SMALL BOWEL TRANSPLANTATION
IMMUNOLOGICAL AND FUNCTIONAL STUDIES

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Bruin, Ronald Wilhelmus Frederik de

Small bowel transplantation: immunological and functional studies /
Ronald Wilhelmus Frederik de Bruin. - [S.l.: s.n.] - III.
Thesis Rotterdam. - With ref. - With summary in Dutch.
ISBN 90-9005489-8 geb.
Subject headings: small bowel transplantation.

Financial support by Sandoz B.V., Uden and B. Braun Medical B.V., Uden for publication of this thesis is gratefully acknowledged.

SMALL BOWEL TRANSPLANTATION

IMMUNOLOGICAL AND FUNCTIONAL STUDIES

DUNNE DARMTRANSPLANTATIE

IMMUNOLOGISCHE EN FUNCTIONELE STUDIES

PROEFSCHRIFT

Ter verkrijging van de graad van Doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. dr. C.J. Rijnvos
en volgens besluit van het College van Dekanen.

De openbare verdediging zal plaatsvinden op
woensdag 3 februari 1993 om 13.45 uur.

door

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geboren te Rotterdam

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PREFACE

Short bowel syndrome is a state of malabsorption and malnutrition after a major resection of the small intestine that may also include some or all of the large intestine. The underlying disorders requiring such a major resection in children include small bowel atresias, malrotation with midgut volvulus, gastroschisis, necrotizing enterocolitis, and extensive aganglionosis. In adults, vascular accidents, traumatic disorders, or diffuse intestinal diseases such as Crohn's disease and radiation enteritis may demand massive intestinal resection.

Total parenteral nutrition is a lifesaving therapy for these patients, indispensable in the early phase of short bowel syndrome. Gradually, varying from a few months to over one year, the remaining intestine adapts to the loss of the bowel and oral intake is progressed until intravenous alimentation can be stopped. Unfortunately, part of these patients never reach this final stage and remain totally dependent on total parenteral nutrition. This chronic, intravenous feeding has many disadvantages. Patients are limited in their lifestyle due to this time-consuming therapy, but moreover at the long-term a number of serious complications such as catheter sepsis and liver impairment may occur. In children these problems are accentuated because of more specific nutritional requirements associated with growth.

Small bowel transplantation would be the treatment of choice for these patients. However, many problems still hamper successful clinical small bowel transplantation. The vigorous and uncontrollable rejection reaction elicited against a small bowel allograft is probably the key event leading to a series of other complications, notably malfunctioning of the graft, sepsis, and peritonitis.

This thesis outlines the problems that surround small bowel transplantation, and presents experiments performed in rats on rejection and graft-versus-host disease, their suppression, and the nutritional function of small bowel grafts, with the ultimate goal to obtain a long-term surviving, properly functioning small bowel transplant.

CHAPTER 1

GENERAL INTRODUCTION

submitted for publication to Transplant. Int.
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Small bowel transplantation (SBT) would be the treatment of choice for patients suffering from the short bowel syndrome. Although in some centers SBT in patients is done with a considerable degree of success (Grant et al. 1990, Todo et al. 1992), it is by no means an established and widely applicable therapy for those with short bowel syndrome. The small bowel is unique among vascularized organ grafts because it not only elicits a vigorous rejection reaction, but is also capable of inducing graft-versus-host disease (GVHD). Rejection of the graft does not only lead to loss of function, but also to bacterial translocation. The risk of fatal sepsis is aggravated by the immunosuppression given to prevent rejection. This chapter describes the history of experimental and clinical SBT, and outlines recent developments and future prospects concerning this theoretically optimal treatment modality for patients who would be dependent on total parenteral nutrition (TPN) for life.

HISTORY

In 1959, Lillehei et al. described for the first time a technique for orthotopic SBT in the dog. Many experimental studies followed, and several patients received a small bowel transplant. Enthusiasm for SBT waned because rejection and sepsis appeared to be insolvable problems, and because TPN was introduced as satisfactory therapy for otherwise untreatable patients. With the introduction of cyclosporine A (CsA) as powerful immunosuppressive agent, a renewed interest in SBT emerged, and it is now on the verge of becoming an established procedure in transplant medicine.

EXPERIMENTAL MODELS OF SMALL BOWEL TRANSPLANTATION

A number of models are used to study SBT, each having its own advantages and drawbacks. Monchik & Russell (1971) first used parent and F1-hybrid rats in SBT. By using rats of 2 inbred parent strains (P) and their offspring (F1) they were able to dissect graft-versus-host disease and rejection. Transplantation from P to F1 produces only GVHD, whereas in the reverse combination only rejection occurs. The relevance of these one-way semi-allogeneic models for the clinical

situation, in which both GVHD and rejection may occur is uncertain (Saat et al. 1989), and hence fully-allogeneic combinations in which the graft is transplanted from P1 to P2 are used to study the possible interaction between GVHD and rejection. Syngeneic transplantation from P1 to P1 can be used to study the effects of ischaemia and lymphatic and neural disruption, while the immunologically induced traumas are circumvented (Table 1). In large animals, syngeneic transplantation finds its equal in autotransplantation, in which the arterial and venous blood supply are divided and reanastomosed, the lymphatics and nerves are disrupted, and the bowel is cut and reanastomosed (Lillehei et al. 1959, Raju et al. 1989, Meijssen et al. 1991[a]).

Table 1. Immunologic reactions after SBT.

Model	GVHD	rejection
P1 → P1	-	-
F1 → P	-	+
P → F1	+	-
P1 → P2	+	+

With respect to the position of the bowel 2 models are used. The bowel can be placed heterotopically; the recipient small bowel remains in situ, and the graft is placed as a Thiry-Vella fistula with both ends of the graft anastomosed as stomas in the abdominal wall of the recipient. The oral end of the graft may also be ligated or placed as a jejunostoma, whereas the distal end is anastomosed end-to-side to the terminal ileum of the recipient bowel.

In the orthotopic model, the recipient small bowel is resected and the graft is placed in continuity with the remaining duodenum and terminal ileum of the recipient. In this model recipient survival is dependent on functioning of the graft. Although orthotopic SBT (OSBT) may be the preferred model (Lee &

Schraut 1986[a], Grant et al. 1991[a]), the reported longer operative time and higher incidence of complications (Kort et al. 1973, Koltun et al. 1987) have resulted in a number of studies in which heterotopic SBT (HSBT) have been used (Watson et al. 1988). In skilled hands, however, operative time and technical success-rate for both techniques is not significantly different (Zhong et al. 1990). Results obtained after HSBT and OSBT are not comparable (Lee & Schraut 1986[a], Grant et al. 1991[a]). After HSBT, rejection of the graft is defined as the development of a palpable abdominal mass and necrosis of stomas (Lee & Schraut 1986[a], Grant et al. 1990). However, the graft may become encapsulated and fibrotic rather than necrotic and perforated, and the recipient may survive despite the graft loss, which makes the end point of rejection more difficult to define (Schraut et al. 1985, Grant et al. 1991[a]). Moreover, probably due to lack of intraluminal nutrients, atrophy of the mucosa occurs (Williamson & Chir 1978), and the permeability of these grafts 7 days postoperatively is significantly higher than after OSBT. Graft survival after HSBT can be prolonged more easily than after OSBT (Grant et al. 1991[a]). In conclusion, OSBT may be the preferred model for both immunologic and functional studies.

Venous drainage of the graft may be in the portal or in the systemic circulation. Although technically more demanding (Shaffer et al. 1988), portoportal drainage is the more physiologic route. Beneficial effects of this route on graft survival have been reported (Kort et al. 1973, Schraut et al. 1985), although they may be of minor importance (Lee & Schraut 1986[a]). Porto-caval shunting may cause metabolic complications, such as a rise in blood ammonia levels, and liver atrophy. The effects of portocaval shunting after SBT appear to be minor, and either type of venous drainage may be used safely (Koltun et al. 1987, Schraut et al. 1986, Shaffer et al. 1989, Kaneko et al. 1991).

Lillehei et al. (1959) were the first to describe a technique for functional bowel transplantation in the dog. After removal of the bowel it was auto-transplanted with good function thereafter. Different models of heterotopic and orthotopic placement of the bowel, with either systemic or portal venous drainage, are used as preclinical models for both immunologic and functional studies. For practical

reasons the pig is also used in SBT (Grant et al. 1988, Kaneko et al. 1991).

HISTOLOGY

The sequence of histologic changes after SBT is well-defined, although slight differences occur, depending on the model studied (Holmes et al. 1971, Monchik & Russel 1971, Madara & Kirkman 1985, Schmid et al. 1989).

In our BN to WAG fully-allogeneic orthotopic total SBT model, early intestinal lesions on days 4-6 posttransplant were characterized by mild infiltration of the lamina propria and submucosa by mononuclear cells and neutrophils and by mild multifocal death of crypt epithelial cells. The number of mononuclear cells increased to moderate over a few day period, while only few neutrophils were seen after 6 days. Crypt cell death was also observed on days 7 to 12 posttransplant, but it never became a prominent feature. Fibrin thrombi in mucosal capillaries were observed with increasing frequency during the course of graft rejection. This was associated with extensive necrosis of the mucosa at 11 and 12 days after grafting. Widespread thrombosis, resulting in ischaemia, is probably the principal cause of graft necrosis. Mononuclear cells accumulated around blood vessels in the mesentery. Eleven to 12 days posttransplant, early changes in the arteries were endothelial hypertrophy, and this was followed by mild intimal proliferation and thrombosis.

The mesenteric lymph node became rapidly depleted of lymphocytes, which were replaced by large mononuclear cells, presumably macrophages, and increasing numbers of fibroblasts.

IMMUNOSUPPRESSION WITH CYCLOSPORINE

Before CsA became available, other means of immunosuppression were used in attempts to prolong small bowel allograft survival. Taylor et al. (1966) used high doses of azathioprine in the artificial model of transplantation of a small segment of small bowel as a Thiry-Vella fistula in the neck of dogs. Only marginal prolongation of graft survival was found. Preston et al. (1966), using the same model, added prednisone to azathioprine and found prolongation of graft

survival from 9 to 27.5 days. Addition of anti-lymphocyte serum (ALS) to this regimen prolonged graft survival to a mean of 38 days (Hardy et al. 1970).

Interest in SBT has rekindled since the introduction of the potent immunosuppressant CsA (Kirkman, 1984). Reznick et al. (1982) first reported graft survival to be prolonged to a mean of 90.6 days after intramuscular (i.m.) administration of 25 mg/kg/day. Many dogs died from pneumonia, perhaps due to the malnourishment caused by chronic rejection of the grafts, or due to this high dose CsA. Discouraging results in both dogs and pigs have also been reported by others (Pritchard & Kirkman 1985).

In both unidirectional and in fully-allogeneic rat models CsA is able to significantly prolong small bowel allograft survival (Schraut, 1988). This is highly dependent on the rat-strain combination used. We have shown that in the BN to WAG rat donor-host combination long-term allograft survival is easy to achieve using short courses of CsA, whereas in the reverse WAG to BN model CsA has only a limited efficacy in prolonging graft survival (de Bruin et al. 1990[a]). In unidirectional P to F1-hybrid models CsA appears to be less effective in preventing GVHD (Kirkman, 1984).

In large animal models prolongation of graft survival is hard to achieve, but continuous intravenous (i.v.) infusion of CsA has been shown to result in long-term allograft survival in pigs, which survived for 122±33 days, without animals that died from rejection (Grant et al. 1988). These results were achieved with 15 mg/kg CsA given intravenously for 7 to 10 days, followed by 30 mg/kg/day orally, tapered to 15 mg/kg over 3 to 4 months. However, high CsA blood levels of approximately 600-700 ng/ml were measured which could lead to toxicity on the kidney (English et al. 1987) and bowel (Crane et al. 1990), and to an unacceptable high risk of developing malignancies (Penn, 1991). Recently, Meijssen et al. (1992[a]) reported that graft rejection in dogs can be prevented with CsA when donor and recipient are fully MHC-matched. CsA dosages in this group were 15 mg/kg i.m. from 1 day before surgery until the end of the first postoperative week, and 30 mg/kg/day orally until day 200 posttransplant thereafter. Recipients of an MHC-matched graft survived for a mean of 211 days

without signs of rejection during CsA therapy. MHC-mismatched dogs survived a mean of 113 days with 4 of 6 animals showing rejection that occurred during CsA treatment.

Pretransplant CsA treatment (pretreatment) of the recipient is associated with a reduced incidence of acute rejection in kidney transplantation (Hong et al. 1991). Moreover, Kahan et al. (1984) and Rogerson et al. (1986) found that low plasma CsA levels in the early postoperative period are associated with a higher incidence of rejection following human renal transplantation. However, in an experimental rat study we were unable to significantly prolong small bowel allograft survival after preloading the recipient with high dose CsA, as compared to postoperative immunosuppression only, although no acute rejection occurred (de Bruin et al. this thesis). It is generally thought that oral administration of CsA should be avoided (Kirkman, 1984), since the disrupted lymphatics of the graft are unable to transport this lipophilic drug to the blood. Although we have shown that absorption of orally administered CsA after total small bowel resection is severely impaired (de Bruin et al. 1992[a]), we found no lowered plasma through levels in the first week posttransplant. This finding is consistent with observations by Aeder et al. (1984) and LaRosa et al. (1989[a]), who found that oral and intraluminal CsA absorption within one week after transplantation did not differ significantly from preoperative or control values. This indicates that there must be an alternative mechanism by which CsA is delivered to the blood. By administering CsA intraperitoneally in normal dogs, Cohen et al. (1983) have shown that some absorption may take place via the peritoneum. It is possible that after transplantation CsA in the lymph leaks into the peritoneal cavity through the disrupted lymphatics, and is subsequently reabsorbed. So even before lymph vessel continuity is reestablished 4 to 10 weeks posttransplant, as is shown to happen in different models (Kocandrle et al. 1966, Rotman et al. 1986, Schmid et al. 1990), orally given CsA is absorbed. It was shown, however, that CsA absorption is highly variable and unpredictable during the early postoperative period, and, moreover, rejection can impair the ability of the graft to absorb CsA (Cohen et al. 1983). Therefore it seems justified to advocate the

parenteral administration as main route, especially in the first months after transplantation. Whether concomitant oral treatment can be of benefit due to local immunosuppressive effects needs to be investigated.

OTHER FORMS OF IMMUNOSUPPRESSION

FK-506 and Rapamycin (RAPA) are both macrolides produced by Streptomyces species, with potent immunosuppressive activity (Chang et al. 1991). FK-506, like CsA, counteracts mitogenic or antigenic stimulation at an early stage of T-cell activation, whereas RAPA intervenes in events more closely related to DNA synthesis. All 3 drugs exert their action via a class of binding proteins known as immunophilins, which possess cis-trans peptidyl prolyl isomerase activity. There is evidence that FK-506 and RAPA bind to the same binding site, whereas CsA binds to the similar, but nonidentical cyclophilin. Probably as a result of this, RAPA has been shown to antagonize the FK-506 induced inhibition of T-cell proliferation, and FK-506 has been shown to antagonize the action of RAPA, although potentation of either drugs may be achieved using equimolar concentrations of the 2 agents. Combinations of CsA and FK-506 or RAPA invariably result in a greater inhibition of mitogen and alloantigen-induced T-cell responses.

RAPA suppresses a wider spectrum of T- and B cell activation pathways than FK-506 or CsA. Interest in these 2 agents for use in SBT is a direct result of the potency of RAPA and FK-506 in prolonging graft survival (Thomson et al. 1990).

FK-506.

Hoffman et al. (1990) performed an extensive study on the use of FK-506 for SBT in rats (Table 2). They showed that long courses of FK-506 are more effective than CsA in the prevention of acute rejection and lethal GVHD in semi-allogeneic models. Short courses of 2 mg/kg on days 0 to 6 posttransplant prevented rejection in the fully-allogeneic ACI to Lew model. In the model used, a 20-fold higher dose of CsA was needed to obtain comparable survival times. No clinical GVHD was observed. Using the BN to Lew rat model, Lee et al.

(1990) found similar results, while Stangl et al. (1991) showed in the same model that FK-506 is able to reverse an ongoing chronic rejection process. We were unable to find superior immunosuppressive effects of short-course FK-506 over CsA using the fully-allogeneic WAG to BN rat-strain combination (de Bruin et al. 1991). Moreover, after FK-506 treatment severe GVHD was seen. This was also observed by Murase et al. (1991[a]) in the BN to Lew combination giving 0.15 mg/kg for 14 days. Hatazawa et al. (1992), on the other hand, observed prolonged survival administering 1 mg/kg/day for 8 weeks in the same model. Masutani et al. (1992) found in a P to F1-hybrid model, that 14 days after HSBT using 0.32 mg/kg/day FK-506 animals showed no histologic signs of GVHD, and Markus et al. (1991) showed that FK-506 is able to reverse ongoing GVHD after bone-marrow transplantation better than CsA and RAPA.

Rapamycin.

Continuous i.v. administration of 0.80 mg/kg of RAPA for 14 days was shown to significantly prolong allograft survival (Strepkowski et al. 1991). Using Lew and (LBN)F1-hybrid one-way models they showed that RAPA is able to suppress both isolated rejection and GVHD, although its effect on GVHD is less potent than on rejection (Kahan et al. 1991). Chen et al. (1992) however found RAPA to be equipotent in suppressing isolated rejection and GVHD in the same model. They also showed that RAPA is synergistically effective with CsA. Similar immunosuppressive efficacy as with CsA was achieved with a 5-fold lower dose of RAPA after fully-allogeneic rat-SBT (Strepkowski et al. 1991). No toxicity was reported from these studies.

Both FK-506 and RAPA may prove to be the more potent, less-toxic immunosuppressants needed for successful clinical SBT, but this has to be evaluated clinically.

IMMUNOMODULATION

It has been widely recognized that preconditioning of the recipient with donor-specific blood transfusions (DST) may lead to prolongation of allograft survival

in both experimental (Marquet et al. 1971) and clinical (Opelz et al. 1976) transplantation. In the BN to WAG rat donor-host combination, DST are very effective in prolonging heart and kidney graft survival (Marquet et al. 1971). However, 3 pretransplant DST had no effect on total or segmental SBT in this model. When DST was combined with low-dose CsA administation, no additional prolongation of graft survival could be measured (de Bruin et al. 1990[b]). Similar results were reported by Fecteau et al. (1992). In contrast, Martinelli et al. (1989) did find a synergistic effect of CsA and DST, and with FK-506, DST acts synergistically as well (Fukuzawa et al. 1991).

These findings indicate that DST may contribute to the prevention of rejection, but more studies are needed to determine whether DST can be a therapeutic option in living-related SBT.

GRAFT-VERSUS-HOST DISEASE AFTER SMALL BOWEL TRANSPLANTATION

Monchik & Russel (1971) first showed that transplantation of the small bowel may produce a lethal GVHD in parent to F1-hybrid rat models. This GVHD shows histologic similarities to that induced by bone-marrow transplantation (Müller-Hermelink & Deltz 1986), and is caused by T-lymphocytes originating from the transplanted gut and its mesenteric lymph nodes (Kirkman et al. 1984[b], Wallander et al. 1989). Clinically, it is characterized by dermatitis, alopecia, a hunched posture, and eventually death of the animal. Observations in these unidirectional GVHD or rejection models are of uncertain relevance to the clinical situation, in which a two-way reaction between rejection and GVHD may occur (Kirkman, 1984). In fully-allogeneic small bowel transplantation, rejection rather than GVHD seems to predominate (Monchik & Russel 1971, Lee & Schraut 1986[b], Frantz et al. 1988). In some fully-allogeneic models, 30 to 50% of the animals show clinically overt GVHD, distinguished from that in the one-way model by its non-lethal, short-lived transient nature. Little is known of the

Table 2: Use of FK-506 in rat small bowel transplantation.

Author	Model[1]	FK-506 dose mg/kg	Additional treatment/remarks	Survival[2] days	GVHD
Hoffmann et al. 1990	HSBT-pc	2,mg/kg, d 3-6		34.9±30.8	none
		2,mg/kg, d 3-6 + 1 mg/kg, d 8-30, qod		50.6±46.5	none
		"	one-way rejection	83.0±82.6	none
		"	one-way GVHD	188.0±72.1	12.5%
Lee et al. 1990	OSBT	2 mg/kg, d 0-4		>180	none
de Bruin et al. 1991	OSBT-pc	2 mg/kg, d 0,1,2,4,6		28.5±6.8se	severe
		2 mg/kg, d 0,1,2,4,6	5 Gy donorirr	31.1±5.7se	none
Fukuzawa et al. 1991	HSBT-pc	0.3 mg/kg, d 0-14	1.0 mg/kg, d -8–4	9.8±2.8	nr
		0.3 mg/kg, d 0-14	1.0 mg/kg, d -8–4 + DST, d-8[3]	62.2±33.6	nr
Murase et al. 1991[a]	OSBT-pp	0.15 mg/kg, d 0-13		44.5 median	mild, self limiting[4]
		0.15 mg/kg, d 0-13		"20-30 days"	severe
Murase et al. 1991[b]	OSBT-pc	0.64 mg/kg, d 0-13		121, median	none
Santiago et al. 1991	HSBT-pc	0.3 mg/kg, d 0-13		6.8±0.8	nr
		1.0 mg/kg, d 0-13		12.4±8.4	nr
		2.0 mg/kg, d 0-13		17.4±4.7	nr
		0.3 mg/kg, d 0-13	donor 2 mg/kg FK, d -3,-2,-1	41.2±3.8	nr
		none	donor 2 mg/kg FK, d -3,-2,-1	12.2±1.9	
Tadeka et al. 1991	HSBT	0.32 mg/kg, d 0-13	minor HC incomp.	80% > 175	nr
		0.32 mg/kg, d 0-13	major HC incomp.	38.0±6.3	nr
Hatazawa et al. 1992	OSBT	1.0 mg/kg/d, 8 weeks, s.c.	">8 weeks"	none	
Stangl et al. 1992	nr	10 mg/kg CsA, d 0-5		27.3±4.8	
		10 mg/kg CsA, d 0-5	FK-506, 2 mg/kg, d 13-15	>270	none
Utsunomiya et al. 1992	OSBT-pc	0.1 mg/kg, d 0-29		13.4± 3.07	nr
		0.3 mg/kg, d 0-29		34.6±12.79	nr
		0.5 mg/kg, d 0-29		32.6±26.16	nr
Yamataka et al. 1992	nr	1.0 mg/kg, d 3-5, i.p.		">20"	nr
		1.0 mg/kg, d 3-5, i.p., + α-ICAM-1, 1 mg/kg, d 1-3		">20"	nr

[1]: Fully allogeneic model, unless otherwise stated. OSBT: orthotopic, HSBT: heterotopic small bowel transplantation, pc: porto-caval venous drainage, pp: porto-portal venous drainage.
nr: not reported. FK was administered intramuscularly, unless otherwise stated.
[2]: Mean survival in days, unless indicated otherwise.
[3]: DST+ donor-specific blood transfusion.
[4]: Murase et al. 1991: mild, self limiting GVHD in BN to Lew combination, severe GVHD in Lew to BN combination.

interaction between rejection and GVHD in these models.

Cohen et al. (1976) investigated the effect of graft-irradiation with 0.5 and 1.5 Gy prior to transplantation in a canine small bowel allograft model. They found that pretreatment with 1.5 Gy leads to rejection of the small bowel allografts in 9.2 days. Pretreatment with 0.5 Gy, however, prolonged graft survival to a mean of 28 days. Therefore they hypothetized that there is a balance between rejection and GVHD, and that the development of subclinical GVHD after 0.5 Gy irradiation results in prolonged graft survival.

Since the early 1960s it has been known that GVHD depresses the host's immunological reactivity (Howard & Woodruff 1961). This is best shown by clinical results obtained with T-cell depleted bone-marrow transplantation. On the one hand, T-cell depletion significantly reduces acute GVHD, on the other hand it substantially increases graft rejection (Storb, 1989). GVHD is also known to be immunosuppressive after experimental spleen-cell-, and small bowel transplantation (Grant et al. 1989).

Histopathologically, GVHD is characterized by a loss of the normal architecture of the spleen, lymph nodes, and thymus (Deltz et al. 1981, Schraut et al. 1986, Grant et al. 1989). This leads to profound immunosuppression with impaired humoral and cell-mediated immune responses (Grant et al. 1989). This immunosuppression probably accounts for the observed in vivo balance between rejection and GVHD.

Diflo et al. (1989) observed a short, sublethal GVHD approximately 4 to 6 weeks after fully-allogeneic transplantation in immunosuppressed animals. Donor-pretreatment with ALS completely eliminated GVHD, but had no effect on graft survival in these immunosuppressed hosts. Gundlach et al. (1989) found that mesenteric lymphadenectomy, a method which has been shown to eliminate GVHD (Pirenne et al. 1990), does not influence the course of acute graft rejection in non-immunosuppressed recipients. CsA was not effective in preventing chronic rejection following mesenteric lymphadenectomy, whereas the same dosage of CsA fully prevented rejection of normal small bowel grafts. They suggested that the absence of an immunosuppressive effect caused by a GVH-

reaction had led to chronic rejection in this model.

We showed that irradiation of the donor with 10 Gy one day before fully-allogeneic SBT in the WAG to BN rat model completely eliminated GVHD, and significantly shortened survival times (Saat et al. 1989). Moreover, CsA treatment of the recipient was unable to completely override this effect (Saat et al. 1991). Pretreatment of the donor with ALS also eliminated clinical GVHD, and led to significantly accelerated rejection (de Bruin et al. 1992[b]). When our recipients of a graft pretreated with ALS received immunosuppressive treatment with CsA no adverse effect on graft survival was seen anymore, whereas clinical GVHD remained suppressed. This important finding is in accordance with earlier findings from our laboratory (Saat et al. 1991). The usefulness of manipulation of this balance for future clinical SBT remains to be established, since it is unclear yet whether this balance theory is a rat strain dependent phenomenon, and even whether GVHD will be a clinical problem.

Prevention of GVHD in SBT often implies reduced immunogenicity of the graft also. In attempts to control both rejection and GVHD, reduction of the immunogenicity, and consequently a diminishing of the number of leukocytes in the graft has been carried out. Moreover, in the recipient the immunosuppression used to prevent rejection also suppresses GVHD, although in semi-allogeneic rat models CsA appears to be less effective in preventing GVHD than it prevents rejection. FK-506 may even enhance GVHD (de Bruin et al. 1991, Murase et al. 1991[a]).

Several donor-pretreatment modalities have been shown to abrogate or diminish clinical GVHD following experimental SBT. Total body irradiation of the donor prior to transplantation completely eliminates GVHD (Monchik & Russel 1971, Lee & Schraut 1985, Deltz et al. 1986) and so does pretreatment of the donor with ALS (Shaffer et al. 1988), mesenteric lymphadenectomy (Deltz et al. 1986), and reduction of the length of the graft (Kimura et al. 1987).

High levels of the cytokine tumor necrosis factor-alpha (TNFα) are measured in rats with lethal GVHD after SBT in a P to F1 GVHD model (Pirenne & Dunn 1991). Blocking of TNFα activity reduces the mortality of GVHD after experi-

mental bone-marrow transplantation, and prevents skin and gut lesions of GVHD (Piguet et al. 1987). This suggests that anti-TNFα antibody therapy could be helpful in reducing the severity or lethality of GVHD. Other cytokines may also be involved in the pathogenesis of GVHD (Mowat, 1989), and are important in graft rejection as well. Anti-cytokine therapy might therefore prove to be valuable in concert with other immunosuppressive modalities.

GRAFT PHYSIOLOGY

The small bowel exhibits nutritional, motor, hormonal and immunologic functions. After transplantation, changes in these functions may be expected for several reasons: the ischaemia, lymphatic disruption, and denervation caused by the transplant procedure, the immunological processes arising after allogeneic transplantation, and the immunosuppressive drugs given. The small bowel transplant must be able to overcome the symptoms of short bowel syndrome. In the adult recipient, long-term prevention of malnutrition and in children normal growth and development followed by long-term prevention of malnutrition are prerequisites for successful clinical small bowel transplantation.

Nutritional function.

A sensitive functional test is whether a small bowel transplant recipient is able to grow and develop normally. Several studies have shown that rats are able to gain weight normally after allogeneic total SBT (Schraut et al. 1987, de Bruin et al. 1992[a]). However, rats grafted with a segmental small bowel transplant may develop impaired nutritional parameters (Schraut et al. 1987), or grow suboptimally (Oki et al. 1989, de Bruin et al. 1992[a]), although Kirsch et al. (1991) have shown that there were no significant differences in growth between sham operated rats, animals with 50% of their bowel (jejunum) intact, and animals transplanted orthotopically with 50% of their total bowel length of jejunum 150 days posttransplant. Moreover, just as the native gut after bowel resection, the transplanted intestine is also capable of adaptation (i.e. increased bowel diameter and increased villus height) (Kirsch et al. 1991). In dogs, conflicting data

have been reported. Long-term surviving allografted dogs maintain their preoperative body weight, but have only slightly better nutritional parameters than dogs with short gut syndrome (Diliz-Perez et al. 1984). Weight after transplantation of a 100-cm long segmental ileal allograft was maintained at 88% of their preoperative weight (Collin et al. 1987). Autotransplanted dogs attained their preoperative weight not until 1 year after transplantation (Raju et al. 1989). Moreover, Ballinger et al. (1962) reported that substantial nutritional disturbances can be expected from lymphatic and neural division alone. Recently it was shown that autotransplanted adult dogs regain their preoperative weight within 1 year after transplantation. One year posttransplant, these animals still had elevated stool moisture content, and developed steatorrhea and impaired D-xylose absorption (Thompson et al. 1992). However, MHC-matched, CsA treated recipients of a 45-65 cm (±25% of total length) long allograft of terminal ileum did not differ in growth or fecal fat content from sham-operated controls, indicating adequate nutritional function (Meijssen et al. manuscript in preparation). In young pigs, total small bowel allografts are able to adequately sustain the recipients' growth at a rate comparable to normal controls (Grant et al. 1988). Porcine segmental jejunal allografts comprizing approximately 25% of the small bowel length are incapable of doing the same; by 180 days after transplantation animals had increased their weight by only 40% (Kimura et al. 1990) as compared to more than 100% in 4 weeks in Grants' animals. From these studies it seems that, from a nutritional point of view, allotransplantation will be possible. Taking into account its limitations (living-related donation, abdominal size), as much bowel length as possible should be transplanted. More studies are needed to determine whether long-term functioning of SBT is feasible in both juvenile and adult recipients as opposed to long-term total parenteral nutrition, and how compromized functions may be restored.

Immune function.

The gut mucosa has an important barrier function to luminal food antigens and pathogens (reviewed by Brandtzaeg et al. 1989). It is constantly challenged by

microbial and food antigens, and its responses to these stimuli must be appropriate; infections must be limited but at the same time the integrity of the vulnerable mucosa must not be compromized. Intestinal immunity was first observed when protective "coproantibodies" were found in stools of orally immunized rabbits. However, the vulnerable gut mucosa should also be protected against potentially harmful reactions against harmless antigens. Indeed, suppressive mechanisms after immunisation with harmless antigens have been observed, and are called "oral tolerance" (Tomasi, 1983). How the suppressive and inductive immunoregulatory mechanisms are established in the intestine is still obscure. The secretory immunoglobulin (Ig) system is the major effector mechanism of mucosal immunity. Approximately 70-80% of all Ig is produced by B-lymphocytes in the mucosa of the small and large bowel. This secretory Ig, which is mainly IgA, is transported to the gut lumen through the epithelial cells of the crypts of Lieberkuhn.

Numerous T-lymphocytes are localized in the gut epithelium. In contrast to the lamina propria T-lymphocytes, these intra-epithelial lymphocytes (IEL) are in direct line with macromolecules in transit across the epithelium (Ernst et al. 1985). Their function is still obscure, but they are thought to play a role in cytotoxic as well as suppressive immune reactions. Apart from these solitary cells, the gut also contains organized solitary lymph nodes, and lymph node aggregates: Peyer's patches. These are probably the major site of antigen presentation and commitment to sIgA synthesis in the mucosal-associated-lymphoid-tissue. Sensitized and committed cells migrate via the mesenteric lymph node and the bloodstream to distant gut. IgA producing cells may also migrate to other mucosal surfaces that are part of the mucosal-associated-lymphoid-tissue, such as the lung, mammary glands, salivary- and lacrimal glands (Tomasi, 1983).

The immune function of bowel transplants is largely unknown. Both in man (Iwaki et al. 1991) and in experimental animals (Lear et al. 1989) it was found that graft-lymphocytes are replaced by cells of recipient origin without the occurrence of rejection. Xia & Kirkman (1990[a]) found no differences in graft

total sIgA production after syngeneic and semi-allogeneic SBT in rats. Immunosuppression with CsA had no effect on the total sIgA production. However, allografted animals treated with CsA failed to produce significant amounts of specific anti-cholera toxin sIgA when challenged with choleratoxin at the time of transplantation. The specific immune response against cholera toxin remained completely suppressed as long as the animals received CsA (Xia & Kirkman 1991). When allografted animals were boosted with choleratoxin 7 days post-transplant, after having been primed 1 week before transplantation, normal sIgA levels were measured (Xia & Kirkman 1990[b]). The presence of (part of) the recipient's colon could be important since it also contributes to gut mucosal immunology. The effect of different immunosuppressive agents on graft immune function needs to be investigated.

Motor function.

The motility of the normal, intact upper gut has been well characterized. The stomach and small intestine display distinct patterns of motility during fasting and feeding. During the fasting or interdigestive period, the upper gut shows a spontaneous and recurrent cyclic pattern of motility, called the migrating motor complex (MMC) or interdigestive myoelectric complex. Feeding interrupts this MMC and induces a less well-defined, none-cyclic pattern of intermittent, low amplitude contractions that persist for a variable period of time dependent on the type and amount of nutrient. These motor patterns are physiologically important. During fasting, the MMC sweeps non-digestable intraluminal debris from the stomach and small intestine ("intestinal housekeeper"). The change in motor pattern caused by feeding is believed to maximize mixing of food and facilitate its absorption.

Schiller et al. (1973) noted alterations in the motility of jejunal segments transplanted in the neck of recipient dogs. In Lew rats that had received a segmental syngeneic SBT, basal electrical rhythm of the graft was not observed for approximately 2 days after transplantation, and did not attain the level of normal rats for at least 3 weeks. MMC were not observed in the transplanted

segment until postoperative day 11, and was constant from postoperative day 16 (Vane et al. 1989). In a dog model of small bowel autotransplantation, in which all extrinsic neural and lymphatic connections to the jejunoileum were transected, the MMC was present, although the coordination between the innervated duodenum and the denervated jejunoileum, present in the normal gut, was lacking (Sarr et al. 1989). The presence of MMC is of importance since its intestinal housekeeper function is thought to be essential in preventing bacterial overgrowth, a situation also encountered after SBT (see below). Feeding or infusing the putative postprandial peptide hormones cholecystokinin or pentagastrin induced a normal "fed" pattern of contractions (Sarr et al. 1989). This implies that the intrinsic nerves of the gut are capable of generating these motor patterns without imput from the central nervous system.

Hormonal function.

A number of hormones are produced by cells scattered diffusely throughout the length of the gut. The physiological role of these peptides is still being evaluated. Intraluminal levels of vasoactive intestinal peptide, somatostatin, and substance P are stable from 4 days after syngeneic SBT to 1 year posttransplant. During rejection, however, lowered levels of these hormones were found (Teitelbaum et al. 1989). LaRosa et al. (1989[b]) reported that SBT does not alter the baseline levels, nor the response to stimuli of serotonin and substance P.

MONITORING OF GRAFT REJECTION

Unlike renal, hepatic, and pancreas grafts, intestinal grafts have as yet no specific distinguishing serum markers to diagnose rejection. Histology remains the gold standard for diagnosis of rejection (Meijssen et al. 1991[a]), although this method has several shortcomings. Full-thickness biopsies from the graft have to be taken from cutaneous stomas to reliably recognize rejection (Millard et al. 1986), which involves the risk of graft perforation. Multiple biopsies are necessary because rejection shows a patchy character and may be easily missed (Dennison et al. 1987, Schmid et al. 1989, Meijssen et al. 1991[b]). In view of these

limitations several small bowel function tests have been studied to determine their usefulness as marker for rejection. Function tests of the graft which need biopsy material from the graft, such as determination of brush border enzyme activity (Schroeder et al. 1990[b], Meijssen et al. 1992[b]) have the same disadvantage of dependence on the presence of an enterostoma. Moreover, they do not detect rejection at an earlier stage than does histology. Functional tests, functional absorption tests, and putative serum markers of rejection must at least be as sensitive and as specific as histology in order to be considered an alternative for histology.

In order to be absorbed, the disaccharide maltose must be split into glucose by the brush border enzyme maltase. This glucose is subsequently absorbed and raises the blood glucose level. In this way, the maltose absorption test can be performed as a glucose tolerance test. Billiar et al. (1984) studied this test in rats and found that a reduction in maltose absorption preceeded histologic changes by 1 to 2 days. They concluded that this test is a reliable, reproducible, and sensitive method to monitor rejection.

Intestinal absorption of water, sodium, glucose, alanine, and lauric acid have also been proposed as early serum markers. These substances require active transport by the mucosa, and significant decreases in their absorption were found when the first changes in histology were present (Dennison et al. 1987).

Intestinal permeability to ^{51}Cr-EDTA is increased during rejection. In rats, a 2-fold increase was measured at the time when minimal histologic signs of rejection were present (Grant et al. 1991[b]). Assessment of urinary ^{51}Cr-EDTA as measure for intestinal permeability has already proved its usefulness permitting early detection and treatment of an acute rejection episode after clinical small bowel-liver transplantation (Grant et al. 1990).

Monocyte-macrophage procoagulant activity is a measure of immune activation of mononuclear cells. Measured in peripheral blood mononuclear cells, elevated levels were observed before histologic changes of allograft rejection, and remained high throughout the course of rejection. This test appears to be an accurate serum marker for detection of rejection (Silverman et al. 1987, Kim et

al. 1990).

The lysosomal acid hydrolase N-acetyl hexosaminidase (NAH) is elevated in serum in association with intestinal ischemia. A study performed in rats suggested that determination of serum NAH is a simple and rapid test which could prove useful as serum marker for small bowel allograft rejection (Maeda et al. 1987). In contrast to this report, Meijssen et al. (1991[c]) found in a dog model that a significant rise in NAH occurred after histologic changes related to acute rejection were visible.

Transepithelial potential difference has been studied to determine its value as diagnostic tool for early detection of rejection. Madara & Kirkman (1985), using an in vitro method, found that a decreased spontaneous transepithelial potential difference, which is an index of baseline active transport resulting from electrogenic sodium absorption and chloride secretion, correlated with histologic signs of rejection. Sodium-coupled glucose absorption, which is an index of villus function, and theophylline-stimulated chloride secretion, which mainly measures crypt cell function, decreased when structural changes indicative of rejection became apparent. Lee et al. (1989) found that changes in basal transepithelial potential difference parallelled, and often preceded histological changes of rejection. Because of the invasive nature of their method, they did not consider it a practical clinical tool. In a model of canine small bowel autotransplantation Meijssen et al. (1991[a]) developed a non-invasive method using a double balloon catheter which was inserted in an enterostomy, and isolated a loop of bowel in which electrophysiological measurements could be performed. It was shown that in vivo electrophysiological parameters provide a useful tool in the assessment of small bowel autotransplants. Reduction of transepithelial potential difference preceded degenerative mucosal changes in the graft. It was subsequently found that, following allotransplantation, electrophysiological parameters correlate with histologic alterations of acute rejection, thus demonstrating that serial monitoring of transepithelial potential differences is a noninvasive method for detecting small bowel allograft rejection that circumvents the disadvantages of histologic monitoring (Meijssen et al. 1991[b]).

SMALL BOWEL TRANSPLANTATION IN MAN

Before CsA became available, attempts at clinical SBT invariably were unsuccessful. Failures were due to technical complications, rejection, GVHD, and sepsis (reviewed in Kirkman, 1984). These discouraging results, and the availability of TPN, led to a diminished interest in SBT.

Several successful SBT have been reported since CsA was introduced. Deltz et al. (1989), reported a case of successful SBT using a segment of 60 cm of jejunum and ileum harvested from the sister of the recipient. Although this graft was not rejected, the patient had severe diarrhea. The graft donor also developed chronic diarrhea. Nine attemps of SBT in 7 children have resulted in 1 successful case in which the recipient is alive for more than 3 years after grafting. In all other patients the grafts had to be removed because of necrosis or rejection was uncontrollable (Goulet et al. 1990). The combined European experience in SBT has been reported recently by Schroeder et al. (1990[a]); from March 1987 until July 1990, 15 SBT were performed in 12 patients. Four grafts are functioning, with patients being independent of TPN. Immunosuppression in these patients was with CsA and prednisone, usually supplemented with azathioprine and ALS. In one patient transient GVHD was encountered. Recently a successful case of multi-organ transplantation including liver, pancreas, stomach, and small bowel, was reported by Margreiter et al. (1992). Two minor rejection episodes of the bowel were encountered. Six months postoperatively all grafted organs functioned normally.

McAllister et al. (1992) performed isolated SBT as well as combined small bowel-liver transplantation and abdominal cluster transplantation; the isolated small bowel transplant had to be removed 15 days posttransplant because of uncontrollable rejection. Two of 3 patients with small bowel-liver transplantation are alive on CsA therapy, whereas the other died from a non-immunological cause. One of 2 patients given an abdominal cluster transplant 1 is well 7 months postoperatively whereas the other has died of a lymphoma. Todo et al. (1992) reported on 1 successful isolated SBT and 4 small bowel-liver transplantations using FK-506 as main immunosuppressant. The function of the transplanted

intestine alone or with accompanying liver has been satisfactory in all 5 patients. Although alimentation was slowly achieved, all patients eventually became completely independent of TPN, and maintained, or gained weight.

In all clinical cases, intestinal continuity was restored in stages. At the first operation, the proximal and distal ends, or the distal end of the graft only, were brought out as enterostomies. This allowed for macroscopic inspection and graft biopsy for monitoring graft rejection. Moreover, early alimentation of the graft or decompression in case of ileus are possible. In a second operation some weeks later intestinal continuity was restored, after which oral feedings could be instituted (Deltz et al. 1989, Grant et al. 1990, Todo et al. 1992).

Although in most cases reported the postoperative course was stormy, these successful cases suggest that SBT has become a clinical reality.

FUTURE PROSPECTS

The reasons why the small bowel graft is particularly vulnerable to rejection are now being delineated. The high rate of sepsis after SBT (Schroeder et al. 1990[a], Grant et al.1991[b]) has several causes: immunosuppression given to the recipient compromises the immunologic barrier function of the bowel wall. Ischemia and rejection increase the permeability of the graft by compromising the physical barrier. This has been shown to lead to bacterial translocation to the host (Grant et al. 1991[b], Browne et al. 1992). Leakage of toxins also occurs in this phase. This may be aggravated by the bacterial overgrowth in the graft encountered after SBT (Browne et al. 1992, Thompson et al. 1992).

Measures maintaining the gut barrier function after SBT will improve its outcome. Better immunosuppressive drugs like RAPA may have a higher therapeutic index than CsA, although some believe that CsA as mainstay of immunosuppressive therapy will provide satisfactory immunosuppression (Schroeder et al. 1990[a], Margreiter et al. 1992).

Several authors suggest an advantage of combined small bowel-liver transplantation, which is nourished by the successful clinical small bowel-liver transplantation (Grant et al. 1990). In rats, SBT performed 17 days following orthotopic liver

transplantation led to long-term survival of the small bowel graft without any immunosuppression, whereas isolated small bowel grafts were rejected in 6 to 9 days (Sarnacki et al. 1992). Similar results were reported by Zhong et al. (1991). However, since not all patients that are candidate for a SBT need a liver graft, it is important to elucidate the mechanism by which the liver "protects" the SB from rejection, and to assess whether the same results may be obtained with an auxilliary liver graft.

Early institution of feeding of the graft helps preventing disuse atrophy (Levine et al. 1974, Riecken et al. 1989), and antibiotics reduce the bacterial load of the gut. Options that bear an experimental character include administration of hormones trophic to the gut such as epidermal growth factor and prostaglandin E_2, or the use of nutrients that are essential to the gut. One such nutrient that, in our opinion, deserves special attention is the amino acid L-glutamine. Several studies have demonstrated that glutamine is the principal fuel for enterocytes (Windmueller, 1982, Souba et al. 1990[a]). There is only limited evidence that glutamine is essential for maintenance of normal intestinal function (Souba et al. 1990[a]). However, the stress of a major operative procedure combined with general anaesthesia is followed by a fall in circulating and muscle glutamine concentrations (Souba & Wilmore 1983). During injury or stress glutamine may be a necessary dietary component to maintain gut structure and function. This is possibly due to the fact that glutamine is essential for nucleic acid biosynthesis and might be especially important during critical illness when the mucosal barrier becomes susceptible to breakdown (Souba et al. 1990[a]). It has been shown, for instance, that glutamine consumption by the intestinal tract is increased by 75% after laparotomy (Souba et al. 1990[a]). It has also been shown that glutamine reduces bacterial translocation from the gut to the mesenteric lymph nodes following abdominal irradiation (Souba et al. 1990[b]). Moreover, glutamine supplementation of standard TPN solutions decreases the villous atrophy associated with long-term i.v. feeding (O'Dwyer et al. 1989), and bacterial translocation following TPN administration is attenuated when glutamine is added to the mixture. This diminished translocation was associated with a normalization of

sIgA levels and a decrease in bacterial adherence to enterocytes, suggesting that glutamine added to the TPN solution enhances gut immune function (Burke et al. 1989, Alverdy et al. 1992). Preliminary results also indicate that glutamine is able to prevent the mucosal atrophy seen after heterotopic SBT (Schroeder et al. 1992). Taken together, these findings indicate that glutamine could have important applications after SBT, worthy of investigating.

Early detection of rejection is of vital importance in preserving barrier function, and may benefit long-term graft function since it could delay and or reduce fibrosis and chronic rejection. Several serum of urinary markers are now availabe which, in conjunction with histology, allow early recognition and treatment of rejection, and new makers are still being sought for. For example, the enzyme diamino-oxidase is a potentially interesting marker for both rejection and long-term graft function (Rose et al. 1991). This enzyme, which is involved in the regulation of polyamine metabolism, and probably regulation of mucosal growth, is produced mainly by mature enterocytes in the villus tip (Rokkas et al. 1990[a]). It may have elevated levels in serum during acute ischemic injury of the small bowel mucosa (Wollin et al. 1981) and hence may be useful in detecting acute rejection. Normally, after i.v. heparin administration a rapid increase of plasma diamino-oxidase is seen (Kobayashi et al. 1969, Luk et al. 1983). These postheparin plasma diamino-oxidase levels are lowered in small bowel mucosal damage (D'Agostino et al. 1991) and could prove to be a marker for graft function, which deserves further study.

Denervation, an inevitable consequence of SBT, causes hypersecretion from the crypts and diarrhea in the early stages after transplantation (Watson et al. 1988, Meijssen et al. 1991[a]). It is thought that long-term impaired motility as a result of denervation, although not hindering passage of food, is associated with bacterial overgrowth. Increased chloride secretion (Meijssen 1991[a], Watson et al. 1988), steatorrhea (Schraut et al. 1988, Raju et al.1989, Thompson et al. 1992), and increased fecal water content (Thompson et al. 1992) are all thought to be long-term consequences of extrinsic denervation of the graft.

Because these obstacles have now been traced, and are taken away or resolved,

and because the first successful clinical cases are being reported, there is reason to be optimistic about the future of clinical SBT.

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CHAPTER 2

MATERIALS AND METHODS

Chapter 2

Rats

Male rats of the inbred Wistar-Agouti (WAG) and Brown Norway (BN) strains were used. The animals were obtained from Harlan-CPB (Austerlitz, The Netherlands), where they were bred under specific pathogen free conditions. The animals were 10 to 14 weeks old when they were used, and weighed between 200-250 g. They were kept under standard laboratory conditions (12 hours light, 12 hours dark) with water and food (Hope farms diet for rat and mouse #1410, Hope farms, Woerden, The Netherlands) ad libitum. The WAG strain is homozygous for the Rt-1^u haplotype (rat major histocompatibility complex), whereas the BN rat is homozygous for the Rt-1^n haplotype. Consequently, organ transplantation between these strains represents an allogeneic situation.

The experimental protocols adhered to the rules laid down in "The Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the E.C. (1986). Specific protocols were approved by the Committee on Animal Research of the Erasmus University, Rotterdam, The Netherlands.

Small bowel transplantation

One-stage total orthotopic small bowel transplantation (SBT) was performed according to a modification of the methods of heterotopic transplantation described by Monchik & Russel (1971) and orthotopic transplantation described by Kort et al. (1973). In brief, the method consists of end-to-side anastomosis of the superior mesenteric artery and portal vein of the graft to the abdominal aorta and caval vein of the recipient, respectively. After resection of the host's small bowel the graft is placed in an orthotopic position by end to end anastomosis of the graft proximally with the host's duodenum and distally with the remaining 1-2 cm of terminal ileum.

In detail, the method is as follows.

Donor

All procedures were performed under clean but nonsterile conditions.

Donors were fasted 24 h before surgery but could drink ad libitum. The donor was anesthetized with ether (diethyether p.a., Merck, Darmstadt, Germany), its abdomen shaved, and taped onto a cork board. The abdomen was opened via a midline incision from pubis to xyphoid. The intestines were visualized by retracting the wound with two T shaped aluminum spatulas. First, the colon was dissected to free the small bowel. This was done by ligating the right and middle colic arteries and veins using 4/0 silk (NC-Silk, B Braun, Melsungen AG, Germany) and dissection of the connective tissue between the small bowel and the colon and colon and pancreas. The superior mesenteric artery was skeletonized, thereby disrupting the intestinal lymphatics and coeliac ganglion. Subsequently the portal vein was visualized and the pyloric- and splenic vein were ligated using 4/0 silk. The donor was systemically heparinized with 200 international units of heparin (Thromboliquine, Organon Technika BV, Boxtel, The Netherlands) per kg body weight given intravenously via the penile vein. Finally, the graft was harvested by cutting the small bowel 1 cm distally from the ligament of Treitz and 1-2 cm proximally from the caecum. The vascular pedicle consisted of the portal vein and superior mesenteric artery including an aortic cuff. The graft was flushed via the superior mesenteric artery with 2 ml of chilled Hanks balanced salt solution (HBSS) and stored in cold HBSS while the recipient was prepared.

Recipient

The abdomen was opened via a midline incision from pubis to xyphoid and the wound retracted with spatulas. The intestines were wrapped in moistened gauze and exteriorized to the left. The abdominal aorta and inferior caval vein were isolated and clamped together with a curved Mosquito hemostat and longitudinal openings of approximately 2 mm were cut distally in the aorta and proximally in the caval vein. End-to-side anastomoses were performed between the superior mesenteric artery and portal vein of the graft and aorta and caval vein of the recipient, respectively, with a continuous suture with 8/0 Mirafil (B Braun, Melsungen AG, Germany). The total ischemic time was approximately 30

minutes. After ligation of its bloodvessels the total SB of the host was resected. The graft was anastomosed end-to-end, proximally with the host's duodenum and distally with the remnant 1-2 cm ileum using a continuous suture with 7/0 silk (Ethicon, Norderstedt, Germany) (Fig. 1). The abdominal wall was closed in one layer with 2/0 silk (NC Silk, B Braun). At the end of the operation the animals received subcutaneously a single dose of 20.000 IU penicillin and 20 mg streptomycin (Depomycine, Mycofarm, de Bilt, The Netherlands).

Segmental small bowel transplantation

Jejunal transplants consisted of 10 cm of proximal jejunum. Ileal grafts were prepared by isolating 10 cm of distal ileum from the small bowel. Like total small bowel grafts, these segments were vascularized by the superior mesenteric artery and portal vein.

Postoperative monitoring

After transplantation, rats received standard rat chow and water ad libitum. The animals were examined for signs of graft-versus-host disease and were weighed three times a week. Weight gain or loss was considered the most important indicator for graft function. All rats that died within 4 days were considered to be technical failures. After death, autopsy was performed to confirm or exclude rejection, using the criteria published by Schraut & Lee (1986). Briefly, acute graft rejection was indicated by signs of peritonitis, a thin-walled distended graft and unimpaired intestinal anastomoses. Usually, the mesenteric lymph nodes of the graft were enlarged.

Histology

For histopathologic analysis tissues were fixed in 3.6% buffered formalin. After dehydrating and embedding, 3-4 μm sections were cut and stained with hematoxilin-floxine saffron.

Fig.1. Model of orthotopic small bowel transplantation. The superior mesenteric artery (SMA) and portal vein (PV) of the graft are anastomosed end-to-side to the recipient aorta (A) and caval vein (CV), respectively.

Chapter 2

GVHD

The occurrence of GVHD after SBT was first described by Monchik & Russel (1971). Clinically it is characterized by redness of ears, snout and paws, followed by scaling, hairloss and diarrhea.

The severity of GVHD was estimated by clinical grading as follows.

Grade 1: Light redness of ears, snout and paws.

Grade 2: Moderate redness of ears, snout and paws; light hair loss and diarrhea.

Grade 3: Severe redness of ears, snout and paws; alopecia, generalized dermatitis and profuse diarrhea.

Cyclosporine A

Commercially available cyclosporine (Sandimmun, Sandoz, Basel, Switzerland) was obtained and dissolved in olive oil to a concentration of 50 mg/ml before intramuscular administration.

Anti-lymphocyte serum

Rabbit-anti-rat anti lymphocyte serum was produced by subcutaneous injection of 10^8 rat thymocytes suspended in complete Freund's adjuvant. Subcutaneous booster injections with 10^8 thymocytes were given after 14, and 28 days. Blood was collected one week after the last immunization, the serum was prepared and decomplemented at 56 °C for 1 hour. The serum was shown to be effective in a rat heart allotransplantation model in which it prolonged graft survival from 8.5±0.5 days to 27±1.1 (MST±SD) days when it was given to the recipient subcutaneously on days 0,1, and 2 after transplantation in a volume of 4 ml/kg.

Irradiation

Whole-body irradiation was performed with a Gammacell-40 ^{137}Caesium irradiation unit (Atomic Energy of Canada, Ltd), 1-4 hr before transplantation.

Other materials and methods are described in the chapters they are relevant to.

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CHAPTER 3

TOTAL ORTHOTOPIC ALLOGENEIC SMALL BOWEL TRANSPLANTATION IN RATS: THE EFFECT OF ALLOGRAFT IRRADIATION COMBINED WITH CYCLOSPORINE-A THERAPY

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Gut 1991; 32: 654-656.

SUMMARY

Rejection and graft-versus-host disease (GVHD) are prominent features in small bowel allotransplantation in rats. Cyclosporine A (CsA) treatment of the recipient and irradiation of the donor were employed to circumvent these phenomena in the WAG to BN rat model. Irradiation of the donor with 5 or 10 Gy did prevent GVHD but resulted in a more vigorous rejection of small bowel allografts in untreated recipients (mean survival times (MST±SE) of 11.5±0.4 (n=8) and 7.5±0.9 (n=11) days respectively, versus 16.6±2.6 days (n=17), ($p < 0.01$). CsA treatment of the recipient (25 mg/kg on days 0,1,2,4 and 6 after transplantation) led to a MST of 38.3±8.5 days (n=10); 20% of the animals developed GVHD. Combined with 5 Gy donor pretreatment, a similar survival was obtained without occurrence of GVHD. However, CsA treatment combined with 10 Gy led to a significant shortening of graft survival (MST 23.1±6.8 days, (n=9)). These results suggest that, although irradiation is very effective in preventing GVHD, high dosages may accelerate rejection by either making the graft more vulnerable to rejection or by completely removing the immunosuppressive effect of GVHD.

INTRODUCTION

Various experimental studies on the rejection of small bowel allografts have shown that under certain immunological conditions, immunocompetent cells in these grafts can cause graft-versus-host disease (GVHD) (Monchik & Russell 1971, Deltz et al. 1981, Schraut et al. 1986, Schraut, 1988). While the occurrence of the GVH-reaction in well-defined inbred systems has been conclusively documented, its significance in outbred systems is uncertain. In a previous study we successfully ameliorated GVHD in the WAG to BN rat model using whole-body irradiation of the donor with 10 Gray (Gy) (Saat et al. 1989). The effectiveness of donor irradiation has been shown previously in a parent to F1-hybrid rat model in which the donors were irradiated with 7, 9.5 and 10 Gy before small bowel transplantation (SBT) (Monchik & Russell 1971, Lee et al. 1985, Deltz et al. 1986). In the present study we investigated the effects of combined cyclosporine A (CsA) therapy and donor irradiation because we had evidence that irradiation of the donor leads to an accelerated rejection of the graft (Saat et al. 1989). We wondered whether CsA therapy could reverse this effect. Earlier we found that CsA, used alone, was not effective in consistently producing long-term survivors in the WAG to BN donor-host combination (Saat et al. 1990). In this study we show that the effect of CsA on GVHD is less pronounced than its effect on rejection, as shown earlier by Kirkman (1984). In large animal models CsA proved to be less successful than in certain rat strain combinations (Craddock et al. 1983, Diliz-Perez et al. 1984, Harmel et al. 1986, Lee et al. 1986, Hatcher et al. 1987, Kirkman et al. 1987, Schraut et al. 1987, Shimazu et al. 1988). Two recent studies in pigs, using ex-vivo irradiation of the transplant with 0.5 Gy, a very low dose, combined with CsA therapy of the recipient showed no effect on host survival time compared to CsA monotherapy (Pritchard & Kirkman 1985, Grant et al. 1988). Because the immunological problems of large animal models resemble the problems in our WAG to BN rat model, this model is highly suitable for investigating the effect of irradiation combined with CsA therapy.

MATERIAL AND METHODS

Animals: a fully allogeneic donor-host model was employed by using inbred WAG ($RT1^u$) rats as donors and inbred Brown Norway ($RT1^n$) rats as recipients as described in chapter 2.

Operative procedures: were performed as described in chapter 2. Briefly, the small bowel was double-tied and cut 1 cm distally to the ligament of Treitz and 1-2 cm proximally to the caecum. The graft was harvested along with the attached vascular pedicles, consisting of the portal vein and the superior mesenteric artery, including an aortic cuff. End to side anastomoses were performed between the graft superior mesenteric artery and the aorta, and the graft portal vein and inferior caval vein, respectively. The host's small bowel was resected and the donor small bowel was anastomosed end to end, proximally with the duodenum, distally with the remnant of the ileum at 1-2 cm from the caecum.

Postoperative care: as described in chapter 2.

Immunosuppression: CsA was administered as described in chapter 2..

Irradiation: was performed as described in chapter 2.

GVHD: the severity of GVHD was estimated by clinical grading as described in chapter 2; Grade 1: light redness of ears, snout and paws. Grade 2: moderate redness of ears, snout and paws; light hair loss and diarrhea. Grade 3: severe redness of ears, snout and paws; alopecia, generalized dermatitis and profuse diarrhea.

Statistics: the survival data were statistically analized using the Wilcoxon rank-sum test for group comparison.

EXPERIMENTAL GROUPS

Six groups were distinguished (see Table 1);

Group 1: Controls, WAG to BN, no immunosuppressive therapy, n=17.

Group 2: Irradiation of the donor with 5 Gy, n=8.

Group 3: Irradiation of the donor with 10 Gy, n=11. The results for this group have been published before (Saat et al. 1989).

Group 4: Administration of 25 mg/kg of CsA on days 0,1,2,4 and 6 after transplantation, n=10.

Group 5: Irradiation of the donor with 5 Gy in combination with 25 mg/kg of CsA given on days 0,1,2,4 and 6 after transplantation to the recipient, n=9.

Group 6: Irradiation of the donor with 10 Gy in combination with 25 mg/kg of CsA given on days 0,1,2,4 and 6 to the recipient, n=9.

RESULTS

Table 2 shows that untreated control rats died after a mean survival time ± standard error of the mean (MST±SE) of 16.6±2.6 days. Six of 17 rats showed grade 1-2 symptoms of GVHD for 3-4 days. In groups 2 and 3, whole-body irradiation of the donor with 5 and 10 Gy respectively resulted in a complete abscense of GVHD. Surprisingly, the survival times in both groups were significantly shorter than those in the control group (11.5±0.4 and 7.5±0.9 days resp. vs 16.6±2.6 days, $P<0.01$). The accelerated graft rejection was more vigorous in group 3 than in group 2 ($P<0.02$). Rats in group 4 that received 25

Table 1. Schedule of graft pretreatment and immunosuppression.

group	n	pretreatment[1]	immunosuppression[2]
1	17	-	-
2	8	5 Gy, in vivo	-
3	11	10 Gy, in vivo	-
4	10	-	CsA
5	9	5 Gy, in vivo	CsA
6	9	10 Gy, in vivo	CsA

1: Whole body irradiation was performed 1-4 hr before transplantation using a Caesium-137 gamma-source.
2: Cyclosporine A was given intramuscularly in doses of 25 mg/kg on days 0,1,2,4 and 6 after transplantation.

mg/kg CsA, survived with a mean of 38.3±8.5 days; 2 of 10 rats developed clinical symptoms of GVHD. Pretreatment with 5 Gy in combination with CsA therapy (group 5), produced no significant difference in survival time when compared with group 4 (47.2±6.8 vs 38.3±8.5 days). However, none of the rats in group 5 showed signs of GVHD or peritonitis. One rat died on day 58 as a result of a late technical complication: stenosis of the proximal intestinal anastomosis. In group 6, irradiation with 10 Gy and CsA therapy resulted in a MST that was significantly shorter than that found in group 4 (23.1±6.8 vs 38.3±8.5 days, $p < 0.01$). At autopsy, 4 of the recipients that died in the first week showed symptoms of peritonitis as a result of a vulnerable, glassy transplant. No GVHD was observed in this group. Most rats in groups 4, 5 and 6 that survived more than 3-5 weeks increased in weight. In comparison with the weight gain observed earlier in a syngeneic (WAG-WAG) study, in which the

Table 2. Effect of donor pretreatment and immunosuppression on the survival of orthotopic small bowel transplantation in rats.

group	survival in days	mean ± SE
1	6,6,6,7,8,8,13[a],14,14,15,15,19[a],21[a], 24,27[a],31[a],47[a].	16.6 ± 2.6
2	10,11,11,11,11,12,12,14	11.5 ± 0.4
3	5,5,6,6,6,6,6,7,10,12,13	7.5 ± 0.9
4	8,11,13,13,21,55,59[b],65,66[a],72	38.3 ± 8.5
5	11,17,31,34,49,55,57,58,72	42.7 ± 6.8
6	5,6,7,7,7,35,39,48,54	23.1 ± 6.8

a: grade 1-2 severity of graft-versus host disease
b: grade 3 severity of graft-versus host disease
group 2 and 3 significantly worse compared with group 1, $p < 0.01$
group 2 significantly better compared with group 3, $p < 0.02$
group 5 not significantly different from group 4
group 6 significantly worse compared with group 4, $p < 0.01$

donor was irradiated with 10 Gy, however, the weight gain was minimal (Saat et al. 1989).

DISCUSSION

This study clearly shows that irradiation of the donor very effectively prevents the occurrence of clinical GVHD. In addition, when GVHD is ameliorated or absent in a fully allogeneic rat model, the acute rejection in untreated recipients is accelerated. This vigorous rejection can be explained by the assumption that irradiation with 10 Gy releases more immunocompetent cells in the peripheral circulation of the recipient than irradiation with 5 Gy. Although we cannot exclude this effect, it is our opinion that the vigorous rejection can only be controlled by CsA therapy when GVHD is not completely abrogated. The mechanisms that are probably responsible for these results have been described in experiments concerning GVHD in bone marrow transplantation (Seddik et al. 1984, Stet, 1987). These studies showed that a GVH-reaction can induce a state of permanent suppression of cell-mediated and of humoral immune responses.
This functional abnormality of T cells or B cells, or both, is probably related to a maturation defect (Seddik et al. 1984). Therefore the absence of GVHD, with its inherent immunosuppressive effect, is the most plausible explanation for the vigorous rejection that occurred in irradiated donors. From studies in a semiallogeneic parent to F1-hybrid rat model it is known that irradiation with 5 Gy, in contrast to 10 Gy, failed to prevent the onset of GVHD (Lee et al. 1985). Extrapolating these data to our results, we presume that the immunosuppressive effect of subclinical GVHD was responsible for the benificial effect on the survival time when donors were irradiated with 5 Gy compared with 10 Gy. Moreover, the abscence of clinical GVHD may be of clinical importance.
Until now it has been uncertain to what degree, if any, GVHD will complicate human SBT. Under the assumption that GVHD will develop after SBT, strategies have to be developed to circumvent this complication. In theory, drug treatment or irradiation of the transplant before surgery would be the ideal method to inactivate donor lymphocytes. Practically, irradiation is the easiest

procedure and can be performed ex vivo without losing its effect (Lee et al. 1985).

Obviously, the major problem in SBT remains the vigorous rejection process and its suppression. CsA will be the most important drug in any standard immunosuppressive regimen. This study gives indirect evidence that irradiation of the donor in combination with CsA therapy is useful only when some immunocompetent cells in the graft, capable of producing a subclinical GVHD whose immunosuppressive effect is beneficial to the survival of the allografts are left. It is necessary to quantify further the donor irradiation dose that will result in the optimal interaction between the occurrence of GVHD and rejection of the graft.

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CHAPTER 4

EFFECTS OF DONOR-PRETREATMENT WITH ANTILYMPHOCYTE SERUM AND CYCLOSPORINE ON REJECTION AND GRAFT-VERSUS-HOST DISEASE AFTER SMALL BOWEL TRANSPLANTATION IN IMMUNOSUPPRESSED AND NONIMMUNOSUPPRESSED RATS

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Transplant. Int. 1992, in press.

SUMMARY

After fully-allogeneic small bowel transplantation (SBT) both graft-versus-host disease (GVHD) and rejection may occur. Donor-pretreatment may prevent GVHD, but this sometimes leads to accelerated graft rejection. To study a possible balance between GVHD and rejection, fully-allogeneic total orthotopic SBT was performed in rats using the WAG to BN donor-host combination. Untreated control grafts were rejected in 16.6±2.7 days (mean±SE), and 35% of the animals had mild, transient GVHD. Pretreatment of the donor with anti-lymphocyte serum (ALS) on days -2 and -1 before grafting, either intravenously or intraperitoneally, completely eliminated the occurrence of clinical GVHD, but led to significantly shortened survival times (12.3±0.8 and 10.3±0.9 days, respectively). Donor pretreatment with 50 mg/kg cyclosporine A (CsA) on days -2, and -1 prolonged graft survival significantly to 22.1 days, but had no significant effect on the incidence of GVHD. Administration of 25 mg/kg CsA on days 0,1,2,4, and 6 after grafting prolonged survival to 38.3 days with no evidence of GVHD. Pretreatment of the donor with ALS combined with the same postoperative short term CsA regimen increased survival to more than 50 days, again with no evidence of GVHD. When CsA was used as both donor pretreatment and postoperative therapy there was no survival advantage compared to the use of postoperative CsA alone. These results show that an in vivo balance between GVHD and rejection exists, and that abrogation of GVHD leads to accelerated rejection. Immunosuppression of the recipient may overrule this accelerated rejection, while preserving the beneficial effect of donor-pretreatment: elimination of clinical GVHD.

INTRODUCTION

As was first shown in the parent to F1 hybrid rat model, transplantation of the small bowel may produce a lethal graft-versus-host-disease (GVHD) (Monchik & Russell 1971). This GVHD shows histological similarities to that induced by bone-marrow transplantation (Müller-Hermelink & Deltz 1986), and is caused by T-lymphocytes originating from the transplanted gut and its mesenteric lymph nodes (Kirkman et al. 1984, Wallander et al. 1989).

In fully allogeneic models of small bowel transplantation (SBT), in which both GVHD and host-versus-graft (rejection) reactions may occur, rejection predominates. However, 30%-50% of the animals transplanted with a total small bowel without immunosuppression show clinically overt GVHD (Saat et al. 1989). This GVHD is characterized by a redness of ears, snout, and paws, and, in more severe cases, also includes dermatitis, alopecia, and a hunched posture of the animals. It is distinguished from GVHD in the one-way model by its nonlethal, short-lived, transient nature. Immunomodulation of the recipient and/or the donor can dramatically alter this picture. From studies in one-way models it is known that the immunosuppressive agent cyclosporine A (CsA) has greater impact on rejection than on GVHD (Kirkman et al. 1984). When CsA is given at a critical dose following fully-allogeneic transplantation, GVHD can be more severe than in untreated animals, while rejection is delayed (de Bruin et al. 1990).

In both the one-way and the fully-allogeneic models of SBT GVHD can be eliminated by reducing the mass of lymphoid tissue in the graft (Lee & Schraut 1985, Deltz et al. 1986). Surprisingly, eliminating GVHD sometimes leads to accelerated graft rejection in fully-allogeneic models (Saat et al. 1991). This finding suggests that there is an immunological balance between rejection and GVHD (Cohen et al. 1976), and that GVHD in some way prevents the development of rejection by attacking the host immune system.

Previous data obtained in our laboratory substantiate this hypothesis. We have shown that irradiation of the donor with 5 or 10 Gray prevents the occurrence of clinical GVHD and leads to accelerated graft rejection. This study also gave

indirect evidence that subclinical GVHD may benefit graft survival (Saat et al. 1991). However, most studies on GVHD following SBT have used one-way models, and further study of the fully-allogeneic model is required to substantiate this hypothesis (Watson & Lear 1989).

The aim of the present study was to determine whether donor-pretreatment with anti-lymphocyte serum (ALS) or CsA could also suppress GVHD, and what effect this pretreatment might have on graft survival following total orthotopic fully-allogeneic SBT.

MATERIAL AND METHODS

Animals: rats of the inbred WAG ($Rt1^u$) and BN ($Rt1^n$) strains were used as donors and recipients, respectively, as described in chapter 2.

Small bowel transplantation: was performed as described in chapter 2. In brief, the total small bowel was harvested from the ligament of Treitz to the terminal ileum, along with a vascular pedicle consisting of the superior mesenteric artery and portal vein. In the recipient the infra-renal aorta and caval vein were clamped and end-to-side anastomoses were performed between the recipient aorta and caval vein and the donor superior mesenteric artery and portal vein, respectively. The recipient small bowel was resected, and the graft was placed in an orthotopic position by end-to-end anastomoses proximally with the host's duodenum and distally with the remaining 1-2 cm of terminal ileum.

Post-operative monitoring: after surgery, animals received standard laboratory rat chow and water ad libitum. The animals were weighed 3 times a week and were inspected daily for signs of clinical GVHD. Three grades of GVHD were distinguished as described in chapter 2. Grade 1: light redness of ears, snout and paws. Grade 2: moderate redness of ears, snout ans paws, light hair-loss and diarrhea. Grade 3: severe redness of ears, snout and paws, alopecia, generalized dermatitis and diarrhea. Rats that died within 4 days from transplantation were considered technical failures. After death, autopsy was performed to confirm or exclude rejection.

Cyclosporine A: commercially available CsA was obtained and dissolved in olive

oil to a concentration of 50 mg/ml before intramuscular administration.

Statistics: The Wilcoxon rank-sum test and the chi-square test were used for statistical analysis of the data.

Anti-lymphocyte serum: was prepared as described in chapter 2.

EXPERIMENTAL GROUPS

The following groups were studied using the WAG to BN fully-allogeneic orthotopic total SBT model;

Group 1: Control group, no immunosuppressive therapy, (n=17).

Group 2: Donor pretreatment on days -2, and -1 prior to transplantation with 4 ml/kg ALS intravenously, (n=7).

Group 3: Donor pretreatment on days -2, and -1 with 4 ml/kg ALS intraperitoneally (n=10).

Group 4: Donor pretreatment on days -2, and -1 with 50 mg/kg CsA, given intramuscularly (n=8).

Group 5: Recipient treatment with 25 mg/kg CsA intramuscularly on days 0, 1, 2, 4, and 6 after transplantation (n=10)

Group 6: Donor pretreatment on days -2, and -1 with 4 ml/kg ALS intravenously, recipient received 25 mg/kg CsA on days 0, 1, 2, 4, and 6 after transplantation (n=7).

Group 7: Donor pretreatment on days -2, and -1 with 4 ml/kg ALS intraperitoneally, recipient treatment with 25 mg/kg CsA on days 0, 1, 2, 4, and 6 after transplantation (n=7).

Group 8: Donor pretreatment on days -2, and -1 with 50 mg/kg CsA intramuscularly, recipient received 25 mg/kg CsA on days 0, 1, 2, 4, and 6 after transplantation (n=9).

RESULTS

Untreated controls (group 1) died from rejection after a mean survival time (MST) ± standard error of the mean (SE) of 16.6±2.7 days. Of these animals, 35% showed grade 1-2 symptoms of GVHD for 3-4 days between 9 and 12 days

Table 1: The effect of donor-pretreatment and recipient immunosuppression on small bowel allograft survival in the fully-allogeneic WAG to BN donor-host combination.

group	donor pretreatment[1]	recipient treatment[2]	survival in days	MST±SE	%GVHD
1	-	-	6,6,6,7,8,9,13[a],14, 14,15,15,19[a],21[a],24, 27[a],31[a],47[a]	16.6±2.7	35
2	ALS iv	-	10,11,11,11,13,14,16	12.3±0.8	0
3	ALS ip	-	5,8,8,9,10,12,12,13 13,13	10.3±0.9	0
4	CsA im	-	13,13[a],14[b],20[a],24,25, 27[a],41[b]	22.1±3.4	62
5	-	CsA im	8,11,13,13,21,55,59[b], 65,66[a],72	38.3±8.5	20
6	ALS iv	CsA im	7,11,13,62,63,69,137	51.7±17.6	0
7	ALS ip	CsA im	7,12,16,62,84,99,104	54.8±16.1	0
8	CsA im	CsA im	9,14,17,22,24[b],25[b], 70[b],79,86	38.4±10.2	33

1: Anti-lymphocyte serum (ALS) was given intravenously (iv) (groups 2 and 6), or intraperitoneally (ip) (groups 3 and 7) on days -2 and -1 prior to transplantation in a volume of 4 ml/kg. Cyclosporine A (CsA) pretreatment was given intramuscularly (im) on days -2, and -1 prior to transplantation at a dose of 50 mg/kg.
2: Recipients were given 25 mg/kg cyclosporine A (CsA) intramuscularly (im) on days 0,1,2,4, and 6 after transplantation.
3: percentage of animals showing clinical signs of GVHD
a: animals showing grade 1-2 GVHD.
b: animals showing grade 3 GVHD.
Survival group 1 vs groups 2, 4, 5, 6, 7, 8: $p<0.01$. Group 1 vs group 3: $p<0.05$, Wilcoxon rank-sum test.

after transplantation (Table 1). Donor pretreatment with ALS, either intravenously or intraperitoneally (groups 2 and 3), successfully prevented the occurrence of GVHD; none of the animals displayed clinical signs of GVHD. Graft survival in these groups was significantly shortened as compared to untreated controls (12.3±0.8 and 10.3±0.9 days respectively, $p<0.05$).

Donor pretreatment with CsA (group 4) significantly prolonged graft survival (MST±SE 22.1±3.4 days, $p<0.01$), while 62% of the animals developed clinical GVHD. Of these, 60% had grade 1-2, and 40% had grade 3 GVHD. CsA given to the recipient at a dose of 25 mg/kg on days 0, 1, 2, 4, and 6 after grafting (group 5) led to a significant prolongation of graft survival time (38.3± 8.5 days, $p<0.01$). Twenty percent of the animals developed GVHD.

Pretreatment of the donor with ALS, either intravenously or intraperitoneally, combined with CsA treatment of the recipient (groups 6 and 7, respectively), led to survival times that were not significantly different from CsA treatment of the recipient alone (51.7±17.6 and 54.8±16.1 days, respectively). None of these animals showed signs of GVHD.

Donor pretreatment with CsA combined with recipient CsA treatment (group 8) gave the same MST as CsA treatment of the recipient alone (38.4±10.2 days). All animals showing GVHD (33%) had grade 1-2 severity.

DISCUSSION

Monchik & Russell (1971) first used parent and F1 hybrid models in SBT. They showed that both unidirectional rejection and GVHD can be induced by small bowel grafts, and that pretreatment of the donor with 7 Gy total body irradiation prior to transplantation completely eliminates GVHD. These findings have been confirmed by other studies (Lee & Schraut 1985, Deltz et al. 1986). Observations in these unidirectional GVHD or rejection models are of uncertain relevance to the clinical situation, in which a two-way reaction between rejection and GVHD can occur (Kirkman, 1984). In fully-allogeneic SBT, rejection rather than GVHD seems to predominate, but little documentation is available on the interaction between rejection and GVHD in these models. In some fully-allogeneic rat

models, the animals die from rejection while GVHD is not clinically present (Monchik & Russell 1971, Lee & Schraut 1986, Frantz et al. 1988). Cohen et al. (1976) investigated the effect of graft-irradiation with 0.5 and 1,5 Gy prior to transplantation in a canine small bowel allograft model. They found that pretreatment with 1.5 Gy leads to rejection of the small bowel allografts in 9.2 days, pretreatment with 0.5 Gy, however, prolongs graft survival to a mean of 28 days. This interesting finding has led to the hypothesis that there is a balance between rejection and GVHD, and that the existence of subclinical GVHD after 0.5 Gy irradiation results in prolonged graft survival.

Since the early 1960s it has been known that GVHD depresses the host's immunological reactivity (Howard & Woodruff 1961). This is best shown by clinical results obtained with T-cell depleted bone-marrow transplantation. On the one hand, T-cell depletion significantly reduces acute GVHD, on the other hand it substantially increases graft rejection (Storb, 1989). GVHD is also known to be immunosuppressive after experimental spleen-cell-, and small bowel transplantation (Grant et al. 1989).

Histopathologically, GVHD is characterized by a loss of the normal architecture of the spleen, lymph nodes and thymus (Deltz et al. 1981, Schraut et al. 1986, Grant et al. 1989). This leads to a profound immunosuppression with impaired humoral and cell-mediated immune responses (Grant et al. 1989). This immunosuppression probably accounts for the observed in vivo balance between rejection and GVHD.

Diflo et al. (1989) observed the occurrence of a short, sublethal GVHD approximately 4 to 6 weeks after fully-allogeneic transplantation in immunosuppressed animals. Donor-pretreatment with ALS completely eliminated GVHD but had no effect on graft survival in these immunosuppressed hosts. Gundlach et al. (1989) found that mesenteric lymphadenectomy, a method that has been shown to eliminate GVHD (Pirenne et al. 1990), does not influence the course of acute graft rejection in non-immunosuppressed recipients. However CsA was not effective in preventing chronic rejection following mesenteric lymphadenectomy whereas the same dosage of CsA fully prevented rejection of normal small

bowel grafts. They suggested that the absence of an immunosuppressive effect caused by a GVH reaction had led to chronic rejection in this model.

In the present study, we showed that pretreatment of the donor with ALS eliminated clinical GVHD and led to significantly accelerated rejection. Intraperitoneal and intravenous administration of ALS were equally effective in preventing GVHD. As Shaffer et al. (1988) have shown that subcutaneous treatment is as effective as intraperitoneal treatment, the route of administration seems to be unimportant.

When our recipients of a graft pretreated with ALS received immunosuppressive treatment with CsA, no adverse effect on graft survival was seen anymore, whereas clinical GVHD remained suppressed. This important finding is in accordance with earlier findings from our laboratory that show that irradiation of the donor with 5 or 10 Gy eliminated clinical GVHD and led to significantly accelerated graft rejection (Saat et al. 1991). When recipients of a graft which was irradiated with 5 or 10 Gy received immunosuppressive treatment with CsA, graft survival was prolonged in both groups, whereas clinical GVHD did not occur.

After CsA treatment of the donor, we found prolonged graft survival but no significant difference in the percentage of animals developing GVHD. However, 2 out of 5 animals that developed GVHD had grade 3 severity. It is known that CsA is more effective against rejection than it is against GVHD (Kirkman et al. 1984). Hence, the prolonged survival we observed could have been due to the immunosuppressive effect of GVHD and the CsA transferred with the graft. Treatment of both donor and recipient with CsA had no beneficial effect on GVHD or rejection compared to recipient treatment alone.

Thus, ALS pretreatment of the donor, combined with CsA treatment of the recipient, results in significantly prolonged graft survival, while clinical GVHD is suppressed. Whether manipulation of this balance will be of use in future clinical SBT remains to be established.

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CHAPTER 5

THE EFFECT OF CYCLOSPORINE A IN SMALL BOWEL TRANSPLANTATION IN RATS IS DEPENDENT ON THE RAT STRAIN COMBINATION USED

This chapter is a modified version of the article:
The effect of cyclosporine A in small bowel transplantation is dependent on the rat strain combination used.
R.W.F. de Bruin, R.E. Saat, E. Heineman, J. Jeekel, and R.L. Marquet.
Transplant. Proc. 1990; 22: 2472-2473.

SUMMARY

Cyclosporine A (CsA) is very effective in preventing the rejection of small bowel allografts in some reported rat models. We have tested the effect of different CsA dosages on the survival of total orthotopic small bowel allografts in the fully-allogeneic WAG to BN and BN to WAG donor-recipient combinations.

In the WAG to BN combination, control animals had a mean survival time (MST) of 16.6±2.7 (SE) days. CsA, 15 mg/kg given on days 0,1,2,4, and 6 after transplantation, did not significantly prolong graft survival times. Interestingly, 50% of the rats developed graft-versus-host disease (GVHD), and 5 rats even exhibited a grade 3 GVH reaction of which they died. CsA 25 mg/kg on days 0,1,2,4, and 6 after grafting led to a MST which was significantly longer than in the control group, although no permanent survival was seen (MST: 38.3±8.5, $p<0.01$, Wilcoxon rank-sum test). When CsA given as in the previous group was followed by a maintenance therapy of 15 mg/kg 3 times a week until day 100, 3 of 9 rats survived for more than 100 days. However, after cessation of CsA therapy the animals died from chronic graft rejection.

In the BN to WAG combination untreated animals showed a MST of 12.2±1.8 days. Fifty percent of the animals showed a mild GVHD occurring between days 5 and 9 posttransplant. The administration of 5 mg/kg CsA on days 0,1,2,4, and 6 after transplantation already evoked a significant prolongation of graft survival. One animal survived for more than 100 days, 1 animal died with a functioning graft 72 days after transplantation, and 3 of 5 animals died after 41.0±11.5 days. After 15 mg/kg, 5 of 8 animals survived permanently (>200 days). This CsA dose also effectively suppressed GVHD. Treatment with 25 mg/kg CsA gave the same MST as in the control group. In this group, no clear-cut rejection could be demonstrated and 1 animal died from functional ileus of the transplant. From this study we conclude that the effectiveness of CsA treatment on the survival of small bowel allografts is dependent upon the donor-recipient combination used, and that "hard" models exist which are relevant in preclinical studies on small bowel transplantation.

INTRODUCTION

Succesful clinical small bowel transplantation (SBT) is hampered by the powerful rejection of the graft. In contrast to the clinical situation, in some rat models long term graft survival is easy to achieve using cyclosporine A (CsA) as a single immunosuppressive agent. In a heterotopic model 15 mg/kg of CsA per day for 7 days resulted in indefinite graft survival in the (LBN)F1-hybrid to Lew combination, and markedly prolonged survival in the Lew to (LBN)F1 combination (Kirkman et al. 1985, Pritchard et al. 1985). Others have reported similar results after prolonged administration of CsA, in doses varying from 15 to 20 mg/kg/day (Harmel et al. 1986, Lee et al. 1986, Hatcher et al. 1987, Schraut et al. 1987). CsA has proved to be much less effective in large animal models. Although statistically significant graft survival is found, long-term survival is only ocasionally achieved (Craddock et al. 1983, Diliz-Perez et al. 1984, Kirkman, 1984).

Previous studies in our laboratory have shown that the BN rat is a high responder to the WAG rat (Niessen et al. 1982). To reduce the costs and difficulties of a large animal model, we sought to determine whether a rat model could be found which exhibited the immunological problems of the large animal model, and thus would not consistently show prolonged small bowel allograft survival after CsA treatment. The present study was undertaken to investigate the effect of different CsA treatment schedules on the survival of small bowel allografts in the "hard" WAG to BN as well as in the "moderate" BN to WAG donor-host combination.

MATERIALS AND METHODS

Animals: rats of the inbred BN ($Rt1^n$) and WAG ($Rt1^u$) strains were used as described in chapter 2.

Intestinal transplantation: transplantation was performed as described in chapter 2. Briefly, the total small bowel, from the ligament of Treitz to the terminal ileum was harvested. The recipients infra-renal aorta and caval vein were clamped and end-to-side anastomoses were performed between the recipient

aorta and caval vein and the donor superior mesenteric artery and portal vein, respectively. The recipient small bowel was resected and the graft was anastomosed proximally with the host's duodenum and distally with the remaining terminal ileum.

After transplantation, rats were weighed 3 times a week and were examined for signs of graft versus host disease (GVHD). After death, necropsy was performed.

GVHD: The severity of GVHD was estimated by clinical grading, as described in chapter 2. Grade I: light redness of ears, snout and paws. Grade 2: moderate redness of ears, snout and paws, light hairloss and diarrhea. Grade 3: severe redness of ears, snout and paws, alopecia, dermatitis and profuse diarrhea.

Statistics: The survival data were statistically analysed using the Wilcoxon rank-sum test.

EXPERIMENTAL GROUPS

WAG to BN donor-host combination;

Group 1: Controls, no treatment (n=17).

Group 2: 15 mg/kg CsA given intramuscularly (im) on days 0,1,2,4 and 6 after grafting (n=14).

Group 3: 25 mg/kg CsA im on days 0,1,2,4 and 6 after grafting (n=10).

Group 4: 25 mg/kg CsA im on days 0,1,2,4 and 6, followed by 15 mg/kg given 3 times a week as maintenance therapy until day 100 (n=9).

BN to WAG donor-host combination;

Group 5: Controls, no treatment (n=10).

Group 6: 5 mg/kg CsA im on days 0,1,2,4 and 6 (n=5).

Group 7: 15 mg/kg CsA im on days 0,1,2,4 and 6 (n=8).

Group 8: 25 mg/kg CsA im on days 0,1,2,4 and 6 (n=6).

RESULTS

Table 1 shows that in the WAG to BN combination control animals had a mean survival time (MST) of 16.6±2.7 (SE) days. Six rats (35%) developed a grade 1-2 manifestation of GVHD. Symptoms became evident on days 9 to 10 posttransplant,

Table 1. Effect of cyclosporine A on the survival of small bowel allografts in the WAG to BN donor-host combination.

treatment	survival in days	MST ± SE[a]
1. -	6,6,6,7,8,8,13[b],14,14,15,15,19[b],21[b],24,27[b],31[b],47[b]	16.6 ± 2.7
2. 15 mg/kg CsA[1]	5,6,7,8,17,19[b],19[c],20[b],20[c],21[c],25[c],26,41,57[c]	20.8 ± 3.8
3. 25 mg/kg CsA[1]	8,11,13[d],13[d],21,55,59[c],65,66[b],72	38.3 ± 8.5
4. 25 mg/kg CsA [1,2]	8,12[d],19,26[c],27[b],51[b],179,212,300	92.6 ± 36.2

a: mean survival time ± standard error of the mean,
b: animals exhibited grade 1-2 GVHD
c: animals exhibited grade 3 GVHD, d: functional ileus of the graft.
1: CsA was given on days 0,1,2,4 and 6. 2: CsA 15 mg/kg 3 times a week until day 100.

and disappeared within 3-4 days (Table 3). A short course of 15 mg/kg CsA (group 2) did not significantly prolong graft survival. Interestingly, 50% of the rats developed GVHD, of which 30 % developed grade 1-2 symptoms, and 70% GVH grade 3 reaction of which they died. The onset of GVHD in this group was approximately 9 days later than in group 1 (Table 3). In group 3, the animals were treated with a higher dose of CsA; 25 mg/kg. The MST was significantly longer than in the control group, although no permanent survival was seen (MST: 38.3 ± 8.5, $p < 0.01$, Wilcoxon rank-sum test). Twenty percent of the animals developed a transient GVHD. At necropsy, 2 animals in this group revealed functional ileus of the graft as cause of death. In group 4, where this high-dose CsA given during the first week posttransplant was followed by a maintenance therapy, 3 of 9 rats survived for more than 100 days. However, after cessation of CsA therapy the animals died from chronic graft rejection. No beneficial effect on the suppression of GVHD was seen either; 33% of the animals developed clinical GVHD.

In the BN to WAG combination untreated animals showed a MST of 12.2 ± 1.8 days (Table 2). Fifty percent of the animals developed a mild GVHD that

Table 2. Effect of cyclosporine A on the survival of small bowel allografts in the BN to WAG donor-host combination.

treatment	survival in days	MST ± SE[a]
5. -	9,10,11[b],12[b],12[b],13,13,13,14[b],15[b]	12.2 ± 0.6
6. 5 mg/kg CsA[1]	18[b],51,54[b],72[c],>100	41.0 ± 11.5
7. 15 mg/kg CsA[1]	10,10,17,>200,>200,>200,>200,>200	>200
8. 25 mg/kg CsA[1]	5,7,10,12,14[d],>200	9.6 ± 1.6

a: mean survival time ± standard error of the mean
b: animals exhibited grade 1-2 GVHD
c: animals exhibited grade 3 GVHD, d: functional ileus of the graft
e: died with functioning graft
1: CsA was given on days 0,1,2,4 and 6

occurred between days 5 and 7 posttransplant and disappeared after 2 days. The administration of 5 mg/kg CsA (group 6) already evoked a significant prolongation of graft survival. One animal survived for more than 100 days, 1 animal died with a functioning graft 72 days after transplantation, and 3 of 5 animals died after 41.0 ± 11.5 days. Fourty percent developed a transient grade 1-2 GVHD. In group 7, 5 of 8 animals survived permanently (>200 days). This CsA dose also effectively suppressed GVHD. Treatment with 25 mg/kg CsA gave the same MST as in the control group. At autopsy, a clear-cut rejection could not be proven, and 1 animal had a functional ileus of the transplant.

DISCUSSION

In the difficult WAG to BN combination we were unable to induce donor specific unresponsiveness, as is seen when heart or kidney transplants are treated with short courses of CsA (Niessen et al. 1982). Long surviving animals in group 4 even rejected their grafts 79, 112, and 200 days after cessation of CsA maintenance therapy, that was given 3 times a week until day 100. Surprisingly, in group 2, 5 animals developed a lethal grade 3 GVHD. This indicates that CsA

evoked a disbalance between GVH and rejection in favour of GVH. Apparently, CsA is less effective in controlling the GVHD than in controlling rejection, a finding reported earlier by Kirkman et al. (1985). The functional ileus seen in some animals after high dose CsA treatment could be a toxic side-effect of CsA. Very little is as yet known about CsA toxicity on the bowel, but it has been shown that CsA may have a deleterious effect on the small bowel microvasculature (Crane et al. 1990).

In the "moderate" BN to WAG combination significant prolongation of graft survival was already seen after a short course of 5 mg/kg CsA. Treatment with 15 mg/kg led to permanent survival of 5 of 8 animals, and totally abolished the occurrence of GVHD. These results are better than those in group 4 of the WAG to BN combination, in which the total amount of CsA given was higher. The poor results obtained in the 25 mg/kg group may be due to CsA toxicity. Since heart allografted rats tolerate this dose very well (Bouwman et al. 1989) this may be a toxic effect on the vulnerable bowel transplant (Crane et al. 1990,

Table 3. Frequency, grade, and duration of GVHD in the various groups.

group	n	GVH grade	GVH grade	clinical GVHD	
		1-2	3	onset[1]	end[1]
1.	17	6	0	9.5 ± 0.3	12.5 ± 0.9
2.	14	2	5	18.0 ± 0.8	23.1 ± 2.9
3.	10	1	1	20.5 ± 2.5	39.0 ± 3.0
4.	9	2	1	26.0 ± 1.7	34.7 ± 8.2
5.	10	5	0	6.2 ± 0.4	8.4 ± 0.5
6.	5	2	0	7.5 ± 1.5	10.5 ± 0.5
7.	8	0	0		
8.	6	0	0		

1: days after transplantation: mean ± standard error of the mean.

Sigalet et al. 1991).

We conclude that the effectiveness of CsA is dependent on the rat strain combination used, and that the WAG to BN combination is a "hard" SBT model which exhibits at least some of the immunological problems seen in large animal models. The BN to WAG combination on the other hand, in which permanent survival can be easily obtained using CsA as single immunosuppressive agent, can be used to study the functional aspects of long surviving animals.

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CHAPTER 6

PRE- AND POSTOPERATIVE TREATMENT WITH CYCLOSPORINE A IN THE MANAGEMENT OF SMALL BOWEL ALLOGRAFT REJECTION AND GRAFT-VERSUS-HOST DISEASE

SUMMARY

Long-term survival of small bowel allografts cannot be obtained on a regular basis since transplantation is hampered by severe rejection. In the WAG to BN rat model we investigated whether pretreatment of the recipient with CsA was able to improve the results obtained with postoperative CsA monotherapy. Previously we have found that 25 mg/kg CsA given on days 0,1,2,4, and 6 followed by 15 mg/kg 3 times a week until day 100 led to long-term survival (>100 days) in only 3 of 9 cases.

Untreated recipients rejected their grafts in 12.8±1.0 days (mean survival time; MST±SE). CsA pretreatment on day -1 prior to transplantation with 50 mg/kg combined with a postoperative course of 25 mg/kg on days 0,1,2,4, and 6 led to a MST of 44±17 days (n=5). When pretreatment was given on days -9,-7,-5, and -3 before transplantation with 25 mg/kg CsA, followed by the same postoperative course as in the previous group, a MST of 79±16 days was found. This pretreatment schedule, combined with a low-dose long-term course of 5 mg/kg CsA on days 0,1,2,4, and 6 after transplantation and subsequently 3 times a week until day 100 gave a MST of 116±33 days. Since in some animals a severe graft-versus-host reaction (GVHD) developed, donors in the next group were irradiated with 5 Gray on the day of transplantation. These grafts, transplanted in recipients that received the same CsA treatment as the previous group, were rejected in 155±15 days. No GVHD was encountered in this group. Without pretreatment, 5 mg/kg CsA given on days 0,1,2,4, and 6 after transplantation and subsequently 3 times a week until day 100 gave a MST of 121±29 days. No statistically significant differences between these groups were found, although only with pretreatment we found consistent long-term survival.

INTRODUCTION

The small bowel is particularly vulnerable to rejection, probably due to the loss of its barrier function. Even mild, reversible rejection is accompanied by bacterial translocation from the graft to the mesenteric lymph nodes and the blood. In a recipient that is receiving immunosuppression, and which probably has a bacterial overgrowth of its small bowel graft (Browne et al. 1992), this may have serious consequences. It is therfore of utmost importance to optimally control rejection after small bowel transplantation (SBT), in order to maintain the gut barrier function. It is thought that cyclosporine A (CsA) is able to act satisfactory as mainstay of immunosuppressive regimen following SBT (Grant et al. 1990, Schroeder et al. 1990). However, experimental results obtained previously in our laboratory using the WAG to BN rat donor-host combination have shown that in this model we were unable to consistently produce long-term allograft survival with postoperative CsA monotherapy (Chapter 5, Saat et al. 1990). Therefore the present study was initiated to investigate whether pretreatment of the recipient with CsA could improve the results obtained with postoperative CsA monotherapy in this rat model of SBT.

MATERIALS AND METHODS

Animals: rats of the WAG ($Rt1^u$) and BN ($Rt1^n$) strains were used as donors and recipients respectively (see chapter 2).

Small bowel transplantation: transplantation was performed as described in chapter 2. In brief, the total small bowel from the ligament of Treitz to the terminal ileum was transplanted with its superior mesenteric artery anastomosed end to side to the recipient aorta, and its portal vein to the caval vein. The recipient small bowel was resected, and the graft was placed orthotopically by end-to-end anastomoses with the host's duodenum and remaining 1-2 cm of terminal ileum.

GVHD: 3 grades were distinguished, as outlined in chapter 2.

Cyclosporine A: see chapter 2.

Irradiation was performed as described in chapter 2.

Histology: terminal ileum of the graft and remnant host ileum were fixed in 3.6% buffered formalin. Three to 4 μm paraffin sections were cut and stained with hematoxylin-phloxine-saffron.

Statistics: the survival data were statistically analysed using Kruskal-Wallis analysis of variance.

EXPERIMENTAL GROUPS

Group 1: Controls, no treatment (n=10).

Group 2: Intramuscular pretreatment with 50 mg/kg CsA 1 day before transplantation, followed by 25 mg/kg CsA im on days 0,1,2,4, and 6 after grafting (n=5).

Group 3: Pretreatment with 25 mg/kg CsA on days -9,-7,-5, and -3 before transplantation, followed by 25 mg/kg CsA on days 0,1,2,4, and 6 after transplantation (n=5).

Group 4: Pretreatment with 25 mg/kg CsA on days -9,-7,-5, and -3 before transplantation, followed by 5 mg/kg on days 0,1,2,4, and 6 and subsequently 3 times a week until day 100 after transplantation (n=9).

Group 5: Recipients were treated as in group 4. Additionally, donors were irradiated with 5 Gray (Gy) on the day of transplantation (n=5).

Group 6: No pretreatment, postoperative CsA consisted of 5 mg/kg on days 0,1,2,4, and 6 and subsequently 3 times a week until day 100 (n=9).

RESULTS

Survival and GVHD;

In a previous study (chapter 5) we found that administration of CsA 25 mg/kg on day 0,1,2,4, and 6 followed by 15 mg/kg 3 times a week until day 100 led to a mean survival time (MST±SE) of 92.6±36.2 days. In the present study, untreated recipients died from rejection in 12.8±1.0 days (Table 1). In group 2, CsA pretreatment on day -1 prior to transplantation with 50 mg/kg combined with a postoperative course of 25 mg/kg on days 0,1,2,4, and 6 led to a MST of 44±17 days. One animal in this group developed mild, grade 1-2 GVHD and 1 had

Table 1. Effect of recipient-pretreatment with cyclosporine on small bowel allograft survival in the WAG to BN donor-host combination.

recipient pretreatment	treatment post-Tx	survival in days	mean±SE	%GVHD[1]
1. -	-	6,10[a]11,12,14[a], 14,15[a],15,15,16	12.8±1.0	40
-	25 mg/kg[2]+ 15 mg/kg	8,12,19,26[c],27, 51,179,212,300	92.6±36.2[5]	11
2. 50 mg/kg, d-1	25 mg/kg[3]	12,15,23[a],85[c],86	44±17	40
3. 25 mg/kg, d-9,-7,-5,-3	25 mg/kg[3]	49[c],65[c],67,71,143	79±16	40
4. 25 mg/kg, d-9,-7,-5,-3	5 mg/kg[4]	12,14,24,35[c],111[c], 186,194,204,269[c]	116±33	33
5. 25 mg/kg, d-9,-7,-5,-3 + 5 Gy donorirr d 0	5 mg/kg[4]	131,131,149,153, 211	155±15	0
6. -	5 mg/kg[4]	6,6,16,145,154 170,178,187,230	121±29	0

1: percentage of animals showing clinical GVHD. a: grade 1; c: grade 3.
2: cyclosporine 25 mg/kg was given intramuscularly on days 0,1,2,4, and 6, followed by 15 mg/kg 3 times a week until day 100.
3: cyclosporine was given intramuscularly on days 0,1,2,4, and 6 after transplantation.
4: cyclosporine was given intramuscularly on days 0,1,2,4, and 6 after transplantation and subsequently 3 times a week until day 100.
Kruskal-Wallis one-way ANOVA analysis found no statistical differences between groups 2,3,4,5, and 6.
5: This group has been previously published in chapter 5.

grade 3 symptoms (Table 2). When pretreatment was given on days -9,-7,-5, and -3 before transplantation with 25 mg/kg CsA, followed by the same post-operative CsA course as in group 2, a MST of 79±16 days was found. Two of 5 animals developed severe grade 3 GVHD. In group 4, this pretreatment schedule, combined with a low-dose long-term course of 5 mg/kg CsA on days 0,1,2,4, and 6 after transplantation and subsequently 3 times a week until day 100 gave a MST of 116±33 days. Since in this group also some animals developed severe GVHD, in group 5 the donors were irradiated with 5 Gray on the day of transplantation. These grafts, transplanted in recipients that received the same CsA treatment as the previous group, were rejected in 155±15 days. No GVHD was encountered in this group.

Table 2. Percentage of animals surviving beyond 100 days.

group	percentage
1.	0%
2.	0%
3.	20%
4.	55%
5.	100%
6.	66%

Without pretreatment, 5 mg/kg CsA given on days 0,1,2,4, and 6 after transplantation and subsequently 3 times a week until day 100 gave a MST of 121±29 days (group 6). By using Kruskal-Wallis one-way ANOVA it appeared that the experimental groups did not differ significantly among each other. However, when comparing the number of animals surviving beyond 100 days, it appeared that only in group 5 all animals lived for more than 100 days (Table 2).

Chronic rejection;

In most long-term surviving recipients in the experimental groups, the cause of death was malnourishment due to chronic rejection of the small bowel trans-

plant. The process of chronic rejection was characterized by an initial normal postoperative course; weight loss in the first 2 weeks was followed by a gradual

Fig. 1a.

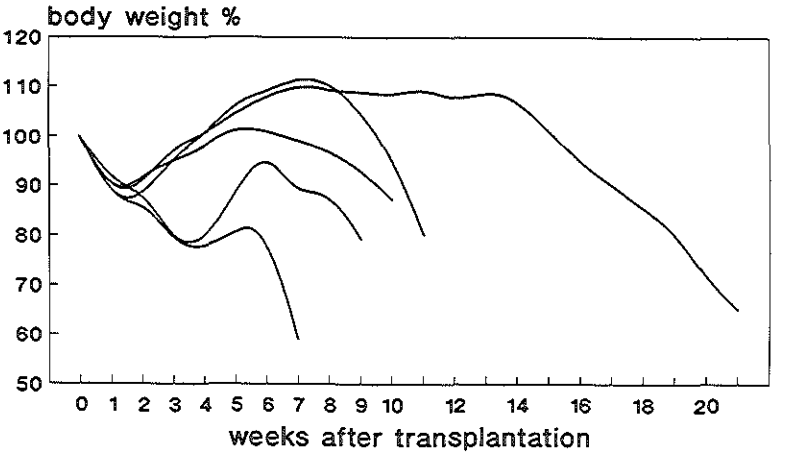

Fig. 1b.

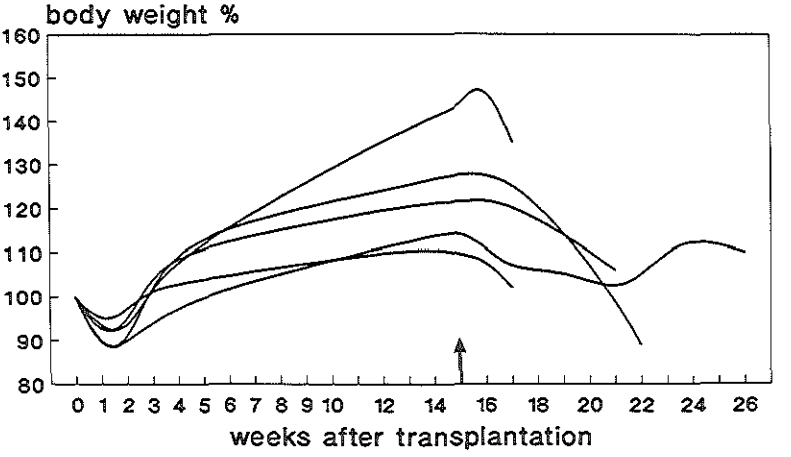

Fig. 1A: Weight curves of recipients pretreated with 25 mg/kg cyclosporine on days -9,-7,-5, and -3 before transplantation followed by 25 mg/kg on days 0,1,2,4,and 6 after transplantation (group 3), and 1B: recipients pretreated with 25 mg/kg cyclosporine on days -9,-7,-5, and -3 before transplantation, followed by 5 mg/kg on days 0,1,2,4, and 6 and subsequently 3 times a week until day 100. Additionally, donors were irradiated with 5 Gray on the day of transplantation (group 5). ↑*: cessation of cyclosporine therapy.*

increase in weight. Subsequently, at various time points for each individual after transplantation, the animals started to lose weight (Figs. 1a & 1b). This weight loss could progress rapidly, leading to the death of the animal in a short while following its onset, or animals could show a gradual but progressive loss of weight. Surprisingly, their clinical appearance was normal. The typical advanced stage of chronic rejection showed a thin, but lively animal with diarrhea. At autopsy, the transplant appeared thick-walled and fibrotic. Histopathologically, moderate to severe villus athropy with mild fibrosis of the muscularis and thickening of the serosa was seen.

DISCUSSION

In the WAG to BN rat model of SBT we have previously shown that long-term survival of small bowel allografts is hard to obtain using CsA monotherapy (Chapter 5). Rejection even occurred after the cessation of long-term CsA therapy given until 100 days posttransplant. We therefore concluded that this model exhibits at least some of the immunological problems seen in large animal models.

In the present study we investigated whether pretreatment of the recipient with CsA could improve these results. Pretreatment with CsA is associated with a reduced incidence of acute rejection in human kidney transplantation (Hong et al. 1991). On the other hand, low CsA plasma levels in the early postoperative period are associated with a higher incidence of rejection (Kahan et al. 1984, Rogerson et al. 1986). Aeder et al. (1991) showed that pretreatment with CsA led to a mitigation of the rejection process compared with CsA therapy beginning at the time of transplantation using a dog model of SBT. Pretreatment of the recipient has also been shown to be effective in prolonging heart xenograft survival in the hamster to rat model (Bouwman et al. 1992).

We were unable to prolong graft survival significantly as compared with post-operative treatment alone. Moreover, pretreatment was associated with the occurrence of severe GVHD. However in group 5, although not statistically significant, uniform long-term graft survival was found. In this group only, all

animals survived beyond the duration of the CsA therapy and no acute rejection or GVHD were seen. Whether this is due to CsA pretreatment combined with a reduction of the immunogenicity of the graft and prevention of clinical GVHD by irradiation of the donor should be investigated further. This could be studied by using markers of gut permeability, such as ^{51}Cr-EDTA (Grant et al. 1991), or lactulose-mannitol (Sigalet et al. 1991[b]), both parameters of gut permeability and early rejection (Grant et al. 1991).

Taking into account the results obtained in chapter 5, and the findings from the present study, it may be speculated that postoperative administration of high dose CsA is detrimental to the survival of small bowel allografted rats. In the present study we found long-term graft survival after low-dose CsA administration, that did not differ significantly from the survival obtained in animals pretreated with CsA and/or high dose postoperative CsA. This hasn't been studied before since higher doses of CsA did not consistently produce long-term survivors. It may well be that we have overdosed CsA postoperatively, leading to toxicity on the small bowel transplant which negatively influenced graft survival. Earlier studies have demonstrated that relatively low doses of CsA are toxic to the bowel, since they compromize the transmural microvasculature (Crane et al. 1990). CsA also affects glucose and fatty acid uptake of normal bowel (Sigalet et al. 1991[a]), and may increase intestinal permeability in both native and transplanted bowel (Sigalet et al. 1991[b]). Pretreatment, however, does not seem to have such an adverse effect.

CsA therapy in all groups seemed ineffective against chronic rejection although it cannot be excluded that prolonging CsA courses beyond 100 days could prevent it. It seems plausible that, as with other organ grafts, long-term small bowel-graft function is jeopardized by this as yet uncontrollable and irreversible process (Tilney et al. 1991).

Considering all results we have obtained with CsA in this model, it appears that postoperative courses of low dose CsA with or without graft irradiation, and maybe pretreatment of the recipient with CsA, are of benefit to the long-term survival of small bowel allografts.

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CHAPTER 7

THE EFFECT OF PRETRANSPLANT DONOR-SPECIFIC BLOOD TRANSFUSIONS ON VARIOUS SEGMENTS OF SMALL BOWEL GRAFTS

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Transplantation 1990; 50: 928-930.

Chapter 7

SUMMARY

The effects of pretransplant donor-specific blood transfusion (DST) on the survival of orthotopic small bowel transplants in rats were investigated in the fully allogeneic BN ($Rt1^n$) to WAG ($Rt1^u$) donor-host combination. Previous studies show that in this combination DST lead to permanent survival of heterotopically transplanted hearts, marked prolongation of kidney grafts, and moderate prolongation of pancreas grafts but have no effect on skin grafts.

Without pretreatment, total small bowel grafts (±45 cm) were rejected in 12.2±1.8 days (mean±SD), and 10-cm segments of jejunum or ileum in 11.2±4.0 and 11.6±0.5 days, respectively. Three DST given on days -21, -14, and -7 before transplantation had no effect on graft survival in any of the groups tested. Total small bowel grafts were rejected in 12.8±2.5 days, and 10-cm long segments of jejunum or ileum in 17.0±7.2 days and 11.5±2.7 days, respectively. Graft-versus-host disease (GVHD), which was mild and transient, occurred in 50% of the nontreated rats engrafted with a total small bowel, in 40% of the animals transplanted with an ileum segment, and in none of the rats that received a jejunal transplant. In the DST- pretreated groups, none of the animals transplanted with a total small bowel or ileum segment and 16.6% of the animals transplanted with a jejunum segment showed clinical signs of GVHD. When DST pretreatment was combined with cyclosporine A (CsA) grafts did not survive any longer than with CsA treatment alone. It is concluded that DST ameliorate GVHD, but do not prolong the survival of small bowel allografts nor act additively with CsA treatment.

INTRODUCTION

The resurgence of interest in small bowel transplantation (SBT) as a treatment for the short bowel syndrome is largely due to the impressive results obtained with cyclosporine A (CsA) in clinical organ transplantation. In several studies on SBT, CsA was the main immunosuppressive agent used. In general, it appears that in large animal models (dog and pig) long term survival can only be achieved using high dosages of CsA (Fujiwara et al. 1987, Grant et al. 1988). Even in some rat models long-term survival is hard to obtain (Saat et al. 1990). In a recent clinical SBT, it was not possible to prevent acute rejection of the graft in 13 days, in spite of agressive quadruple therapy, which gave rise to serious side effects (Grant et al. 1989). Other attempts of clinical SBT have also failed to yield long-term surviving grafts with good function (Grant, 1989). Apparently, small bowel grafts are vigorously rejected, and there is an urgent need for adequate immunosuppression.

Pretransplant donor-specific blood transfusions (DST) may lead to a prolongation of allograft survival in both experimental (Marquet et al. 1971) and clinical (Opelz et al. 1973) transplantation. The mechanisms underlying this well-known phenomenon are still not fully elucidated, but suppressor cells (Singh et al. 1987), anti-idiotypic antibodies (Phelan et al. 1989), clonal deletion of cytotoxic T-cell precursors (van Twuyver et al. 1989), and metabolites of arachidonic acid (Perez et al. 1989) have all been shown to be involved, depending on the model used.

Previous studies in our laboratory have shown that DST induce T-suppressor cells, leading to permanent heart graft acceptance and marked prolongation of kidney graft survival. This induction of donor-specific unresponsiveness would be one way to improve the results of SBT. Reduction of the immunogenicity of the graft by reducing its length, i.e., transplantation of a segment of 15-30% of the total length of the small bowel offers another avenue to approach the problem of ameliorating rejection (Kimura et al. 1987).

The present study was undertaken to investigate the effect of DST on the survival of small bowel grafts, varying in length and site of origin, and to

evaluate the effect of CsA in combination with DST in a fully allogeneic donor-host combination.

MATERIALS AND METHODS

Animals: rats of the inbred BN ($Rt1^n$) and WAG ($Rt1^u$) strains were used as described in chapter 2.

Blood transfusions: 1 ml of heparinized whole blood was administered intravenously on days -21, -14 and -7 prior to transplantation.

Small bowel transplantation: orthotopic total SBT was performed as described in chapter 2. Briefly, the total small bowel, from the ligament of Treitz to the terminal ileum was harvested along with its vascular pedicle, consisting of the portal vein and superior mesenteric artery. In the recipient the infrarenal aorta and inferior caval vein were clamped, and end-to-side anastomoses were performed between the recipient aorta and caval vein and donor superior mesenteric artery and portal vein, respectively. The recipient small bowel was resected and the graft was anastomosed proximally with the host's duodenum and distally with the remaining terminal ileum.

Jejunal transplants consisted of 10 cm of proximal jejunum. Ileal grafts were prepared by isolating 10 cm of distal ileum from the small bowel.

Postoperative monitoring: as described in chapter 2. In this study only grade I GVHD (light redness of snout, ears and paws) was observed.

Cyclosporine A: (Sandimmune, Sandoz, Basel, Switzerland) was dissolved in oil to a concentration of 25mg/ml and administered intramuscularly (i.m.) in a volume of 0.1-0.2 ml.

Statistics: Wilcoxon rank-sum test and the chi-square test were used for statistical analysis of the data.

EXPERIMENTAL GROUPS

BN rats were used as donors and WAG rats as recipients, except in the syngeneic group (2) in which WAG rats were used both as donor and as recipient;

Group 1: Control group; transplantation of total small bowel (45 cm). No pre, or

postoperative treatment (n=10).

Group 2: Control group, syngeneic transplantation of 10 cm of proximal jejunum (n=3).

Group 3: Transplantation of 10 cm proximal jejunum, no treatment (n=6).

Group 4: Transplantation of 10 cm distal ileum, no treatment (n=5).

Group 5: Total small bowel graft (45 cm), recipient pretreated with 3 DST (n=9).

Group 6: Transplantation of 10 cm proximal jejunum, recipient pretreated with 3 DST (n=6).

Group 7: Transplantation of 10 cm distal ileum, recipient pretreated with 3 DST (n=8).

Group 8 : Total small bowel graft, recipient treated with 5 mg/kg CsA i.m. on days 0, 1, 2, 4 and 6 after grafting (n=5).

Group 9: Transplantation of total small bowel, recipient pretreated with 3 DST and 5 mg/kg CsA on days 0, 1, 2, 4 and 6 after grafting (n=4).

RESULTS

Survival times of different segments, no treatment

The animals in group 1 rejected their grafts between 9 and 15 days, with a mean survival time (MST±SD) of 12.2±1.8 days. Animals transplanted with a 10-cm jejunal or ileal segment (groups 3 and 4) rejected their grafts with a MST of 11.2±4.0 and 11.6±0.5 days, respectively. This is not significantly different from group 1. There was however a difference in the occurrence of GVHD. In group 1 50%, and in group 4 40% of the rats developed a mild transient grade I GVHD. The onset of clinical GVHD was between days 5 and 11 posttransplant and lasted for approximately 3 days. The group grafted with the jejunal transplants (group 3) showed no signs of GVHD ($p<0.05$, chi-square test)(Table 1).

Effects of DST

Pretransplant administration of 3 DST had no significant effect on graft survival, neither in the total small bowel transplanted group, nor in the jejunal- or ileal-

Table 1. Survival times of various segments of small bowel transplants.

group	survival in days	MST± SD[1]	%GVHD[2]
1. BN to WAG	9,10,11[a],12[a],12[a],13,13,13,14[a],15[a]	12.2±1.8	50%
2. WAG to WAG jejunal graft	>100,>100,>100		
3. BN to WAG jejunal graft	6,9,10,12,12,18	11.2±4.0	0%
4. BN to WAG ileal graft	11,11[a],12[a],12,12	11.6±0.5	40%

1: mean survival time ± standard deviation
2: percentage of animals showing clinical signs of GVHD.
a: animals showing clinical signs of GVHD.

engrafted groups. The MST of the pretreated animals that received a total small bowel, a jejunal, or an ileal segmental graft were 12.8±2.5, 17.0±7.2, and 11.5±2.7, respectively (Table 2).

A significantly smaller number of rats in the transfused groups showed clinical signs of GVHD ($p<0.05$, group 1 versus groups 5 and 7). In groups 5 and 7 none of the rats, and in group 6 one rat (16.6%) exhibited a grade I GVHD.

Effects of DST and CsA

A short course of CsA led to significantly prolonged survival times in comparison with untreated, or DST treated animals. In group 8, 3 out of 5 animals died with an MST of 40.1±20.0 days; 1 animal died 72 days after transplantation with a functioning graft, and 1 animal survived beyond 200 days. Survival times in animals receiving the combination of pretransplant blood transfusions with a posttransplant course of CsA (group 9) did not differ significantly from those obtained in group 8; 3 out of 6 animals died with an MST of 26.7±3.2 days, and 3 out of 6 animals survived for more than 100 days (Table 2). In group 2 WAG rats transplanted with a syngeneic, 10-cm long small bowel graft thrived well and

Table 2. Effect of donor-specific blood transfusions and cyclosporine A on graft survival.

group	survival in days	MST±SD[1]	%GVHD[2]
5. 3X DST[3]	10,10,11,12,12,14,14,14,18	12.8±2.5	0%[6]
6. 3X DST[3] jejunum segment	7,14,15,15,25[a],26	17.0±7.2	16.6%
7. 3X DST[3] ileum segment	6,9,11,13,13,13,13,14	11.5±2.7	0%[6]
8. CsA[4]	18[a],51,54[a],72[5],>200	>79.6±70.3	40%
9. DST[3]+CsA[4]	23,28[a],29,>200,>200,>200	>113.3±95.0	16.6%

1: mean survival time ± standard deviation
2: percentage of animals showing clinical signs of GVHD
3: donor-specific blood transfusions: 1 ml of heparinized whole blood was given intravenously on days -21,-14 and -7 before transplantation
4: Cyclosporine A was given intramuscularly on days 0,1,2,4 and 6 after grafting at a dose of 5 mg/kg.
5: died with functioning graft
6: $p<0.05$ versus group 1, Chi-square test
a: animals showing clinical signs of GVHD

showed a steady increase in weight (Table 1).

DISCUSSION

Theoretically, segmental grafts have immunological advantages over total small bowel grafts: both the number of immunocompetent cells transplanted and the organ-specific antigenic load are reduced, thus ameliorating GVHD and rejection. In this study no beneficial effect of length reduction on the rejection process could be demonstrated. This is in accordance with results obtained by Kimura et al. (1987), and Stangl et al. (1989) who also found no differences in graft rejection times, irrespective of the length or the site of origin of the graft.

In rats transplanted with a jejunal segment, which includes a short segment of

the mesentery and only about 50% of the mesenteric lymph nodes as compared to a total small bowel or an ileal graft, no GVHD occurred. In 50% of the rats grafted with a total bowel and 40% of the animals transplanted with an ileum segment, clinical GVHD developed. This is in contrast to observations made by Kimura et al. (1987) who found no relation between the length or the segment of bowel that was transplanted, and the occurrence of GVHD.

In this study, donor-specific blood transfusions had no effect on graft survival in any of the groups tested. Previous studies in our laboratory have shown that DST in this model lead to permanent heart graft survival (Marquet et al. 1971), marked prolongation of kidney graft survival (Marquet et al. 1971), and very moderate prolongation of pancreas graft survival (Spillenaar-Bilgen et al. 1990) but have no effect on skin graft survival (Marquet et al. 1971). DST-induced T-suppressor cells are involved in this model that can impose unresponsiveness upon naive recipients (Singh et al. 1987). Possibly, segmental grafts of 20% of the small bowel are still highly immunogenic and a further decrease in antigenic load is mandatory to allow the blood transfusion effect to emerge.

An alternative explanation is that the immunogenicity of the graft is effectively reduced, but that the bowel is more vulnerable to rejection than heart or kidney grafts, which are able to withstand rejection episodes.

Surprisingly, significantly fewer rats in the DST-pretreated groups showed clinical signs of GVHD as compared to the control groups. This finding implies that DST may have induced some form of immunosuppression, reflected in the abscence of GVHD, but not in the survival time, again an indication for the vulnerability of the graft. Rolstad (1976) has shown that recipient cells are required for the development of a GVH reaction in a parent to F1 one-way model, and he postulated that these cells contributed to the GVH reaction by secreting mitogenic and blastogenic factors. In our model, a DST-induced alteration of the recipient responsiveness may have contributed to the abscence of clinical GVHD.

Five mg/kg CsA given on days 0,1,2,4 and 6 after grafting significantly prolonged graft survival. Using this treatment schedule 60% of the animals showed

prolonged survival, while 40% survived permanently. Consequently, it should be possible to obtain improved graft survival when additional treatment would act additively or synergistically. We found no positive effects of combined treatment with DST and CsA on graft survival. Martinelli et al. (1988), who also found no effect of DST on small bowel allograft survival, did find a synergistic effect of DST combined with low dose CsA. This difference may be explained by the different donor-host combination used. They used the ACI to Lew combination, starting with CsA on the day of transfusion, and proceeding with low-dose CsA until 30 days after transplantation.

From our data we conclude that preconditioning with DST ameliorates GVHD, and does not have a beneficial effect on the survival of small bowel allografts. Combined DST and CsA treatment does not improve the outcome of graft survival in the model employed. These data suggest that preoperative donor-specific blood transfusions have no effect on small bowel transplant survival using these inbred strains of rats.

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CHAPTER 8

FULMINANT GRAFT-VERSUS-HOST DISEASE AFTER FK-506 TREATMENT IN FULLY ALLOGENEIC SMALL BOWEL TRANSPLANTATION

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Transplant. Proc. 1991; 23: 3257-3259.

SUMMARY

Multiple studies have demonstrated that FK-506 is effective in preventing the rejection of cardiac, renal, and hepatic allografts. Although it is shown that rejection and graft-versus-host disease (GVHD) following small bowel allografting are also suppressed by FK-506, additional studies are needed to determine the role for FK in small bowel transplantation (SBT). We studied the effectiveness of FK-506, as compared to cyclosporine A (CsA), as an immunosuppressant in small intestinal transplantation. Fully-allogeneic-total-orthotopic-SBT was performed in the WAG to BN donor-host rat combination. Control animals died from rejection with a mean survival time (MST) of 12.8±1.0 days, (group 1, n=10), 40% of the animals exhibited a mild, transient GHVD (grade 1-2) consisting of mild redness of ears, snout, and paws.

CsA 15 mg/kg given intramuscularly on days 0,1,2,4, and 6 after grafting gave a MST of 61.4±4.1 days (group 2, n=5), none of the animals showed clinical GVHD.

FK-506, 2 mg/kg given on days 0,1,2,4, and 6 after grafting gave a MST of 28.5±6.8 days, (group 3, n=8). 88% of the animals had severe clinical GVHD, consisting of severe redness of ears, snout and paws, inflamed neck, and hunched posture. Although in some animals clinical signs of GVHD subsided, histopathologic examination revealed severe GVHD-like lesions at the time of death. Other animals died during clinical GVHD.

Additional treatment with 5 Gray donor-irradiation on the day of transplantation abolished clinical GHVD completely, but did not lead to prolongation of graft survival when compared to FK treatment alone, (group 4, n=7, MST 27.6±2.4 days).

This study shows that FK is less effective in controlling GVHD than in controlling rejection. The most striking finding however is that FK-506 treatment allows the development of previously unseen fulminant GVHD. This finding may have important impications for its use in clinical SBT.

INTRODUCTION

Since its discovery in 1984 (Kino et al. 1987), the macrolide FK-506 has been shown to be a potent immunosuppressive drug in experimental and clinical organ transplantation (Thomson, 1990). Whereas the effects of FK-506 in heart, kidney, and liver transplantation have been thoroughly investigated, its effects on experimental small bowel transplantation (SBT) remain relatively unexplored. Results obtained in SBT with cyclosporine A (CsA) are poor (Marquet & Ingham-Clark 1991), and rejection remains the limiting obstacle. Better immunosuppressive drugs may give new impetus to clinical SBT. The present study was undertaken to compare the effects of FK-506 and CsA on rejection and graft-versus-host disease (GVHD) after fully-allogeneic SBT in rats.

MATERIALS AND METHODS

Animals: rats of the inbred WAG ($Rt1^u$) and BN ($Rt1^n$) strains were used as donors and recipients respectively, as described in chapter 2.

Small bowel transplantation: was performed as described in chapter 2. In brief, the total small bowel was harvested from the ligament of Treitz to the terminal ileum, along with a vascular pedicle consisting of the superior mesenteric artery and portal vein. In the recipient the infra-renal aorta and caval vein were clamped and end-to-side anastomoses were performed between the recipient aorta and caval vein and the donor superior mesenteric artery and portal vein respectively. The recipient small bowel was resected, and the graft was placed in an orthotopic position by end-to-end anastomoses proximally with the host's duodenum and distally with the remaining 1-2 cm of terminal ileum.

Post-operative monitoring: after surgery, animals received standard laboratory rat chow and water ad libitum. The animals were weighed 3 times a week and were inspected daily for signs of clinical GVHD. Three grades of GVHD were distinguished. Grade 1: light redness of ears, snout and paws. Grade 2: moderate redness of ears, snout ans paws, light hair-loss and diarrhea. Grade 3: severe redness of ears, snout and paws, alopecia, generalized dermatitis and diarrhea. Rats that died within 4 days from transplantation were considered technical

failures. After death, autopsy and histologic examination were performed.

Cyclosporine A: commercially available CsA (Sandimmune, Sandoz, Basel, Switzerland) was obtained and diluted in olive oil to a concentration of 50 mg/ml before intramuscular administration.

FK-506: FK-506 was a gift from the Fujisawa Pharmaceutical Co, Osaka, Japan. FK-506 was dissolved in absolute methanol, and subsequently diluted 10 times in olive oil to a concentration of 5 mg/ml.

Histology: the following tissues of the recipients were sampled for histopathologic analysis: terminal ileum of grafted bowel, remnant ileum of recipient, thymus, submandibular salivary gland, liver, spleen, mesenteric-, cervical-, and axillary lymph nodes, lip, and ear. These tissues were fixed in 3,6% buffered formalin. Three to 4 μm paraffin sections were cut and stained with hematoxylin-phloxine-saffron.

EXPERIMENTAL GROUPS

The following groups were studied in the fully-allogeneic WAG to BN model;

Group 1: Control group, no immunosuppressive therapy, n=10.

Group 2: Recipients were treated with 15 mg/kg CsA on days 0, 1, 2, 4, and 6 after transplantation, n=5.

Group 3: Recipients were treated with 2 mg/kg FK-506 on days 0, 1, 2, 4, and 6 after grafting, n=8.

Group 4: Recipients received FK-506 as group 3, additionally donors were irradiated with 5 Gray on the day of transplantation, n=7.

RESULTS

Graft survival and clinical GVHD

Untreated recipients in group 1 died from rejection in 12.8±1.0 days (mean survival time, MST ± standard error of the mean, SE) (Table 1). Fourty percent of the animals had a mild transient GVHD (grade 1-2) that usually became visible on day 9 posttransplant, and lasted approximately 3 days.

Table 1: Effect of FK-506 on rejection and GVHD following orthotopic total small bowel transplantation in the WAG to BN rat model.

treatment	survival in days	MST±SE	%GVHD[d]
-	6,10[a],11,12,14[a],14,15[a], 15,15,16	12.8±1.0	40
CsA 15 mg/kg[c]	48,59,60,69,71	61.4±4.1	0
FK-506 2 mg/kg[c]	20,21[b],21[b],21[b],22[b],23[b], 24[b],76[b]	28.5±6.8	88
5 Gy donor irr + FK-506 2 mg/kg[c]	21,23,25,26,28,30,65	31.1±5.7	0

a: animals showed mild (grade 1-2) clinical GVHD
b: animals had severe (grade 3) GVHD
c: cyclosporine A (CsA) and FK-506 were given intramuscularly on days 0,1,2,4, and 6 after transplantation
d: percentage of animals showing clinical GVHD

CsA 15 mg/kg, given on days 0, 1, 2, 4, and 6 after transplantation (group 2) led to a MST of 61.4±4.1 days. None of the animals showed clinical signs of GVHD. FK-506 treatment (group 3) gave significantly shortened survival times when compared to CsA treatment. Although acute rejection did not occur in this group the MST±SE was 28.5±6.8 days. Seven out of 8 animals developed severe clinical (grade 3) GVHD (Table 2). These animals not only developed a severe redness of ears, snout, and paws, but also had a severely inflamed ventral neck region, a hunched posture, and tip-toed on their hind legs. In most animals clinical GVHD started between day 13 and 16 posttransplant, and lasted until 17 to 25 days posttransplant. Donor-pretreatment with 5 Gray gamma-irradiation on the day of transplantation completely abolished clinical and histologic GVHD. The MST was 31.1±5.7 days, which was not significantly different from FK-506 treatment alone.

Table 2: Rejection and graft-versus-host disease after FK-506 treatment.

survival days	clinical GVHD[1] days posttx	histological GVHD[2] at time of death	histological rejection	cause of death
20	-	nd	nd	eci
21	16 - 19	severe	minimal[3]	GVHD
21	16 -	nd	nd	rejection (macroscopically)
21	13 - 17	mild	severe	rejection (histologically)
22	16 - 22	moderate	minimal	GVHD
23	6 - 23	nd	nd	rejection (macroscopically)
24	13 - 24	moderate	minimal	severe pneumonia
76	15 - 25	none	chronic	mild chronic rejection

FK-506 was given at a dose of 2 mg/kg on days 0, 1, 2, 4, and 6 after grafting.
nd = not done.
1: all animals had severe clinical GVHD, characterized by severe redness of ears, snout, paws, and a severely inflamed ventral neck region.
2: histologic GVHD was characterized by dermatitis of the skin of ear and lip, infiltration of the portal triads of the liver, apoptosis of few bile ductular epithelial cells, few apoptotic salivary gland duct epithelial cells, and depletion of lymphocytes in thymus and spleen. Moderate and mild GVHD lesions were limited to the skin of ears and lip.
3: there was minimal evidence of graft rejection, characterized by scattered apoptosis of crypt epithelial cells.

Histology

Lesions of severe GVHD were characterized by marked acanthosis of the epidermis of ears and lip with scattered dyskeratotic keratinocytes. The dermis was infiltrated by a moderate number of macrophages and lymphocytes mixed with few neutrophils. Portal triads of the liver were infiltrated by a moderate number of mononuclear cells and there was apoptosis of a few bile ductular epithelial cells. A few apoptotic epithelial cells were seen in the submandibular salivary gland duct epithelium. Moderate and mild GVHD lesions were less severe and limited to the skin of ears and lip. The thymus was depleted of

lymphocytes. There was necrosis and depletion of lymphocytes in the splenic white pulp and lymph nodes. Animals with GVHD had minimal evidence of graft rejection, characterized by apoptosis of scattered crypt epithelial cells. After donor pretreatment no signs of histologic GVHD could be demonstrated, all grafts were rejected.

DISCUSSION

Most studies on the immunosuppressive capacities of FK-506 have shown that it is a highly potent agent in preventing allograft rejection. Hoffman et al. (1990), performed an extensive study on the use of FK-506 for small intestine transplantation in rats, and found that long courses of FK-506 are more effective than CsA in the prevention of acute rejection and lethal GVHD in one-way Parent to F1-hybrid models. Short courses of 2 mg/kg given on days 0-6 were effective in preventing rejection in a fully-allogeneic model and the survival times were significantly longer than after CsA treatment. No clinical GVHD was observed.

In our study, we were unable to find superior immunosuppressive effects of FK-506 over CsA. Graft survival after a short course of FK-506 was significantly worse when compared to CsA treated animals. Moreover, FK-506 permitted the development of severe clinical GVHD, with the corresponding histopathologic lesions including dermatitis and lymphoid depletion of thymus and spleen. After donor-irradiation no clinical GVHD was observed, but this had no effect on graft survival. The immunosuppressive effects of mild GVHD we observed in earlier studies (Saat et al. 1989) did not occur after FK-506 treatment. The observed GVHD was clearly harmful to the recipient.

Taken together the severe GVHD that follows FK-506 treatment in our model, and the recent findings indicating that FK-506 may accentuate heart graft coronary disease (Arai et al. 1991), one of the main lesions of chronic rejection, we feel there is reason to stay cautious about the use of FK-506 as mainstay of future immunosuppressive protocols in SBT.

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CHAPTER 9

FUNCTIONAL ASPECTS OF SMALL BOWEL TRANSPLANTATION IN RATS

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Scand. J. Gastroent. 1992; 27: 483-488.

SUMMARY

Although clinical small bowel transplantation (SBT) is still severely hindered by rejection of the graft, prolonged graft survival can be achieved using cyclosporine A (CsA) in several experimental models of SBT. In an immunologically quiescent phase after transplantation, the important question arises whether a small bowel (SB) allograft has enough functional capacity to maintain a normal nutritional status. We investigated the functional capacity of orthotopic SB transplants, and evaluated the ability of the total small bowel transplant to absorb orally given CsA in the early postoperative period, and the effect of this oral CsA treatment on allograft survival as compared to intramuscular administration. Between 3 and 7 months postoperatively, recipients of syngeneic-, and allogeneic total SB transplants and as syngeneic jejunal segmental grafts had significantly decreased serum triglyceride levels. Total serum protein and albumin concentrations, serum cholesterol values, fecal fat excretion, and percentage of split fatty acids were normal. One year after transplantation weight in the groups transplanted with a total SB graft was not different from age-matched untreated controls. Animals grafted with a segmental graft, however, showed a significantly impaired growth and had not reached a normal weight 1 year after transplantation. Growth was also significantly impaired after near-total SB resection. These animals had to be killed because of their poor condition. CsA absorption after SBT equalled that in normal controls. Graft survival after intramuscular treatment was significantly better than after oral treatment.

INTRODUCTION

Small bowel transplantation (SBT) would be the therapy of choice for patients with the short bowel syndrome. These patients are now being treated with total parenteral nutrition. Although great improvements have been made with the use of this life support system, problems such as recurrent catheter sepsis, thrombosis of veins, and liver damage will eventually result in patient morbidity and mortality (Grosfeld et al. 1986). For these patients a safe SBT would greatly enhance the quality of life or could even be life-saving.

Clinical SBT is still severely hampered by rejection of the graft; of several recently transplanted patients only few have a long-term functioning graft (Schroeder et al. 1990). At the moment clinical SBT can not be considered as a true therapeutic option (Watson & Lear 1989). Although several experimental studies on the function of small bowel grafts have been performed, there is as yet no conclusive answer as to whether a total small bowel graft has enough functional capacity to maintain a normal nutritional status, or to sustain normal growth and development in juvenile subjects. This question becomes even more urgent when segmental SBT is considered. In living-related transplantation, in which only partial resection of the donor bowel is possible, and paediatric transplantation in which there is size dissimilarity in abdominal space between donor and acceptor, or in attempts to reduce graft-versus-host-disease, segmental transplantation would be the treatment of choice.

Changes in the function of the graft can be anticipated because of the transplant procedure itself, and the immunological processes which will take place. It is thought that cyclosporine A (CsA) therapy after transplantation should be given parenterally instead of orally because of the impaired capacity of the disrupted lymphatics of the transplant to absorb fat and fat soluble substances (Schraut, 1988). The aim of the present study was to investigate the effects of ischaemia, neural-, and lymphatic division as a result of the transplant procedure, and of rejection damage as a result of allogeneic transplantation on the function of small bowel grafts. Secondly, we investigated whether a transplant is able to absorb orally given CsA, and what effect this route of administration has on graft

survival as compared to intramuscular treatment.

MATERIALS AND METHODS

Animals: rats of the inbred BN ($Rt1^n$) and WAG ($Rt1^u$) strains were used as described in chapter 2.

Small bowel transplantation: SBT was performed as described in chapter 2. In brief, the total small bowel was harvested from the ligament of Treitz to the terminal ileum, along with a vascular pedicle consisting of the superior mesenteric artery and portal vein. Segmental grafts consisted of ±10 cm of proximal jejunum. In the recipient the infrarenal aorta and caval vein were clamped and end-to-side anastomoses were performed between the recipient aorta and caval vein and the donor superior mesenteric artery and portal vein respectively. The recipient small bowel was resected, and the graft was placed in-continuity by end-to-end anastomoses, proximally with the host's duodenum and distally to the remaining terminal ileum.

Postoperative monitoring: all rats were weighed 3 times a week during the first month, weekly until 3 months posttransplant and every other week during the rest of the follow-up period. All animals that died within 4 days were considered technical failures.

Immunosuppression: CsA was administered as described in chapter 2.

Biochemistry determinations: total serum protein concentration was measured by means of the Biuret method and albumin levels by means of the bromcresol-green method (Boehringer Mannheim GmBH). Triglyceride levels were determined colorimetrically, using the triglycerides GPO PAP system (Boehringer Mannheim GmBH). The Monotest cholesterol (Boehringer Mannheim GmBH) was used to measure serum cholesterol levels. Fecal fat excretion and percentage of split fatty acids, were determined using the method of van de Kamer et al. (1949). The pH of feces was measured using a standard pH electrode. Plasma CsA trough levels were measured using the cyclo-Trac SP RIA kit (INCSTAR Corp. Stillwater, Minnesota, USA).

Statistics: for statistical analysis of the nutritional data analysis of variance

(ANOVA) followed by a Student-Newman-Keuls t-test were performed using SPSS/PC+. Survival data were analysed using Wilcoxons' rank-sum test.

EXPERIMENTAL GROUPS

Experimental groups to study functional variables;

Group 1: normal healthy WAG rats (n=5).

Group 2: syngeneic total SBT (length approximately 45 cm) from WAG to WAG rats (n=5).

Group 3: syngeneic segmental SBT (±10 cm of proximal jejunum) from WAG to WAG rats (n=3).

Group 4: fully allogeneic transplantation of the total small bowel (length approximately 45 cm) from BN to WAG rats (n=7). To prevent rejection, recipients were treated with CsA, 15 mg/kg given intramuscularly on days 0,1,2,4, and 6 after transplantation.

Group 5: near total small bowel resection in WAG rats. The remnant of the terminal ileum (± 5 mm) was anastomosed to the duodenum (n=3).

Experimental groups to study CsA absorption and allograft survival;

Group 6: normal healthy BN rats treated with CsA, 25 mg/kg orally on days 0,1,2,4, and 6 (n=4).

Group 7: fully allogeneic transplantation of the total small bowel from WAG to BN rats. CsA, 25 mg/kg was administered orally on days 0,1,2,4, and 6 after transplantation (n=5).

Group 8: short bowel controls; BN rats underwent near total small bowel resection and subsequent anastomoses of the bowel remnants. The animals were given CsA, 25 mg/kg orally on days 0,1,2,4, and 6 after surgery (n=2).

Group 9: fully allogeneic transplantation of the total small bowel from WAG to BN rats. CsA, 25 mg/kg was given intramuscularly on days 0,1,2,4, and 6 after transplantation (n=10).

Plasma trough levels of CsA were measured at regular intervals in groups 6, 7, and 8.

RESULTS

Nutritional variables

Between 3 and 7 months after transplantation the following parameters were studied: body weight, total serum protein, albumin and triglyceride levels, cholesterol values, amount of fecal fat, percentage of split fatty acids in feces, and the pH of the feces.

Total serum protein and albumin levels were equal in all the groups tested (Table 1), except in the short bowel control animals (group 5), in which all the nutritional parameters were significantly lowered. Serum triglyceride levels, however, were significantly reduced as compared with normal, healthy WAG rats (group 1) in the syngeneic total small bowel transplanted animals (group 2), the animals transplanted with 10 cm of proximal jejunum (group 3), the group transplanted with an allogeneic total small bowel treated with CsA (group 4), and the short bowel control group. There were no significant differences in cholesterol levels between the groups examined. Fecal fat excretion in group 2 was decreased. There were no differences in the percentage of split fatty acids in feces among any of the groups. The pH of the feces in all the groups was $\geq$ 6.3.

Recipient growth

Fig. 1 shows the weight curves of the small bowel resected rats (group 5) and the animals transplanted with a syngeneic total small bowel from WAG to WAG (group 2) in which no rejection occurs. There is a clear benefit from syngeneic SBT in terms of growth as compared to animals without a small bowel. Although one short bowel control rat even increased in weight the two others only lost weight. Despite their short bowel status, the animals lived on standard laboratory rat chow for a long time. Eventually these animals had to be killed on days 28, 61, and 196 after surgery because of their poor condition.

Up to 1 year after transplantation, there were no significant differences in growth among normal age-matched WAG rats and syngeneic and allogeneic total small bowel transplanted animals (Fig. 2). The animals transplanted with a jejunal segmental graft were significantly impaired in their growth and, in contrast to the other transplanted groups had not reached a normal weight after 1

Table 1. Nutritional status after small bowel transplantation. Mean values ± standard deviation and statistical significances.

group	serum protein (g/l)	serum albumin (mg/ml)	serum cholest (mmol/l)	serum triglyc (mmol/l)	fecal fat (g/100g)	fecal fatty acid (% split)
1.	69.0±1.3	37.4±0.5	1.6±0.1	1.2±0.3	3.2±0.2	70±2
2.	65.0±1.5	35.0±2.6	1.9±0.4	0.9±0.2*	2.3±0.4*	73±9
3.	67,1±1.3	33.4±1.1	1.5±0.1	0.6±0.2*	2.9±0.4	78±4
4.	n.d.	34.2±1.8	1.5±0.3	0.9±0.1*	3.0±0.5	71±11
5.	35.4±8.2*	27.9±6.8*	0.4±0.2*	0.4±0.1*	n.d.	n.d.

Functional parameters were studied between 3 and 7 months after transplantation.
Group 1: normal healthy WAG rats; group 2: syngeneic total small bowel transplantation from WAG to WAG rats; group 3: syngeneic segmental small bowel transplantation from WAG to WAG rats; group 4: fully allogeneic transplantation of the total small bowel from BN to WAG rats; and group 5: near total small bowel resected WAG rats.
*: $p<0.05$, Student-Newman-Keuls t-Test compared with group 1.

year.

CsA absorption after oral administration in SBT

Twenty-four hours after transplantation and after CsA administration high levels were measured (Fig. 3). In normal, healthy BN rats the mean level was 765 ± 47 (mean ± SE) ng/ml, in transplanted animals 882 ± 270 ng/ml, and in short bowel controls 14 ± 4 ng/ml. On days 2, 4, and 6 after CsA administration even higher levels were found both in the normal animals (group 6) and in the transplanted animals (group 7). The small bowel resected animals (group 8) failed to absorb considerable amounts of CsA.

The effect of oral CsA administration on graft survival was inferior to intramusculair treatment, the former giving a mean graft survival time (MST) of 24.2 ± 16 days, the latter a MST of 38.3 ± 27 ($p<0.05$, see Table 2).

Table 2. Small bowel allograft survival after oral and intramuscular cyclosporine treatment.

treatment[1]	survival in days	MST ± SD
oral	8,8,27,36,43	24.2 ± 16
intramuscular	8,11,13,13,21,55,59,65,66,72	38.3 ± 27.0[2]

1: cyclosporine was given at a dose of 25 mg/kg on days 0,1,2,4, and 6 after transplantation.
2: $p < 0.05$ versus group 2, Wilcoxon rank-sum test.

DISCUSSION

In this study we show that SBT is able to overcome the symptoms of a short bowel syndrome. The nutritional status of the animals transplanted with a syngeneic total small bowel graft was significantly better than that of animals that underwent near-total small bowel resection. Moreover, the transplanted animals grew whereas short bowel controls showed a severely impaired development, or were unable even to maintain their preoperative body weight. This is in accordance with results obtained in dogs by Diliz-Perez et al. (1984), and in rats by Oki et al. (1989), and Kimura et al. (1987), the only other studies in which a short bowel group was investigated. We could not confirm the earlier finding by Sonnino et al. (1989) that in BN rats the cecum has to be resected to create a lethal short bowel syndrome after extensive small bowel resection. In their study, however, 3 cm of small bowel was left, whereas in our study only about 1 cm remained. This underlines the importance of near-total resection, since only a few cm of preserved small bowel apparently enables sufficient adaptation to sustain the animal's growth.

There were no significant differences in total serum protein and albumin levels among the different groups. In contrast to Raju et al. (1989), who found normal total serum protein levels in spite of decreased albumin levels caused by an increase in globulin in a dog model of autotransplantation, the normal total

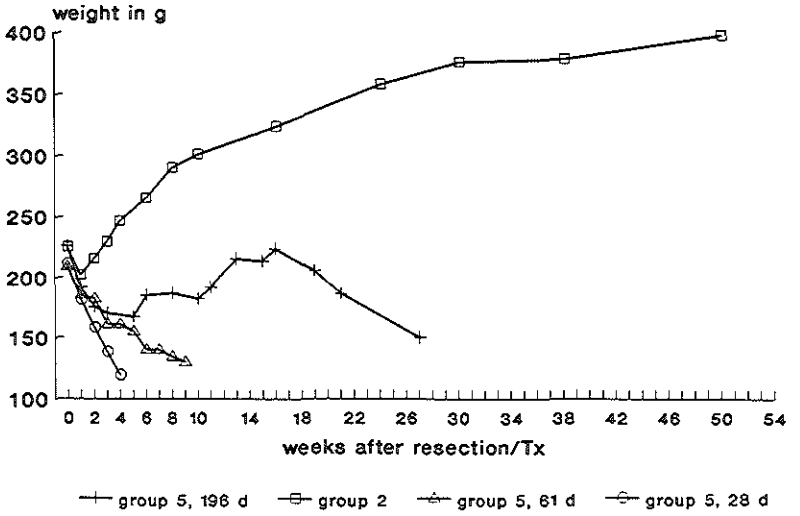

Fig.1. Growth curves of animals transplanted with a syngeneic total small bowel graft (group 2, n=5), and animals that underwent near-total small bowel resection (short bowel control group, n=3). Because of the great interindividual variation small bowel resected animals are depicted individually.

serum protein levels in our study are attributable to serum albumin itself.

Schraut et al. (1987) also found in a rat model that serum albumin levels were equal to those in normal rats in segmental and total small bowel grafts 12 months postoperatively. Normal serum albumin levels were also found in a rat total small bowel transplant group but, in contrast to our findings, they were significantly lowered in segmental transplants (Oki et al. 1989). As has been previously reported by others (Oki et al. 1989, Raju et al. 1989) serum cholesterol values were normal in all the transplanted groups. Serum triglycerides were significantly lowered in all the experimental groups, but neither fecal fat excretion nor the percentage of split fatty acids in feces were altered. Others have found normal triglyceride levels both in segmental and in total small bowel grafts (Schraut et al. 1987, Raju et al. 1989).

Both fecal fat excretion and percentage of split fatty acids in feces were unimpaired, except in the syngeneic total small bowel transplanted animals, in which, for unknown reasons, fecal fat excretion was decreased. Others have

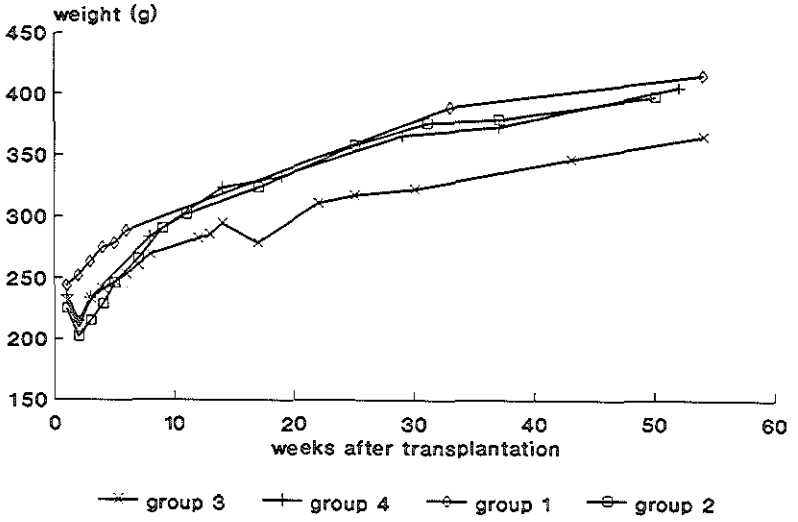

Fig.2. Growth curves of normal, healthy WAG rats (group 1, n=5), syngeneic (group 2, n=5), and allogeneic (group 4, n=7) total small bowel transplanted animals, and animals transplanted with a 10 cm syngeneic jejunal segmenal graft (group 3, n=3).

found an increased fecal fat excretion in rats (Schraut et al. 1987), and dogs (Diliz-Perez et al. 1984). In this study only triglyceride levels appeared to be a moderately sensitive marker of an impaired bowel function.

The weight curves of the animals did not correspond well with the above-mentioned nutritional variables. After an initial weight loss in the first 2 weeks after transplantation the syngeneic total small bowel grafted and allogeneic long-surviving animals began to grow steadily. At 30 weeks after transplantation, there were no differences in weight between the controls and the total small bowel transplanted animals, which confirms results obtained by Lee & Schraut (1986). The animals with a 10 cm syngeneic jejunal segment (± 20% of total length), however, showed a significantly impaired growth. Weight after 30 weeks and after 1 year was significantly less than that in unoperated controls or total small bowel transplanted groups. This shows that although the nutritional parameters did not differ significantly among each other, the weight curves did.

In this rat model of SBT, therefore, weight of the transplanted groups appears to

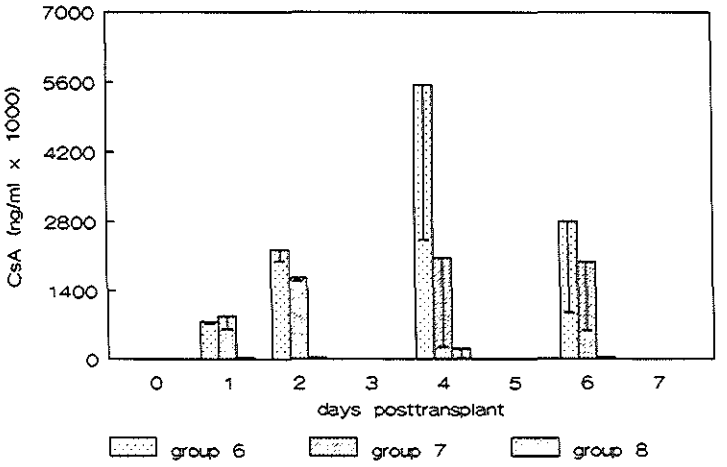

Fig.3. Cyclosporine plasma trough levels after oral administration; 25 mg/kg was given in a volume of 0,1-0,2 ml on days 0,1,2,4, and 6 after transplantation. Group 6: normal healthy controls (n=4), group 7: allogeneic total small bowel transplantation (n=5). Group 8: animals which underwent near total small bowel resection (n=2).

be the most important indicator for graft function. We are aware of the fact that weight gain alone might not be the optimal parameter for growth since it does not reflect the body composition of the animal. Possibly, total body composition analysis using total body electrical conductivity, will prove to be a useful tool (Bracco et al. 1983). The (near) normal nutritional parameters, however, at least give an indication that body composition of the animals might be acceptable.

Another important conclusion that we draw from this study is that the function of syngeneic segmental grafts is impaired when compared with syngeneic and even allogeneic total small bowel transplants. In clinical SBT, segmental transplantation will often be the treatment of choice when living-related transplantation or pediatric transplantation is considered. Strategies must therefore be developed to increase the functional capacity of segmental grafts. The importance of this is further stressed by findings in large animal models which show that both after autotransplantation (Raju et al. 1989), and after allotransplantation of the entire small bowel (Diliz-Perez et al. 1984), at best the

animals attained their preoperative weight, but never exceeded it.

It is generally thought that CsA absorption after transplantation will be impaired because the disrupted lymphatics of the graft are unable to transport this lipophilic drug to the blood (Schraut, 1988). Here we show that CsA absorption after total small bowel resection is severely impaired, which stresses the importance of this site for its absorption. Consistent with findings by Aeder et al. (1984), and LaRosa et al. (1989), who found that oral and intraluminal CsA absorption within 1 week after transplantation did not differ significantly from preoperative or control values, oral CsA absorption directly after transplantation in our study gave the same plasma levels as in untreated controls. This indicates that there must be an alternative mechanism by which CsA is delivered to the blood. By administering CsA intraperitoneally in normal dogs, Cohen et al. (1983) have shown that some absorption may take place via the peritoneum. It is possible that after transplantation CsA in the lymph leaks into the peritoneal cavity through the disrupted lymphatics, and is subsequently reabsorbed. So even before lymph vessel continuity is re-established 4 to 10 weeks posttransplant, as is conclusively shown to happen (Kocandrle et al. 1966, Schmid et al. 1990), CsA can be administered orally. This reabsorption probably does not account for all the CsA absorbed, since lower levels are found after intraperitoneal than after oral administration (Cohen et al. 1983). It was shown that CsA absorption is highly variable and unpredictable during the early postoperative period, and, moreover, rejection can impair the ability of the graft to absorb CsA (Cohen et al. 1988). It therefore seems justified to advocate parenteral administration as the main route, even when lymphatic connections between donor and recipient are established. Whether concomitant oral treatment can be of benefit owing to local immunosuppressive effects in the graft needs to be investigated.

In conclusion, this study shows that SBT is able to overcome the symptoms of a short bowel syndrome. Nutritional variables after syngeneic and allogeneic transplantation are unimpaired, except for plasma triglyceride levels. Recipient growth seems to be the most important parameter for graft function in this model and reveals that segmental jejunal grafts are unable to sustain a normal

growth of the recipient. Oral CsA can be absorbed by small bowel grafted animals as efficiently as by normal controls. Immunosuppression is better after intramuscular administration.

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CHAPTER 10

GENERAL DISCUSSION

Chapter 10

GENERAL DISCUSSION

The small bowel allograft has proven to be one of the most difficult organs to transplant; it is highly immunogenic, and lymphocytes originating from the mesenteric lymph nodes and Peyer's patches have the potential to induce graft-versus-host disease (GVHD). Nevertheless, since small bowel transplantation (SBT) is considered the only causal therapy for patients with the short-bowel syndrome, a considerable effort is being made by several groups of investigators to make SBT a standard procedure for those with short bowel syndrome who would depend on total parenteral nutrition for life.

The studies described in this thesis were performed to investigate various immunological and functional aspects of SBT, with the ultimate goal to obtain a long-term surviving, properly functioning small bowel transplant.

In the fully-allogeneic rat model both rejection and GVHD may occur following SBT. In most models however, rejection predominates. In our BN to WAG rat donor-recipient combination rejection takes place in approximately 12 days in untreated recipients of a small bowel allograft (de Bruin et al. 1990[a], Chapter 7, this thesis). Fifty percent of these animals develop a mild, transient form of GVHD that starts some 6 days after grafting and lasts for approximately 3 days. In the reverse WAG to BN donor-host combination graft rejection usually takes place in 17 days (de Bruin et al. 1990[a]). Thirty-five percent of these animals develop GVHD that begins 9.5 days after transplantation and lasts about 4 days. Abrogation of GVHD was obtained by irradiation, or by anti-lymphocyte serum treatment of the donor. These modalities effectively prevented GVHD, but at the same time graft rejection was accelerated. These findings were reported also following bone marrow transplantation, in which effective anti-rejection therapy is associated with an increased incidence of GVHD (Storb, 1989). These findings have led to the hypothesis that a balance exists between rejection and GVHD, and that the prescence of GVHD ameliorates the rejection process (Cohen et al. 1976, Storb et al. 1989, Saat et al. 1991, de Bruin et al. 1992). This is further supported by the finding that combined liver/small-bowel transplantation induces GVHD and leads to permanent graft acceptance in a rat strain combination in

which SBT alone leads to rapid rejection without occurrence of GVHD (Zhong et al. 1991). It is uncertain whether this balance can be manipulated in man in order to use the immunosuppressive effects of GVHD. We have shown that irradiation of the donor with 5 Gray, a dose which fails to prevent the onset of GVHD in a parent to F1 model (Lee & Schraut, 1985), prevented the occurrence of clinical GVHD in a fully-allogeneic model. However, when recipients of these grafts were treated with cyclosporine A (CsA) similar survival data were obtained as in recipients that had received untreated grafts. This is in contrast with survival data after donor irradiation with 10 Gray when CsA failed to prolong survival as efficiently as in the previously mentioned groups. It thus seems possible to manipulate GVHD in such a way that its immunosuppressive properties are maintained, while the clinical symptoms are prevented. At present, no such delicate immunosuppression is known in the human situation and until this is the case we argue that suppression of GVHD by pretreating the donor, or graft, is preferred since GVHD is unlikely to be of benefit to a SBT patient, and its immunosuppression is uncontrollable and unpredictable. Moreover, by pretreating the graft its immunogenicity is concomitantly reduced, thereby prolonging graft survival time (Williams et al. 1988). We have found that GVHD may be more severe in immunosuppressed than in untreated recipients (de Bruin et al. 1990[a], de Bruin et al. 1991, Chapters 5 and 7, this thesis). After pretreatment of the recipient with CsA some animals display a severe form of GVHD, probably because graft lymphocytes are "privileged" since they are transferred to a host in which the lymphocytes are already in contact with CsA. This is not the only mechanism, because severe GVHD was also seen after postoperative administration of CsA or FK-506 only (de Bruin et al. 1990[a], de Bruin et al. 1991, Murase et al. 1991). Although these observations are interesting from an immunological point of view, they may have little impact on clinical transplantation, since it is now firmly established that pretreatment of the donor or the graft can effectively prevent the occurrence of GVHD (de Bruin et al. 1992[a]).

CsA as immunosuppressant prolonged graft survival in the WAG to BN as well

as the BN to WAG model, but in the WAG to BN model it was hard to obtain consistent long-term survival using moderate to high doses of CsA (de Bruin et al. 1990[a]). Surprisingly, low doses of CsA gave better results; with a low-dose maintenance therapy of 5 mg/kg CsA until day 100 posttransplant, high responder BN recipients of a WAG-graft had a mean survival time of 121 days, with 67% of the animals surviving long-term (Chapter 6, this thesis). This could mean that the high dosages used previously were toxic to the bowel graft and hence negatively influenced graft survival. Indications for this are the reported toxicity of CsA on both large and small bowel (Innes et al. 1988, Crane et al. 1990, Sigalet et al. 1992) and the clinical findings that patients with a stable graft do not reject their graft despite relatively low doses of CsA or FK-506 (Goulet et al. 1992, Todo et al. 1992). We feel therefore that the use of low-dose CsA needs more attention and that CsA may prove to be an effective drug in SBT.

Specific immunomodulation as a means of improving graft survival could also benefit small bowel allograft survival. Therefore several investigators have tested the effect of donor-specific blood transfusions (DST) on the survival of small bowel allografts. Unlike in other organ grafts, DST alone did not improve the survival of SBT (Martinelli et al. 1988, de Bruin et al. 1990[b], Fecteau et al. 1992, Fukuzawa et al. 1991). A synergistic effect between DST and CsA (Martinelli et al. 1988) and FK-506 (Fukuzawa et al. 1991) has been reported, although we (de Bruin et al. 1990[b]) and others (Fecteau et al. 1992) found no synergism. These data are inconclusive as to whether DST are beneficial to small bowel allograft survival and this needs further attention.

Matching of donor and recipient for MHC-antigens may also be helpful in ameliorating the rejection response. This has been convincingly shown by Meijssen et al. (1992). They showed in a dog model that CsA treatment led to a significant prolongation of graft survival in fully MHC-matched donor-recipient combinations as compared to MHC-unidentical combinations. One clinical SBT has been performed in a well-matched donor-recipient combination; 5 episodes of acute rejection occurred, but good graft function was obtained long-term, using CsA as main immunosuppressant (Deltz et al. 1990). Therefore tissue

matching may prove helpful in improving the outcome of SBT.

Because a small bowel transplant is denervated, has suffered ischemia and reperfusion injury, its lymphatics are disrupted, and is under immunologic attack of the host, changes in its function may be expected. Indeed, changes have been found, among others, in stool fat excretion (increased, Schraut et al. 1987), motility (impaired, Sarr et al. 1989), bacterial colonization (overgrowth, Browne et al. 1992), and permeability (increased, Sigalet et al. 1992). Whether or not these changes have an impact on the well-being and weight course of the recipient on the long-term is still uncertain. Rats transplanted with a total small bowel isograft or allograft grow normally, and the only child who has a functioning transplant is growing and developing normally (Goulet et al. 1992). However, rats grafted with segmental small bowel transplants may develop impaired nutritional parameters (Schraut et al. 1987), or grow suboptimally (Oki et al. 1992, de Bruin et al. 1992[b]). In view of this it seems reasonable to transplant as much bowel length as possible.

No standard protocol for preservation of the small bowel graft has been developed, nor is it known what maximal ischemic time is tolerable. In a recent series of successful SBT a simple preservation method proved to be appropriate; simple core cooling of the graft with University of Wisconsin solution without extensive flushing of the capillary bed, followed by immersion in an ice bath. Even intraluminal washing was omitted, leaving the succus entericus in the graft, with no consequent infection from this practice (Todo et al. 1992). Reported ischemic times are up to 16 hours (D'Allesandro et al. 1992).

Restoration of graft barrier function in order to prevent bacterial translocation, and prevention of rejection with either new, more potent drugs or with a combination of drugs and specific immunomodulation of donor and recipient are needed to improve the successrate of SBT. An early marker of rejection could obviate the need for graft biopsies. On the long-term, chronic rejection may become the major limiting factor of good graft function.

It should be noted that in most successful clinical cases not only the small bowel but also the liver was grafted. The rejection process seems to be less aggressive

in these combined-graft recipients. It is important to elucidate the mechanism by which the small bowel is protected from rejection in these cases, since this may be an important step forward.

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SUMMARY, SAMENVATTING

SUMMARY

Small bowel transplantation (SBT) would be the treatment of choice for patients with short-bowel syndrome who depend life-long on total parenteral nutrition. Although this latter therapy is life-saving for these patients, it is by no means ideal, since it is associated with significant morbidity and mortality from complications such as hepatic failure and sepsis.

The small bowel graft is unique among vascularized organ grafts in that it includes large amounts of lymphoid tissue. This has two potentially negative consequences; it may increase the immunogenicity of the graft, and alloreactive cells in the graft have the potential to induce graft-versus-host-disease (GVHD). Unfortunately, these problems still hamper successful clinical SBT as an established therapy. In this thesis experiments are described performed with the aim to study rejection and GVHD, their possible interaction, and strategies for their prevention after SBT. Moreover, some functional aspects of SBT were studied.

In Chapter 3 it was found that irradiation of the donor with 5 or 10 Gray (Gy) eliminated clinical GVHD, but significantly shortened the survival time of small bowel allografts in untreated recipients. Cyclosporine A (CsA) treatment of the recipient combined with 10 Gy donor-irradiation led to significantly reduced graft survival time as compared with CsA treatment alone, whereas after irradiation with 5 Gy, survival times equalled those without graft irradiation. We concluded that irradiation effectively prevents GVHD, and that rejection is accelerated when GVHD is absent in this fully-allogeneic model. Therefore it was postulated that high dosages of irradiation could either increase the vulnerability of the graft, or, by eliminating GVHD, remove its immunosuppressive effect.

In Chapter 4 it was shown that treatment of the donor with anti-lymphocyte serum eliminated clinical GVHD, and, as was also found in Chapter 3, this led to significantly shortened survival times. Postoperative CsA treatment of the recipient overruled this accelerated rejection and led to graft survival times comparable to those found with CsA treatment alone. In this combined

treatment group no clinical GVHD was observed. CsA used for both donor pretreatment and postoperative therapy had was no survival advantage or effect on GVHD compared to postoperative CsA treatment alone. The results obtained in Chapters 3 and 4 show that there is a balance between rejection and GVHD, and that abrogation of GVHD leads to accelerated rejection. Immunosuppression given to the recipient may overrule this and lead to long-term graft survival without GVHD.

In Chapter 5 it was found that it was difficult to achieve long-term graft survival using CsA monotherapy in the WAG to BN donor-recipient combination. Long-surviving animals that had received CsA maintenance therapy until day 100 posttransplant died from chronic rejection after cessation of CsA therapy. In contrast, unresponsiveness could easily be induced in the BN to WAG donor-host combination. Fifteen mg/kg of CsA given on days 0,1,2,4, and 6 after transplantation led to permanent graft acceptance in 60% of the cases. We concluded that the effect of CsA on rejection and GVHD following SBT is dependent on the rat strain combination used. The WAG to BN combination exhibits some of the immunological problems seen in large animal models, whereas long-term graft survival can be easily induced in the BN to WAG model.

In the WAG to BN donor-recipient combination, pretreatment of the recipient with CsA did not lead to statistically significant prolongation of graft survival, and was associated with the occurrence of severe GVHD. It was found, however, that with previously untested low-dose CsA maintenance therapy it was possible to obtain long-term graft survival. It was concluded that postoperative low-dose CsA maintenance therapy with or without donor-irradiation is of benefit to the long-term survival of small bowel allografts (Chapter 6).

In the BN to WAG model, donor-specific blood transfusions given to the recipient are very effective in prolonging the survival of heart and kidney allografts. In Chapter 7 it was found that pretreatment of the recipient with 3 donor-specific blood transfusions on days -21, -14, and -7 before transplantation had no effect on graft survival after transplantation of a total small bowel or a

10 centimeter long segment of jejunum or ileum. Combined treatment of the recipient with donor-specific blood transfusions and CsA did not improve graft survival compared with CsA alone. Unexpectedly, after preconditioning with donor-specific blood transfusions, fewer rats showed signs of GVHD. It was concluded that donor-specific blood transfusions do not have an effect on small bowel allograft survival, but ameliorate GVHD.

In the WAG to BN donor-host combination, the immunosuppressive agent FK-506 appeared to be less effective in controlling rejection and GVHD than CsA. After administration of 2 mg/kg FK-506 on days 0,1,2,4, and 6 after grafting, mean survival was prolonged to 28.5 days, and 88% of these animals developed fulminant GVHD. Additional irradiation of the donor with 5 Gy prevented GVHD, but did not lead to better survival times (Chapter 8).

In Chapter 9 we showed that SBT is able to overcome the symptoms of short bowel syndrome. Growth of animals transplanted with a syngeneic or allogeneic total small bowel graft equalled that of healthy control animals. Since transplantation of a segment of bowel may offer some advantages, animals transplanted with a 10 centimeter long jejunal graft were studied. These animals showed significantly impaired growth. CsA absorption after oral administration in the early postoperative phase was not significantly different from unoperated controls, but graft survival after intramuscular CsA administration was significantly better than after oral administration.

Chapter 10 generally discusses the significance of the results presented in Chapters 3 to 9, and gives some leads to further investigations.

SAMENVATTING

Transplantatie van de dunne darm is theoretisch de beste behandeling voor patiënten met het "short bowel" syndroom. Dit ziektebeeld, dat ontstaat na uitgebreide darmresectie, wordt gekenmerkt door diarree, steatorrhoe, malnutritie en gewichtsverlies. Sinds 1968 worden deze patiënten behandeld met totale parenterale voeding. Bij een deel van de patiënten adapteert de restdarm, waardoor de patiënt uiteindelijk geheel of gedeeltelijk enteraal gevoed kan worden. Bij een subgroep van deze patiënten treedt geen adaptatie op, waardoor zij levenslang afhankelijk zijn van totale parenterale voeding. Deze therapie is echter verre van ideaal vanwege de complicaties die ermee geassocieerd zijn, zoals leverfalen en sepsis. Voor deze patiënten zou dunne darmtransplantatie uitkomst bieden.

Echter, de dunne darm bevat veel lymfoid weefsel in de Peyerse plaques, de mesenteriale lymfklieren, en de darmwand. Hierdoor is de darm een zeer immunogeen orgaan, en bovendien zijn alloreactieve cellen in het transplantaat in staat om in de ontvanger van dat transplantaat een zogenaamde graft-versus-host reaktie (GVHR) te veroorzaken. Deze problemen staan klinische toepassing van dunne darmtransplantatie als standaardtherapie voor mensen met het "short bowel" syndroom nog steeds in de weg.

In dit proefschrift worden experimenten beschreven die uitgevoerd zijn in de rat teneinde afstoting en GVHR, de mogelijke interactie tussen deze processen en de beïnvloeding ervan te bestuderen. Daarnaast zijn nog enkele functionele aspecten van dunne darmtransplantatie bestudeerd, met het uiteindelijke doel om dunne darmtransplantatie in de mens mogelijk te maken.

In hoofdstuk 1 wordt een overzicht gegeven van de huidige stand van zaken in het dunne darmtransplantatie onderzoek.

In hoofdstuk 3 wordt beschreven dat na bestraling van de orgaandonor met 5 of 10 Gray (Gy) de GVHR onderdrukt werd, maar dat tegelijkertijd een significante verkorting van de overlevingstijd van het transplantaat werd gevonden. Behandeling van de transplantaat ontvanger met het immunosuppressivum cyclosporine A (CsA), gecombineerd met bestraling van de

donor met 10 Gy, leidde tot een verkorte overlevingstijd ten opzichte van CsA behandeling alleen. CsA behandeling gecombineerd met 5 Gy bestraling van de donor leidde tot eenzelfde overlevingstijd als behandeling met CsA alleen. In deze groep werd echter geen klinische GVHR meer gezien. Er werd gekonkludeerd dat bestraling van de donor effectief de GVHR kan onderdrukken, en dat dit leidt tot een versnelde afstoting van het darmtransplantaat. De mogelijke oorzaak hiervan is dat hoge doses bestraling het transplantaat kunnen beschadigen, of dat het immunosuppressieve effect dat veroorzaakt wordt door GVHR, bij afwezigheid van GVHR een snellere afstoting mogelijk maakt.

In hoofdstuk 4 werd gevonden dat behandeling van de donor met anti-lymfocyten serum (ALS) de GVHR onderdrukte en dat, zoals ook in hoofdstuk 3 werd gevonden, tegelijkertijd de overlevingstijd korter werd. Behandeling van de ontvanger met CsA, in combinatie met voorbehandeling van de donor met ALS, leidde tot een overlevingstijd vergelijkbaar met CsA behandeling alleen, en ook hier werd geen GVHR meer gezien. Wanneer CsA zowel aan de donor als aan de ontvanger werd toegediend, werd geen betere transplantaatoverleving of verandering in optreden van GVHR gezien in vergelijking tot CsA behandeling van alleen de ontvanger. De resultaten die beschreven zijn in de hoofdstukken 3 en 4 geven aan dat er een balans bestaat tussen afstoting en GVHR; wanneer de GVHR wordt onderdrukt treedt er een versnelde afstoting op, die echter teniet kan worden gedaan door de ontvanger te behandelen met CsA. Dit leidt dan tot een langdurige overleving in afwezigheid van klinische GVHR.

In de WAG naar BN-rat donor-ontvanger combinatie bleek het moeilijk om langdurig overlevende dieren te krijgen met CsA behandeling alleen. Dieren die langdurig hun transplantaat behielden onder CsA onderhoudstherapie, stierven alsnog aan de gevolgen van chronische afstoting wanneer deze onderhoudstherapie 100 dagen na transplantatie gestaakt werd. In de omgekeerde BN naar WAG donor-ontvanger combinatie kon al met een dosering van 15 mg/kg CsA gegeven op dagen 0,1,2,4, en 6 na transplantatie langdurige (>200 dagen) overleving verkregen worden in 60% van de gevallen.

Er werd gekonkludeerd dat het effect van CsA op afstoting en GVHR afhankelijk is van de donor-ontvanger combinatie die wordt gebruikt. In de WAG naar BN combinatie worden gedeeltelijk dezelfde problemen gezien als in het grote proefdiermodel, namelijk een moeilijk te beheersen afstotingsreaktie, terwijl in het omgekeerde model gemakkelijk langdurige transplantaatoverleving bereikt kan worden (hoofdstuk 5). In het WAG naar BN model leidde ook voorbehandeling van de ontvanger met CsA niet tot significant langere overlevingstijden vergeleken met postoperatieve CsA behandeling alleen. Wel trad bij voorbehandeling een ernstige vorm van GVHR op. In deze studie werd ook gevonden dat met een lage onderhoudsdosering van 5 mg/kg CsA langdurige transplantaatoverleving verkregen kon worden, en kon worden gekonkludeerd dat een lage dosis CsA als onderhoudsdosering alleen, of gecombineerd met bestraling van de donor, een langdurige transplantaatoverleving kan garanderen (hoofdstuk 6).

Donor-specifieke bloedtransfusies gegeven aan de ontvanger vóór transplantatie, leiden in het BN naar WAG model tot een langdurige verlenging van de overleving van hart-, en niertransplantaten. In hoofdstuk 7 wordt beschreven dat toediening van donor-specifieke bloedtransfusies aan de ontvanger niet leidt tot een langere darmtransplantaatoverleving. Het "bloedtransfusie effect" werd noch gevonden na transplantatie van de gehele dunne darm, noch na transplantatie van 10 centimeter jejunum of ileum. Gecombineerd met CsA therapie werd geen betere transplantaatoverleving gevonden dan na CsA behandeling alleen. Wel trad na toediening van donor-specifieke bloedtransfusies bij minder dieren GVHR op.

In de WAG naar BN combinatie bleek het immunosuppressivum FK-506 minder werkzaam te zijn tegen afstoting en GVHR dan CsA. Twee mg/kg FK-506 gegeven op dagen 0,1,2,4, en 6 na transplantatie gaf een gemiddelde overlevingstijd van 28,5 dagen. Achtentachtig procent van deze dieren ontwikkelden een ernstige vorm van GVHR. Wanneer de donor bestraald werd met 5 Gy werd geen GVHR meer gezien, maar de gemiddelde overleving werd niet beter (hoofdstuk 8).

Samenvatting

In hoofdstuk 9 wordt aangetoond dat een dunne darm transplantaat de symptomen van het short bowel syndroom kan genezen. De groei van dieren die een syngeen of allogeen darmtransplantaat hadden ontvangen was gelijk aan die van gezonde, niet getransplanteerde dieren. Omdat transplantatie van een deel van de dunne darm in sommige gevallen voordelen heeft, werden ook dieren bestudeerd die getransplanteerd waren met een transplantaat van 10 cm jejunum. Deze dieren groeiden slechter dan dieren die een gehele darm hadden ontvangen.

In de vroege postoperatieve fase verschilde de absorptie van oraal toegediende CsA niet van die in gezonde controle dieren. De immunosuppressie was na intramusculaire toediening echter beter dan na orale toediening.

In hoofdstuk 10 worden de resultaten besproken die gevonden werden in de hoofdstukken 3 t/m 9, en worden enkele suggesties voor verder onderzoek gedaan.

APPENDICES

LIST OF ABBREVIATIONS

ALS	anti-lymphocyte serum
CsA	cyclosporine A
DST	donor-specific blood transfusion
GVHD	graft-versus-host disease
GVHR	graft-versus-host reaktie
Gy	Gray
HBSS	Hanks balanced salt solution
HSBT	heterotopic small bowel transplantation
IEL	intra-epithelial lymphocyte
Ig	immunoglobulin
im	intramuscularly
ip	intraperitoneally
iv	intravenously
kg	kilogram
mg	milligram
MHC	major histocompatibility complex
ml	milliliter
MMC	migrating motor complex
MST	mean survival time
NAH	N-acetyl hexosaminidase
OSBT	orthotopic small bowel transplantation
PP	Peyer's patch
RAPA	Rapamycin
SB	small bowel
SBS	short bowel syndrome
SBT	small bowel transplantation
SD	standard deviation
SE	standard error of the mean
TNF-α	tumor necrosis factor alpha
TPN	total parenteral nutrition

NAWOORD

Op deze plaats wil ik diegenen die een essentiële bijdrage aan de totstandkoming van dit proefschrift hebben geleverd danken. Met name: mijn co-promotor dr. Richard Marquet. Beste Richard, toen ik zo'n 9 jaar geleden onder jouw hoede kwam was het allerminst de bedoeling dat ik zou promoveren, en ik was dat ook geenszins van plan. Door jouw stimulerende manier van begeleiden, het steunen van mijn eerste schreden op het pad van de wetenschap, en vervolgens door mij de vrijheid te geven serieuze activiteiten in die richting te ontplooien is het er, min of meer vanzelfsprekend, toch van gekomen. Zonder jouw hulp en toewijding was dit proefschrift er niet geweest. Bedankt!

Prof. dr. J. Jeekel, mijn promotor, voor zijn konstruktieve bijdragen aan de opzet van het onderzoek en aan het manuscript van dit proefschrift.

Prof. dr. R. Benner, Prof. dr. J.C. Molenaar en dr. W. Weimar voor hun bereidheid in de kleine commissie zitting te nemen, en voor hun snelle beoordeling van het manuscript. Drs. Co Hagoort voor zijn opmerkingen en adviezen over het engels.

Iedereen die de afgelopen jaren als medewerker van het Laboratorium voor Chirurgie of als lid van de Marketto's heeft bijgedragen aan het scheppen van het (wetenschappelijke) klimaat waarin het zo prettig werken was. Zonder volledig te zijn wil ik met name noemen: Ronald Saat, arts. Beste Ronald, na jou introduktie op het lab van "GVH is goed voor U" moet het lastig geweest zijn afscheid te nemen van een zo goed lopend onderzoek. Bedankt voor de enthousiaste start van het onderzoek, en voor het ter beschikking stellen van je data. De heer Fred Bonthuis, Eelco Bouwman, arts , Maarten Meijssen, arts, en dr. Marcel Scheringa voor hun betrokkenheid bij het onderzoek en de hechte samenwerking. Dr. Erik Heineman voor zijn stimulerende rol binnen de darmtransplantatiegroep. Drs. Monique Wolvekamp, nou ja....laat maar. Dr. Harm HogenEsch voor het beoordelen van de histologische preparaten en mevrouw Joke van 't Hoff voor de gastvrijheid tijdens de vetbepalingen.

Gerda Bosman en Marcel Scheringa, bedankt voor jullie steun als paranymf.

Pa en ma, bedankt voor jullie belangstelling en steun.

Caroline, je gaf niet alleen kleur aan dit boekje. Bedankt.

CURRICULUM VITAE

Ronald W.F. de Bruin werd geboren op 6 juni 1960 te Rotterdam. De middelbare schoolopleiding aan de Openbare Scholengemeenschap Prins Alexanderpolder te Rotterdam werd in 1977 afgesloten. Datzelfde jaar begon hij aan het van Leeuwenhoek Instituut te Delft de MBO opleiding tot technisch mikrobiologisch analist, welke in 1980 werd afgesloten met het behalen van het diploma. In datzelfde jaar begon hij aan het van 't Hoff Instituut te Rotterdam de HBO opleiding tot medisch mikrobiologisch analist, waar in 1982 het diploma werd behaald. Sinds maart 1983 is hij in dienst van de afdeling Algemene Heelkunde (hoofd: Prof. dr. J. Jeekel), Academisch Ziekenhuis Dijkzigt te Rotterdam werkzaam op het Laboratorium voor Experimentele Chirurgie van de Erasmus Universiteit. In 1988 werd begonnen met het onderzoek naar dunne darmtransplantatie bij de rat dat, na het behalen van het diploma HTO-ingenieur afstudeerrichting medische mikrobiologie in 1992 aan de Hogeschool Rotterdam en Omstreken, werd afgerond met deze dissertatie.