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Long-term small bowel allograft function induced by short-term FK 506 application is associated with split tolerance

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Abstract Functional long-term allograft survival after experimental small bowel transplantation (SBT) is limited by chronic rejection. Initial application of high-dose FK 506 has been shown to induce stable long-term graft function. In order to examine whether this long-term function is associated with donor-specific tolerance, we analyzed the functional status of recipient T cells in vivo and in vitro. One-step orthotopic SBT was performed in the allogeneic Brown Norway (BN)-to-Lewis rat strain combination. FK 506 was given daily at a dose of 2 mg/kg from days 0–5 in the rejection model and from days 0–9 in the long-term functional model. Mean survival time in the rejection model was 98 ± 2.8 days. Histological examination of these small bowel allografts disclosed signs of chronic rejection. In contrast, all animals of the long-term functional model survived long term (> 250 days) without clinical signs of chronic rejection. The latter model, furthermore, produced evidence of donor-specific tolerance. Whereas heterotopic Dark Agouti (DA) hearts were rejected regularly within 7 days, BN hearts survived indefinitely (> 70 days). In vitro, mixed leukocyte reactivity of CD4 + T cells was similarly strong against donor (BN) antigens as against third-party (DA) antigens. The split tolerance revealed by our in vivo and in vitro results enabled acceptance of both the small bowel allograft without signs of chronic rejection and of donor-specific heart allografts.

Key words Small bowel transplantation · Split tolerance · FK 506 · Rat

Introduction

Immune reactivity against an allograft is determined by the immunogenicity of the grafted cells and the presence of recipient allospecific T cells [1]. We investigated the capacity of allospecific T cells to initiate damage to the small bowel allograft after an initial high-dose application of FK 506. For this purpose we established two small bowel transplantation (SBT) models in the MHC incompatible Brown Norway (BN)-to-Lewis (LEW) rat strain combination, one model leading to chronic rejection of allografts and the other to stable long-term allograft function.

The aims of this study were to examine whether FK 506-induced long-term survival after SBT is associated with donor-specific tolerance and whether small bowel recipients show a specific downregulation of allospecific recipient T cells. To do this we analyzed the functional status of recipient T cells from the long-term surviving animals in vivo and in vitro.

Materials and methods

Animals

Inbred male rats of the strains BN, LEW, and Dark Agouti (DA) were purchased from Charles River (Sulzfeld, Germany) and Harlan Winkelman (Borchen, Germany). The rats weighed 180–220 g

and were maintained under controlled conditions. All animals received care according to national guidelines for animal care (German law on the protection of animals).

Immunosuppression and experimental groups

FK 506 was supplied in powder form by Fujisawa (Munich, Germany). It was diluted in sterile saline at a standard concentration of 2 mg/kg body weight and was daily administered intramuscularly in the hind limb. LEW recipients of BN small bowel allografts received FK 506 injections: group 1, the rejection model, from days 0–5 and group 2, the long-term functional model, from days 0–9. LEW animals receiving small bowel isografts served as syngeneic controls (group 3).

Small bowel transplantation

One-step orthotopic SBT was performed as described previously [2]. Animals dying within 7 days after transplantation were considered to be technical failures and excluded from the study.

Intra-abdominal heterotopic heart transplantation

Long-term survivors were used as recipients for donor-type (BN) or third-party (DA) secondary heart grafts. Heterotopic transplantation of primarily vascularized cardiac grafts was performed according to the method of Ono and Lindsey [3].

Postoperative monitoring

After transplantation, all animals were examined daily for signs of graft failure. Animals were killed when they demonstrated a weight loss of 2% on 6 successive days.

Flow cytometry

Flow cytometric analysis was performed as described elsewhere [4]. Immunocompetent donor cells derived from the small bowel graft were detected by the monoclonal antibody (mAb) OX-27 (stained MHC class I antigen on BN cells) and recipient-derived leukocytes within the small bowel graft by the mAb NDS-60 (stained MHC class I antigen on LEW cells). NDS-60 was the kind gift of Dr. M. Dallman, Department of Biology, Imperial College of Science Technology and Medicine, London, UK; OX-27 was purchased from Serotec, Oxford, UK.

Mixed leukocyte reaction (MLR)

Ten thousand γ-irradiated allogeneic or syngeneic spleen dendritic cells (20 Gy) were co-cultured with 10^5 and 0.5×10^5 purified CD4 + responders of spleen and peripheral lymph nodes, respectively, in 200 µl complete RPMI 1640 medium in U-bottomed microtiter plates (Greiner Labortechnik, Frickenhausen, Germany). The culture medium was supplemented with 10% heat-inactivated FCS, 5 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 2×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, and 1% non-essential amino acids (all reagents from Life Technologies, Karlsruhe, Germany). Cells were isolated as described previously [4]. The plates were incubated for 3 days at 37 °C with 5% CO_2 and with 0.5 µCi per well of [^{3}H] thymidine (Amersham, Braunschweig, Germany) for the final 6 h of culture. T cell proliferation was measured by [^{3}H] thymidine uptake. All experiments were set up in hexaplicates, and the results are expressed as mean cpm ± standard deviation.

Table 1 Experimental groups and graft function time. Group 1, chronic rejection model; group 2, long-term functional model; group 3, syngeneic controls. (*BN* Brown Norway, *LEW* Lewis)

Group	*n*	Strain combination	FK 506 treatment	Graft function (days)
1	6	BN-to-LEW	Day 0–5	98 ± 2.8[a]
2	6	BN-to-LEW	Day 0–9	> 200
3	6	LEW-to-LEW	None	> 200

[a] MST ± SD

Results

Clinical outcome and histopathological evaluation

Orthotopic SBT was performed with BN rats as donors and LEW rats as recipients. After an initial postoperative slight weight loss, all animals regained weight rapidly and grew at a rate comparable to syngeneic controls. Late weight loss in group 1, the rejection model, correlated with the onset of chronic rejection between days + 80 and + 100, posttransplantation (Table 1). Animals of group 2, the long-term functional model, survived long term without any signs of chronic rejection. These animals were killed between days 200 and 250; histological examinations revealed normal small intestinal structure. Graft mesenteric lymph nodes (MLN) were sometimes depleted of lymphoid cells and a low infiltration was present at the base of villi.

Detection of cell migration

Infiltration of the small bowel graft by recipient cells was observed in both models. On day + 3, recipient-derived immunocompetent cells had infiltrated the lymphoid compartments of the graft, for example lamina propria and MLN. Even within the intraepithelial compartment, cells were nearly completely of recipient origin by day + 100 (Fig. 1). Small numbers of donor cells from the graft were detectable in the recipient's spleen and MLN up to day + 60. There was no evidence of a persistent chimerism beyond day + 60. Further, in another transplantation model, where small bowels from male BN rats were grafted to female LEW recipients, there was no evidence of microchimerism beyond day + 80 as evaluated by PCR using rat Y-chromosome (Sry)-specific primers (not shown). This method can detect a level of microchimerism between 0.1 and 0.01% [5].

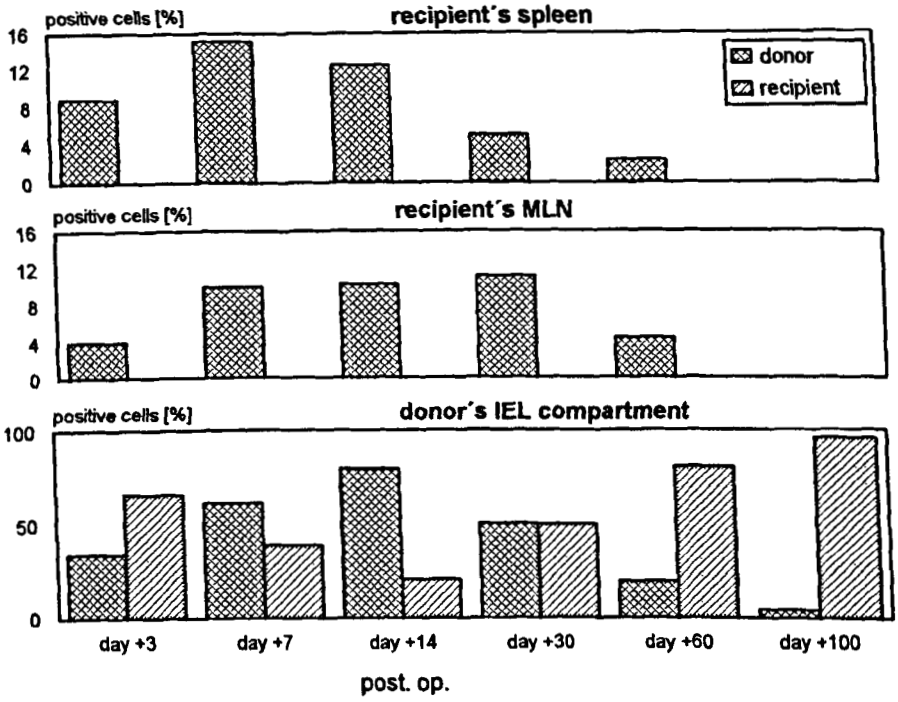

Fig. 1 Flow cytometric analysis of immunocompetent donor [Brown Norway (BN)] and recipient cells [Lewis (LEW)] with the monoclonal antibodies OX-27 (stained MHC class I on BN cells) and NDS-60 (stained MHC class I on LEW cells) on different postoperative days ($n = 5$ animals per time point). Donor cells from the graft were detected in spleen and mesenteric lymph nodes (*MLN*) of the recipients. Donor cells decreased over time, whereas recipient cells infiltrated the graft as shown for the intraepithelial compartment (*IEL*)

Table 2 Evidence of donor-specific tolerance in the long-term functional model (group 2) as indicated by heterotopic heart allografts. Donor-specific *(BN)* hearts survived indefinitely (> 70 days), whereas third-party Dark Agouti *(DA)* hearts were regularly rejected within 7 days. Syngeneically grafted controls (group 3) rejected both hearts within 8 days. Results from three different experiments

Group	Strain combination	FK 506 treatment	BN heart survival (d)	DA heart survival (days)
2	BN-to-LEW	Day 0–9	> 70	6.8 ± 0.9
3	LEW-to-LEW	None	7.8 ± 0.9	6.7 ± 0.5

Evidence for donor-specific tolerance in vivo

Animals of group 2 were assessed for donor-specific tolerance in vivo by intra-abdominal heterotopic cardiac allografts. Donor-specific (BN) cardiac grafts were permanently accepted, whereas third-party (DA) grafts were promptly rejected in a normal fashion within 7 days (mean survival 6.8 ± 0.9 days). All non-functional grafts showed myocyte necrosis and mononuclear cell infiltration which are signs compatible with acute rejection. Donor cardiac grafts, by contrast, exhibited normal histology without evidence of acute rejection (Table 2).

Donor-specific tolerance did not correspond with the absence of alloreactive T cells

To determine whether the transplantation tolerance induced under FK 506 treatment in vivo also produced unresponsiveness to donor antigens in vitro, MLRs were performed. Recipient CD4 + T cells isolated from spleen and peripheral lymph nodes were stimulated with donor (BN) and third-party (DA) antigens. This induced no significant reduction in proliferation against BN antigens (Table 3). The response was comparable to that against third-party antigens. In summary, normal alloreactivity against donor-specific antigens was demonstrated in vitro, in spite of perfectly accepted small bowel grafts without evidence of dysfunction.

Discussion

In the present paper we provide evidence of the induction and maintenance of donor-specific tolerance after an initial application of high-dose FK 506 that prolongs small bowel allograft survival for more than 250 days. A second antigen boost by donor-specific heart grafts did not abrogate this state of in vivo unresponsiveness. Recipient T cells failed to trigger graft rejection in vivo, but they did show a strong proliferative response against donor-specific (BN) antigens in vitro.

The ultimate goal of clinical transplantation is the induction of specific transplantation tolerance that results in graft acceptance and function without the need for

Table 3 In vitro detection (mixed leukocyte reaction) of alloreactive T cells in the long-term functional model (group 2) by [^{3}H] thymidine uptake. Purified CD4+ T cells from spleen and peripheral lymph nodes *(PLN)* were similarly stimulated with donor *(BN)* and third-party *(DA)* antigens. Further, the response was comparable to that of the controls. Results from three different experiments

Group	Strain combination	FK 506 treatment	Source of CD4+ T cells	Stimulator cells	
				BN	DA
2	BN-to-LEW	Day 0–9	Spleen	33587 ± 6045[a]	43969 ± 5031
			PLN	32078 ± 7035	30880 ± 5031
3	LEW-to-LEW	None	Spleen	36680 ± 6112	38230 ± 5098
			PLN	33510 ± 8051	40415 ± 4032

[a] T cell response in cpm

lifelong immunosuppression. The major problems following SBT that are attributable to the strong immunogenicity of the small bowel are: (1) the need for strong immunosuppression to prevent rejection reactions, (2) infectious and lymphoproliferative disorders resulting from generalized immune paralysis through strong immunosuppression, and (3) sepsis-related complications caused by the loss of barrier function when the immunosuppression is not strong enough. Thus, induction of immunological tolerance seems to be particularly important for the small bowel.

Induction and maintenance of transplantation tolerance appears to be based on a variety of mechanisms, including clonal deletion [6], clonal anergy [7], cell-mediated suppression [8], and infectious tolerance [9, 10]. The precise mechanisms of donor-specific tolerance in the long-term functional model are as yet poorly understood. We could show that T cells isolated from these long-term survivors remain functionally capable of responding directly to donor antigens in vitro. They recognize intact allo-MHC molecules on the surface of allogeneic stimulator cells [11]. Donor cells from the small bowel graft had migrated to the recipient's lymphatic compartments and were responsible for this direct alloactivation of recipient T cells in vivo. We could demonstrate that these so-called passenger leukocytes disappeared completely between days + 60 and + 100 in both SBT models. We therefore postulate that the indirect pathway where T cells recognize alloantigens after processing and presentation by antigen-presenting cells may be responsible for the destructive process in the rejection model. There is evidence that indirect allorecognition may be the dominant pathway in chronic rejection [12]. The hypothesis is that activation of CD4 + T cells by indirect allorecognition leads to cytokine secretion that provides the necessary signals for the growth and maturation of different effector cells of rejection, namely CD8 + T cells, natural killer cells, macrophages, and B cells [13].

Little is known about the immunological events that promote the switch from immune responsiveness to non-responsiveness in long-term survivors after SBT. The two transplantation models differed in the duration of FK 506 application. In contrast to animals in the rejection model, animals in the long-term functional model received an additional 4 days of immunosuppression. Whereas the animals of the rejection model had limited allograft function up to day + 98, animals in the long-term functional model survived long term (> 250 days). The direct and indirect T cell activation against donor-specific antigens appears to be completely inhibited in vivo, probably by environment-dependent factors, for example, soluble factors or cell–cell interactions, as demonstrated by the subsequent acceptance of donor-specific heart allografts in vivo. However, in vitro these factors were not present and allogeneic T cells proliferated in the presence of donor-specific antigens.

The indirect mode of allorecognition is also important for delivering tolerance. Oluwole et al. [14] described the induction of transplantation tolerance to rat cardiac allografts by intrathymic inoculation of allogeneic soluble peptides. We have evidence that small bowel recipients sensitized with allopeptides show prolonged allograft survival (unpublished data). However, the coherence between the indirect pathway of allorecognition and rejection, as well as tolerance induction after SBT, remains largely unclear and needs further clarification.

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