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BRIEF REPORT

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Endotoxin in the peripheral blood during acute intestinal allograft rejection

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Abstract Intestinal rejection is associated with increased gut permeability and bacterial translocation. The present study examined endotoxin and proinflammatory cytokines in the peripheral circulation during acute intestinal rejection. Heterotopic intestinal transplants were performed using Lewis rats ($RT1^l$) as donors and DA rats ($RT1^a$) as recipients. DA rats with intestinal isografts were used as controls. Serum samples were obtained at sacrifice on postoperative days (POD) 7 and 14. Lipopolysaccharide (LPS) was measured using the limulus amoebocyte lysate assay. Interleukin-1 (IL-1) and 6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured using bioassays. Large amounts of LPS were detected in the serum of intestinal allograft recipients concurrent with the development of graft rejection. Serum IL-6 and TNF-α levels were significantly elevated in the allograft recipients on both POD 7 and 14 when compared to DA isografts ($P < 0.05$). Serum IL-1 activity was not detected in the allograft or isograft recipients at either of the two time points. Further studies are warranted to determine the role of intraluminal bacteria and their products in the pathophysiology of intestinal allograft rejection.

Key words Intestinal transplantation, endotoxin, rat cytokine · Small bowel transplantation, endotoxin, rat Endotoxin, small bowel transplantation, rat

Introduction

Despite recent advances in immunosuppression, rejection continues to be a major barrier to small bowel transplantation (SBT) [21]. The factors contributing to the increased frequency and severity of intestinal rejection are poorly defined. The large amounts of bacteria in the small bowel are a unique feature of intestinal allograft. We have shown that early intestinal rejection is characterized by a breakdown of the gut barrier function and bacterial translocation [4]. We hypothesized that the release of lipopolysaccharide (LPS) during intestinal rejection could stimulate graft-infiltrating macrophages to produce proinflammatory cytokines that may augment the rejection process. The present study was undertaken to examine endotoxin and proinflammatory cytokines in the peripheral circulation during acute intestinal rejection.

Materials and methods

Heterotopic intestinal transplants were performed using Lewis rats ($RT1^l$) as donors and DA rats ($RT1^a$) as recipients. DA rats with isografts were used as controls. The donor aorta was sutured to the side of the recipient's aorta. The donor portal vein was anastomosed to the side of the recipient's vena cava. Both ends of the intestinal graft were exteriorized as stomas. The stomas were examined daily for clinical evidence of graft rejection. Five animals in each group were sacrificed on postoperative days (POD) 7 and 14. Serum samples were obtained and the grafts were examined histologically at sacrifice.

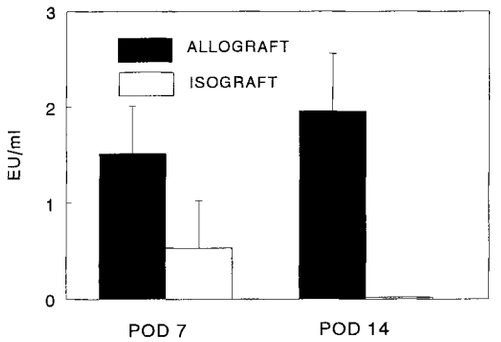

Fig. 1 Serum LPS concentration after small bowel transplantation

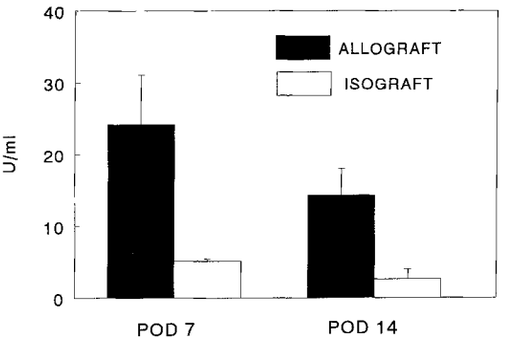

Fig. 2 Serum IL-6 activity after small bowel transplantation

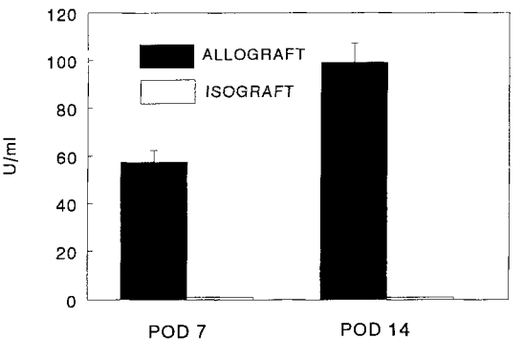

Fig. 3 Serum TNF-α activity after small bowel transplantation

The limulus amoebocyte lysate assay was used to quantitate serum levels of LPS [7]. Sterile serum samples were diluted 1:10 with pyrogen-free water (Sigma, St. Louis, Mo., USA) and incubated at 75 °C for 5 min; then, further serial dilutions were prepared and assayed in 10 × 75 mm glass tubes (baked at 180 °C for 3 h). Formation of a hard gel that permitted complete inversion of the tube without disruption of the gel was defined as a positive result. Serum LPS levels were interpolated from a LPS standard control (*E. coli* 0111: B4, Sigma). The result is expressed as endotoxin unit/ml (EU/ml).

Interleukin-1 (IL-1) activity was measured using the LBRM-TG6 cell line [6]. Briefly, serial dilutions of serum or a human rIL-1 standard (Bioproducts for Sciences, Indianapolis, Ind., USA) were prepared in 96-well culture plates (Linbro Flow Laboratories, McLean, Va., USA) with RPMI-1640 medium (Sigma). The mitomycin C-treated LBRM-TG6 cells (2×10^4) were added to each well along with 50 µl PHA (3 µg/ml). The cultures were incubated at 37 °C for 24 h, followed by the addition of CTLL-2 cells (1×10^4), and the plates were further incubated for 24 h. The proliferation of CTLL-2 cells was measured using a colorimetric assay [13]. IL-6 activity was determined using the B9 cell line [23]. Serial dilutions of serum or a human rIL-6 standard were prepared in 96-well culture plates with Iscove's medium (Gibco, Burlington, Ontario), and B9 cells (1×10^4) were added to each well. The plates were incubated for 3 days at 37 °C and the resulting cell proliferation was determined using a colorimetric assay [13]. Tumor necrosis factor-α (TNF-α) activity was assayed using the L929 cell line. L929 cells (1×10^4) were plated in 96-well culture plates and incubated for 24 h. The cells were treated with actinomycin D (Sigma) at a final concentration of 1 µg/ml for 2 h. Serial dilutions of serum or a human rTNF-α were added to each well and cultured for 18 h. Cell death was determined using a colorimetric assay [13]. The activities of IL-1, IL-6, and TNF-α are expressed as U/ml. The data are expressed as mean ± SD of five animals in each group and compared using Student's *t*-test with a *P* value less than 0.05 as the level of significance.

Results

Histological examination of the intestinal allografts revealed heavy lymphocytic infiltration with blunted villi on POD 7 and full-thickness necrosis of the allografts on POD 14. None of these changes was present in the isografts. The animals with intestinal allografts had no clinical or histological evidence of graft-versus-host disease (GVHD). High levels of LPS were present in the serum of the animals with rejecting intestinal allografts on POD 7 (1.52 EU/ml) and 14 (1.96 EU/ml; $P > 0.05$). Three of five animals in the isograft group had detectable LPS on POD 7, but the mean level was significantly lower than that in the allograft group ($P < 0.05$). By POD 14, serum LPS activity was not detected in the isograft controls whereas the LPS levels remained high in the allograft group (Fig. 1). Serum TNF-α and IL-6 levels were also elevated in the allograft recipients on POD 7 and 14 compared with the isograft controls ($P < 0.05$; Fig. 2, 3). The TNF-α activity on POD 14 was significantly higher than that on POD 7 ($P < 0.05$), whereas the difference in IL-6 levels was not statistically significant. IL-1 activity was not detected in either the allograft or the isograft recipients on either POD 7 or 14.

Discussion

This study has demonstrated increased levels of circulating LPS and proinflammatory cytokines during acute intestinal allograft rejection. Unlike other solid organ grafts, the intestine is continuously exposed to an environment of anaerobic and gram-negative bacteria. Endotoxemia after intestinal transplantation was first reported 20 years ago following 12-h preservation of canine small

bowel grafts [22]. Transient endotoxemia was later reported after gut transection, denervation, or isograft transplantation in the rat [1]. More recently, Nogushi et al. have documented the presence of IL-6, TNF-α, and IL-2 in the serum of rejecting small bowel/liver or isolated small bowel allografts in humans [15].

LPS has many biological effects that could contribute to intestinal graft damage including: (1) activation of neutrophils to produce elastase and superoxide ions that damage endothelial cells [9], (2) increased surface expression of the adhesion molecules such as CD11-CD18 that promote the adherence of leukocytes to the endothelium [9], and (3) stimulation of macrophages to release proinflammatory cytokines such as TNF-α, IL-1, and IL-6 [14, 17]. TNF-α is cytotoxic to intestinal epithelial cells [3]; it also damages gut barrier function [16], activates neutrophils, stimulates procoagulant activity, and increases the expression of MHC antigens [11]. TNF-α may play a central role in the pathogenesis of intestinal allograft rejection since treatment with anti-TNF-α antibodies has prolonged small bowel allograft survival in rats [19]. IL-6 and IL-1 are important costimulatory cytokines for T cell activation [5]. IL-6 interferes with gut barrier function and promotes acute phase responses [16]. Despite overlapping functions of IL-1 and TNF-α, the present study failed to detect IL-1 activity in the serum of rats with intestinal allografts using a bioassay. In contrast, Northern blot analysis has shown increased gene transcription for IL-1 in rejecting mouse intestinal allografts [18]. The discrepancy between these two observations may be due to the post-transcriptional regulation, the local release of IL-1 [12], or poor sensitivity of the bioassay used in the present study.

The present data support the notion that the release of LPS during the breakdown of the gut barrier may augment the rejection process by stimulating the production of proinflammatory cytokines. It is also possible that proinflammatory cytokines produced during graft rejection may, in turn, damage the gut barrier function and promote bacterial translocation since allograft rejection itself is associated with the expression of a variety of cytokines. Studies have shown elevated serum levels of TNF-α and IL-6 during rejection of renal allografts [11] and an increased expression of TNF-α, IL-6, and IL-1β gene transcripts in rejecting liver allografts in humans [10].

The concentrations of LPS and proinflammatory cytokines in the peripheral circulation after SBT may be affected by whether the intestinal allograft is drained into the systemic circulation or into the portal circulation. The liver is an efficient scavenger of LPS and cytokines in portal blood [2]; these substances are normally only released into the peripheral circulation when the liver is saturated or severely diseased. Clearance of LPS and cytokines by the native liver may explain why rat intestinal allografts that are drained into the portal vein survive longer than grafts that are drained into the vena cava [20].

In conclusion, we have demonstrated LPS and proinflammatory cytokines in the peripheral blood during acute intestinal rejection. Further studies are warranted to see if there is a cause and effect relationship between these events. Experiments are currently under way in our laboratory to determine if intestinal rejection can be prevented or ameliorated by treatment with anti-endotoxin antibodies or non-toxic LPS [8].

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