Continuous Endotoxin Infusion Suppresses Rat Spleen Cell Production of Cytokines

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Abstract. **Endotoxin, i.e., lipopolysaccharides, was continuously infused into rats at a nonlethal dose by means of an implanted osmotic pump for up to 2 weeks. The pump was connected to the jugular vein by a polyethylene catheter. Administration of endotoxin via the pump compromised the ability of spleen cells to produce the lymphokines interleukin 1 and tumor necrosis factor after stimulation *in vitro* with endotoxin. In addition, the ability of the spleen cells to produce α/β-interferon in response to endotoxin *in vitro* was also examined, as was the capability of the spleen cells to produce γ-interferon following stimulation with concanavalin A. Suppression of the expected interleukin 1 and tumor necrosis factor production by spleen cells from rats continuously infused with endotoxin was observed. There was also a moderate effect on interferon production, but this was much less. These results provide further findings indicating the unresponsiveness of spleen cells to lipopolysaccharides, as well as to a nonspecific plant mitogen, following continuous infusion of endotoxin into rats via an implanted osmotic pump. Additional studies are needed to determine the mechanisms involved in such suppression.** [P.S.E.B.M. 1992, Vol 199]

Bacterial endotoxins, i.e., lipopolysaccharides (LPS), have marked effects on the physiological function of many systems, including the immune response system. Endotoxins can markedly stimulate or suppress immunity (1–3). For example, macrophages, an important cell type of the lymphoid system, may be activated by LPS and show heightened phagocytic activity, but may also release a wide variety of cytokines, including interleukin 1 (IL-1) and tumor necrosis factor (TNF). In this regard, LPS, as well as other immune stimulators, is usually given as a bolus injection. However, infection of individuals with gram-negative organisms results in the release of endotoxins in a continuous manner. We have studied the effects of LPS on a wide variety of physiological functions of rats after continuous infusion over periods of days using a subcutaneously implanted osmotic pump (4, 5). The pump was implanted under the skin in the dorsal region of the rat for up to 1 week and connected by tubing to the jugular vein. Slow infusion of pyrogen-free saline for 42 hr provided for a postsurgical recovery period followed by infusion of a nonlethal dose of endotoxin in an attempt to reproduce effects associated with continuous release of endotoxins from the site of an *in vivo* infection. In these experiments, alterations of some metabolic function, as well as some hemodynamic and cardiovascular parameters such as blood pressure and myocardial function, occurred in the infused rats. Also, it was found that continuous infusion of endotoxin markedly affected the immune response, as determined by blastogenic assay of spleen cells stimulated *in vitro* with various mitogens, including the LPS itself (6). The continuous infusion of LPS resulted in a continued and long-lasting depression of blastogenic responsiveness of the splenocytes. Spleen cells from these LPS-infused rats were also found to be unresponsive in regards to *in vitro* production of the cytokines IL-1 and TNF. As shown in this report, a marked depression of production of these cytokines by spleen cells from endotoxin-infused rats occurred, as compared with cytokine production by spleen cells from control rats.

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Materials and Methods

Animals. Male Sprague-Dawley rats, weighing approximately 300–350 g each, were used for these studies. They were purchased from Charles River Co., Cambridge, MA, and kept in plastic rat cages with wire mesh lids. They were fed commercial rat food and allowed water *ad libitum*. All animals were acclimated to the animal care facility for 1 week before use.

Alzet Pump. Alzet 2-ml pumps were purchased from Alza Corp., Palo Alto, CA. Each pump measured 1.4 × 5 × 1 cm in diameter and weighed approximately 15 g when empty. The pumps had a reservoir volume of 2 ml and delivered their content at a nominal rate of 10 μl/hr for up to 7 days.

Endotoxin. Phenol-water-extracted *Escherichia coli* LPS was obtained from Difco Laboratories, Detroit, MI (6). For use, the endotoxin was dissolved in pyrogen-free saline at a concentration of 1.0 μg/ml. Similar results were obtained with different LPS preparations from *E. coli*.

Pump Implantation. Each animal was operated on as described previously and had an osmotic pump placed under the dorsal skin (4–6). The pump was connected via a prefabricated tubing to the right jugular vein. As described previously, the pump first dispensed saline for 42 hr, and then a nonlethal dose of endotoxin in a continuous manner at a rate of 10 μl/hr. The total level of endotoxin in the blood of the animals was determined by a semiquantitative, photometric Limulus lysate assay and showed that within 6 hr of the onset of endotoxin administration, a concentration of about 1 μg of LPS/ml was present in the serum (5, 6). This level persisted throughout the time of administration of the endotoxin. Control animals received endotoxin by a single bolus injection of 20 μg of LPS subcutaneously 10 days before sacrifice and testing their spleen cells for cytokine formation.

Blastogenic Assay. Individual rats were sacrificed and their spleens were obtained. Spleen cells were prepared by teasing into RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) containing 10% fetal calf serum (Hyclone Laboratory, Logan, UT) and antibiotics. The resulting cell suspensions were washed several times and resuspended to a concentration of 2×10^6 viable nucleated cells/ml, as determined by trypan blue stain technique with a hemocytometer (6–8). To each well of 96-well microtiter plates (Costar, Cambridge, MA) were added 10^6 spleen cells and 0.1 ml of sterile medium. Medium alone was added as a control to some wells. All cells were tested in triplicate. The test wells received either 10 μg of *E. coli* LPS/ml or 5 μg of concanavalin A (Con A)/ml (Sigma). The cultures were incubated for 48 hr at 37°C in an atmosphere of 95% air and 5% CO_2, pulsed with [^{3}H]thymidine, and incubated further for 18 hr at 37°C. The amount of radiolabel taken up by the cells was determined by collecting the cells on glass fiber filters as described previously (7, 8). The Δcpm was calculated as the amount of radioactivity taken up in the stimulated cultures minus the amount of radioactivity of control cultures without stimulator. The stimulation index was also calculated in some cases by standard formula by comparing the cpm for mitogen-treated cells minus cpm of control unstimulated cultures divided by the cpm of unstimulated cells × 100 (7, 8). In all cases, each experimental point was based on results of spleen cells from three to five animals and each experiment was repeated at least three times. Differences between cultures in each group were less than 10%.

IL-1 Assay. Spleen cells from the rats were cultured at a concentration of 10^6 cells/ml for 18 hr in the presence of 10 μg of *E. coli* LPS. After incubation, cultures were centrifuged at 1000*g* for 30 min and cell-free supernatants were collected. The supernatants were then tested for IL-1 activity by the standard C_3H/HeJ mouse thymocyte proliferation assay (8–11). For this purpose, graded amounts of culture supernatants were added to 5×10^6 fresh thymocytes in microtiter plates. The cells were then incubated for 18 hr at 37°C and treated with 0.5 μCi of [^{3}H]thymidine for 18 hr at 37°C. The amount of radiolabel taken up into the thymocytes was taken as evidence of IL-1 present in the culture supernatants. As controls, supernatants from nonstimulated cells were used. In addition, antibody to IL-1α (Genzyme, Cambridge, MA) was added to the wells to neutralize activity as evidence of the presence of IL-1α (11). Nearly all the IL-1 induced in the spleen cell cultures was neutralized by a 1/20 dilution of this antibody, indicating that it was a product of macrophages in the cell cultures. Data were expressed as Δcpm obtained by subtracting the background cpm from the cpm in the test sample and the mean ± SE calculated for three or more experiments. An IL-1 standard obtained from J. Oppenheim, NIH, was used to determine units/ml and results presented as percentage of this control.

TNF Assay. Cell culture supernatants, in the amount of 0.1 ml, were added to 10^6 WEHI 164 tumor cells obtained from ATCC and the cells were readily lysed by rTNF, as described previously (12–14). To determine whether the active factor lysing the cells was indeed TNF, anti-TNF-α serum (Genzyme) at a dilution of 1/20 was used for neutralization tests with control rTNF (Amgen Co., Palo Alto, CA) to block the activity of specific TNF (12–14). The TNF activity was neutralized by this serum, indicating that TNF-β (or lymphotoxin) was not present (13).

γ-Interferon Assay. The antivirus activity of culture supernatants was measured exactly as described previously using L929 cells challenged with vesicular stomatitis virus (15–17). One unit of interferon (IFN)

was calculated as the reciprocal of the dilution of a sample in a well which protected 50% of the cells in the monolayer from the virus-induced cytopathogenic effect. As a positive control, standard rIFN-α/β calibrated against a reference reagent provided by the National Institute of Allergy and Infectious Disease was used. In addition, the type of IFN present in the samples was determined by neutralization with specific anti-IFN antibodies (15).

Results

Continuous infusion of endotoxin into rats markedly suppressed the ability of their spleen cells to respond to LPS by blastogenesis (Table I). Spleen cells from rats infused with endotoxin from approximately 2 to 7 days showed a markedly diminished blastogenic response to LPS as compared with controls. Furthermore, suppressed blastogenic responses were also manifest when Con A, a nonspecific mitogen known to affect mainly T cells, was used for simulation of the spleen cells (Table I). Spleen cells from rats given a bolus injection of endotoxin responded in an enhanced manner.

In order to determine mechanisms whereby suppression occurred in endotoxin-infused animals, experiments were performed in which spleen cells from the same rats were tested for their ability to produce lymphokines *in vitro* (Table II). Spleen cells from rats injected with a bolus of endotoxin 10 days earlier showed production of IL-1 and TNF in their culture supernatants as compared with control animals that did not receive endotoxin. In contrast, spleen cells from mice given endotoxin in a continuous manner via a pump showed a continuing and markedly depressed production of IL-1 and TNF. For example, animals with an implanted pump dispensing LPS showed 1 day later a 45–55% decrease in cytokine formation by their spleen cells as compared with untreated controls. By 5 days, spleen cells from infused animals showed an even more marked suppression of cytokine production when cultured and appropriately stimulated. Suppression was maximum 7 days after LPS infusion and continued even when endotoxin was no longer infused, i.e., Days 7–10 (Table II).

Marked suppression of cytokine formation did not appear to extend to γ-interferon activity. This interferon is thought to be a product of T cells and α/β-interferon is considered a product mainly of macrophages. Thus, spleen cells from LPS-infused rats were examined for interferon production when stimulated *in vitro* with LPS, which induces mainly α/β-interferon. Con A was used to stimulate other cultures, since this mitogen induced T lymphocytes to produce γ-interferon. As is apparent in Table III, interferon induced by either LPS or Con A was only slightly to moderately altered for the first few days after endotoxin infusion, as shown by testing culture supernatants of spleen cells from LPS-infused rats as compared with those from nontreated control animals. Con A induction of interferon was either not depressed or even slightly increased early after infusion, and there was only a moderate reduction when the rats were infused with endotoxin for 3 or even 5 days. However, by Day 7, γ-interferon production became undetectable. Production of α/β-interferon was only moderately affected at the same time (Table III). Spleen cells from rats given a single bolus injection of endotoxin 10 days prior to sacrifice

Table I. Effect of LPS Treatment of Rats on Blastogenic Responsiveness of Spleen Cells

Rat treatment[a]	Blastogenic response[b]	
	LPS	Con A
None (control)	6.3 ± 1.2	18.9 ± 3.5
LPS bolus[c]	12.2 ± 2.3[d]	29.6 ± 4.5[d]
LPS infusion[e]		
1 day	5.9 ± 2.1	19.4 ± 4.2
3 days	3.1 ± 0.9[d]	10.3 ± 1.9[d]
5 days	2.3 ± 0.8[d]	4.1 ± 2.2[d]
7 days	1.9[d]	2.3 ± 0.8[d]
10 days	<1.0[d]	1.6 ± 0.9[d]

[a] Groups of rats were given no treatment, as control, or LPS either as bolus or by continuous infusion.
[b] Blastogenic response of spleen cells (10^6 cells) stimulated with LPS (10 μg/ml) or Con A (5 μg/ml) for 48 hr. Results are average stimulation index ± SE for spleen cells from indicated rat group.
[c] Rats were injected with 20 μg of LPS subcutaneously 10 days before testing spleen cells.
[d] Significantly different than controls; $P < 0.01$ determined by Student's *t* test.
[e] Rats were implanted with an osmotic pump dispensing saline for 42 hr, then LPS (10 μg/hr) continuously from 42 hr to 7 days, and no infusion from 7 to 10 days.

Table II. Effect of LPS Treatment of Rats on Spleen Cell Production of Cytokines

Rat treatment[a]	Cytokine[b]	
	IL-1α	TNF-α
LPS-bolus	138 ± 64	183 ± 47
Infusion		
3 days	72 ± 16[c]	62 ± 48[c]
5 days	5 ± 3[c]	12 ± 10[c]
7 days	<2[c]	<5[c]
10 days	<2[c]	<5[c]

[a] Groups of three rats each given LPS either as a bolus (20 μg sc) 10 days before or continuously by implanted osmotic pump from 42 hr to 7 days.
[b] Spleen cell suspensions tested for IL-1 or TNF production after *in vitro* stimulation of 10^6 cells with 10 μg LPS/ml; results given as percentage of controls (no LPS administration) ± SE (IL-1 activity averaged 9721 ± 1320 cpm and TNF averaged 945 ± 65 units/ml for normal control spleen cells; anti-IL-1α (Genzyme) at a 1/20 dilution inhibited IL activity of control or LPS-treated rats >90%, while anti-TNF-α antibody at 1/20 dilution inhibited TNF activity >95%.
[c] Significantly different from control activity of spleen cells from nontreated normal rats; $P < 0.01$ determined by Student's *t* test.

Table III. Effect of LPS Treatment of Rats on Spleen Cell Production of Interferon

Day of treatment[a]	LPS induced[b] (units/ml)		Con A Induced[b] (units/ml)	
	α/β	γ	α/β	γ
None (controls)	56	24	20	100
LPS bolus	140	60	40	200
LPS infusion				
3 days	120	20	20	160
5 days	50	10	20	140
7 days	40	0	20	140

[a] Groups of three rats each were either untreated controls or were given LPS as a bolus injection (20 μg) 10 days earlier or from an osmotic pump continuously (saline from 0 to 42 hr and LPS [10 μg/ml] from 42 hr to 7 days).
[b] Spleen cells (10^6) stimulated *in vitro* with 10 μg/ml of LPS or 5.0 μg/ml of Con A for 18 hr and supernatants assayed for IFN activity; anti-α/β or anti-γ-antibody (1/20) dilution inhibited control α/β- or γ-IFN >95% respectively.

showed a slight to a moderately enhanced level after LPS or Con A stimulation *in vitro*.

Discussion and Conclusions

The results of this study indicate that not only is the blastogenic responsiveness of rat spleen cells markedly depressed following continuous infusion of endotoxin, but there is also a marked depression of the ability of the cells to produce the cytokines IL-1 and TNF, both associated with macrophage activation by LPS (9, 10, 12–14, 18, 19). Spleen cells from normal rats, when stimulated with LPS *in vitro*, produced soluble factors with the characteristics of IL-1 or TNF, as determined by conventional biologic assay with target cell cultures responsive to these factors. Furthermore, neutralization studies with antibody indicated that the activity associated with IL-1 could be neutralized by specific anti-IL-α antibody, and activity considered due to TNF was in turn neutralized by specific anti-TNFα antibody (data not shown).

Rats given a single bolus injection of LPS were stimulated so their spleen cells underwent heightened blastogenesis in the presence of either LPS or Con A. Their spleen cells were also stimulated to produce heightened amounts of IL-1 and TNF. However, the kinetics of cytokine formation in these control rats were not studied. It is possible that cytokine function was first depressed, then increased to normal and, finally, at 10 days, enhanced. It was also found that spleen cells from rats continuously infused with LPS and stimulated *in vitro* with the mitogen Con A, which activates mainly T cells, were able to produce essentially normal interferon levels. It is accepted that Con A stimulates spleen cells to produce γ-interferon mainly by activated T lymphocytes. In contrast, when LPS was used as the stimulator *in vitro*, only a moderate deficiency of α/β-interferon production was noted. This interferon is considered a product of macrophages (15, 17). By 7 and especially 10 days after LPS infusion began, production of even γ-interferon was markedly depressed. Thus, from these data, it appeared likely that the continuous infusion of LPS "exhausts" lymphoid cells in the spleen so that they do not produce cytokines normally.

It should be noted that the spleen cells from infused rats were exposed continuously to an environment in which endotoxin was present. Even several days after endotoxin infusion ceased, there still was detectable endotoxin in the plasma of the animals, as shown by the Limulus lysate assay (data not shown). The lymphoid cells in the spleen of endotoxin-infused animals were unresponsive in regard to production of monokines when stimulated *in vitro* with additional LPS. Furthermore, even when not stimulated *in vitro*, the cells were not producing detectable amounts of IL-1 or TNF. This suggests that it is possible that the *in vivo* presence of LPS is sufficient to cause the cells to become noncompetent to produce these monokines without further *in vitro* stimulation. Furthermore, it was evident that when a stimulatory dose of LPS was added to the cells *in vitro*, they were unable to produce detectable amounts of IL-1 or TNF.

Previous studies had shown that LPS-infused rats became tolerant to bolus injection of a nonlethal dose of endotoxin 7–21 days later and resistant to a lethal dose of LPS (unpublished data). The background blastogenesis by these spleen cells, however, was not different from the background response of spleen cells from nontreated control rats (unpublished). Such results support the belief that cells from LPS-treated animals become functionally tolerant to further stimulation with LPS *in vitro*. In contrast, spleen cells from rats given a bolus injection of endotoxin 10 days earlier were responsive to additional stimulation with endotoxin *in vitro* in terms of producing heightened amounts of IL-1 and TNF, as compared with cells from nontreated control rats. It is of interest that when Con A was used as the *in vitro* stimulator, rather than LPS, essentially normal or even enhanced levels of interferon were produced by the cultures, at least for the first few days. However, this appeared to be due mainly to γ-interferon, as shown by appropriate neutralization tests with specific antiserum. However, when the cells were stimulated with LPS *in vitro*, there was only a moderate reduction in their ability to produce α/β-interferon. In all experiments, equal numbers of spleen cells were used for the *in vitro* cultures, regardless of whether the cells were obtained from normal, bolus-injected, or continuously infused animals. The cell cultures contained similar populations (i.e., lymphocytes and macrophages) and had similar viability (i.e., about 95%).

It is possible, but unlikely, that the trypan blue

stain assay, which indirectly measures membrane integrity, may be less sensitive than direct measurement of a cell's capacity to grow, which could have been compromised by continuous exposure to LPS. It is noteworthy that after administration of endotoxin continuously via the osmotic pump, there was a marked increase in spleen size, as well as number of cells in the spleen. For example, 7 days after implantation of a pump and infusion of endotoxin for 5 days, there was essentially a doubling in size of the spleen and a large increase in the number of lymphocytes and macrophages in this organ (20). There was also an increase in the number of germinal centers in the spleen, a change compatible with an active immune response. However, there was no disproportional shift in the percentage of the major cell populations in the spleen. Nevertheless, it is possible that a spleen cell suspension from a continuously endotoxin-infused rat has increased numbers of lymphoid cells that are defective in immune capability. Nevertheless, numerous studies have shown that endotoxin given by injection once or several times over a course of a few days enhances, rather than depresses, a wide variety of immune responses, including antibody formation and lymphokine production. Such effects by endotoxins are considered to be a hallmark of LPS on immunity.

The observation in the present study that spleen cells from LPS-infused rats are depressed in their ability to produce and/or secrete lymphokines important in immune-response regulation, IL-1, and TNF suggests that this unresponsiveness of the cells to produce these lymphokines has occurred because of the endotoxin, even though the cells did not appear to be damaged or nonviable. Endotoxin is known to influence various metabolic parameters of cells (1–3). Therefore, cells from animals exposed continuously to endotoxin may have a defect in lymphokine production because of such metabolic effects. This could be related to increased susceptibility to morbidity or mortality in individuals infected with a gram-negative bacterium-releasing endotoxin in a continuous manner. Suppression of immune responsiveness is often seen in such infection and such suppression may be related to inhibition of lymphokine production. Additional studies are in progress to examine the mechanisms involved.

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