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ORIGINAL ARTICLE

The influence of surgery, immunosuppressive drugs, and rejection, on graft function after small bowel transplantation: a large-animal study

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Abstract In this study we assessed functional changes (motility and absorption) of intestinal allografts in a large-animal model of orthotopic small bowel transplantation in swine. Studies were performed on non-rejecting animals in the early and late stages after transplantation and after induction of different grades of acute rejection. Immunosuppression consisted of oral FK506 and mycophenolate mofetil. In each study group we regulated drug administration, in terms of dosage and timing, in order to induce different grades of acute rejection or to prevent it. Migrating myoelectrical complexes were recorded in fasting animals so that motility could be assessed. Mucosal biopsy of the allograft and D-xylose absorption tests were performed on the same day as the motility study. In the early stages following intestinal transplantation, we observed in non-rejecting animals a slightly increased graft motility and a marked carbohydrate malabsorption. Recovery of the carbohydrate absorption capacity occurs within 2 months, but the persistence of diarrhea leads to partial malabsorption and to a lack of normal weight gain. Motility reduction correlates with the grade of acute rejection and becomes significant at a later stage, when rejection is severe. Allograft carbohydrate absorption, on the contrary, is markedly reduced in all rejecting pigs, irrespective of the grade of rejection. In summary, the early functional impairment of non-rejecting animals has multifactorial causes due to surgery and immunosuppression (drug toxicity), and its occurrence suggests the need for specific guidelines for clinical early postoperative enteral feeding. The functional studies adopted here are helpful in defining the grade of functional impairment with or without acute rejection; however, they are not useful for early detection of ongoing acute rejection of the small bowel graft.

Keywords Intestinal transplantation · Motility · Absorption · Acute cellular rejection · Experimental model

Introduction

Intestinal transplantation has recently become an accepted therapy for selected patients with short-bowel syndrome or other forms of irreversible intestinal failure [1, 13, 37]. However, results in terms of long-term patient and graft survival are still not comparable to those of other single abdominal-organ transplantations

[36]. After intestinal transplantation, surgical and immunological factors can affect the enteric function of the transplanted gut, especially during the first 2–3 postoperative months. They include denervation, lymphatic disruption, ischemic injury, and immune phenomena.

Most of the effects of ischemia-reperfusion injury are transient, reversible, and recover within the first 2 weeks [9, 19, 37]. The surgical technique leads to extrinsic denervation, disruption of intrinsic neural continuity, and lymphatic transection. There is evidence that after small-bowel transplantation (SBTx) fasting motility is controlled by the intrinsic nervous system, but is temporally dissociated with that of the native intestinal remnant [28]. Extrinsic sympathetic re-innervation can occur partially during the first year after transplantation, but its efficacy on motility control is expected to be poor [20]. Likewise, the lymphatic vessels can regenerate within a few weeks after transplantation without major improvement in absorption. Moreover, it has been demonstrated that circulating hormones do not alter temporal coordination of intestinal motor patterns [28].

Among the affecting factors, acute cellular rejection (ACR) is the most life threatening, and its prevention or early recognition is crucial to a successful intestinal transplant. Stojanovic et al. have recently shown in an experimental study that acute rejection is detected earlier by in vivo microscopy than by histology [33]. In another clinical study, Kato et al. applied zoom video-endoscopy for the monitoring of rejection. The more accurate assessment of the graft mucosa provided by this method enabled the timely initiation of rejection treatment [16].

The understanding of the functional changes occurring in the early and late stages after intestinal transplantation and caused by ACR could be useful in defining: (a) causes of malabsorption and dysmotility symptoms, (b) guidelines for the enteral feeding of transplant patients, and (c) early functional indicators of intestinal rejection. In this study we have assessed the functional changes (motility and absorption) of intestinal allografts in a large-animal model of orthotopic SBTx in swine. Studies were performed on non-rejecting animals early and late after transplantation and after induction of different grades of acute rejection.

Materials and methods

Animals and study groups

Only animals that did not develop technical complications from the intestinal transplant procedure or from electrode implantation were included in the study. Therefore, studies were performed on 38 outbred piglets (mean weight 25 ± 7 kg) divided into five experimental groups according to the grade of ACR (Table 1): group I ($n = 12$), normal controls; group II ($n = 8$), SBTx with no or mild ACR at day 15; group III ($n = 6$), SBTx with no or mild ACR at day 60; group IV ($n = 8$), SBTx with moderate ACR; group V ($n = 4$), SBTx with severe ACR. All animals received humane care in compliance with the 'principles of laboratory animal care' formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Intestinal transplantation

The surgical procedure of SBTx has been described previously [2]. Briefly, general anesthesia was achieved with 3% isoflurane. The donor small bowel, excepting the duodenum and 5 cm of the terminal ileum, was isolated with its vascular pedicle and excised, cold perfused through the aorta with UW solution, and immersed in iced normal saline, without intraluminal flushing. After excising the recipient small bowel, we re-vascularized the graft by end-to-end anastomoses of donor and recipient superior mesenteric vessels. Intestinal continuity was restored with a side-to-side host duodenal-graft jejunal anastomosis, with exteriorization of a 10-cm jejunal 'chimney' segment for visual monitoring and postoperative mucosal biopsies of the stoma. A distal end-to-end anastomosis between graft ileum and the retained stump of recipient ileum left the recipient ileo-cecal valve and entire colon intact. A gastrostomy was performed to allow for enteral feeding and controlled tacrolimus and mycophenolate mofetil (MMF) administration.

Immunosuppression

Immunosuppression consisted of oral FK506 (tacrolimus, Prograf) alone or in combination with MMF (CellCept). In each study group we adapted the drug administration, in terms of dosage and timing, in order to induce different grades of ACR or to prevent it (Table 1). In particular, although i.m. induction with FK506 was similar in groups II and III, trough levels after the start of enteral drug administration were maintained between 15 and 25 ng/ml in group II and between 5 and 15 ng/ml in group III. In this latter group, enteral MMF was added to FK506 from the first postoperative day. Group-IV animals were immunosuppressed only during the first 5 postoperative days

Table 1 Study groups

Group	*n*	SBTx	Immunosuppression		
		Model	Tacrolimus (mg/kg per day)	MMF (mg/kg per day)	Length of treatment
I	12	Control (no SBTx)	–	–	–
II	8	No/mild ACR	0.3i.m./0.6 p.o.	–	15 Days
III	6	No/mild ACR	0.3i.m./0.6 p.o.	20 p.o.	60 Days
IV	8	Moderate ACR	0.3i.m.	–	5 Days
V	4	Severe ACR	–	–	–

with i.m. FK506, while group-V animals did not undergo immunosuppression. At the time of functional studies immunosuppression was halted, and in all animals the FK506 levels were below the detectable range.

Postoperative care

Following total parenteral nutrition for 3–5 days, enteral feeding was started gradually through the gastric stoma while the animals were also allowed to eat graded aliquots of food. All animals resumed a complete oral food intake (two full doses of balanced pig chow daily) within 7 days. Weight was measured weekly or more frequently. Prophylactic cephalosporin was given intramuscularly for the first 7 days. Peptic ulcer prophylaxis was undertaken with daily ranitidine for 7 days. Infections thereafter were treated with antibiotics chosen on the basis of culture results.

Morphological study

Stomal biopsies were performed every 7 days, or earlier if rejection was suspected. In addition, full-thickness biopsies of the graft were taken after reperfusion and at re-laparotomy/autopsy. The formalin-fixed, paraffin-embedded samples were stained with hematoxylin and eosin, Movat's pentachrome stain, and Alcian blue–PAS. The grading system established by Murase et al. [23] was used to quantify grades of ACR (Fig. 1).

- (0): no evidence of ACR.
- (1): mild. Edema, dilated lacteal vessels, blunted villi, minimal infiltration of activated lymphocytes in the lamina propria.
- (2): moderate. Same as (1) but more advanced (including more generalized leukocyte infiltration) plus epithelial necrosis and denudation.
- (3): severe. Same as (2) but more severe including transmural leukocyte infiltration plus widespread hemorrhagic necrosis of the mucosa.

Motility study

In all transplant groups except group III, eight bipolar electrodes were implanted during transplantation. On the 60th postoperative day or later, the animals of group III were brought into the operating room where, under general anesthesia, they underwent exploratory laparotomy with a 10-cm ileal segment resection of the graft for full-thickness morphological studies, implantation of the bipolar electrodes, and placement of a Port-a-Cath catheter in the external jugular vein. Group-I animals included non-transplant, non-immunosuppressed pigs that underwent the same surgical procedure with ileal resection, implantation of bipolar electrodes, placement of a central vein catheter and gastrostomy.

The eight bipolar electrodes were implanted on the antimesenteric border of the small bowel to monitor myoelectrical activity at the following levels: descending duodenum and first loop of the native jejunum remnant (E_1, E_2), and then every 50 cm in an aboral direction along the graft (E_3–E_8), the first being placed 5 cm from the side-to-side jejuno-jejunostomy. Migrating myoelectrical complexes (MMCs) were recorded twice (on two different days with a 2-day to 3-day interval) in fasting animals, by use of a multichannel recorder (Neuro 20, Battaglia, Rangoni, Italy) with a time constant of 0.03 s, and simultaneously stored by a computerized system that automatically calculated the number of spikes per unit of time by means of original software [10]. The different phases of the MMCs were defined according to criteria established previously in our laboratory [11], i.e., phase III (activity front) was defined as the period of maximal activity lasting more than 3 min and migrating over at least three electrode sites. The MMC period was defined as the time interval between the end of two consecutive activity fronts at the same electrode.

Absorption study

D-xylose is a monosaccharide of the pentose family. It is absorbed into the small bowel through a passive mechanism. The D-xylose test is used for the evaluation of carbohydrate malabsorption and is generally considered to be a good index of mucosal damage. D-xylose was given orally through the gastrostomy at the standard dose of 0.5 g/kg, and its serum levels were measured with a Beckman spectrophotometer at time 0 and 30, 60, 90, and 120 min after administration. Blood samples were taken from a catheter placed in a central vein.

Timing of functional study and statistics

In each group both the D-xylose absorption test and MMC recordings were performed on the day on which the desired rejection grade was proven by biopsy, and repeated two days later. As a consequence, both functional studies were carried out in the following ranges of postoperative days: days 7–14 for group I, days 14–20 for group II, days 64–70 for group III, days 15–30 for group IV, and days 7–15 for group V. Data were expressed as mean ± SD. Means between groups were compared over time with the two-way ANOVA test for repeated measures (Stata 5, Stata-Corp, College Station, Tex., USA). A P value of below 0.05 was considered to be statistically significant.

Results

Clinical outcome and histopathology

The postoperative course of groups II and III animals was uneventful, and they reached the 15th and 60th postoperative days, respectively, without developing serious complications. However, all animals in both groups suffered persistent diarrhea in 45% (mean) of the postoperative days. In the long-term surviving group (group III), mean body weight variation from day 0 to day 60 was 0%, while in the non-transplant control group I, animals doubled their weight during the same time period (+100%, $P < 0.01$).

All biopsy specimens taken at protocol time-intervals during the postoperative course were rejection free or presented minimal–mild rejection in both groups II and III (Fig. 1). Full-thickness histopathological examination of the transplanted intestines at day 60 showed an intact wall structure and, when compared with normal intestines, more blunted villi and some dilated lymphatic vessels. In group IV, moderate rejection occurred between the 15th and the 30th post-transplant day. In group V, severe rejection was already present at biopsy after the first postoperative week (Fig. 1).

Motility study

Figure 2 shows the results of the motility study in all five groups. Compared with normal controls (group I), myoelectrical activity increased in the rejection-free transplanted intestines either at an early (at day 15—group II) or late (at day 60—group III) stage after transplantation. The increased grade of acute rejection correlates with a progressive reduction in myoelectrical activity. As a consequence, there is a statistically significant difference in the mean number of spikes/h between group V vs groups II and III, respectively.

The visual computer-plot comparison between groups II and III vs group V shows the different motility patterns. In the non-rejecting animals (groups II and III), normal MMCs were easily detected, MMC cycling was found to be independent of spike activity in the residual part of the native jejunum, and the MMC period in the graft was slightly shorter than with group-I normal controls (50 ± 4 vs 61 ± 11 min, respectively, NS); moreover, the velocity of MMC propagation in the graft was higher than in normal intestine (26 ± 3 vs 31 ± 5 cm/s, respectively, NS). The MMC pattern was absent in group-IV and group-V animals.

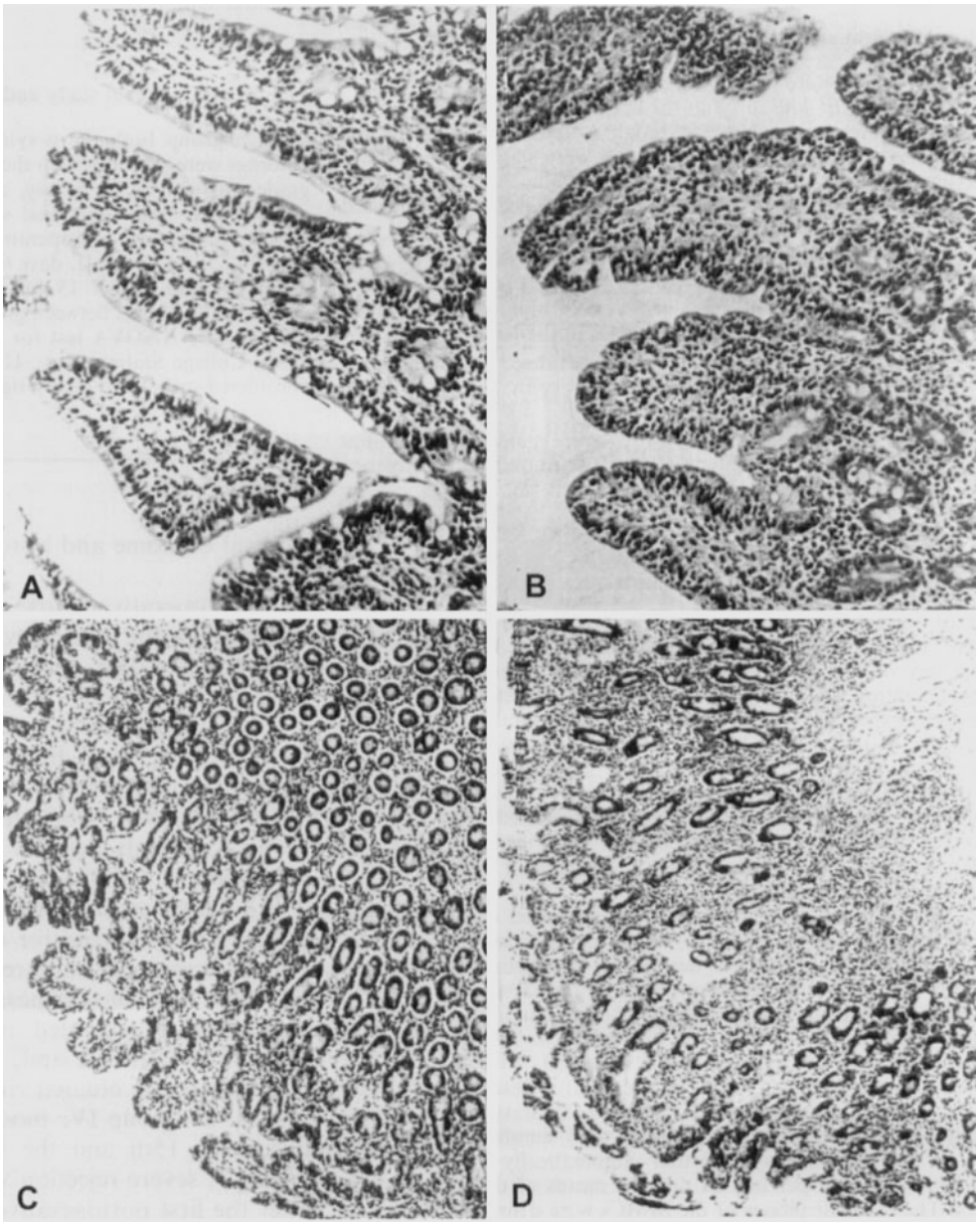

Fig. 1A–D Histopathology of the transplanted intestine, at mucosal biopsy, with different grades of ACR. **A** Normal intestine, **B** mild ACR, **C** moderate ACR, **D** severe ACR

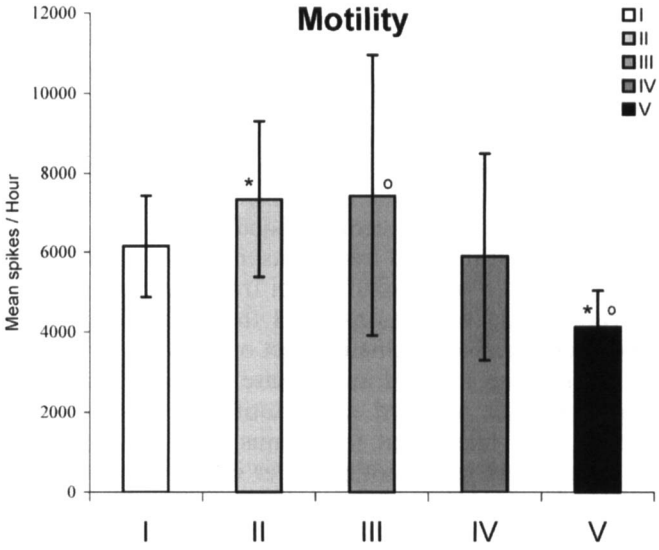

Fig. 2 The mean number of spikes/h (expressed as a mean bar ± SD bar for each group) is the computerized expression of myoelectrical activity of the intestine; therefore, it quantifies intestinal motility. If ACR is absent or mild, intestinal motility is increased either in the early (group II) or late (group III) stages after SBTx. Reduction of intestinal motility occurs when ACR is moderate (group IV) and becomes significant in the event of severe ACR (group V). $*P < 0.05$ group II vs group V, $°P < 0.05$ group - III vs group V

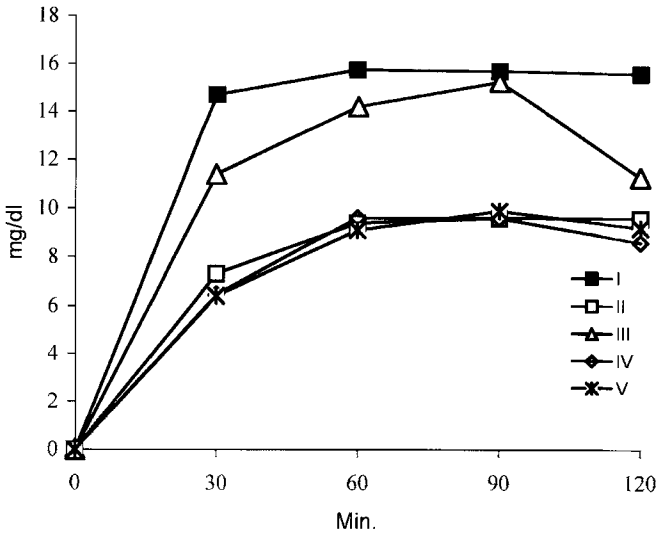

Fig. 3 The D-xylose absorption curves demonstrate a marked reduction in carbohydrate absorption early after SBTx (groups II, IV, and V) without any difference in ACR grading. In non-rejecting animals, the absorptive capacity returns to almost normal values at 2 months after transplantation (group III). SD bars have been omitted for clarity

Absorption study

The D-xylose curves (Fig. 3) show a reduced absorption in all transplant groups when compared with normal controls, the only exceptions being the non-rejecting long-surviving recipients (group III), whose absorption capacity returned to almost normal values during the first 90 min of evaluation. Thereafter, their absorption capacity decreased faster than in controls. In this study, statistical analysis failed to detect any significant differences due to the variability of the values within each group.

Discussion

This study synthesizes several years of experimental work on intestinal transplantation. In a previous study we achieved long-term survival after total SBTx in a model of swine, using for the first time a combined therapy of low-dose FK506 and MMF [3]. This double-drug regimen is currently being used in clinical intestinal and multi-visceral transplantation [24, 36, 37].

The opportunity to have at our disposal a large, healthy, long-surviving animal model, never achieved by others [25], allowed us to carry out preliminary morphological and functional studies of the intestinal graft at a late stage after transplantation [4] and also further assessments of graft function in different clinical situations. In the present study we compared intestinal graft motility and carbohydrate absorption in the early and late stages after transplantation and during different grades of ACR.

Large-animal models of intestinal graft rejection

Three different experimental models of intestinal graft acute rejection are feasible in pigs. A mild ACR can usually be detected in the mucosal biopsies of animals treated with chronic baseline immunosuppression and is characterized by a mild inflammatory infiltrate, lacteal-vessel enlargement, and edema (Fig. 1b). This picture was present in the majority of the protocol biopsies performed in animals of groups II and III, remained unchanged during the follow-up period, and never reached clinical significance.

After a short course of immunosuppression (FK506 for the first 5 postoperative days), the animals showed a moderate ACR that occurred slowly within the first month after transplantation (group IV). The morphological picture was characterized by a shortening of the villi, flattening of the epithelium, dilation of the crypts, edema, and cellular infiltration in the lamina propria and lamina muscularis mucosae (Fig. 1c). The clinical consequence was mainly bacterial translocation and the development of progressive systemic sepsis, loss of weight, watery diarrhea, emaciation, and death.

We induced severe ACR within 10 days of intestinal transplantation by withholding any form of immuno-

suppression. Its development was fast and sudden, with the destruction of the mucosal layer and lamina muscularis mucosae and massive cellular infiltration of the muscle layer (Fig. 1d). One week after a normal postoperative course the animals suddenly became lethargic, showed signs of sepsis, developed hemorrhagic diarrhea and died within 2–3 days.

All three models are reliable for functional studies. They clinically reflect the outcome of the three different grades of ACR occurring in humans after SBTx. Moderate or severe ACR are devastating events, and the combined occurrence of rejection and sepsis requires very complicated management. As a consequence, during the first 3 months after intestinal transplantation, more than 25% of the transplanted grafts are lost mainly due to immunological and infective complications [36, 37]. One of the possibilities for the avoidance or limiting of bacterial translocation (and the resulting sepsis) during ACR is the early recognition of the oncoming rejection and its immediate treatment. An understanding of the functional modification occurring in the different stages of ACR may be of some help not only in correlating them with malabsorption and dysmotility symptoms, but also in defining a possible strategy for early diagnosis of ACR using functional tests.

Motility and absorption in the transplanted gut

Our results show that early after intestinal transplantation (day 15), in the absence of clinical symptoms of ACR (no or mild ACR at mucosal biopsy), graft motility was slightly increased, while carbohydrate absorption was halved. Four reasons can be put forward to explain these findings: ischemia-reperfusion injury, intestinal denervation, lymphatic disruption, and immunosuppressive drug toxicity.

Ischemia-reperfusion injury has been studied extensively in intestinal grafts. Absorptive capacity for water, electrolytes, and nutrients decreases because of mucosal damage and increased intestinal permeability. In most cases, the injury is reversible shortly after transplantation. A recent clinical study showed the presence of high brush-border enzyme activity as early as 2 weeks after intestinal transplantation [17], suggesting that intraluminal digestion is not altered by ischemic injury. However, chronic histopathological changes of the small bowel due to ischemia-reperfusion injury have been described in rats [7]. In our study, recovery from ischemic injury within 2 weeks and absence of chronic alterations after 2 months were confirmed by graft histopathology [3].

It is generally agreed that intestinal denervation and lymphatic disruption are important factors influencing motility and absorption early after transplantation. However, both surgical factors should not interfere in sugar absorption. Lipid but not carbohydrate absorption is impaired by lymphatic disruption [29, 39]. Moreover, using denervated intestine in canine models, Sarr et al. showed that denervation did not affect the absorption of glucose [30].

Immunosuppressive drugs may play an important role in early functional impairment of the intestinal graft, also because the recommended therapeutic dose in small-bowel transplant recipients is higher than that administered in other solid-organ transplantations. In a previous study we demonstrated that FK506 and erythromycin exert a prokinetic effect on a normal intestine [8]. Both drugs induced an increase in phase-II activity similar to that reported with motilin [5]. As a consequence, it is likely that tacrolimus may contribute to early increased graft motility after intestinal transplantation. From this study, we cannot say whether the prokinetic activity of tacrolimus alone is responsible for the long-term persistence of intestinal hypermotility at day 60 after transplantation, since the lack of extrinsic nerve regeneration could also explain the phenomenon.

Different studies have also shown that tacrolimus can influence intestinal absorption. Henke et al. [14] and Madsen et al. [22] showed that tacrolimus decreases rat mitochondrial energy production in vitro and in vivo, respectively. More recently, Gabe et al. have demonstrated a marked inhibition in cellular energy production, a concomitant increased intestinal permeability leading to endotoxemia, and an impaired intestinal absorptive function in clinically stable liver-transplanted patients on tacrolimus [12]. One of the conclusions of this latter study was that, since intestinal integrity is maintained by cellular ATP levels, their decrease due to tacrolimus exposure may explain the impaired carbohydrate absorption. Our experiments in pigs, which were limited to assessing passive carrier-mediated transport (D-xylose test), do not support this conclusion. In healthy animals treated with high doses of tacrolimus (blood levels up to 50 ng/ml), the D-xylose absorption curve was similar to that in the untreated controls (data not shown). Moreover, in the transplant animals in this study, after 60 days of receiving chronic tacrolimus treatment, their monosaccharide absorption returned to normal values.

MMF has some potential toxic effect on the GI-tract, and diarrhea is the most common clinical side effect of this drug. MMF selectively inhibits the proliferation of T and B lymphocytes acting on the pathway for de novo purine synthesis [32]. Nucleotides (purines and pyrimidines) and their related metabolic products play key roles in many biological processes. One example is the beneficial effect on intestinal trophism, such as promotion of intestinal growth, protection against diarrhea, and stimulation of epithelial/mucosal barrier function. The use of MMF is effective in the inhibition of the immunological functions of nucleotides, but may pre-

dispose the intestine to metabolic impairment because of the reduction of nucleotide trophic activity [6]. This fact, associated with the slightly increased motility, may explain why, at 2 months after transplantation, group-III animals who received MMF in combination with FK506 continued to be affected by diarrhea resistant to pharmacological treatment. Consequently, animals were unable to gain weight, when compared with healthy controls, in spite of the improved absorption. Moreover, data from clinical experience show that more than 75% of pediatric intestinal transplant recipients in whom MMF was omitted had a normal growth rate [21, 34].

One important observation to emerge from this study is the partial recovery of carbohydrate absorption during the first 2 months after intestinal transplantation. The absorption curve of D-xylose in group III shows a similar peak value and a faster decay rate than that in healthy controls (group I). The regeneration of lymphatic vessels that occurred within 2 weeks in experimental studies in rats [31] may explain the long-term improvement in lipid, but not carbohydrate, absorption capacity. To induce a faster recovery of the original lipid absorptive function, microsurgical lymphatic reconstruction has been experimentally proposed, with encouraging results [18, 35]. Reparation of architectural changes caused by ischemia-reperfusion injury and adaptation to a lower chronic immunosuppressive regimen may explain the potential aptitude of a non-rejecting graft to progressively resume normal carbohydrate absorption.

Experiments in microsurgical nerve coaptation performed in rats [35] have been more complex and less effective. Little is known on spontaneous extrinsic nerve regeneration after intestinal transplantation. However, the absence of temporal coordination of the cyclic motor pattern between the native intestinal remnant and the transplanted gut after 2 months correlates with other experimental studies [28] and may prove the lack of nerve regeneration.

The impaired early post-transplant absorption and its progressive recovery have some clinical implications. Parenteral nutrition should be continued until a normal or near-normal nutrient assimilation is achieved [26]. A modification of enteral feeding to meet the changing clinical needs and, thus, the absorptive status of the transplanted intestine, has been suggested [15]. Enteral feeding should be initiated early after transplantation, if possible within the first week [27], to accelerate the restoration of normal mucosal trophism. An elemental diet is recommended at first, followed by a semi-elemental diet. Since dietary lipid absorption is affected by interruption of lymphatic drainage, a lipid-enriched diet should be introduced with caution after a few weeks, when lymphatic regeneration may have occurred [17]. All these recommendations fit in with our experimental findings.

Finally, in our study, motility reduction correlated with the grade of ACR and became significant at a later stage, when rejection was already severe. Allograft carbohydrate absorption, on the contrary, was markedly reduced in all transplant pigs, irrespective of the grade of ACR. These findings demonstrate that the functional studies adopted here are not useful for early detection of ongoing acute rejection of the small-bowel graft.

In conclusion, early after intestinal transplantation, before rejection sets in, the slightly increased graft motility and the marked carbohydrate malabsorption have multifactorial causes, which are due to surgery and immunosuppression (drug toxicity). The recovery of carbohydrate absorption capacity occurs within 2 months, but the persistence of diarrhea leads to partial malabsorption and to a lack of normal weight gain. This study raises the question of whether tacrolimus and MMF should be the recommended immunosuppressive drugs for intestinal transplantation. To date, tacrolimus is considered to be the baseline drug of choice, and, as yet, there are no alternative drugs with such a powerful immunosuppressive effect [13, 36, 37]. Moreover, if tacrolimus is used in combination with another effective drug, its dosage can be reduced, to avoid toxic effects. MMF is a safe and useful drug in kidney and liver transplantation. However, in intestinal transplantation MMF may contribute to affect the enteric function and to enhance diarrhea and malabsorption, as also observed clinically [38]. For this reason, other drugs should be tested in combination with tacrolimus to improve the immunological control of ACR and to reduce toxic effects on the small-bowel allograft.

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