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Suppressed *in Vitro* Blastogenic Responsiveness of Rat Spleen Cells after Continuous Infusion of Endotoxin by an Implanted Osmotic Pump (42578)

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Abstract. Continuous infusion of a gram-negative bacterial endotoxin in relatively small doses into rats by means of an implanted osmotic pump was studied. The model system was designed to examine the effects of endotoxin on the blastogenic response of spleen cells to the endotoxin itself and to a nonspecific T-cell mitogen, concanavalin A (Con A). Rats were implanted with an osmotic pump which delivered saline for the first 42 hr to provide postsurgical recovery before the onset of endotoxin infusion. Previous studies had shown that during the first 1–4 days after administration of endotoxin marked alterations of metabolism and some changes in physiologic parameters such as blood pressure and *in vitro* myocardial performance occurred. In the present study the blastogenic responsiveness of spleen cells to endotoxin itself as well as to the nonspecific T-cell mitogen Con A was markedly decreased after several days of continuous administration of endotoxin. Control animals receiving only saline for the same period of time showed a similar depression of blastogenic responsiveness to the lipopolysaccharide (LPS), as well as to Con A, however, with a delay of 2–4 days before comparable levels of suppression became evident. These results indicate that marked alterations of immune competence as measured by blastogenesis of spleen cells to *Escherichia coli* LPS and to a mitogen such as Con A may occur after implantation of an osmotic pump, with or without continuous infusion of endotoxin. Further studies seem warranted to determine the role of the foreign body reaction to the osmotic pump as well as to the endotoxin administered by the pump.

Bacterial endotoxins are derived from the outer membranes of gram-negative organisms and have been studied extensively in terms of their effects on the physiology and biochemistry of recipient individuals (1–3). Alterations of immune function have also been studied in much detail in endotoxin-treated individuals. In most cases, endotoxin, either crude lipopolysaccharide (LPS) somatic antigens from gram-negative bacteria or purified components, has been injected as a single bolus into experimental animals at various times prior to or during assays for either physiological or immunological responsiveness (1, 4, 5). Recently, this laboratory has developed a model system based on continuous infusion of bacterial endotoxin into rats via an implanted osmotic pump over a period of days (6). The pump is implanted under the skin in the dorsal region of the rat and osmotic pressure forces a slow infusion of saline and then nonlethal doses of endotoxin into the rats' jugular veins for a period of up to a week.

Although alterations of metabolic functions and some physiologic parameters were observed in rats infused with endotoxin, only minimal effects occurred in control animals with pumps containing saline only (7–9). Many investigators have demonstrated that individual doses of endotoxin given at a single time prior to, simultaneously with, or after administration of a wide variety of antigens may cause alterations of immune functions in terms of either kinetics or magnitude (10, 11). Since it appeared likely that endotoxin administered in a continuous manner through the osmotic pump more closely approximated real pathophysiological conditions following infection with gram-negative bacteria, it was of interest to evaluate possible immunological changes induced by an endotoxin administered in such a manner. The results of the present study clearly show that continuous infusion of nonlethal doses of endotoxin into rats by means of an implanted osmotic pump

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causes a marked decrease in the ability of spleen cells, over a period of days, to respond to the endotoxin by blastogenesis after *in vitro* stimulus with LPS. In addition, blastogenic response to concanavalin A (Con A), a non-specific T-cell mitogen, was also suppressed. Surprisingly, however, control rats which received continuous infusion of saline only showed a similar, but somewhat delayed, suppression of these blastogenic responses. Other control animals in which a pump was implanted and immediately removed showed no suppression of blastogenic responses.

Materials and Methods. *Animals.* Adult Sprague–Dawley rats, weighing 300–350 g each, were purchased from Harlan Co. (Houston, TX) and from Charles River Co. (Cambridge, MA). They were kept in groups of two in plastic rat cages and fed commercial rat food and water *ad libitum.* They were acclimated to the laboratory for at least 1 week before use.

Osmotic pump. Alzet 2-ml osmotic pumps were purchased from Alza Corp. (6). Each pump measures 1.4 × 5.1 cm, weighs 5.1 g empty, has a reservoir volume of 2 ml, and delivers the reservoir content at a nominal rate of 10 μl/hr for 7 days. The pumps were used as described previously (6).

Endotoxin. Escherichia coli endotoxin (026: B8 lipopolysaccharide B) was purchased from Difco Laboratories (Detroit, MI). The endotoxin dosage which resulted in noticeable morbidity but no lethality was 0.1 mg/100 g body wt per day.

Osmotic pump implantation. Each animal was operated on as described previously and an osmotic pump was placed under the dorsal skin (6). The pump was connected through a prefabricated capillary tubing coil into the right jugular vein. All animals given fluids through the osmotic pump received sterile isotonic saline for the first 42 hr. Those animals receiving endotoxin had the LPS administered from 42 hr up to 5 days thereafter. Rats designated as "saline controls" received sterile isotonic saline throughout the duration of the experiment.

Preparation of spleen cells. The spleen was removed from each animal under ether anesthesia and weighed. Single cell suspensions from a portion of each individual spleen were prepared using a Stomacher 80 lab blender (Tekmar Co.). Cell suspensions were washed three times in HBSS and resuspended in RPMI 1640 medium containing 10% fetal calf serum, 2-mercaptoethanol (10^{-5} *M*), and antibiotics. Viable cell counts were determined using trypan blue exclusion technique and cell concentrations were adjusted to 2×10^6 cells/ml.

Blastogenic assay. Spleen cell suspensions in 0.1-ml volumes were placed in 96 well microtiter plates. An additional 0.1 ml of medium combining Con A (5 μg/ml) or *E. coli* LPS (10 μg/ml) was added to each well. The plates were incubated for 48 hr at 37°C in 95% air and 5% CO_2. At that time, plates were pulsed with 0.5 μCi [^{3}H]thymidine and incubated for an additional 18 hr. The cells were harvested on glass fiber filters and the number of counts per minute was determined by standard liquid scintillation counting.

Endotoxin assay. The circulating levels of endotoxin in plasma samples of each rat were measured by a kinetic spectrophotometric method (12). Amebocyte lysates were obtained from Associates of Cape Cod, Inc. (Woods Hole, MA).

Results. Spleen cells from control rats responded well to stimulation *in vitro* with *E. coli* LPS (Table I, Fig. 1). In all cases there was a strong stimulation index after 3 days of incubation of the cells *in vitro* with the dose of stimulator used. When rats were implanted with an osmotic pump and given saline only, there was still a marked stimulation of the spleen cells obtained 1–2 days thereafter. However, when rats were given saline for 42 hr and endotoxin for 1–2 days thereafter, there was a marked decrease in the amount of radioactivity taken up into the spleen cells after incubation *in vitro* for 72 hr with the *E. coli* LPS. By the third day after infusion of LPS (5 days after pump implantation) there was essentially no blastogenic response. Rats given LPS for 5 days, i.e., 7 days after implantation of the pump, showed no response. Rats rested for 3 days without further administration of endotoxin failed to recover their blastogenic responsiveness. It is noteworthy, however, that control animals given saline alone in the pump, without endotoxin, also showed an equal depression of blastogenic responsiveness by their spleen cells several days after implantation of the pump and administration of saline (Table I, Fig. 1).

The spleen size of rats increased continu-

TABLE I. EFFECTS OF CONTINUOUS INFUSION OF STERILE SALINE OR ENDOTOXIN INTO RATS BY AN OSMOTIC PUMP ON BLASTOGENIC RESPONSIVENESS OF SPLEEN CELLS TO LIPOPOLYSACCHARIDE

	Blastogenic responses of spleen cells[a]						
	Saline infusion			LPS infusion			
Days after surgery[b]	Unstimulated (cpm ± SD)	+LPS (cpm ± SD)	SI	Duration of LPS infusion (days)	Unstimulated (cpm ± SD)	+LPS (cpm ± SD)	SI
None (controls)[c]	8,261 ± 630	113,175 ± 10,910	13.7		—	—	—
2	11,106 ± 2,650	61,037 ± 12,650	4.8	0.25	5250 ± 730	37,346 ± 1,230	7.1
5	7,118 ± 653	41,267 ± 2,780	6.5	3	5360 ± 976	4,288 ± 536	−0.8
6	8,216 ± 320	5,751 ± 621	−0.7	5	5140 ± 396	2,056 ± 139	−0.4
10[d]	6,733 ± 486	3,366 ± 410	−0.5	5	5264 ± 465	2,632 ± 289	−0.5

[a] Counts per minute (cpm) ± SD, for spleen cells from three or four rats per group given only saline or saline followed by endotoxin for an indicated length of time. SI, stimulation index.

[b] Groups of rats implanted with osmotic pump and given saline only for 2, 5, or 7 days or saline for 42 hr and then infused with bacterial endotoxin at a dose of 0.1 mg/ 100 g body wt/day for 6 hr, 3 days, or 5 days.

[c] No surgery performed prior to removal of the spleen.

[d] No infusion from 7 to 10 days.

ously from the onset of endotoxin infusion (Day 2 after surgery). Rats administered sterile saline alone by the pump showed a small but still consistent spleen weight increase—compared to the spleen weight of control, nonoperated rats—from Day 5 on through Day 10 after surgery (Fig. 2A). However, indexing spleen weight to total body weight revealed essentially unchanged values in saline-infused rats. In contrast, the spleen weight of rats receiving endotoxin infusion showed a continued increase throughout the course of the experiment even when the ratio of spleen weight to body weight was determined (Fig. 2B).

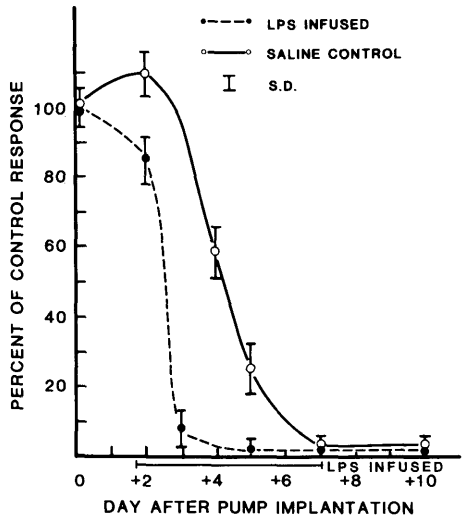

FIG. 1. Blastogenic responses of spleen cells from rats infused for indicated times with saline only or with saline for 42 hr and endotoxin from 42 hr to 7 days by implanted osmotic pump. Each point represents response of spleen cells from three or four rats per group as a percentage of control responses.

As is evident in Table II, the decrease in splenic blastogenic responsiveness of endotoxin-treated rats was also evident with Con A as the stimulator *in vitro*. A greater decrease occurred by Days 2 and 3 after endotoxin infusion. Rats given saline for 4–7 days also showed a decreased response to Con A. By 10 days after implantation of the pump containing either saline or endotoxin all animals showed essentially no splenic response to the mitogen.

The circulating level of endotoxin measured after 6 hr, 3 days, and 5 days of continuous endotoxin infusion was (in terms of means ± SE) 0.076 ± 0.003 (n = 3), 0.108 ± 0.014 (n = 5), and 0.060 ± 0.03 (n = 4) μg/ml of plasma. None of the plasma samples obtained at matched time points from saline-infused rats contained any detectable amount of endotoxin.

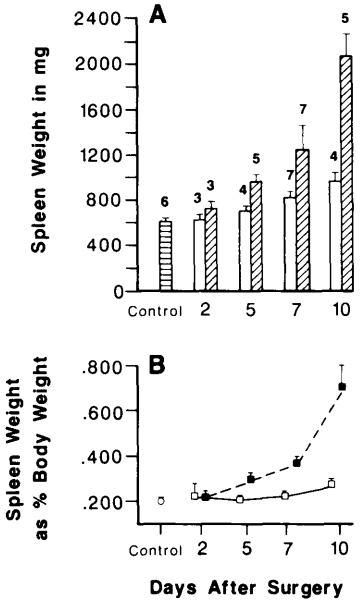

FIG. 2. Spleen weight of nonoperated control and saline- and endotoxin-infused rats at various times after surgery for pump implantation and catheter placement. Bars represent means and one standard error. (A) Spleen weight in milligrams □, Saline infused; ▒, ET infused. Numbers in parentheses refer to the number of animals in each group. (B) Spleen weight as a percentage of body weight. □, Saline infused; ■, ET infused.

Discussion and Conclusion. Endotoxins from gram-negative bacteria have been utilized over the last 3–4 decades to study a wide variety of physiologic functions in septicemic and/or infected animals and have been used in models concerning the nature and mechanism of immune dysfunction, including the effects of bacterial adjuvants on immunity. In a typical experiment endotoxin is administered to an animal as a single inoculum or occasionally as multiple inocula at various times before or during testing of physiologic or immunologic parameters. Administration of endotoxin by a bolus injection, however, is not similar to an actual infection, i.e., the continuous release of a bacterial product(s) from the site of infection. In this regard, the recent development of osmotic pumps has permitted continuous infusion of endotoxins or other test substances over a relatively long time period in a continuous manner.

In this laboratory such an osmotic pump has been utilized to infuse a constant amount of bacterial endotoxins into rats intravenously. A number of studies have been performed showing that rats develop altered physiologic parameters such as myocardial function decrease, a small decrease in blood pressure, and changes in various metabolic patterns (13, 14). However, after a few days of continuous infusion of endotoxin, and while endotoxin is still present in the circulation of the animals, most physiologic parameters return to normal. Thus it was of interest to study immunologic function in such rats, i.e., animals receiving continuous infusions of endotoxin.

TABLE II. EFFECTS OF CONTINUOUS INFUSION OF SALINE OR ENDOTOXIN ON BLASTOGENIC RESPONSES OF RAT SPLEEN CELLS TO LIPOPOLYSACCHARIDE AND CONCANAVALIN A

Days after surgery[a]	Spleen cell blastogenic response (SI)[b]			
	Saline infusion		LPS infusion	
	LPS	Con A	LPS	Con A
None (control)	14.6 ± 2.3	108.6 ± 12.3	—	—
2	13.9 ± 1.6	112.5 ± 11.5	7.2 ± 1.2	47.2 ± 5.8
5	6.5 ± 1.9	37.8 ± 6.5	<1.0	1.8 ± 0.3
7	<1.0	1.5 ± 0.1	<1.0	<1.0
10[c]	<1.0	<1.0	<1.0	<1.0

[a] Time in days after implantation of osmotic pump in rats and administration of saline or *E. coli* endotoxin; LPS-treated animals received saline for 42 hr and then were infused with *E. coli* LPS from +42 hr to +7 days. Saline-treated rats received only saline throughout the experiment.

[b] Mean values of SI ± SD, for spleen cells from three or four rats per group at indicated times after administration of saline or LPS. Cultures were stimulated either with 10.0 μg *E. coli* LPS or with 5 μg Con A and SI was determined 72 hr later by [^{3}H]thymidine uptake test.

[c] Animals given no infusion from +7 to +10 days.

In these initial experiments, as reported here, endotoxin in a relatively small concentration was infused continuously into rats by means of an osmotic pump. In order to provide a postsurgical recovery period before the onset of the continuous endotoxin infusion, saline was infused through a coil attached to the pump for the first 42 hr. Afterward endotoxin was infused so that the animals had a constant level of 1 μg endotoxin per milliliter plasma throughout the remaining 5 days of endotoxin infusion (6). Administration of a single bolus of endotoxin usually results in a marked increase of blastogenic responsiveness of spleen cells from the animals to the endotoxin itself (11). Addition of a single inoculum of LPS usually results in an enhanced response. However, in the present situation continuous infusion of endotoxin resulted in a depressed response to endotoxin, as well as to the mitogen Con A. There was also an increase in spleen weight, but the maximum increase did not occur until about 5–7 days after endotoxin was administered (7–10 days after the beginning of the experiment and implantation of the pump).

As a control, animals were given sterile saline alone for the 7-day period and followed for a total of 10 days measuring the same parameters, i.e., spleen weight, circulating plasma levels of endotoxin (none was detected in control animals given saline alone), and determination of the blastogenic responses of spleen cells to endotoxin and to a nonspecific mitogen. Rats given saline alone for several days by continuous infusion from the osmotic pump showed a depression of the bastogenic response to the *E. coli* LPS. This suppression was nonspecific, since the animals also showed suppression of blastogenesis to Con A. However, these rats showed only a modest, if any, change in spleen size. Thus, despite some delay, suppression of blastogenic responses to the mitogen occurred in control animals receiving saline by continuous infusion from the osmotic pump. This was most evident on Days 5 to 10 after implantation.

As to the possible relationship between spleen size and blastogenic response, no valid conclusions can be drawn at this time either by us or by other investigators. By way of analogy we can refer to the extensive literature on leukemia virus infection and spleen size alteration vs immunosuppression (15). Marked increases in spleen size due to leukemia virus infection may or may not be reflected in altered immune responsiveness of spleen cells. Similarly, there are many reports of endotoxin injection increasing spleen size and altering splenic cellularity; however, the accompanying modulation of the immune response is not uniform. The mechanisms underlying these phenomena are largely unknown.

Control experiments were also performed in which the animals were sham operated; i.e., an osmotic pump was implanted and within 15 min, the pump was removed. These animals, when tested 4–5 days later for splenic blastogenesis, showed normal responses (data not shown). Thus surgery itself did not suppress the blastogenic responsiveness of spleen cells to LPS or Con A. However, the continuous presence of the foreign body, i.e., the osmotic pump, containing either endotoxin or saline, seemed related to the suppression noted. Although the LPS administered in a continuous manner by the pump affected the blastogenic responsiveness of spleen cells to the specific endotoxin as well as to the nonspecific mitogen in a negative manner, similar suppression, although somewhat delayed, still occurred when the pump dispensed sterile saline alone. Since the saline used was endotoxin free and the saline-infused animals had no detectable levels of endotoxin circulating in their plasma throughout the experiment, the results cannot be attributed to a lower dose of endotoxin being infused from the saline-filled pumps.

In order to test whether some component could leach from the pump over time, we "soaked" pumps in medium for 7 days and tested the resulting fluids for immunosuppressive activity—at time points matched to the sampling times of the pump implantation/infusion experiments—and found none.

Our results also serve as a "caveat" for the widespread practice of implanting various devices into patients for a multitude of therapeutic purposes. Such interventions may significantly contribute to the development of compromised immunocompetence often observed in such patients. Further studies are obviously necessary to ascertain whether implantation of a foreign body, including com-

ponents of the osmotic pump, has a negative influence on the immune response system and the mechanisms involved.

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