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Effect of small bowel transplantation, denervation and ischaemia on rat intestinal microflora

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Abstract The effects of denervation and warm ischaemia on quantitative and qualitative changes in small intestinal microflora following rat heterotopic small-bowel isotransplantation were assessed. Animals with Thiry-Vella fistula, but without transplants, acted as controls. Thirty and 40-fold increases in bacterial colony counts were seen in the isografts compared to controls at 2 and 7 days, respectively ($P < 0.05$). Aerobic faecal organisms predominated at 2 and 7 days, but an overgrowth of *Flavobacterium meningosepticum* occurred at 28 days in the transplanted and host bowels. The effect of warm ischaemia on intestinal microflora was assessed by the application of a microvascular clamp to the superior mesenteric artery for 90 min. The effect of denervation was assessed following microsurgical division of all nervous tissue around the superior mesenteric artery. After 7 days, lengths of jejunum and ileum were removed and intraluminal microflora assessed. The number of bacterial colonies isolated from the ileum in the warm ischaemia group was six times greater than the number in the control group, whereas no significant changes were seen in the upper bowel. In contrast, denervation led to a slight, but consistent, decrease in colony counts. These findings suggest that the increase in bacterial numbers in an isografted small bowel primarily results from warm ischaemia rather than from mesenteric denervation, and that physical aspects of the procedure may affect the development of sepsis following small-bowel transplantation.

Key words Small-bowel transplantation, rats · Intestinal microflora, small-bowel transplantation · Bacterial translocation, small-bowel transplantation

Introduction

Following transplantation the effects of rejection, immunosuppression and graft-versus-host disease (GVHD) leave the patient vulnerable to the development of sepsis by commensal organisms. Patients receiving an intestinal allograft are additionally exposed to possible bacterial translocation from the transplant and are at particular risk if they are suffering from protein malnutrition [3, 20]. In the early clinical cases of small-bowel transplantation, it is likely that death resulted from sepsis following translocation of organisms across mucosa damaged by rejection [8, 9]. We have previously demonstrated that rejection and GVHD are associated with shifts in intestinal microflora towards potentially pathogenic organisms that may pose a major threat for the development of sepsis [19]. Although transplantation of the small bowel inevitably involves cold ischaemia and denervation, the precise effects of these factors on mucosal integrity and bacterial populations within the small bowel have not previously been studied. In most current experimental models and in clinical small-bowel trans-

plantation, the graft is initially placed out of continuity with the native intestine and is therefore deprived of normal luminal content. These factors may affect the number and type of organisms within the bowel.

The aim of this study was to assess the quantitative and qualitative effects of heterotopic small-bowel isotransplantation on intestinal bacterial microflora in rats, and to investigate the effects of warm ischaemia and denervation on the bacterial milieu within the small gut. Physical effects of the transplantation procedure on the presence of pathogenic organisms in the small-bowel graft may influence the development of sepsis following fully allogeneic transplantation. Denervation cannot be avoided, but if ischaemia leads to bacterial overgrowth, then measures could be taken to minimise the injury.

Materials and methods

The effect of transplantation on intestinal microflora

The effect of transplantation on intestinal microflora was assessed using inbred male DA rats weighing 200–250 g (B & K Universal, Hull, UK). Heterotopic vascularised accessory small-bowel isotransplantation was carried out ($n = 26$) using a modification of the technique of Monchik and Russell [16]. The entire small bowel from the ligament of Treitz to the ileocaecal valve was harvested from each donor. The small-bowel vasculature was flushed with 10 ml Marshall's hypertonic citrate solution at 4 °C, and the intestinal lumen was cleared with 20 ml 0.05 % chlorhexidine solution. The harvested bowel was immersed in cold saline at 4 °C. The portal vein and the superior mesenteric artery with a cuff of aorta were anastomosed end-to-side to the recipient inferior vena cava and infrarenal aorta. The two ends of the transplanted bowel were exteriorised as stomas in the right flank to form a Thiry-Vella fistula. Each animal received 10 ml of normal saline and 1 mg gentamicin subcutaneously following the procedure. No other antibiotics were given thereafter. Post-operative analgesia was provided by subcutaneous buprenorphine. The total cold ischaemic time was 2 h.

To control for the effect on intestinal microflora of taking a loop of bowel out of continuity, a second group of 23 animals was studied. A 15-cm centrally placed loop of native intestine was separated from the rest of the small bowel and flushed with 20 ml 0.05 % chlorhexidine. Both ends were exteriorised as stomas to form a Thiry-Vella fistula, as in the group of transplant recipients. The residual small intestine was re-anastomosed. In this group the Thiry-Vella fistula did not undergo cold ischaemia or denervation, but reproduced the loss of continuity with the normal intestine, which was the feature of the heterotopic transplantation model. Peri-operative medication and post-operative treatment were the same in the transplantation and control groups. All animals recovered rapidly from the procedures and were eating normally and passing faeces within 12 h. All rats received standard, cereal-based laboratory chow that was sterile on routine aerobic culture. The animals from the transplant and Thiry-Vella fistula groups were sacrificed at 2, 7, 14 and 28 days after operation. In each animal the transplant or Thiry-Vella fistula and the native small bowel were harvested separately and flushed with 10 ml sterile saline. The effluent was collected aseptically.

The effect of warm ischaemia and denervation on intestinal microflora

The effect of warm ischaemia on intestinal microflora was assessed by exposing the superior mesenteric artery, using minimal dissection and applying a Scoville-Lewis microvascular clip to the main trunk ($n = 7$). Complete occlusion of the artery was confirmed by an increasingly dusky appearance of the small gut. The clamp was removed after 90 min to permit intestinal reperfusion, the abdomen was closed and the animals were allowed to recover.

The effect of denervation on intestinal microflora was assessed by performing a laparotomy and exposing the superior mesenteric artery ($n = 6$). All tissue surrounding the superior mesenteric artery and aorta was divided as far as the coeliac artery above and the renal arteries below, thus clearing the superior mesenteric artery of all peritoneum, nerves and lymphatics. The warm ischaemic and denervation experimental animals were sacrificed 7 days later. Small intestine was obtained from the warm ischaemic and denervation groups and normal DA rats (control) and cut into four equal lengths (upper jejunal, lower jejunal, upper ileal and lower ileal loops). Each loop was flushed with 10 ml sterile saline solution, and the intraluminal bacterial content was assessed.

Bacterial isolation

The effluents were mixed thoroughly and serially diluted 1 in 10 to a dilution of 10^{-11}. One-millilitre samples of each of five dilutions were placed onto Columbia Horse Blood agar and MacConkey salt-free agar plates (Becton Dickinson, Oxford, UK) for aerobic culture. Similar volumes were plated onto pre-reduced fastidious anaerobic blood agar with neomycin (FFA, Becton Dickinson) and placed into BBL Gas Pak anaerobic jars (Becton Dickinson). All plates were incubated at 37 °C for 48 h. The colonies were counted on individual plates with a minimum of 30 colonies per plate. The average number of colonies was calculated and the extrapolated final colony count was expressed as bacterial colonies per gram dry weight of bowel tissue [15]. Dry weight was assessed following desiccation of strips of bowel tissue. This method was chosen to prevent differing water content secondary to oedema from influencing the results.

In each of the animal groups the frequencies of the occurrence of species were recorded and presented as percentages for both the Thiry-Vella fistula and the native intestine. For all experiments, the groups were statistically compared using the Wilcoxon rank sum test on $\log_{10}$ data.

Bacterial identification

After 48 h representative colonies were selected and replated to obtain pure isolates that were identified by routine laboratory methods [5]. Further identification was based on API 20E, API 20NE, API Staph and ATB 32A for anaerobes (Analytical Profile Index, Biomerieux, Basingstoke, UK).

Results

At no stage were anaerobes isolated from the specimens in this study, although anaerobic organisms were present in faeces. These findings suggest that either anaerobes were not present or that they were in such small numbers in the jejunum and ileum that they could not be isolated.

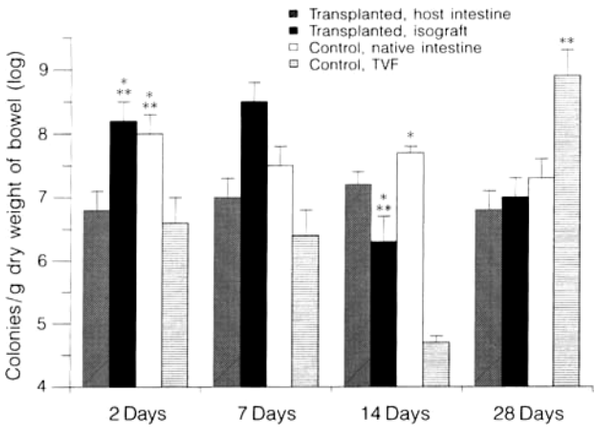

Fig. 1 Bacterial colony counts in host and graft bowel following isotransplantation, and in native small bowel and Thiry-Vella fistulae following intestinal isolation. Data are expressed as mean ± SEM. * $P < 0.05$ comparing host with graft, and Thiry-Vella fistulae with control intestine, ** $P < 0.05$ comparing isograft with Thiry-Vella fistulae, and host intestine with native intestine (Wilcoxon rank sum test)

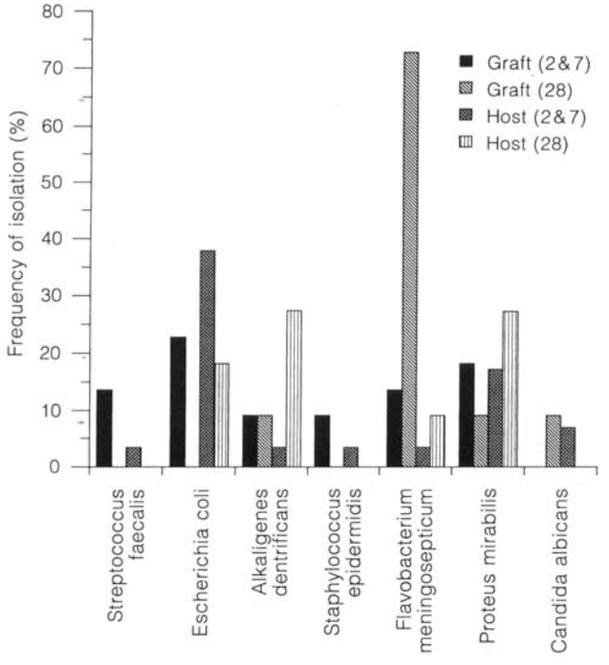

Fig. 2 Frequency of isolation of bacterial species from host and graft intestines following isotransplantation

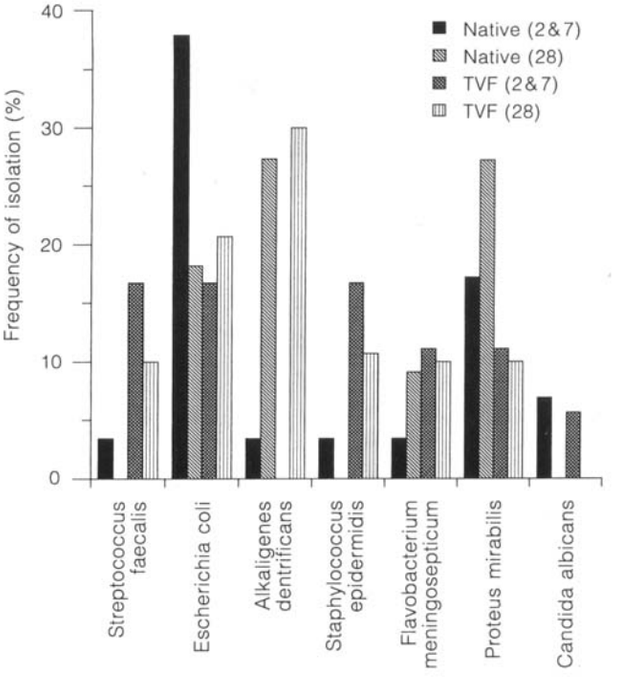

Fig. 3 Frequency of isolation of bacterial species from the native intestine and the Thiry-Vella fistulae following intestinal isolation

The effect of transplantation on intestinal microflora

By 48 h after transplantation, there was a 30-fold increase in the number of colonies present within the isograft compared to the host intestine. A similar pattern was observed in animals studied at day 7 with a 40-fold increase in colonies in the isografted bowel. After 14 days the number of bacterial colonies in the graft fell to a level similar to those isolated in the native intestine, and the colony counts were virtually identical at 28 days (Fig. 1). In contrast, in the control animals with Thiry-Vella fistulae, lower numbers of bacterial colonies were isolated from the loops than from the residual native bowel on days 2, 7 and 14. However, on day 28 there was a massive overgrowth in the Thiry-Vella fistulae that differed significantly from that of the isografts (Fig. 1).

The species profiles in the native intestine, grafts and Thiry-Vella fistulae in both the control animals and the transplant recipients were virtually identical at 2 days and 7 days. Consequently, the data for each loop at both points in time have been combined in Figs. 2 and 3. The pattern was still very mixed at 14 days, but with fewer organisms (data not shown). The bacterial species profile changed dramatically at 28 days, with a decrease in the number of species isolated and a considerable overgrowth of the gram-negative organism *Flavobacterium meningosepticum* (Figs. 2, 3). The frequency of this organism in the graft increased from 14 % to 73 %. *Flavobacterium meningosepticum* was isolated from both host and graft intestines in all eight animals in the transplant group and was the sole organism found in two of the host intestinal loops and five of the graft loops. The only other bacteria isolated from these animals were *Staphylococcus epidermidis, Alkaligenes denitrificans* and *Candi-*

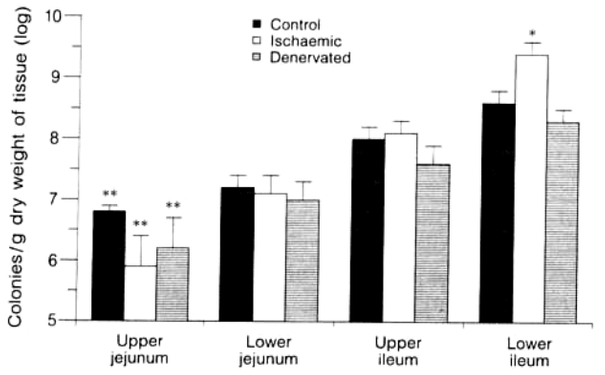

Fig. 4 Effect of warm ischaemia and denervation on bacterial colony counts in small intestine. Data are expressed as mean ± SD. $P < 0.05$ comparing the three upper jejunal groups with both their upper and lower ileal equivalents, * $P < 0.05$ comparing the warm ischaemic group with the control in the lower ileum (Wilcoxon rank sum test)

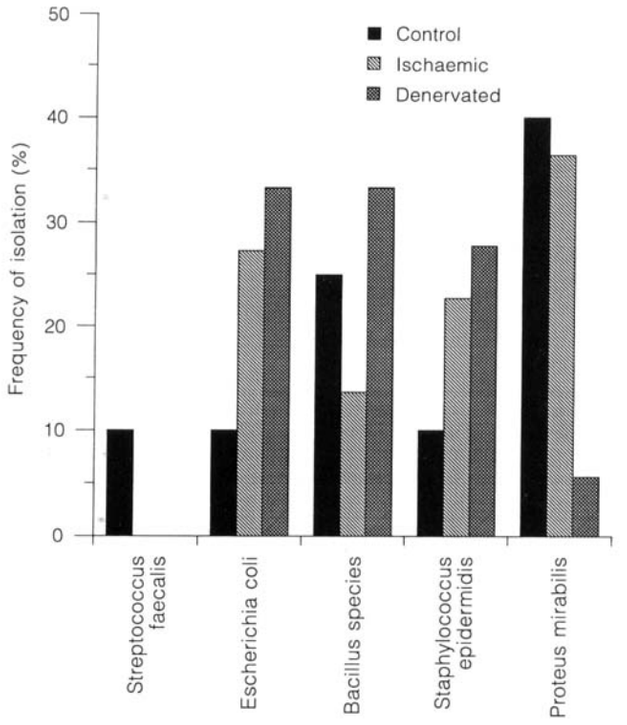

Fig. 5 Frequency of isolation of bacterial species from the proximal small intestine of control, warm ischaemic and denervated animal groups. Results obtained on day 7

da albicans. Organisms more associated with intestinal microflora, *Escherichia coli, Streptococcus faecalis* and *Proteus mirabilis* were not present.

In the control animals this change of pattern was not seen. A wide variety of organisms were isolated from the native intestine and the Thiry-Vella fistula at 2 and 7 days. At 14 days a similar profile of six organisms was found in the native intestine, but only two bacteria, *Proteus mirabilis* and *Bacillus sp.,* were isolated from the control Thiry-Vella fistula. These two organisms are entirely consistent with normal bowel flora. By 28 days there were fewer species in both the native bowel and the Thiry-Vella fistula. All organisms were normal intestinal commensals. No organism dominated to the exclusion of other bacteria, although *Alkaligenes denitrificans,* which is found in faeces, represented nearly a third of the organisms isolated in both gut loops.

The effect of warm ischaemia and denervation

There was a gradual increase in the number of bacterial colonies progressing down the small intestine in both the experimental and control groups. The differences between adjacent loops (i.e. upper and lower jejunum, upper and lower ileum) were too small to reach statistical significance, but significant changes in the number of bacterial colonies were seen in all groups when the upper jejunal loops were compared with the ileal loops (Fig. 4).

The number of bacterial colonies isolated from the lower ileum in the warm ischemia group was six times greater than that of the control group ($P < 0.05$), whereas no significant changes were seen in the upper small bowel. In contrast, there was a slight, but consistent, decrease in colony counts throughout the length of the intestine in the denervated group when compared to the controls.

A mixture of organisms comprising mainly *Proteus mirabilis* and *Bacillus sp.* was seen in the upper half of the gut in control animals, with smaller populations of *Escherichia coli, Streptococcus faecalis* and *Staphylococcus epidermis* (Fig. 5). In comparison, *Staphylococcus epidermidis* was isolated far more frequently from the proximal small bowel of the warm ischaemia and denervation groups. In both these groups *Escherichia coli* was also isolated more frequently (Fig. 5).

The distribution of bacterial species in the distal small bowel of the control group was similar to the distribution in the proximal gut (Fig. 6). Small differences occurred in the proportional frequency, notably an increase in *Escherichia coli* and *Staphylococcus epidermidis.* The proportions of *Proteus mirabilis, Staphylococcus epidermidis* and *Escherichia coli* in the warm ischaemia group were virtually identical to those in controls, but there was a slight increase in *Bacillus sp.* (Fig. 6). In the denervated intestine there was a large increase in *Bacillus sp.* with a matching reduction in *Escherichia coli.* The proportion of *Staphylococcus epidermidis* was similar to that in the controls (Fig. 6).

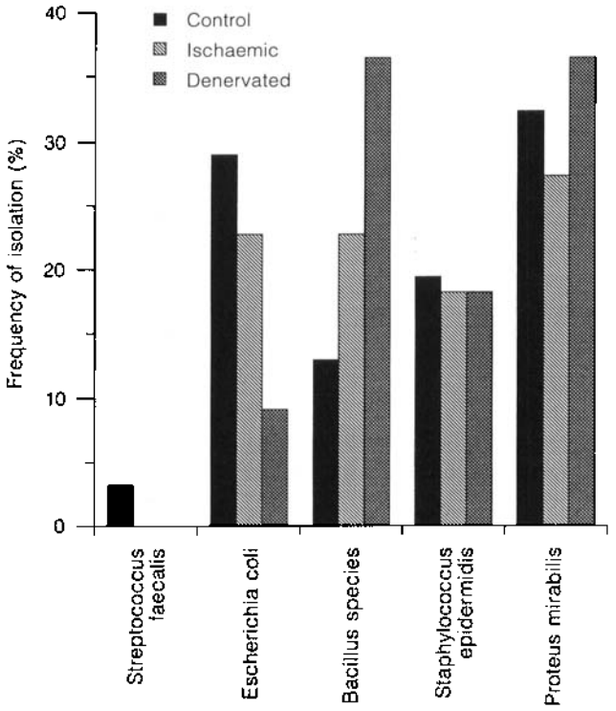

Fig. 6 Frequency of isolation of bacterial species from distal small intestine of control, warm ischaemic and denervated animal groups. Results obtained on day 7

Discussion

Translocation of bacteria into host mesenteric lymph nodes and spleen has been reported following rejection in a uni-directional model of orthotopic small-bowel transplantation in the rat [11]. Bacterial translocation into recipient tissues correlated with the change in intestinal permeability and breakdown in gut barrier function [11].

This study demonstrates that isotransplantation of small bowel in the rat, a model free of rejection and GVHD, leads to an increase in the number of bacteria within the grafted loop compared to the number in the host intestine. Furthermore, a striking change in the profile of the microflora in the grafted loop is seen in favour of the potentially pathogenic organisms *Flavobacterium meningosepticum* and *Alkaligenes denitrificans*. The *Flavobacteria* are commonly present in fresh or sea water and in soil and foodstuffs, but are not recognised as normal human intestinal flora. *Flavobacterium meningosepticum* is undoubtedly pathogenic to man and is a known cause of meningitis in neonates [13, 18] and of pneumonia in adults [7]. *Alkaligenes* are often found in human faeces and may cause bacteraemia [17].

The bacterial population changes observed after transplantation were not seen in the isolated loops of animals without transplants. One explanation for these differences could be an indirect effect of the inevitable denervation of the bowel following graft removal from the donor animal. Hypersecretion from the crypts occurs following denervation, which may add to the substrate pool. It is also possible that the dyskinetic action of peristalsis due to the loss of neural input produces decreased bowel transit and a stagnation effect that encourages bacterial overgrowth. However, our data suggest otherwise, with a slight fall in bacterial numbers in all areas of the small intestine following denervation of the mesentery. These findings are not statistically significant, but are consistent and suggest that there is a cleansing effect of the hypersecretion and a decrease in transit time. The animals in this group all produced loose stools.

The grafted bowel loop undergoes a period of ischaemia, which is unavoidable despite attempts to protect it by hypothermia and rapid restoration of the blood flow by early transplantation. Villous tip sloughing and disruption of the mucosal surface of the small intestine occur following harvesting and cold storage. The increase in bacterial numbers seen in this study following a period of warm ischaemia may be a direct result of the increased availability of substrate from dead tissue sloughed off into the lumen. A statistically significant increase in the number of bacterial colonies was demonstrated in the ileum of the warm ischaemia group compared to the control animals. *Staphylococcus epidermidis* proliferated in both the warm ischaemia and denervated groups. *Staphylococcus epidermidis, Candida albicans* and *Pseudomonas aeruginosa* are implicated in bacterial translocation and are recognised as significant causes of infection in seriously ill patients in intensive care [14]. In this study the effects of ischaemia and denervation on intestinal microflora were separated by applying a microvascular clamp to the mesenteric artery in situ, and the effect of warm ischaemia on intraluminal bacteria may be somewhat different from the consequences of cold ischaemia. Bacterial overgrowth with potentially pathogenic organisms, as demonstrated in the ischaemic group of animals in this study, is a cause for concern, and attempts to reduce ischaemia in the graft may decrease the risk of bacterial overgrowth following transplantation.

The animals used in this study had none of the immunological problems of rejection or GVHD, were healthy throughout the study and received no immunosuppressive therapy. In clinical small-bowel transplantation heavy immunosuppression is required, which may promote the development of infection. The limited clinical data show small-bowel transplant patients to be at risk of widespread septic complications [4, 10, 21]. Antibiotics disturb the ecological balance of the gut [6] and may encourage bacterial translocation to the mesenteric lymph nodes of mice [1]. Malnutrition, intravenous feeding and immunosuppression are known to enhance this process in humans [12]. Combinations of

antibiotics with immunosuppressive drugs can alter intestinal bacterial flora. In such cases bacterial translocation may be followed by death from systemic sepsis [2]. Therefore, the choice of antibiotic therapy during small-bowel transplantation as a prophylactic against infection must be considered carefully.

In summary, these data show that the physical effect of small-bowel transplantation in the rat leads to quantitative and qualitative changes in the small-bowel microflora in favour of potentially pathogenic organisms. These changes appear to result from ischaemia rather than from denervation. Such physical effects may influence the development of sepsis following transplantation, as recent work has demonstrated that rejection and GVHD are associated with bacterial translocation into recipient tissues [19]. A better understanding of the consequences of ischaemia and denervation on intraluminal bacterial profiles following small-bowel transplantation may help to reduce the overgrowth of potentially pathogenic organisms, their translocation into recipient tissues and the incidence of sepsis.

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