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The Effect of Endotoxin and Endotoxin Tolerance on Inflammation Induced by Mycobacterial Adjuvant

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Peptidoglycan, the substance in mycobacteria thought to be responsible for inducing adjuvant arthritis, and endotoxin (lipopolysaccharide or LPS) share many inflammatory properties. Since repeated administration of LPS produces tolerance, i.e., resistance to the toxic and inflammatory effects of LPS, we tested whether LPS and/or LPS tolerance might influence inflammation due to mycobacterial adjuvant. Male Sprague-Dawley rats were injected with *Escherichia coli* LPS or saline intraperitoneally and then challenged with 100 μg killed *Mycobacteria butyricum* (adjuvant) in the footpad. A single dose of 100 μg LPS three or 24 hours before adjuvant markedly, but transiently, reduced the local footpad swelling that begins within hours of the adjuvant injection and histologically resembles a sterile abscess. Animals that received multiple doses of LPS and were therefore tolerant or animals that received LPS 72 hours before adjuvant demonstrated adjuvant-induced footpad swelling nearly equal to controls. The anti-inflammatory effect of LPS was transient since footpad swelling in all groups was nearly comparable six days after the adjuvant injection and LPS failed to inhibit consistently the arthritis that develops two or more weeks after adjuvant injection. These studies establish that LPS can markedly inhibit the prodrome of adjuvant arthritis (footpad swelling due to *M. butyricum*), that inhibition of this prodrome does not prevent the subsequent development of arthritis, and that LPS tolerance diminishes this anti-inflammatory effect of LPS.

INTRODUCTION

Lipopolysaccharide (LPS or endotoxin) induces inflammation in experimental animals. Direct or indirect inflammatory effects of LPS include fever [1], activation of the complement [2], coagulation [3], and kinin cascades [4], increased vascular permeability [5], increased prostaglandin synthesis [6], and activation of polymorphonuclear leukocytes [7], lymphocytes [8], monocyte-macrophages [6], and platelets [9].

Less well appreciated is that LPS may be anti-inflammatory as well. In 1955, Humphrey [10] noted that polysaccharides from gram-negative bacteria could inhibit reversed passive Arthus reactions in rabbit skin but believed that the effect might be an artefact due to hypotension. Subsequent investigators have demonstrated that endotoxin can inhibit active Arthus reactions in guinea pig and rabbit [11], and it can reduce the cellular infiltrate that follows the intraperitoneal injection

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of a variety of irritants into mice [12]. Pyrogen, usually in the form of endotoxin, was believed to be an effective form of therapy for arthritis in man [13]. Experiments in our own laboratory have begun to explore the mechanism of LPS-induced anti-inflammatory effects [14]. These studies in rabbits indicate that intravenous LPS inhibits the chemotactic responsiveness of polymorphonuclear leukocytes to complement-derived peptides without inhibiting the ability to respond to another chemoattractant, n-formyl-methionyl-leucyl-phenylalanine [14].

Adjuvant arthritis is a widely employed model for experimental arthritis in rats [15–20]. In susceptible strains, this arthritis can be readily induced by an injection of killed mycobacteria in mineral oil (adjuvant) [19]. The arthritogenic component of the mycobacteria is believed to be peptidoglycan (PG), since PG from a variety of sources other than mycobacteria induces a similar arthritis [18]. PG and LPS share a number of inflammatory properties. For example, both induce fever [21], activate complement, activate macrophages [22], act as an adjuvant [23], and cause gelation of limulus lysates [24]. Repeated injections of LPS induce a state of refractoriness to both its toxic effects, such as lethargy, fever, and diarrhea [25], and at least some of its inflammatory effects [5,26,27]. For example, in rabbits a single injection of LPS induces ocular vascular permeability [5], the generation of chemotactic activity in the aqueous humor [27], and a rise in aqueous humor prostaglandin E_2 [27]. Rabbits tolerant to endotoxin by virtue of a daily injection for five days show no change in ocular vascular permeability, no generation of chemotactic activity in the aqueous humor, and no rise in aqueous humor prostaglandin E_2 subsequent to intravenous LPS [5,27]. Similarly, 100 μg of LPS injected intraperitoneally to rats induces eye inflammation [26], but multiple injections of LPS inhibit this inflammatory change [26]. Although known as tolerance, this state should not be confused with immunologic tolerance, since tolerance to LPS may result from an immune response rather than the lack of an immune response [25].

We therefore studied whether LPS and/or LPS tolerance could inhibit inflammation due to mycobacterial adjuvant. We demonstrate that a single injection of LPS potently but transiently inhibits the local footpad swelling that develops soon after the mycobacterial injection. LPS does not consistently affect the systemic arthritis that develops two weeks after adjuvant injection. Since animals tolerant to LPS show relative resistance to inflammation induced by LPS, and since PG and LPS share inflammatory effects, we hypothesized that animals tolerant to LPS would show a marked reduction in their inflammatory response to PG. Paradoxically, LPS tolerance diminished the anti-inflammatory effects of a single dose of LPS.

METHODS

Animals

Male Sprague-Dawley rats (160–200 g) were purchased from Simonsen Laboratories (Gilroy, CA) and fed standard laboratory chow.

Reagents and Experimental Protocol

Killed *Mycobacteria butyricum* and *Escherichia Coli* 055:B5 LPS (phenol extract) were purchased from Difco (Detroit, MI). The *M. butyricum* was ground with a mortar and pestle and suspended in a mineral and enema oil mixture. It was injected in a volume of 0.1 ml (1 mg mycobacteria/ml) into a hind footpad on the first day of study (Day 0). LPS was suspended in pyrogen-free saline (McGaw Laboratories) and stored at $-20°C$ until use. It was injected intraperitoneally in a volume of 0.1 ml

(1 mg/ml) in a variety of schedules, as given in the results. This dose of endotoxin produced transient lethargy and diarrhea in virtually all animals. It is fatal to approximately 10 percent of animals. Control animals received intraperitoneal injections of sterile, pyrogen-free saline. Footpad diameter as measured with appropriate calipers was used as an index of arthritis in the uninjected footpad or as a quantitation of soft tissue swelling in the injected footpad [15,17]. Readings were done by an observer unaware of the treatment the rats had received. Readings taken in this fashion were reproducible and comparable determinations were obtained if the footpad measurements were repeated by a second independent observer.

Complement Determination

Total hemolytic complement titers were measured in rat serum using a microtiter assay [28]. Sheep erythrocytes were sensitized with heat-inactivated rabbit anti-sheep hemolysin (Difco) and suspended in gelatin-veronal buffer, pH 7.35, at a concentration of 5×10^7 cells/ml. Hemolysis was determined after 60 minutes of incubation of sensitized sheep erythrocytes with 0.025 ml aliquots of serial, twofold dilutions of rat serum.

Histology

The paw was fixed in 10 percent neutral buffered formalin, sectioned, and stained with hematoxylin and eosin for histologic examination.

Cell Counts

Total leukocyte and platelet counts were determined by a coulter counter. Neutrophil counts were calculated based on the percentage of neutrophils relative to total white cell count present on a smear of peripheral blood.

RESULTS

A single dose of 100 μg *E. coli* LPS potently inhibited the local footpad swelling that develops after an injection of *M. butyricum*. As shown in Table 1, the increment in footpad size 24 hours after mycobacteria injection was reduced from 3.92 ± 0.22 mm in the control rats to 1.90 ± 0.22 mm in the rats treated with LPS three hours before adjuvant ($p < .0005$). LPS 24 hours before adjuvant also had a marked effect in reducing footpad swelling ($p < .0005$), but minimal benefit was seen when LPS was given 72 hours before adjuvant. The beneficial effect of LPS was abrogated if LPS tolerance was induced by a schedule of eight injections over an 11-day period,

TABLE 1
Effect of LPS on Soft Tissue Swelling 24 Hours After Adjuvant Injection Locally

	Controls	Single-Dose LPS			Multiple-Dose LPS
Time of last LPS injection relative to adjuvant	–	−3 hours	−24 hours	−72 hours	−3 hours
Footpad swelling (mm) (mean ± SE)	3.92 ± .22	1.90 ± .22	1.73 ± .31	3.34 ± .36	3.82 ± .23
Number of animals	24	8	7	6	8
p value relative to control		< .0005	< .0005		

Multiple-dose animals received 10 μg, 50 μg, and six 100 μg doses of LPS over an 11-day period up to and including the day adjuvant was given.

even if the last dose of LPS was three hours before the adjuvant (Table 1). These animals were tolerant since, with this schedule of injections, they failed to develop the lethargy and diarrhea normally seen after 100 μg *E. coli* LPS.

The anti-inflammatory effects of LPS were not paralleled by either depletion of total hemolytic complement or by neutropenia; 24 hours after 100 μg of LPS, no change could be detected in hemolytic complement titers and neutrophil counts rose from 2,755 ± 1,685/mm³ to 11,804 ± 5,436/mm³ (n = 3). Platelet counts 24 hours after endotoxin did diminish from 726,000 ± 38,000/mm³ to 254,000 ± 19,000/mm³ (n = 3).

The effect of LPS on the local footpad swelling was transient. As shown in Fig. 1, swelling increased daily in the adjuvant-injected footpad despite endotoxin pretreatment. By day 6 after the adjuvant, the reduction in swelling in the adjuvant-injected footpad was no longer significant. Furthermore, endotoxin pretreatment did not consistently affect the arthritis that develops roughly two weeks after the adjuvant injection. Using increment in footpad diameter in the uninjected hindfoot as an index of arthritis, we were unable to find a regimen of single or multiple LPS injections that consistently inhibited arthritis (Fig. 2).

In addition, LPS treatment did not consistently affect adjuvant arthritis once the joint disease was established. LPS treatment for established arthritis was greatly limited by the inability of rats with adjuvant arthritis to tolerate endotoxin. 100 μg *E. coli* LPS was fatal to more that 50 percent of rats with active adjuvant arthritis, which parallels the increased lethality of LPS in animals with mycobacteria infections [29] or those that have received Bacille Calmette-Guerin vaccine [30]. As shown in Fig. 3, 10 μg of LPS after single or multiple doses did not affect the arthritis due to mycobacterial injection.

Histologically the adjuvant injection induces a sterile abscess with a predominant polymorphonuclear cell infiltrate 24 hours after injection. The histologic appearance of footpads from control and LPS-treated rats did not differ significantly, i.e., LPS did not appear to influence the degree of cellular infiltrate qualitatively.

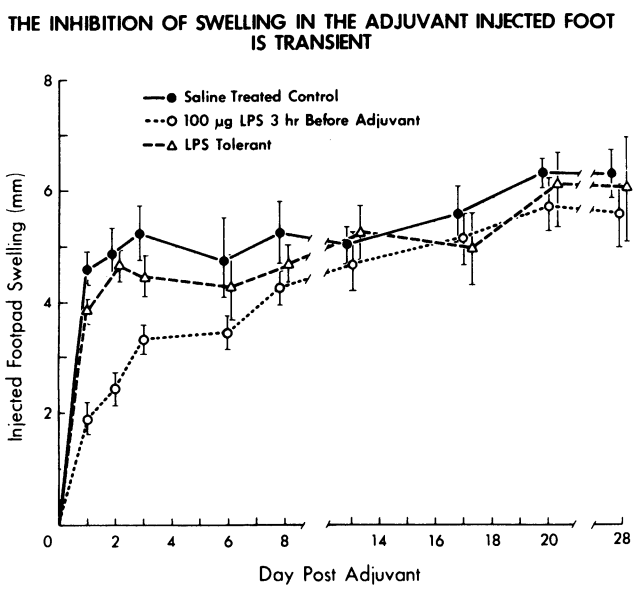

FIG. 1. The inhibition of swelling in the adjuvant-injected foot is transient. Although the rats (n = 9) receiving a single injection of LPS had markedly less swelling at the adjuvant injection site initially, by day 6 differences between the controls (n = 7), LPS-tolerant rats (n = 7), and single-dose treated rats are no longer significant. Values given are mean ± SEM.

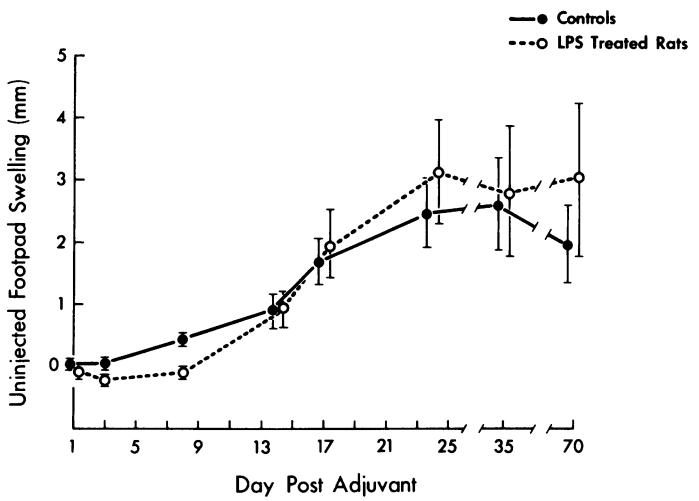

FIG. 2. LPS fails to inhibit adjuvant arthritis. Rats (n = 9) that received 100 μg of LPS three hours before adjuvant did not have any reduction in the arthritis or footpad swelling that affects the uninjected opposite hindfoot. For controls, n = 7. Values represent mean ± SEM.

DISCUSSION

These studies establish that LPS can markedly but transiently inhibit inflammation due to mycobacterial adjuvant. We are unaware of a previous study that demonstrates that LPS is anti-inflammatory in rats. The effect of LPS could not be demonstrated if LPS was given in multiple doses or if it was given 72 hours before the adjuvant. LPS did not consistently inhibit the subsequent arthritis in any treatment regimen.

In 1962 Wood and Pearson reported that lipopolysaccharide could inhibit adjuvant arthritis [20]. The LPS could only be given in mineral oil, not saline, in order to be effective. It was injected 15 to 40 days before the adjuvant and could be derived from gram-positive or gram-negative bacteria. Many factors make the Wood and

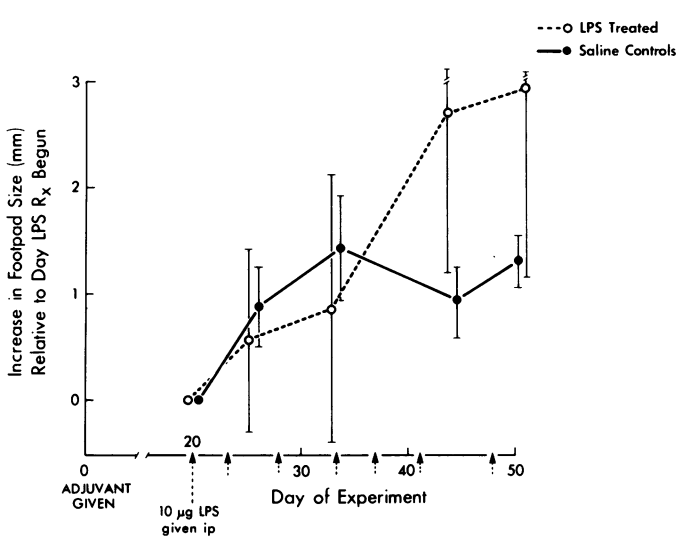

FIG. 3. LPS fails to reduce inflammation once adjuvant arthritis is established. Two groups of eight rats were treated either with LPS or saline. The two groups were initially comparable in terms of severity of joint disease. Neither single nor multiple doses of LPS reduced the progressive swelling in the uninjected footpad. Values represent mean ± SEM.

Pearson study not comparable to the present one, including the definition of lipopolysaccharide, which most authorities now reserve for use in reference to gram-negative bacteria. Transfer studies have clearly established adjuvant arthritis as an immunologic disease [16]. Although LPS is normally considered an adjuvant itself, LPS administration during certain times can also inhibit an immune response [13]. It is possible that the effects that these investigators observed relate to immunologic effects of LPS. Our observations pertain more to LPS effects on acute inflammation in which neutrophils rather than mononuclear cells appear to be primarily involved. Our study demonstrates that the acute inflammation induced by *M. butyricum* can be markedly diminished without affecting the subsequent immunologic inflammation.

Our own studies should be compared with the work of several investigators who have studied the effect of endotoxin on active bacterial infection or the effect of endotoxin on the leukocytic response to bacteria or bacterial products [32–36]. Depending on its dose and time of administration, LPS is capable of inhibiting the migration of PMN in response to an intradermal or intraperitoneal inoculum of bacteria and it can also enhance the lethality of such an infection [32–36]. Of special relevance to our own study is the observation of Delaunay and Pages that 1 mg of endotoxin given systemically to guinea pigs inhibited the diapedesis of leukocytes normally seen locally after the intradermal injection of acid-fast bacilli [32]. However, our study differs significantly from that reported by these investigators. In addition to studying a different species with a lower dose of endotoxin, our own histologic examination failed to show that the reduction in swelling could be attributed to reduced polymorphonuclear leukocyte extravasation. No prior study has evaluated the effect of endotoxin tolerance on this anti-inflammatory effect of LPS or evaluated the relationship between inhibition of the initial footpad swelling with the subsequent arthritis.

The possible relationship between endotoxin, adjuvant arthritis, and Reiter's syndrome was an added impetus for the present studies [37]. Reiter's syndrome is a reactive arthropathy that may be preceded by a gram-negative dysentery [38] which in turn can result in endotoxemia [39]. Reiter's syndrome is a multisystem disease that includes eye, bowel, genitourinary, and cutaneous manifestations in addition to a characteristic arthropathy. Rats with adjuvant arthritis may develop joint, eye, skin, bowel, and genitourinary disease that histologically, clinically, and radiographically resembles Reiter's syndrome [19]. Although LPS is anti-inflammatory, its failure in rats to inhibit the arthritis of adjuvant disease is compatible with the observation in man that Reiter's syndrome may begin subsequent to endotoxemia.

Having previously observed that endotoxin tolerance prevents endotoxin-induced ocular vascular permeability [5] and reasoning that endotoxin and mycobacteria (i.e., peptidoglycan) might induce swelling by a similar mechanism, we predicted that endotoxin tolerance would inhibit mycobacterial inflammation more than a single dose of endotoxin. To our surprise LPS tolerance abrogated the anti-inflammatory effects of endotoxin. This, however, should be interpreted with caution. LPS tolerance can broadly be divided into an early form that appears to be a pharmacologic tachyphylaxis and a later form due to the production of neutralizing antibodies [25]. The 11-day tolerance regimen used in these studies may have allowed production of antibodies such that the effective dose of LPS was less than 100 μg. The diminished reduction in footpad swelling in LPS-tolerant rats might merely be a dose-dependent effect. Our studies cannot rule out this possibility but in ex-

amining the effect of LPS on reversed passive Arthus reactions in rabbit skin, we have also noted that LPS tolerance diminishes the anti-inflammatory effects of LPS, even when the tolerance was established with a regimen designed to minimize the development of neutralizing antibodies [Rosenbaum JT, Howes EL, Goldstein IM: Endotoxin tolerance diminishes the anti-inflammatory effects of endotoxin. Fed Proc 41:558, 1982 (abstract)]. Intriguingly, Verghese and Snyderman have observed that C3H/HeJ mice, which are genetically "tolerant" to toxic effects of LPS, also are refractory to anti-inflammatory effects of LPS [12]. Other investigators have found that LPS-tolerant rabbits are not protected from fever induced by synthetic peptidoglycan [21].

These studies do not establish the mechanism of this LPS-mediated anti-inflammatory effect. Neutropenia and complement depletion seem unlikely possibilities. Hypotension or intercurrent illness are also unlikely since 24 hours after this dose of LPS, the rats behave normally. In the rabbit, we have established that LPS given *in vivo* inhibits the response of neutrophils to complement-derived chemotactic stimuli [14]. However, the neutrophil exudation evident even in the LPS-treated rats suggests that this mechanism is not the major explanation for the results reported here. Other possible mechanisms include a stress-induced release of adrenal steroids and/or catecholamines [40] or a stimulation of prostaglandin production [41]. Given in large doses exogenously, prostaglandin E can inhibit inflammatory changes such as vascular permeability [42]. By inducing inflammation, LPS might induce a tachyphylaxis to some of the mediators released by this inflammation. It is hoped that further studies can clarify the mechanism by which LPS can be both inflammatory and anti-inflammatory as well.

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