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A short course of high-dose cyclophosphamide induces long-term survival of intestinal allografts in mice

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Abstract Several transplant programs have recently added cyclophosphamide (CyP) to their immune suppression protocols in an attempt to reduce intestinal graft rejection rates. The present study was undertaken to confirm the benefits of this drug in a murine small bowel transplant model. A short course of monotherapy with CyP 20 mg/kg per dose resulted in a mean survival time (MST) of 17.5 ± 3.6 days, compared with a MST of 7.5 ± 0.7 days in the untreated controls ($P < 0.01$). Cyclosporin A (CsA) 30 mg/kg per day produced comparable survival rates when used as monotherapy (MST: 14.2 ± 1.3 days) or in combination with CyP 20 mg/kg per dose (MST: 21.3 ± 5.1 days). Treatment with high dose CyP (40 mg/kg per dose) completely prevented graft loss in 8 of 10 animals (MST: 72.5 ± 5.3 days, $P < 0.01$). However, adding CsA abrogated the induction of long-term survival achieved by CyP alone (MST: 23 ± 0.4 days). These data have important implications for the use of CyP in clinical transplantation.

Keywords Small bowel transplantation · Mice · Cyclophosphamide · Rejection · Immunosuppression

Abbreviations *CyP* Cyclophosphamide · *GVHD* Graft-versus-host disease · *HPS* Hematoxylin-phyloxin-saphronin · *MST* Mean survival time · *SBT* Small bowel transplantation

Introduction

Several programs have recently added antiproliferative agents, such as cyclophosphamide (CyP), to their immunosuppressive protocols for intestinal transplantation in an effort to reduce the high rejection rates associated with this procedure [15, 17]. CyP is an attractive drug for induction therapy because of its immunosuppressive potency [1, 19] and its ability to induce tolerance in rodent transplant models [10]. However, there are no experimental data confirming the effectiveness of CyP in preventing intestinal graft rejection.

In this study, we applied short-course treatment of CyP to a model of small bowel transplantation (SBT) in mice. Our data suggest that protocols using CyP for intestinal grafting will have to be designed carefully to maximize the benefits of this agent and minimize its toxicity.

Table 1 Intestinal graft survival in balb/c recipients

Donor	Treatment	Individual survival (days)	MST ± SE
BALB/c	None	> 80 × 5	80 ± 0
BALB/c	CyP 40 mg/kg	32[a], > 80 × 3	68 ± 12
C57BL/6	None	5, 7, 7, 8, 9, 9	7.5 ± 0.7
C57BL/6	CyP 20 mg/kg	9, 10, 12, 20, 22, 32	17.5 ± 3.6[c]
C57BL/6	CyP 40 mg/kg	30[a], 55[b], > 80 × 8	72.5 ± 5.3[c]
C57BL/6	CsA 30 mg/kg	11, 12, 13, 13, 17, 19	14.2 ± 1.3[c]
C57BL/6	CsA 30 mg/kg + CyP 20 mg/kg	14, 19, 22, 23, 26	20.8 ± 2[c]
C57BL/6	CsA 30 mg/kg + CyP 40 mg/kg	22, 23, 23, 24	23 ± 0.4

[a]Animal died of pneumonia
[b]Infarcted graft
[c]$P < 0.01$ versus untreated allografts, Mann-Whitney U-test

Materials and methods

Male BALB/c (H-2d), and C57BL/6 (H-2b), and CBA/H (H-2 k) mice weighing between 25–30 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed at the animal facilities of the University of Western Ontario in accordance with the guidelines of the Canadian Council on Animal Care [2]. In all animal experiments "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985) were followed. Anesthesia was induced by intraperitoneal injection of pentobarbital 65 mg/kg and subcutaneous administration of buprenorphine 0.1 mg/kg and atropine 0.04 mg/kg.

Heterotopic small bowel transplants were performed as described previously [21]. Briefly, the proximal jejunum was isolated. The portal vein was mobilized by dividing the pyloric and splenic vein. The aorta was exposed. Right renal artery and celiac trunk were ligated. The graft was perfused with cold, lactated Ringer's solution via the infrarenal aorta. The portal vein was divided at the liver hilum. The graft was removed with a patch of the aorta and stored in lactated Ringer's solution at 4 °C. End-to-side anastomoses were carried out between donor aorta and recipient aorta, and between donor portal vein and recipient inferior vena cava using 11/0 nylon suture. Both ends of the graft were exteriorized as stoma. The native gut remained intact. Animals that died within 4 days after undergoing transplantation were excluded from the study (< 20 %).

CyP powder (Procytox, Horner, Montreal, Quebec) was dissolved in sterile water for injection (Abbott Laboratories, Montreal, Quebec) and stored at 4 °C for maximal 3 days. CyP was injected intravenously through the tail vein on postoperative day (POD) 0, 2, 4, and 7. CsA powder was dissolved in Intralipid 10 % (Clintec, Mississauga, Ontario) and stored at 4 °C for a maximum of 2 days. CsA was administered intramuscularly daily from POD 0–6 and every other day thereafter.

The animals were followed for up to 80 days, the predefined end-point of the study. Their stomas were assessed daily with an operation microscope. The animal was killed if there was evidence of irreversible graft rejection, manifested by necrosis of the stoma. Additional mice (n = 3–4) were killed on POD 10, 20, and 40 to examine the sequential histology of the graft. At autopsy, the abdominal cavity was inspected macroscopically. Portions of the graft were fixed in 10 % paraformaldehyde, embedded in paraffin, cut in 3–5 μm sections, and stained with hematoxylin-phyloxin-saphronin (HPS). Rejection was graded from 0–2 (0 = normal, 0.5 = mild change, 1 = moderate change, 1.5 = marked change, and 2 = severe change) for the following changes: cryptitis, lymphocytic infiltration, loss of goblet cells, mucosal erosion, blunting of villi, and vasculitis [20]. Histological sections of skin, liver, native intestine, and spleen were assessed for signs of graft-versus-host disease (GVHD) as described previously [6].

Skin grafts were performed in three BALB/c mice with long-term surviving allografts (> 80 days) [8]. Two graft beds were prepared on the back of the recipient mouse. Trunk skin of C57BL/6 or CBA/H (third party) was sutured to the recipient. The grafts were covered with a bandage for 7 postoperative days. Rejection was defined as total necrosis of the skin graft.

Survival data are reported as the mean survival time (MST) ± standard error (SE). Survival times were compared using the Mann-Whitney U Test. The mice killed on POD 10, 20, and 40, to obtain tissue samples for histological examination, were excluded from the survival analysis. Scores for histological criteria of rejection were summarized as median and statistically analyzed by Mann-Whitney U Test. Samples with a P-value less than 0.05 ($P < 0.05$) were considered significant.

Results

Table 1 presents the survival times with the various immune suppressive protocols. Table 2 summarizes the histology of allografts. All of the treated and untreated isografts had a normal stomal mucosa and clear fluid output throughout the 80 days observation period. Except for mild mucosal atrophy, the histology of the isografts was normal, including the animals that were treated with CyP. Isograft recipients that received CyP had no significant signs of drug toxicity.

Untreated C57BL/6 allografts were rejected by POD 9 (MST: 7.5 ± 0.7 days). Histology showed severe rejection. Survival of allografts treated with CyP 20 mg/kg per dose was significantly prolonged, compared to untreated allografts (MST: 17.5 ± 3.6 days, $P < 0.01$, Mann-Whitney U test). CsA 30 mg/kg prolonged graft survival to MST of 14.2 ± 1.3 days ($P < 0.01$). Long-term survival (> 80 days) was achieved in 8 of 10 animals treated with CyP 40 mg/kg per dose ($P < 0.01$, versus untreated allografts). The prolonged survival achieved with CyP 40 mg/kg per dose alone was abrogated by the co-administration of CsA 30 mg/kg per day (MST: 23 ± 0.4 days). The latter grafts developed severe rejection, characterized by rapid destruction of the intestinal mucosa, beginning at around POD 18. Moreover, treatment with CyP 40 mg/kg per dose and CsA was toxic: 50 % of the animals in this group had to be excluded from the survival analysis because they developed intolerable drug side effects, such as prolonged diarrhea and lethargy during the first 4 days after surgery.

Table 2 Median histological scores of intestinal allografts

Treatment	Time of histology	No.	Cryptitis	Lymphocyte infiltration	Mucosal erosion	Blunting of villi	Goblet cell loss	Vasculitis
None	Rej.	6	1	1.5	1.5	1.5	2	0.5
CyP 20 mg/kg	Rej.	6	0.5	2	1.5	1.5	2	0
CyP 40 mg/kg	POD 10	4	0[a]	0[a]	0[a]	0[a]	0.5[a]	0
	POD 20	3	1	1	0[a]	1	2	0
	POD 40	4	0.5[a]	0.5	0[a]	0	0.5[a]	0
	POD 80	5	0.5[a]	0[a]	0[a]	0.5[a]	0[a]	0[a]
CsA 30 mg/kg	Rej.	6	1.5	1.5	2	2	2	1
CyP 20 mg/kg + CsA 30 mg/kg	Rej.	5	0.5[a]	1.5	1.5	2	2	1
CyP 40 mg/kg + CsA 30 mg/kg	Rej.	4	0.5	2	1.5	2	2	1

[a] $P < 0.05$ vs. untreated allografts, Mann-Whitney U test

The group of allografts treated with CyP 40 mg/kg per dose developed a transient rejection at day 20 that spontaneously resolved. During this time, their stoma output increased and the mucosa became pale, but the villi remained intact and there was no necrosis of the stoma. Corresponding to the macroscopic observations, sequential histology revealed a normal appearance on POD 10, mild to moderate rejection on POD 20, and only minor abnormalities on POD 40 (Figure 1). Of note, mucosal erosion was absent in these grafts despite the presence of other characteristic signs of rejection ($P < 0.05$). The histological findings could not be attributed to toxic effects of CyP, since the sequential analysis of isografts receiving the same treatment showed normal graft architecture throughout the observation period.

The histology of long-term surviving grafts revealed only mild cryptitis and mucosal atrophy on POD 80. One animal died of pneumonia on POD 30 with a normal graft, another died of graft necrosis due to infarction on POD 55. To test whether donor-specific tolerance was induced, three animals with long-term surviving allografts after treatment with CyP 40 mg/kg per dose were challenged with C57BL/6 (donor strain) and CBA/H (third party) skin grafts. The third party grafts were rejected within 12 days, whereas rejection of the donor specific skin grafts was delayed to 15, 16, and 19 days. There was no clinical and histological evidence of GVHD in any of the study groups.

Discussion

This study demonstrates that a short course of high-dose CyP induces prolonged survival of murine intestinal grafts. To the best of our knowledge, this is the first report of long-term survival (> 80 days) of murine intestinal allografts using chemical immunosuppression [3, 4, 12]. Interestingly, this benefit is lost if CsA is co-administered with CyP.

There are only few previous papers on the effectiveness of immune suppressive drugs for SBT in mice [3, 4, 12]. For example, Chen et al. examined the effect of rapamycin and cyclosporine on rejection and graft-versus-host disease after SBT in mice [4]. Rapamycin given for 14 days in high doses prolonged survival of recipients (BALB/c) of fully allogeneic allografts (C57BL/6) to a MST of 27 days. Similar results were achieved using CsA 4 mg/kg per day (MST: 35.2 days). Because animals with heterotopic intestinal grafts can survive despite rejection of the graft [21], results are difficult to compare with our study, which used graft survival as the endpoint. Newell and co-workers employed tacrolimus to treat intestinal grafts in a semi-allogeneic strain combination [12]. A short course of tacrolimus delayed the onset of rejection and decreased the severity of rejection for up to 28 days after transplantation. Long-term survival was not assessed.

The immunological properties of CyP are complex and have been the subject of extensive research [1]. The interest in CyP as immunosuppressive drug after transplantation has focused on two qualities. First, CyP has a preferential effect on B-lymphocytes, which makes it attractive for the treatment of antibody-mediated rejection [11]. Second, a preoperative bolus of CyP, given after antigen stimulation by donor spleen cell injection, deletes activated cell clones and induces robust tolerance in murine transplant models [10]. We have recently demonstrated that a short postoperative course of CyP without prior spleen cell infusion also induces donor specific tolerance of heart allografts in mice [5]. The induction of tolerance in this model is associated with a transient depletion of leukocyte counts and a reduction of antibody production, followed by a deviation of the immune response towards Th2 cytokines.

After an initial rejection-free period, the animals treated with CyP 40 mg/kg per dose had an episode of transient rejection at day 20 that resolved spontaneously without additional immunosuppression (Figure 1). These findings may also suggest that the long-term immunosuppressive effect of CyP is likely to be caused by an active immunological process rather than by clonal

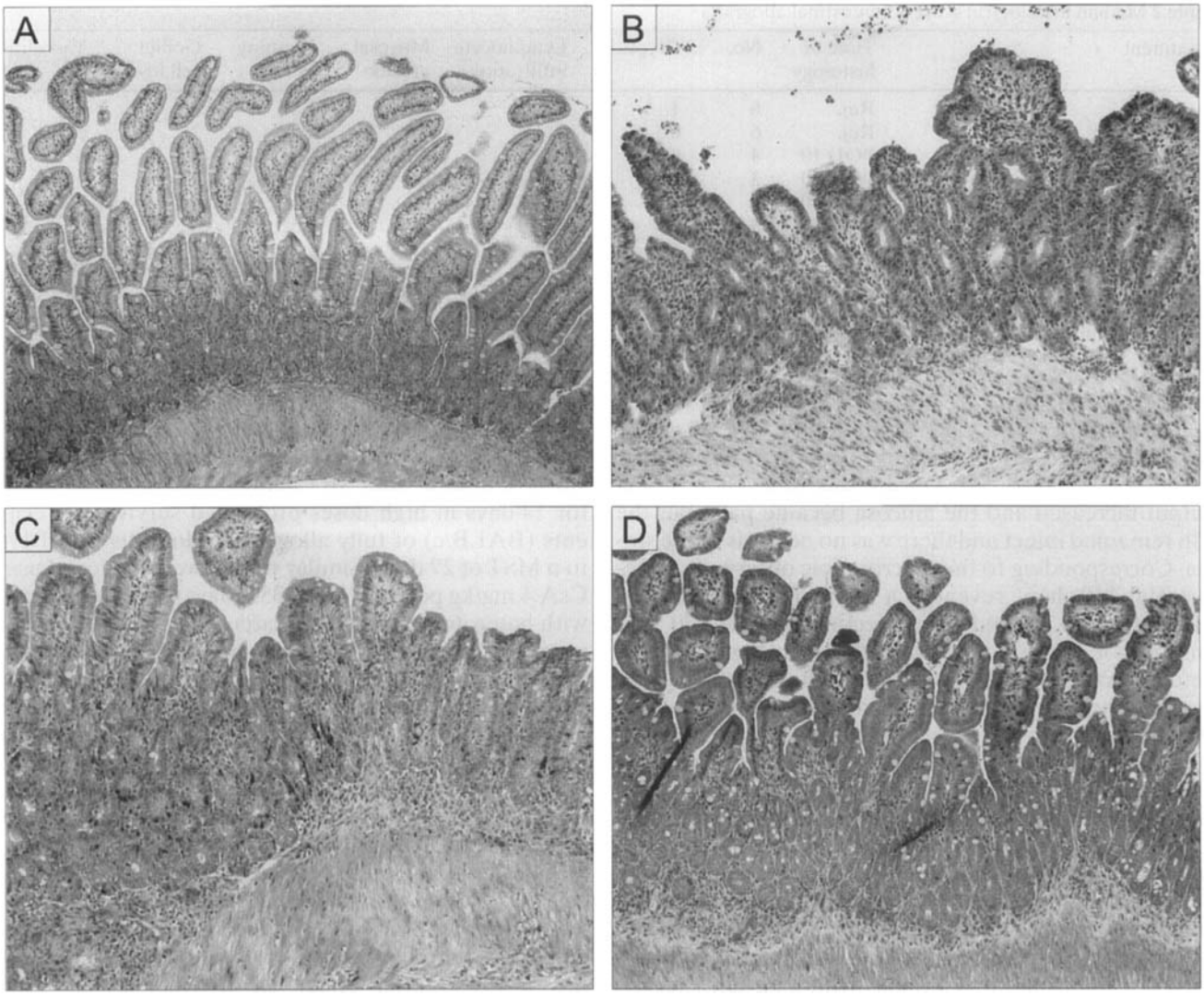

Fig. 1 A–D Sequential histology of intestinal allografts treated with CyP 40 mg/kg per dose on POD 0, 2, 4, and 7. **A** Normal graft on POD 10; **B** moderate rejection on POD 20; **C** mild changes on POD 40; and **D** normal histology on POD 8

deletion. In contrast to the results with heart transplantation, donor specific tolerance was not induced after SBT, despite normal graft histology on POD 80. These data provide further evidence that it is more difficult to prevent rejection of the small bowel graft than other solid organ transplants.

Depending on the timing of administration, CyP can enhance or abrogate GVHD [9, 18]. For example, the injection of CyP into F1 hybrids before administration of allogeneic, parental cells results in lethal GVHD [9]. In contrast, CyP given to F1 hybrids after allogeneic parental cells prevented GVHD [18]. In the present experiments, we did not find clinical or histological evidence of GVHD in any of the animals with fully allogeneic bowel transplants. However, studies using a semiallogeneic combination (parental grafts to F1 hybrids) may address this issue more specifically.

CsA alone or with CyP was relatively ineffective in preventing intestinal graft rejection in our model (Table 1). Resistance to CsA treatment in mice has also been observed after cardiac transplantation [16]. The combination of CyP 40 mg/kg per dose and CsA was toxic, and it abrogated the long-term survival achieved with CyP alone. Two mechanisms may contribute to this detrimental effect. First, CsA might interfere with the deletion of cytotoxic lymphocytes by blocking their proliferation in response to donor antigens [13]. Second, CsA might interfere with the CyP-mediated shift towards a Th2 cytokine profile and/or the production of the immune regulatory cells that prevent rejection [14].

These data have important implications for the use of CyP for intestinal grafting. Our findings suggest that

simply adding CyP to existing immune suppressive protocols may increase toxicity without reducing graft rejection rates. This hypothesis is consistent with data from Reyes et al. that documented increased rates of rejection when CyP was used in pediatric recipients of intestinal transplants [15]. Recently, Gross et al. reported two cases of post-transplant lymphoproliferative disease (PTLD) after SBT that were treated with temporary withdrawal of tacrolimus-based immunosuppression followed by intravenous administration of CyP and steroids. Only one patient developed transient mild acute rejection [7].

The results of our experimental study suggest that to maximize CyP's benefits for SBT, it may be necessary to give this drug as the sole induction agent and then try to maintain immune suppression and/or promote long term graft acceptance using immune suppressive drugs that do not block the calcineurin pathway.

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