surgery,
Chiba University School of Medicine,
Chiba, Japan

[1] *Present address:*
Department of Surgery, Chiba University
School of Medicine, Chiba, Japan

[2] *Present address:*
Department of Surgery,
Chang Gung Memorial Hospital,
123 Ta-Pei Road, Niao-Sung, Kaohsiung,
Taiwan Fax: + 886 7 732 4855

Abstract The tolerance induced by orthotopic liver transplantation [DA ($RT1^a$) rats to PVG ($RT1^c$) rats] can be prevented by total body irradiation of the donor rat. Reconstitution of the irradiated donor with DA splenic leukocytes reintroduces this tolerance. To investigate the major histocompatibility complex (MHC) specificity of passenger leukocytes, irradiated DA donors were reconstituted by third-party BN ($RT1^n$) splenic leukocytes. The reconstitution with BN splenocytes re-established DA-specific tolerance in PVG recipients, as confirmed by subsequent DA cardiac allografting, while BN hearts were rejected with second-set tempo. To determine which cell components play an important role in re-establishing liver graft tolerance, DA splenic leukocytes were further purified into three types: T, B, and adherent cells. Only "T-cell-enriched" preparations restored liver graft tolerance in three out of five PVG recipients. These results suggest that passenger leukocytes of differing MHC types can help to induce liver-specific tolerance and that T cells in the liver graft may be essential to regulate tolerance induction.

Key words Liver transplantation, passenger leukocytes, rat · Passenger leukocytes, liver transplantation, rat · Rat, liver transplantation, passenger leukocytes · Tolerance, liver transplantation, rat

Introduction

Donor-derived passenger leukocytes, in particular dendritic cells, provide the trigger for allograft rejection since these cells may cause sensitization of host T cells to donor antigens [5, 14, 15]. Total body irradiation (TBI) of the donor has previously been reported to be an effective method for significantly reducing passenger leukocytes and prolonging allograft survival for rat pancreas islet, kidney, heart, and liver transplantation in several rejector combinations [6, 21, 22, 23, 28, 30]. However, recent studies [26, 29] show that the removal of passenger leukocytes by TBI of the donor appears to be associated with not only the suppression of rejection, but also the deletion of spontaneous rat liver allograft acceptance. Sun et al. [29] have demonstrated, in a non rejector rat orthotopic liver transplantation (OLT) model, that TBI of the donor rats prevents spontaneous

allograft acceptance. They have also shown that TBI of the donor is successful in removing T and B lymphocytes, most dendritic cells, and monocytes/macrophages from the liver. These results suggest that TBI-depleted populations of passenger leukocytes in the donor liver, and not exclusively dendritic cells, protect the transplanted liver and have an important role in spontaneous liver graft acceptance in the induction of tolerance. Therefore, we speculate that, in low responder or tolerogenic combinations, more complex interactions occur between donor passenger leukocytes and the recipient immune system, leading to a decrease in the alloresponsiveness of the recipient.

We report here that the reconstitution of irradiated rats with splenic leukocytes can re-establish the induction of tolerance in a tolerogenic rat OLT model but not in a rat heterotopic heart transplant (HHT) model. The main purpose of the present study was to investigate the MHC specificity of reconstituted passenger leukocytes and to identify which fraction of the spleen cells has the ability to re-establish liver graft tolerance. We also observed the level of migration of donor cells into the recipient PVG spleen following OLT with irradiated and repopulated donors.

Materials and methods

Animals

Male DA ($RT1^a$), PVG ($RT1^c$), and BN ($RT1^n$) rats weighing 250–300 g were obtained from the Animal Resources Centre (Perth, Western Australia).

Preparation of spleen cells

A spleen from a naive DA or BN rat was passed through a 60-mesh stainless steel sieve, and the suspension was washed twice by centrifugation in Dulbecco's modified Eagle's medium (DMEM; Commonwealth Serum Laboratories, Melbourne, Australia) containing 10 % fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia). After the red cells were lysed with 0.8 % NH_4Cl 10 mM TRIS treatment for 10 min, the spleen cell suspension (SPCs) was passed through 200-nylon mesh screens and then washed three times. This fraction contained an average of 1.5×10^8 cells per spleen and was used as "splenic leukocytes" to reconstitute the irradiated rat. To further divide the spleen cells into "T-cell-enriched", "B-cell-enriched" and "adherent cell-enriched" preparations, 20 ml of SPCs was resuspended in DMEM in 10 % FCS and incubated in a plastic 70 cm^2 petri dish (Disposable Products, South Australia) at 37 °C for 2 h. The nonadherent cells were gently rinsed with warm medium, and the adherent cells were collected by vigorous pipetting. "B-cell-enriched" populations were recovered by vigorous pipetting of DMEM through the column. All cell populations were washed, counted, and prepared at appropriate concentrations for injection.

One and two-color staining for flow cytometry was performed on freshly isolated leukocytes from the spleen. After washing in phosphate-buffered saline (PBS) containing 2 % FCS and 0.0 % sodium azide, the cells were incubated in 100-µl volumes on ice with optimal concentrations of the biotinyated antibodies OX-42 (DX11b), OX-8 (CD8), WW3/25 (CD4), and RLN-9D3 (B cells; CALTAG, San Francisco, Calif.). The excess antibody was removed by washing the cells in PBS three times and centrifuging for 5 min at 500 g. Flow cytometric analysis was performed on a FACScan (Becton Dickinson, San Jose, Calif.). A forward scatter acquisition gate was used to exclude erythrocytes, dead cells, and cell debris.

Orthotopic liver transplantation (OLT) using donor grafts from irradiated or reconstituted rats

Kamada's cuff method [11] was used for OLT. In group 1, "untreated" livers taken from naive DA rats were implanted into PVG recipients. In group 2, to assess the effect of irradiation on liver graft viability, the "irradiated" DA livers were implanted into naive DA recipients. The DA rats were lethally irradiated with 1000 rads with a 20 MEB Electron (Varian, Clinac 850, USA). In group 3, the DA rats were irradiated as in group 2; then, 24 h later, the irradiated DA livers were harvested and transplanted into naive PVG recipients. In groups 4 and 5, DA rats received the same dose of irradiation as in group 2; however, immediately after irradiation, they were injected via the penile vein with 1.5×10^8 purified leukocytes extracted from the spleens of naive DA (group 4) or BN (group 5) rats. The "irradiated and reconstituted" livers were removed and implanted into naive PVG recipients 24 h later. All rats surviving longer than 60 days in groups 1, 4, and 5 underwent DA or BN cardiac allografting to confirm the specificity of tolerance. In addition, several rats were sacrificed on various days after OLT in order to obtain liver samples for histological analysis.

Heterotopic heart transplantation (HHT) with donor grafts from irradiated or reconstituted rats

Heterotopic hearts were transplanted as reported previously [9]. The heart grafts were harvested at the same time as the livers from the donor rats already described. In group 6, untreated hearts taken from naive DA rats were implanted into naive PVG recipients. In group 7, irradiated DA heart grafts were harvested from the same donors used for group 3 and transplanted into naive PVG recipients. In group 8, irradiated and reconstituted DA heart grafts were harvested from the same donors used for group 4 and implanted into naive PVG recipients.

Cell components responsible for induction of tolerance in OLT (irradiated and reconstituted DA liver into PVG recipient)

To determine which cell components of DA splenic leukocytes play an important role in the re-establishment of tolerance observed in group 4, the irradiated rats were reconstituted by three different cell-enriched populations: T cells (5×10^7, group 9), B cells (5×10^7, group 10) and adherent cells (3×10^7, group 11) extracted from naive DA spleen cells. These cell numbers were determined for each population according to the average ratio of T, B, and adherent cells isolated from one untreated spleen in our hands. The DA livers from these irradiated and reconstituted DA rats were implanted into naive PVG recipients. All other conditions and experimental procedures were similar to those in group 4.

Table 1 Effect of donor irradiation and reconstitution on liver and heart allografts

Groups	Treatment of DA donor	Implanted organ	Recipient	Survival days
1	Untreated	Liver	PVG	> 60 (× 6)
2	Irradiated (1000 rads)	Liver	DA	> 60 (× 6)
3	Irradiated (1000 rads)	Liver	PVG	5, 7, 8, 11, 14, 17, 18*
4	Irradiated + reconstituted (DA splenic leukocytes, 1.5×10^8)	Liver	PVG	> 60 (× 6)
5	Irradiated + reconstituted (BN splenic leukocytes, 1.5×10^8)	Liver	PVG	16, 18, > 60 (× 6)
6	Untreated	Heart	PVG	7 (× 2), 8 (× 7), 9 (× 3)
7	Irradiated (1000 rads)	Heart	PVG	7, 8, 10
8	Irradiated + reconstituted (DA splenic leukocytes, 1.5×10^8)	Heart	PVG	7, 8, 9

* $P < 0.05$ vs group 1 (Wilcoxon rank test)

Immunofluorescence assay for detection of DA class I in PVG recipient spleen

Immunohistochemical assays were performed with minor modifications as previously described [15]. Briefly, spleen samples taken on days 1, 7 and 15 from groups 1, 3, and 4 were immediately frozen in liquid nitrogen and stored at –70 °C until used. Frozen sections were cut to 6 μm in thickness in a cryostat, fixed with –20 °C 1:1 acetone/methanol, and blocked with 5 % low-fat milk powder in TRIS (hydroxymethyl) aminomethione-buffered saline (TBS; pH 7.4). The sections were then treated with a directly labelled fluorescein in isothiocyanate (FITC)-conjugated mAb that reacts only with DA ($RT1^a$) MHC class I (mAb C3; Pharmingen, San Diego, Calif.). The FITC-conjugated mAb was diluted to a ratio of 1:100 in TBS, incubated with the section for 1 h at 37 °C, washed with TBS, and mounted in a solution of 50 mg of *N*-propyl-gallate (Sigma) in 200 μl 2 M TRIS and 800 μl of glycerol. Immunofluorescence was observed with an Olympus fluorescence microscope (Model BH2-RFL, Tokyo, Japan). The mAb C3-labelled cells were counted in five representative fields of view (magnification × 400), and the average number was expressed as the number of labelled cells per mm^2 of recipient splenic tissue.

Statistical analyses

The Kaplan and Meier survival curves were compared by means of the Wilcoxon rank test. The differences were considered to be statistically significant if the *P* values were less than 0.05.

Results

The different immunological roles of donor irradiation and reconstitution with splenic leukocytes in liver and heart transplantation

It has previously been demonstrated that PVG rats grafted with DA livers naturally overcome rejection and that tolerance is induced [9], and this was confirmed in group 1. However, TBI (1000 rads) of DA donor rats 24 h prior to OLT prevented the induction of tolerance in PVG recipients [26], as shown in our group 3 (Table 1). Group 2 showed that graft viability was not affected by irradiation. We also confirmed our previous findings that the reconstitution of the irradiated DA donor rats with 1.5×10^8 splenic leukocytes can re-establish the induction of tolerance (group 4).

To investigate the effect of irradiation and repopulation on other organ transplants, irradiated and/or reconstituted hearts were transplanted heterotopically. TBI of the donor did not prolong graft survival (group 7, Table 1). Reconstitution of the irradiated donor (group 8) also had no effect on heart graft survival compared to that of untreated controls (group 6).

MHC specificity of tolerance induced by the irradiated liver allograft reconstituted with splenic leukocytes

In order to investigate whether third-party splenic leukocytes have the ability to protect DA hepatocytes, the irradiated DA donor rats were reconstituted with BN ($RT1^n$) splenic leukocytes (1.5×10^8). Six out of eight recipient PVG rats bearing irradiated DA livers reconstituted with BN splenic leukocytes survived longer than 60 days (group 5, Table 1). The OLT rats in groups 1 and 4 (PVG rats with DA type passenger leukocytes) surviving for longer than 60 days accepted DA hearts but rejected BN hearts at the normal tempo, indicating that DA donor-specific tolerance had been established (Table 2). The surviving OLT rats in group 5 (PVG recipient with DA type liver reconstituted with BN splenic leukocytes) underwent HHT, to identify in which MHC type tolerance was induced. Untreated DA or BN hearts ($n = 3$) were implanted into group 5 PVG recipients surviving longer than 60 days. DA hearts were accepted and BN hearts were rejected, not at the normal tempo (8 days), but with second-set tempo (5 days), as shown in Table 2.

Cell population of reconstituted passenger leukocytes responsible for re-establishment of the induction of tolerance

To demonstrate which cell preparation of splenic leukocytes has the ability to protect liver grafts and induce donor specific tolerance, DA splenic leukocytes were puri-

Table 2 Survival of cardiac allografts in PVG recipients surviving longer than 60 days after OLT

PVG recipients (treatment of donor liver)	DA cardiac allograft survival in days Mean ± SD *(n)*	BN cardiac allograft in survival in days Mean ± SD *(n)*
Naive	8.1 ± 0.7 (12)	8.0 ± 0.8 (8)
Group 1 (untreated)	> 60 (3)*	8.3 ± 9.6 (3)
Group 4 (irradiated + reconstituted with DA splenic leukocytes)	> 60 (3)*	8.0 ± 1.0 (3)
Group 5 (irradiated + reconstituted with BN splenic leukocytes)	> 60 (3)*	5.0 ± 1.0 (3)**

* $P < 0.05$ vs control (naive) (Wilcoxon test)
** $P < 0.05$ vs control (naive) (Wilcoxon test)

Table 3 The effect of donor reconstitution with different cell populations

Groups	Type of reconstituted cells in irradiated DA donor	Recipient	Survival days
9	Splenic "T-cell-enriched" (5×10^7)	PVG	30, 55, > 60 (× 3)[a]
10	Splenic "B-cell-enriched" (5×10^7)	PVG	5, 6, 6, 12, 21**
11	Splenic "adherent cell-enriched" (3×10^7)	PVG	7, 12, 12, 16, 20**

[a] Each of three surviving OLT rats accepted a DA heart, but not a third-party BN heart
** $P < 0.05$ vs group 1 (Wilcoxon rank test)

fied into three different cell-enriched populations of T cells (5×10^7), B cells (5×10^7), and adherent cells (3×10^7), and then injected into the irradiated DA donor. The T, B, and adherent cell populations were determined to be > 90 %, 65 %–80 %, and > 90 % pure, respectively, by flow cytometry. "T-cell-enriched" suspensions contained 60 % CD4, 30 % CD8, 5 % B cells; "B-cell-enriched" suspensions contained 70 % B cells, 15 % CD11 b, and 5 % CD4; and "adherent cell-enriched" populations contained 60 % CD11 b, 20 % B cells, and 10 % CD4 of surface marker-positive lymphocytes. Repopulation with B cells or adherent cells immediately after irradiation did not reintroduce liver allograft acceptance and caused a significant reduction in liver allograft survival in five PVG recipients (group 10 or 11, $P < 0.05$ vs group 1; Table 3). Histological findings revealed that the cause of death was consistent with rejection of the implanted liver (data not shown). However, when the irradiated DA rats were inoculated with a splenic "T-cell-enriched" population, all five rats in group 9 survived longer than 30 days, and DA-specific tolerance was induced, as shown by heart grafting on three of the rats (Table 3).

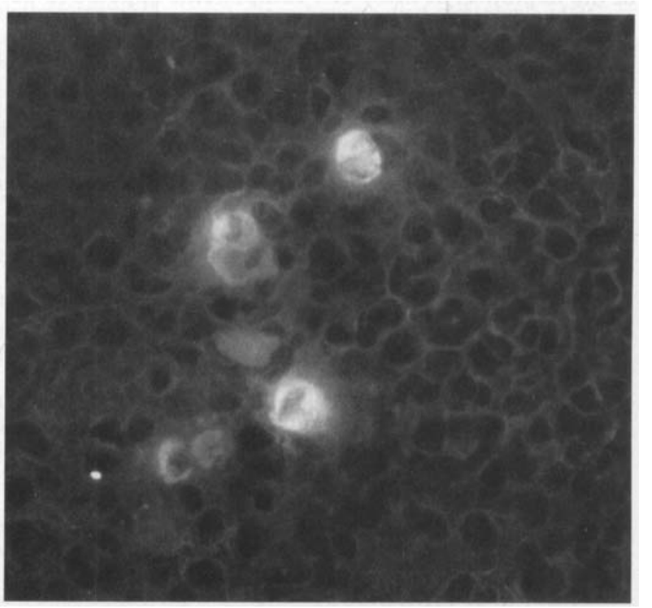

Fig. 1 Detection of donor passenger leukocytes in the spleen of a PVG recipient at POD 7 after OLT. Immunofluorescence staining of DA class I with mAb C3 (magnification × 400)

Detection of cells expressing donor class I antigen in the spleen of PVG recipient after OLT

An example of immunofluorescence staining is shown in Fig. 1, with the labelling of DA type class-I-positive cells in the PVG recipient's spleen. The number of DA type class I-positive cells in the PVG spleens in group 1 was relatively high at post-operative day (POD) 1 and gradually increased until POD 15, as shown in Table 4. In group 3, the spleen of the PVG recipient bearing the irradiated DA liver showed a remarkably reduced number of DA type class I-positive cells. In group 4, the number of DA class I-positive cells in the PVG recipient spleen was also much lower than that in group 1. Despite the lower number of DA class I-

Table 4 The average number of mAb FITC-labelled donor (DA) cells per mm^2 in the spleens of PVG recipients[a]

Groups	Treatment of donor	Labelled cells per mm^2 (mean ± SD)		
		POD 1	POD 7	POD 15
1	Untreated	17.0 ± 5.0	21.0 ± 3.8	26.0 ± 4.4
3	Irradiated (1000 rads)	4.6 ± 0.5	5.2 ± 0.9	4.0 ± 1.3
4	Irradiated + reconstituted (DA splenic leukocytes, 1.5×10^8)	7.2 ± 1.2	3.1 ± 0.7	12.0 ± 2.0

[a] Spleen samples were taken from PVG recipients at 1, 7, and 15 days after OLT

positive cells observed in this group within the PVG spleen at POD 7 ($3.1 \pm 0.7/mm^2$), the number of positive cells detected increased by POD 15, although the levels never reached those of the untreated control group 1.

Discussion

It has been demonstrated [22,29,30] that TBI depletes the donor of most passenger leukocytes, including T, B, dendritic, and monocyte/macrophage cells. The kinetics of the depletion of these cell components appears to be different [8, 29], and one previous study showed that the number of interstitial dendritic cells was undiminished even 24 h after lethal irradiation [8]. Using a naturally tolerogenic combination of rats in OLT, we have demonstrated, as have others [29], that TBI of the donor fails to induce tolerance in these recipients. However, unlike other studies using parking of the liver [27, 29], reconstitution of the irradiated donor with bone marrow cells [31], or injection of cells into the recipient, we reestablished liver-induced tolerance simply by injecting naive donor type spleen cells into the irradiated donor 24 h prior to transplantation [26]. Furthermore, our present study shows that splenic "T-cell-enriched" populations may play an important role in the restoration of liver graft acceptance, although we should further investigate the involvement of radio-resistant dendritic cells [8,29] or intrahepatic hematopoietic stem cells [2,20] in the induction of tolerance. This is consistent with other clinical trials and experimental studies that have shown that donor T cells can play a critical role in preventing allogenic marrow graft rejection [18, 19].

In the present study, we show that the effect of TBI and/or reconstitution is specific to liver transplantation and cannot be observed in HHT models in which the same donor is used. The different effects of passenger leukocytes on allograft survival in liver and heart transplantation may simply be due to the remarkable difference in the numbers of passenger leukocytes. Alternatively, the type of passenger leukocytes may be different in the two types of organ grafts carrying donor-derived cells, as the function or characterization of donor passenger leukocytes may independently and respectively be modulated by the existence of a liver or a heart allograft. Although we performed HHT 24 h after TBI of the donor in the combination DA-to-PVG, others have shown that an increased interval between TBI and transplantation in other combinations appears to prolong allograft survival [6, 20, 28].

Reconstitution with third-party BN splenic leukocytes of the DA donor after TBI can restore DA-specific tolerance in PVG recipients. This may be associated with the previous finding that rejection is naturally overcome in a fully allogeneic combination of BN donor liver into PVG recipients [10], as well as OLT (DA-PVG), and we confirmed the permanent acceptance of BN liver transplants in five PVG recipients. However, precisely what it is that allows third-party splenic leukocytes with an MHC type differ from that of the donor hepatocytes to reintroduce tolerance remains to be investigated further. The specificity of tolerance reintroduced by TBI and the reconstitution of the donor appears to depend on the MHC of donor hepatocytes rather than on that of passenger leukocytes. We speculate that such an effect of passenger leukocytes in low responder or tolerogenic combinations may be mediated by nonspecific immunosuppressive factors, as we have recently reported [4, 7, 17].

However, PVG recipients were sensitized to BN ($RT1^n$) antigens, as shown by the rejection of heart grafts within 6 days. Following OLT (DA-PVG) and subsequent HHT (DA or BN heart allografts), both DA and BN antigens promoted an allogenic response in the PVG recipient. We speculate that the DA liver graft selectively deleted the PVG anti-DA T cells only against DA type surface antigens. However, the PVG anti-BN T cells may not have been deleted, and the persistence of the immune system's ability to respond to BN antigen may have resulted in second-set rejection. The abrogation of the host immune response by deletion of alloreactive T cells by the liver graft may be one mechanism of liver graft tolerance [3, 12]. This is supported by the notion that the liver is the site for deletion of activated T cells [1, 9].

In the normally tolerogenic DA-to-PVG combination, there is long-term persistence of donor passenger leukocytes that have migrated into the recipient spleen [13, 16]. Previous studies have shown that TBI of the donor can reduce the number of radiosensitive passenger leukocytes and prevent the induction of tolerance in tol-

erogenic rat OLT models [26,29]. This correlates well with our immunofluorescence assay results, which show a markedly reduced number of migrating DA class I-positive cells in the spleen of the PVG recipient bearing the irradiated DA liver. Reconstitution of the irradiated donor restored tolerance; however, the number or migrating cells observed was significantly lower than that of the untreated tolerogenic model (Table 4). We believe that there may be no relationship between the induction of tolerance and the total number of migrating passenger leukocytes observed in the recipient spleen, especially in the early phase after OLT.

In the clinical setting of liver transplantation, large numbers of donor lymphocytes are regularly transferred to the recipient by liver grafting, and these cells represent a distinct population consisting predominantly of preactivated T and NK cells [24, 25]. Our preliminary results suggest that the "T-cell-enriched" fraction of passenger leukocytes in the donor liver may possess immunomodulatory properties inhibiting the alloreactive potency of the recipient's immune system in a rat tolerogenic OLT model. Further studies to characterize the liver passenger leukocytes specifically involved in the induction of tolerance may suggest a new strategy for selective immunomodulatory therapy for allograft rejection.

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