Lipopolysaccharide Pretreatment of Cyclosporine-Treated Rats Enhances Cardiac Allograft Survival

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Lipopolysaccharide (LPS), the endotoxin in gram-negative bacterial cell walls, is a major factor in septic shock. Tumor necrosis factor (TNF) appears immediately after LPS release or LPS injection in rats, but when these animals have LPS reinjected for up to 7 days, TNF production is inhibited. Because inhibiting TNF with anti-TNF antibodies prolongs cardiac allograft survival and is synergistic with cyclosporine (CsA), enhanced graft survival could result from inhibiting TNF via LPS pretreatment. Accordingly, heterotopic rat heart transplants were performed in: I, untreated controls; II, LPS pretransplant treatment; III, LPS post-transplant treatment; IV, low-dose CsA post-transplant treatment; V, CsA post-transplant treatment and PBS (LPS vehicle); or VI, LPS pretransplant treatment and low-dose CsA post-transplant treatment, using Brown Norway (BN) donors and Lewis (LEW) recipients. Rejection was defined by a lack of contractions. Results showed that while LPS pre- or post-treatment alone had little allograft survival effect, LPS pretreatment combined with CsA significantly prolonged survival vs control or CsA alone (22.0 ± 1.6 days vs 6.8 ± 0.6 days or 13.4 ± 1.1 days; $P < 0.001$). Primary MLRs of LPS-pretreated LEW splenocytes cocultured with irradiated BN splenocytes had significantly less [^{3}H]thymidine incorporation than untreated LEW splenocytes (3671 ± 349 vs 7828 ± 14 cpm). TNF assays of untreated and PBS-treated LEW spleen cells cocultured with irradiated BN spleen cells had 1.3 and 1.1 pg of TNF/10^6 cells, respectively, in 2 hr, but no TNF from LPS-pretreated LEW cells was detected. These results suggest that LPS-enhanced allograft survival may be due to TNF inhibition and lymphocyte suppression, providing insight into immunosuppressive mechanisms. © 1993 Academic Press, Inc.

INTRODUCTION

Immunosuppression using cyclosporine (CsA), an inhibitor of interleukin-2, has made cardiac transplantation accepted therapy for end-stage cardiac failure. Cardiac transplantation offers a return to an acceptable lifestyle; however, rejection and immunosuppression remain as major morbidities. The role of cytokines, specifically tumor necrosis factor (TNF), in rejection has brought new insight into immunosuppression. TNF induces tumor cell necrosis and regression by upregulating immune responses and exerting cytotoxic actions on transformed cells [1]. TNF recruits immune cells to a site of antigenic challenge, activates lymphoid cells, augments the expression of MHC antigens, and induces the production of intermediate cytokines. TNF modulates transplant rejection by elicitation and activation of T-cell lymphocytes and macrophages [2] and by mediating the actions of cytotoxic T-cells [3]. Further evidence for TNF's role in rejection is demonstrated by successful anti-TNF strategies resulting in extended transplant allograft survival [4–7].

Lipopolysaccharide (LPS), the endotoxin in gram-negative bacterial cell walls, upregulates TNF production under many conditions, including septic shock. TNF appears immediately after LPS release or LPS injection in rats, but when these animals have LPS reinjected for up to 7 days TNF production is inhibited [8]. As many successful anti-TNF strategies have resulted in extended cardiac allograft survival and are synergistic with CsA, enhanced graft survival could result from inhibiting TNF via LPS pretreatment. This present study investigated the timing, mechanism, and potential importance of LPS pretreatment induction of TNF inhibition and its relationship to immunosuppression for cardiac transplantation.

MATERIALS AND METHODS

Animals and Reagents

Inbred male Lewis (LEW; RT-1^1) and male Brown Norway (BN; RT-1^n) rats weighing 150 to 250 g were

obtained from Harlan Sprague Dawley, Inc., (Waltersville, MD). LPS (from *Esherichia coli* serotype 0111:B4; Sigma, St. Louis, MO) was prepared in PBS (phosphate-buffered saline). CsA was obtained from Sandoz Pharmaceuticals (East Hanover, NJ). Recombinant human TNF-α (Genzyme, Cambridge, MA) was used for TNF assays.

Culture Medium

RPMI 1640 medium with 2 mM L-glutamine (Gibco, Grand Isaland, NY) was supplemented with 10% heat-inactivated fetal calf serum (Gibco), 0.1 mM nonessential amino acids, 10 mM Hepes buffer, 100 μg/ml penicillin, 100 μg/ml streptomycin (Gibco), and 5×10^{-5} M 2-mercaptoethanol (Sigma) (complete medium).

Transplantation

Adult male BN rats were anesthetized and mechanically ventilated. A midline incision was made from the neck to the xyphoid. The left superior vena cava, left carotid, and left and right subclavian arteries were ligated. The right carotid artery was cannulated with a catheter for heparinization and perfusion of the donor. The right superior and inferior vena cavae and left lung were ligated and divided. The heart–lung preparation was removed while being retrograde perfused through the right carotid artery cannula. The perfusate was a modified Krebs–Ringers solution, aerated with 95% O_2 and 5% CO_2 at a perfusion pressure of 80 cm of H_2O and a temperature of 37°C. Preparation of the donor heart was completed by occlusion of the descending aorta and ligation and division of the right lung at the hilum. The donor heart was continuously perfused while awaiting and during transplantation and remained in normal sinus rhythm. Next, the recipient LEW rat was anesthetized and a midline incision was made from the sternum to the mandible, dividing the left sternocleidomastoid for exposure. The left common carotid artery was isolated, the distal portion ligated, and the proximal artery occluded. The descending aorta of the donor was isolated, the distal portion ligated, and the proximal artery occluded. The descending aorta of the donor was anastomosed (end to side) to the recipient left carotid artery. After completion of the arterial inflow, the venous anastomosis was performed from the left pulmonary artery of the donor heart to the left external jugular vein of the recipient. Continuous perfusion of the donor heart was terminated with restoration of blood flow into the beating donor heart from the recipient. Total perfusion time was routinely 45 min. The donor heart was observed for rhythm disturbances and the anastomoses were inspected for hemostasis. This heterotopic heart transplant technique, without ischemia or reperfusion is accomplished with >90% survival and has been reported previously [9].

Protocols

Six treatment protocols were examined: I, controls received no immunosuppression. Experimental groups included: II, LPS pretreatment intravenously (1 mg/kg on Day -7 and 5 mg/kg from Day -6 to Day -3); III, LPS post-treatment (1 mg/kg iv $\times$ 5 days from Day 0 to Day 4): IV, low-dose CsA intramuscularly (1.5 mg/kg/day $\times$ 14 days, 1/10 the dose of CsA required to permanently prevent rejection in this model) [4, 10, 11]; V, low-dose CsA and PBS; or VI, low-dose CsA and LPS pretreatment, using BN donors and LEW recipients. No animal received any other immunosuppression. The animals recovered and were monitored on a daily basis with palpation to determine the continued viability of the grafted heart. Rejection was determined by the absence of palpable contractions in the transplanted heart and confirmed by autopsy and selective histologic examination. Student's t test analysis was used to compare differences between experimental groups. Differences were considered to be statistically significant at a confidence limit of 95% ($P < 0.05$). Loss of graft function within 48 hr of transplant was considered a technical failure (<5%) and omitted from further analysis. 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 Animals" prepared the National Institutes of Health (NIH pub. No. 80–23, revised 1978).

Mixed Lymphocyte Reactions

The effect of LPS pretreatment on the primary immune response was examined in one-way mixed lymphocyte culture, with splenic lymphocytes from LEW rats $\pm$ LPS treatment as responders and irradiated BN spleen cells as stimulators. LEW rats were pretreated with 1 ml PBS from Day -7 to Day -3 or LPS 1 mg/kg on Day -7 and 5 mg/kg from Day -6 to Day -3. Spleen cells were isolated on Day 0. Spleens were processed through a 100-gauge stainless steel mesh and suspended in medium consisting of RPMI 1640. Erythrocyte-free suspension was obtained following brief treatment with sterilized water for 20 sec and then isotonicity was recovered by adding equal volume of 2$\times$ PBS immediately. Equal numbers (3×10^5) of responding cells and irradiated (3000 rads, ^{137}Cs; J. R. Shepard, Glendale, CA) allogenic stimulator cells were planted in quadruplicate in 96-well flat-bottom microtiter plates (Costtar, Cambridge, MA) in complete medium at 37°C in a 5% CO_2 atmosphere. For the TNF production studies, the supernatants were collected after 2 hr incubation and stored at -20°C. For proliferation studies, the cultures were incubated for 5 days and subjected for the final 18 hr of incubation to 1 μCi/well of [^{3}H]thymidine (NEN, Wilmington, DE). The cultures were then harvested and [^{3}H]thymidine incorporation was determined using a Beckman LS 6000 LL scintillation system.

TABLE 1

Allograft Survival Data of the Effect of LPS Treatment and CsA upon Cardiac Allograft Survival

	Pretreatment Rx	Post-transplant Rx	n	Allograft survival
I	None	None	10	6.8 ± 0.6
II	LPS	None	5	8.0 ± 1.7
III	None	LPS	5	$9.6 \pm 0.8^*$
IV	None	CsA	6	$14.8 \pm 1.0^*$
V	PBS	CsA	5	$13.4 \pm 1.1^*$
VI	LPS	CsA	6	$22.0 \pm 1.6^{*,**}$

Note. Control group recipients: I, received no immunosuppression. Experimental groups included: II, LPS pretreatment (1 mg/kg intraperitoneally from Day −7 to Day −3 pretransplant); III, LPS post-transplant × 5 days; IV, low-dose CsA (1.5 mg/kg/day × 14 days); V, CsA and LPS vehicle (PBS); VI, CsA and LPS pretreatment.

* $P < 0.05$ vs control.

** $P < 0.01$ vs CsA.

TNF Assay

The amount of TNF-α in MLR conditional medium was determined by using the WEHI-164 clone 13. An equal volume (100 μl) of conditional medium in serial twofold dilutions or TNF-α standard dilutions were added in 96-well microtiter plates. WEHI-164 cells were seeded at a density of 5×10^4 cells/well in a 100-μl volume of RPMI 1640 containing 1% FCS and incubated at 37°C for 20 hr in a humidified CO_2 incubator. MTT tetrazolium was added and the cells were further incubated for 4 hr at 37°C. Isopropanol with 0.04 N HCl was added. After dissolving the dark blue forazan crystals, the plates were read on a BIO-Kinetics reader using a test wavelength of 550 nm. Data were expressed as units/ml of TNF-α calculated by Macintosh ELISA Analysis. To confirm that the cytotoxic activity was due to TNF, positive samples were neutralized with a polyclonal rabbit anti-murine TNF antiserum that crossreacts with rat TNF, and the WEHI assay was then repeated. All positive samples rested with the anti-TNF antiserum were negative, confirming that the bioactivity was due to TNF.

RESULTS

Graft Survival

Untreated LEW recipients rejected BN grafts at 6.8 ± 0.6 days (Table 1). Intramuscular administration of 1.5 mg/kg CsA to LEW rats from Day 0 to Day 14 caused a significant prolongation of BN graft survival to 14.8 ± 2.4 days. When the recipients were pretreated with LPS alone, mean survival time of grafts had a slight, but not significantly prolonged, graft survival (8.0 ± 1.7 days). A significant prolongation of graft survival was obtained from LPS post-treated recipients (9.6 ± 0.8 days, $P < 0.05$ vs control). PBS (vehicle control) pretreatment
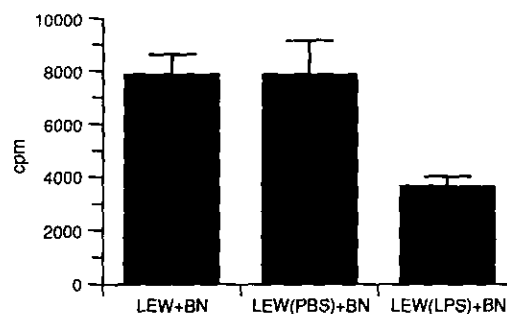

FIG. 1. Effect of LPS pretreatment on lymphocyte proliferation, as denoted by radiolabeled thymidine incorporation. LEW and BN spleen cells (5×10^6/well) were cultured for 5 days.

plus low-dose CsA post-treatment had no effect on graft survival (13.4 ± 1.1 days) vs low-dose CsA alone (14.8 ± 2.4 days). However, LPS pretreatment combined with low-dose CsA significantly prolonged graft survial to 22.0 ± 1.6 days and was more effective than either PBS plus CsA or CsA alone.

MLR Responses

LEW rats were given LPS 1 mg/kg on Day -7 and 5 mg/kg/day from Day -6 to Day -3 or 1 ml PBS (vehicle) from Day -7 to Day -3. Spleen cells were isolated on Day 0. Spleen cells from LPS-pretreated, PBS-injected, and normal, control untreated rats were stimulated with irradiated BN spleen cells. The results (Fig. 1) showed that spleen cells from LPS-pretreated rats had significantly less [^{3}H]thymidine incorporation than those from normal or PBS-injected rats (3671 ± 347 vs 7828 ± 814 and 7952 ± 1233, respectively; $P < 0.05$). TNF-α production in the MLRs was measured. Normal and PBS-treated LEW spleen cells cocultured with irradiated BN spleen cells showed 1.3 and 1.1 pg of TNF/10^6 cells, respectively, in 2 hr, but no TNF from LPS-pretreated LEW cells was detected (1.3 and 1.1 pg of TNF/10^6 cells) (Fig. 2).
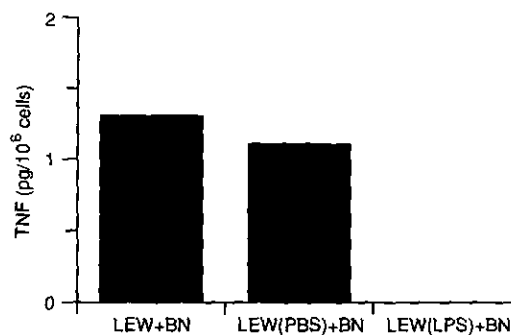

FIG. 2. Effect of LPS pretreatment on TNF production from lymphocyte proliferation assays, using cells obtained 5 days after *in vivo* transplantation. Splenocytes from LEW rats were cocultured with irradiated splenocytes from BN rats for 2 hr.

DISCUSSION

Tumor necrosis factor, found in lymphocytes, macrophages, and other immune active cells, mediates many inflammatory and immune events beyond necrosis of tumor cells [1–3]. The role of TNF in allograft rejection has long been postulated, as lymphotoxin (TNF-β) was noted in rejected human renal allografts [10] and TNF-α demonstrated in rejected rat cardiac allografts [11]. Elevated TNF has been detected in patients who have undergone liver, renal, and bone marrow transplants rejection [3, 6, 11, 12]. Although the exact role of TNF in allograft rejection is unknown, our previous study [4] demonstrated that TNF is an important stimulus to the MLR. Using lymphocytes from the present strains of rats (BN vs LEW), TNF was found to dose-dependently augment the MLR at physiologic TNF levels (12.5 to 125 pg/ml). This present study confirmed this role of TNF in augmenting lymphocyte proliferation, as TNF inhibition by LPS pretreatment led to less proliferation by MLR (Fig. 1).

Our previous studies have demonstrated that a single intraperitoneal injection of anti-TNF antibody at the time of transplant or up to 72 hr later significantly delayed cardiac allograft rejection. Immunohistochemical analysis demonstrated a specific TNF fluorescence staining pattern for TNF associated with a mononuclear cell infiltrate, indicating local synthesis of TNF during rejection [4]. This confirms others results that antibody therapy against TNF enhanced cardiac allograft survival in rats (Buffalo rat donors and LEW recipients), while administration of TNF itself to recipients conversely accelerated the time to rejection [7]. In another study, we demonstrated that anti-TNF antibody in combination with low-dose (subtheraputic dose) CsA was significantly more effective than either drug alone [13]. Others have also shown less allograft TNF, lower serum TNF levels, and less infiltration of the allograft with cytotoxic T-lymphocytes by use of anti-TNF antibodies and low-dose CsA to synergistically prolong graft survival [14]. These studies indicated that TNF plays an important role in the immune rejection process; therefore, inhibiting TNF production and its activity may prolong allograft survival.

In this study, we hypothesized that enhanced graft survival could result from inhibiting TNF via LPS pretreatment. LPS, the endotoxin in gram-negative bacterial walls, has been extensively studied as a major factor in the pathogenesis of bacterial infection. The action of LPS is mediated almost solely by TNF, as monocytes activated by LPS have cytotoxicity completely blocked by antibody to TNF-α [15]. LPS activity inhibitors also prevent LPS-evoked TNF release and monoclonal antibodies directed against LPS can decrease TNF production and enhance sepsis survival in vivo [16]. Interestingly, LPS has the property of inducing hyporesponsiveness or tolerance to its own effects [17], which has been demonstrated in man and experimental animals. TNF appears almost immediately upon LPS injection in mice, but when such animal are injected again with LPS, they are unresponsive and do not produce TNF again, at least for 7 days [8]. If an allograft were transplanted during this time of TNF unresponsiveness, it may prolong allograft survival. While LPS pretreatment alone did not effectively enhance transplant survival, preoperative LPS treatment combined with low-dose CsA significantly prolonged allograft survival compared with CsA alone (22.0 ± 1.6 days vs 14.8 ± 1.0 days; $P < 0.01$). The present study also showed that splenocytes harvested from LPS-treated LEW rats cultured with irradiated BN splenocytes for 5 days showed a significant decrease in thymidine incorporation compared with untreated normal LEW rat cells. Furthermore, no detectable TNF was found from spleen cells of LPS-treated LEW rats cocultured with irradiated BN splenocytes, while cocultured spleen cells from normal and vehicle control rats produced TNF. These results confirmed that TNF was not produced from these LPS-pretreated spleen cells and suggested that LPS pretreatment not only inhibits TNF production from a second LPS stimulation, but also suppresses the responses of spleen cells to allogeneic stimulation.

The regulatory mechanisms for the activation and/or inhibition of macrophage production of TNF by LPS are not resolved. It has been demonstrated that when monocytes are stimulated by LPS at 1 μg/ml for 4 to 6 hr, MLR supernatants contain TNF at an average of 60 μg/ml. Pretreatment of monocytes with LPS at 10 ng/ml for 3 days results in cells refractory to subsequent stimulation by LPS at 1 μg/ml and no TNF production, which correlates with our study. In the refractory desensitized cells, production of all cytokines is downregulated with a 10-fold reduction in protein production [18]. Furthermore, in a study of isolated macrophages, pretreatment with low amounts of LPS results in nearly complete suppression of TNF secretion. This desensitization appeared to be regulated at the transcript level, with a reduction in mRNA [19]. These experiments correlate with in vivo observations that two injections of LPS spaced 5 hr apart in rabbits cause TNF levels to rise in the blood only after the first LPS injection. This hyporesponsiveness was induced by LPS doses 1000 times less than those required to induce TNF production [20].

This study utilized the inhibition of TNF induced by LPS pretreatment in combination with low-dose CsA to enhance cardiac allograft survival. TNF production/expression, as demonstrated in MLRs between these two immunologically incompatible strains of rats (Fig. 2) confirm that TNF was not produced by these LPS-pretreated cells. Furthermore, lymphocyte proliferation was depressed in our model with LPS pretreatment (Fig. 1). These studies demonstrated a synergistic effect against allograft rejection from CsA and inhibition of TNF by LPS pretreatment, interacting at different lev-

els of the immune response, as inhibition of TNF decreases lymphocyte proliferation and perhaps blocks the toxic action of released cytokines, whereas CsA acts primarily as an anti-IL-2 agent, blocking the release of interferon-γ, depressing recruitment of monocytes. These results confirm TNF and cytokine interactions as playing an important role in the immune response and enhance our understanding of the mechanism of TNF in allograft rejection.

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