Adrenergic modulation of splenic macrophage cytokine release in polymicrobial sepsis


Departments of Physiology and Surgery, The Burn and Shock Trauma Institute, Loyola University Medical Center, Maywood, Illinois 60153

Submitted 10 December 2003; accepted in final form 4 May 2004

Deng, Jiangping, Kuzhali Muthu, Richard Gamelli, Ravi Shankar, and Stephen B. Jones. Adrenergic modulation of splenic macrophage cytokine release in polymicrobial sepsis. Am J Physiol Cell Physiol 287: C730-C736, 2004. First published May 19, 2004; 10.1152/ajpcell.00562.2003.—Enhanced adrenergic stimulation and catecholamine release are important components of the pathophysiology of sepsis. Under physiological conditions, adrenergic stimulation has been shown to be a negative regulator of proinflammatory cytokine production through increasing IL-10 production. Here we have investigated if adrenergic stimulation similarly inhibits TNF-α and IL-6 production by splenic macrophages isolated from a polymicrobial sepsis model. Male B6D2F1 mice were subjected to sham (S), laparotomy (Lap), and cecal ligation and puncture (CLP) under anesthesia. Splenic macrophages were isolated 72 h after the initial injury and were stimulated with endotoxin (LPS) in the presence and absence of epinephrine. Compared with S and Lap, splenic macrophages from the CLP group produced significantly less TNF-α and IL-6 and more IL-10 when stimulated with LPS. Macrophage cultures from CLP animals incubated with either epinephrine or IL-10 for 2 h had significantly reduced TNF-α and IL-6 release in response to LPS. However, similar cultures pretreated with IL-10 antibody before the addition of exogenous epinephrine failed to reverse the attenuation of LPS-stimulated cytokines. Pretreatment of macrophage cultures with β2- (ICI-118551) but not β1-adrenergic (atenolol) receptor antagonists reversed the epinephrine-mediated cytokine attenuation following LPS treatment. Data are also presented that demonstrate the involvement of protein kinase A activation with adrenergic agonist but not with IL-10 stimulation. Taken together, these findings suggest that adrenergic mechanisms may influence peripheral tissue macrophage inflammatory cytokine response following trauma and sepsis, independent of the effects of IL-10.

catecholamines; sympathetic; cecal ligation and puncture; bacterial endotoxin; β-antagonist

ALTHOUGH CRITICAL TRAUMA AND its sequelae of septic complications are major causes of mortality in the first 4 decades of life, the pathophysiology remains largely elusive (1, 2, 33). An important but poorly understood aspect of host responses to infectious challenge and traumatic injury is the interaction of sympathetic nerves and the immune system.

Sympathetic activation resulting in the release of catecholamines has been documented in both experimental (13, 14, 39, 40) and clinical (3) studies involving sepsis. However, much of the direct evidence for increased neurotransmitter and hormone release in sepsis has come from experimental paradigms involving spleen, heart, bone marrow, and plasma (13, 14, 39, 40). Our laboratory has previously correlated increased catecholamine release in the bone marrow and enhanced myelopoiesis during sepsis (39). More recently, our laboratory has also demonstrated that bone marrow monocytes developing under the influence of increased sympathetic activation resulting from sepsis are functionally different in terms of their cytokine responses (8). Although sympathetic activation is known to occur in sepsis and may be important for cardiovascular and metabolic compensation, several lines of investigation also suggest that sympathetic activation may mediate immune modulation in sepsis. Maestroni (22, 23) has recently demonstrated that catecholamines modulate the migration and maturation of dendritic cells, which are an important component of both innate and adaptive immune responses and very likely have an important role in the development of sepsis. Equally important is the work of Sanders and coworkers (16, 30, 32) who have presented evidence suggesting that catecholamines may be pivotal in the modulation of Th1 and Th2 cell interactions.

The spleen is one of the lymphoid organs that not only plays an important role in immune regulation following infection but also is highly innervated with sympathetic nerve terminals and, therefore, is an essential target organ for studies involving interactions between the neural and immune systems. Although stimulation of sympathetic nerves to the spleen can mediate the rapid movement of stored red blood cells and leukocytes into the circulation (24, 43), the effect of intense stimulation on innervated immune cells (10) during conditions of sepsis has not yet been elucidated. The potential influence of sympathetic stimulation on immune cell function should be considered in light of the importance of the spleen as an immune organ and its fundamental role in bacterial clearance through antibodies and macrophages, as well as by bacterial endotoxin detoxification (21). Indeed, the significance of the spleen in immune function is highlighted by the incidence of overwhelming postsplenectomy infection, and such occurrences have raised important questions regarding the conditions that warrant splenectomy (12).

In the present study, we have used a murine animal model of surgical trauma and sepsis to assess cytokine-sympathetic neural interactions in splenocyte macrophage cells under closely controlled in vitro conditions. Our goal was to explore macrophage cytokine release by using endotoxin provocation and to define how adrenergic stimulation could modify pro- and anti-inflammatory cytokine interactions, both before and after conditions of injury-induced sepsis. We tested the premise that adrenergic stimulation would enhance anti-inflammatory cyto-
kine release that, in turn, would lead to attenuation of proinflammatory cytokine production. We report that adrenergic stimulation can induce anti-inflammatory cytokine release such as IL-10; however, adrenergic agonist-mediated attenuation of proinflammatory cytokine release may be independent of IL-10.

MATERIALS AND METHODS

Animals. B6D2F1 male mice, weighing 25–30 g, were purchased from Jackson Laboratories (Bar Harbor, ME). Before the start of the experiments, mice were allowed to acclimatize for 7 days after arrival at our Comparative Medicine Facility, under a controlled temperature (20–22°C) and humidity (20–40%) environment, with a 12:12-h light-dark cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Loyola University Medical Center.

Experimental septic peritonitis. The procedure of cecal ligation and puncture (CLP) with entry into the peritoneum was used as a model of “polymicrobial sepsis,” as originally described by Wichtermann et al. (44). In brief, mice were randomly divided into sham (S), laparotomy (Lap), and CLP groups. All of the animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Whereas the S group was not surgically manipulated, the Lap and the CLP groups were subjected to a midline ventral laparotomy with exposure and isolation of the cecum. The Lap group was not subjected to any further surgical intervention. In the CLP group, the cecum was tied off ~1 cm from the end and punctured with a single hole by using a 21-gauge needle. All animals were resuscitated with 2 ml of sterile saline administered subcutaneously. Surviving animals were euthanized 72 h postinjury, and splenic macrophages were isolated for further studies.

Preparation of splenic macrophage cultures. Splenic macrophages were prepared from each of the three treatment groups 72 h after surgical procedures by placing excised spleens in a plastic culture dish containing HBSS and extracting cells with the use of a rubber-tipped plastic 20-ml syringe plunger. Splenocytes were treated with ACK lysing buffer to remove red cells, washed with additional HBSS, counted, and suspended in RPMI-1640 medium with 10% heat-inactivated FBS at 3 × 10^6 cells/ml in six-well plates (Costar, Cambridge, MA). After 2-h incubation at 37°C with 5% CO_2_, nonadherent cells were removed from the culture plate by gentle washing with warm HBSS. Fresh RPMI-1640 with 10% FBS covered the adherent spleen cells, which were then used in cell treatment protocols, as described below.

Cell treatment protocols. 1) To determine the cytokine responses, freshly prepared splenic macrophages were treated with bacterial endotoxin (LPS) from E. coli (Difco, Detroit, MI) at 200 ng/ml for 18 h, and conditioned media were collected and stored at −80°C until analysis. 2) The influence of IL-10 on LPS-mediated cytokine response was determined by 2-h incubation with IL-10 (10 ng/ml), with or without IL-10 antibody (PharMingen, San Diego, CA), followed by LPS for 18 h. 3) The influence of epinephrine on LPS-mediated cytokine response was determined by 2-h incubation with variable concentrations of epinephrine (10^-6 to 10^-12 M; Sigma Chemical, St. Louis, MO), with or without adrenergic antagonists (Atenolol, selective β_1_, or ICI 118,551, selective β_2_ antagonist; Sigma Chemical) followed by LPS for 18 h. 4) To determine the ability of epinephrine to stimulate the release of IL-10 in the absence of endotoxin, macrophages from different treatment groups were incubated with epinephrine (10^-6 M) for 2, 4, 8, 24, or 48 h. 5) The influence of epinephrine to indirectly influence LPS-mediated cytokine response by stimulating IL-10 release was determined by 2-h incubation with epinephrine (10^-6 M; Sigma Chemical), with or without IL-10 antibody (PharMingen), followed by LPS for 18 h. 6) To test whether epinephrine or IL-10 inhibits TNF-α production through cAMP-stimulated protein kinase A activation, splenic macrophages were treated with or without the protein kinase A inhibitor H-89 (10^-6 M; Sigma Chemical) for 1 h, followed by either epinephrine or IL-10 for 2 h, after which LPS was added and maintained for 18 h.

Total cellular protein was assayed by bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL), and the cytokine levels were standardized to the cellular protein concentration.

Cytokine determination. IL-6, IL-10, and TNF-α levels in conditioned media were determined by standard ELISA techniques (BioSource, Camarillo, CA). All cytokine concentrations are reported as picograms per milligram of protein.

RESULTS

Animal mortality and sepsis. S and Lap treatments did not result in any mortality by 72 h, when all animals were euthanized. CLP animals displayed an average of 30% mortality by 72 h, with most deaths occurring between 48 and 72 h.

Endotoxin-evoked cytokine responses of splenocyte-derived macrophage cells: modulation by epinephrine and IL-10. The effect of polymicrobial sepsis on splenic macrophage cytokine production and its modulation by epinephrine was investigated in mice 72 h following CLP. Figure 1 describes the influence of S, Lap, and CLP treatments on LPS-stimulated TNF-α production. TNF-α responses in cells taken from CLP-treated mice were reduced to 70% of that observed in S and Lap-treated animals (P < 0.05). To determine whether adrenergic agonists or IL-10 modulated TNF-α production, splenic macrophages were pretreated with either epinephrine or IL-10 for
2 h before stimulation with LPS. Exogenous addition of both epinephrine and IL-10 resulted in a significant reduction in TNF-α release (P < 0.05), but, at this single concentration, IL-10 treatment resulted in greater reduction than epinephrine. The reduction of TNF-α release with epinephrine appeared to be greater in the CLP group (23% of that with LPS alone) than that in S (34%) and Lap (35%).

Figure 2 describes the influence of S, Lap, and CLP treatments on LPS-stimulated IL-6 production. IL-6 responses in cells taken from CLP-treated mice were reduced to ~60% of those from S- and Lap-treated animals (P < 0.05). To determine whether adrenergic agonists or IL-10 modulated IL-6 production, splenic macrophages were pretreated with either epinephrine or IL-10 2 h before stimulation with LPS. Exogenous addition of both epinephrine and IL-10 resulted in a significant reduction in IL-6 release (P < 0.05), but epinephrine and IL-10 treatment reduced IL-6 production by the same percentage in all three groups (see percentage values in Fig. 2).

Because sepsis increases circulating levels of epinephrine (18), we explored the possibility that CLP altered the sensitivity of epinephrine to inhibit TNF-α release by conducting dose-response experiments. These results are presented in Fig. 3 and confirm that CLP treatment resulted in decreased TNF-α release in response to endotoxin. These results also demonstrate that endotoxin-stimulated TNF-α release was inhibited in all groups to the same extent (40–45%) by 10⁻⁶ M epinephrine, but all groups were insensitive to 10⁻⁹ M epinephrine. Thus polymicrobial sepsis (CLP) reduced the maximal TNF-α response to endotoxin stimulation but did not alter the sensitivity of epinephrine-mediated inhibition of endotoxin stimulation.

Figure 4 describes results of IL-10 responses following LPS provocation, including the results of epinephrine pretreatment. Splenic macrophage release of IL-10 was increased approximately twofold in the CLP group compared with that in both S and Lap groups (P < 0.05). However, pretreatment with epinephrine did not significantly alter the IL-10 response in any of the treatment groups.

Epinephrine stimulation of IL-10 release in the absence of endotoxin. Figure 5 presents results of epinephrine stimulation of IL-10 release in splenocyte macrophages from animals...
subjected to S, Lap, and CLP treatment. IL-10 levels from the 24-h time point represent the maximal differences observed where IL-10 release from the CLP group was threefold that of the S and Lap groups ($P < 0.05$).

IL-10 antibody treatment and responses to endotoxin with or without epinephrine. Figures 6 and 7 describe the results of experiments that tested the influence of epinephrine to indirectly inhibit TNF-α and IL-6 production through the release of IL-10 in splenic macrophages. Because there was maximal release of IL-10 as well as maximal epinephrine-mediated inhibition of proinflammatory cytokine release in splenic macrophages following CLP treatment, the results presented in these figures involve macrophages taken exclusively from CLP-treated animals. In both Figs. 6 and 7, four different experimental conditions are contrasted, and each set of experiments was conducted with or without the presence of IL-10 antibody. Comparisons labeled “Endotoxin” indicate that the presence of the IL-10 antibody did not influence either the TNF-α or IL-6 response to 18 h of LPS exposure. Comparisons labeled “Epinephrine + Endotoxin” suggest that epinephrine-induced inhibition of LPS-stimulated TNF-α and IL-6 is not altered by the presence of the IL-10 antibody, and the comparisons labeled “IL-10 + Endotoxin” serve as a positive control ($P < 0.05$), demonstrating that inhibition of TNF-α and IL-6 release with LPS by IL-10 can be relieved with IL-10 antibody. These results suggest that epinephrine inhibition of LPS-stimulated TNF-α and IL-6 release may involve direct inhibition via adrenergic mechanisms.

Adrenergic blockage of epinephrine-mediated inhibition of cytokine release following endotoxin stimulation. Figure 8 presents results of LPS-stimulated TNF-α in cells taken from CLP-treated animals in the presence of β-adrenergic agonists and antagonists. Epinephrine inhibition of this response (TNF-α with and without epinephrine pretreatment) was abolished in a dose-response manner by pretreatment with a β2-adrenergic antagonist but was not altered by β1-adrenergic antagonists (note comparisons with $P < 0.05$).

Protein kinase A involvement in epinephrine and IL-10 inhibition of endotoxin-stimulated TNF-α release. Figure 9 presents results of LPS-stimulated TNF-α release in cells from CLP-treated animals in the presence of protein kinase A inhibitors before stimulation with either epinephrine or IL-10.
Epinephrine inhibition of the endotoxin-mediated TNF-α release was abolished by pretreatment with PKA inhibitor H-89. In contrast, IL-10 inhibition was not altered by the PKA inhibitor, suggesting that the epinephrine- but not the IL-10-induced response is mediated by the action of cAMP.

**DISCUSSION**

The present study clearly shows that epinephrine is a potent downregulator of LPS-stimulated TNF-α and IL-6 production by ex vivo cultures of splenic macrophages. Because epinephrine treatment induces significant amounts of IL-6 production, independent of LPS, it is possible that epinephrine could mediate its inhibitory effect of proinflammatory cytokine production through IL-10. IL-10 has been previously shown to significantly negate the production of TNF-α and IL-6 in macrophages (9, 11), and our results confirm these findings in using exogenous IL-10. However, our studies with anti-IL-10 antibodies suggest that stimulation of endogenous IL-10 production by epinephrine alone was insufficient to account for the inhibitory effect of epinephrine on LPS-stimulated proinflammatory cytokine production by splenic macrophages.

Although our observed lack of effect of IL-10 to attenuate proinflammatory cytokine release is in contrast to previous works, suggesting their physiological actions (37, 38), the critical in vivo concentration determining this effect is not known. It can be argued that our ex vivo culture conditions may not accurately represent the in vivo milieu. A much higher concentration of IL-10 could be potentially achieved in the microenvironment that includes both release and site of action. Therefore, the physiological action of IL-10 within the spleen during sepsis remains a possibility. Interestingly, both IL-6 and TNF-α production in response to LPS were significantly attenuated in CLP, whereas IL-10 levels were augmented. These findings are consistent with the concept of a progression from proinflammatory to antiinflammatory cytokine responses in developing sepsis (19, 28).

In contrast to our findings, Schwacha et al. (34) have reported ex vivo LPS-stimulated proinflammatory cytokine responses by splenic macrophages to be exaggerated 7 days after burn injury. These responses, however, involve conditions of thermal injury without infection and may represent a different sequela of responses (34). These authors have attributed the heightened proinflammatory cytokine responses to decreased IL-10 receptor expression, and, therefore, it is important to relate these findings to our data. Although we did not measure them in the present study, a decrease in functional macrophage IL-10 receptors following CLP might explain the lack of IL-10 influence on LPS-stimulated proinflammatory cytokine response. However, similar findings of the lack of IL-10 influence in the uninjured control group (S) seem to make this possibility less likely.
Our pharmacological findings demonstrate that adrenergic inhibition of LPS-mediated cytokine release could be reversed in a dose-dependent manner with selective $\beta_2$-adrenergic blockade, thus suggesting a clear mechanism for the observed action of epinephrine. Furthermore, our epinephrine dose-response data suggest that inhibition of LPS-stimulated cytokine release occurs at plasma concentrations approximating those reported for mice and at much lower concentrations than would be expected to occur in polymicrobial sepsis based on previous studies in conscious rats (5, 18).

To appreciate the significance of our results, we must view our findings within the framework of other studies involving adrenergic modulation of endotoxin-stimulated TNF-α, IL-6, and IL-10 release in both human and animal tissues. Monocytes have been demonstrated to release IL-10 in response to adrenergic stimulation (45), and this response is greatly augmented in the presence of bacterial endotoxin (37, 38). TNF-α and IL-6 release in whole blood, presumably by monocytic cells, has been shown to be inhibited by catecholamines (4, 41, 42), and similar results were found with the use of a monocytic cell line (35). Although catecholamines have been reported to suppress endotoxin-mediated TNF-α release in liver tissue and Kupfer cells, $\beta$-adrenergic stimulation in these preparations has been reported to increase IL-6 release (20). Similar to our present findings, $\beta$-adrenergic inhibition of IL-6 has also been reported in spleen tissue (36); however, in the kidney and the skin, $\beta$-adrenergic stimulation has been reported to increase IL-6 (15, 25, 26). Collectively, these findings suggest that adrenergic modulation of cytokine release appears to be tissue specific, but how such regulation may be altered during conditions of sepsis is largely unexplored.

Bergmann et al. (4) have examined the adrenergic modulation of endotoxin-induced cytokine release in human whole blood taken from septic patients. Like our findings, they report attenuation of TNF-α and IL-6 production in sepsis; however, the adrenergic suppression of this release is maintained. They also demonstrate increased IL-10 release under conditions of severe sepsis, but, in contrast to our findings, IL-10 release was not increased by epinephrine. Furthermore, prolonged septic shock ablated the ability of epinephrine to inhibit IL-6 release and markedly reduced the epinephrine attenuation of TNF-α. Direct comparisons between these clinically based findings and our results using experimental sepsis, although important, are complicated by differences in the cell source of cytokines, the severity of sepsis, and the possible effect of therapeutically administered catecholamines in the clinical group. However, these findings do suggest the potential importance of catecholamines to modulate cytokine release in the development of sepsis and septic shock, and additional experimental paradigms would seem well suited to extend the clinical observations under controlled conditions.

Although our data demonstrate that epinephrine and IL-10 have a common result, the suppression of TNF-α and IL-6 release, the cell signaling mechanisms involved appear to be quite contrasting. Our results clearly show that, whereas adrenergic stimulation-mediated suppression involves protein kinase A and thus cAMP production, IL-10 suppression of TNF-α does not. Furthermore, previous studies have demonstrated that dibutyryl cAMP inhibits TNF-α synthesis and that this involves the cAMP response element binding protein and interference of the NF-κB nuclear signaling pathway (7, 27, 29). In contrast, cellular mechanisms by which IL-10 has been shown to inhibit TNF-α production are more diverse. These include the absolute requirement for STAT-3 transcription factor (31), inhibition of gene translation through blockade of p38 mitogen-activated protein kinase activation (17), as well as posttranslational mechanisms involving the destabilization of TNF-α mRNA (6).

In summary, our work suggests that increased adrenergic activation could play a dual role in sepsis. While on the one hand it may meet the well-recognized rheological and metabolic needs in sepsis, on the other it may signal an anti-inflammatory phase by ameliorating proinflammatory cytokine production and stimulate anti-inflammatory cytokine production. How catecholamines in sepsis among other biological parameters may alter the balance between pro- and anti-inflammatory cytokines may hold the key to patient outcome.

GRANTS

This work was supported by National Institute of General Medical Sciences Grants R01 GM-61746 (S. B. Jones) and R01 GM-56424 (R. Shankar).

REFERENCES

3. Benedikt CR and Grahame-Smith DG. Plasma noradrenaline and adrenergic inhibition of IL-6 has also been reported in spleen tissue (36); however, in the kidney and the skin, $\beta$-adrenergic stimulation has been reported to increase IL-6 (15, 25, 26). Collectively, these findings suggest that adrenergic modulation of cytokine release appears to be tissue specific, but how such regulation may be altered during conditions of sepsis is largely unexplored.


