Early-Phase Endotoxin Tolerance: Induction by a Detoxified Lipid A Derivative, Monophosphoryl Lipid A

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After a sublethal exposure to lipopolysaccharide (LPS) or to lipid A, which is that portion of the LPS molecule associated with endotoxicity, a transient period ensues during which a normally responsive individual is rendered hypersensitive to LPS-induced toxicity. This period has been defined as early-phase endotoxin tolerance. Recently, a nontoxic derivative of lipid A from Salmonella typhimurium, monophosphoryl lipid A (MPL), was isolated and purified. In this study, we assessed the ability of MPL to induce early endotoxin tolerance. Injection of MPL resulted in a dose-dependent stimulation of both serum colony-stimulating factor and serum interferon, indicators of in vivo LPS responsiveness. In contrast, MPL failed to induce the symptoms of endotoxicity which are normally seen after injection of even sublethal amounts of intact endotoxin or lipid A preparations. Injection of MPL on day 0 reduced significantly the amount of LPS-induced serum colony-stimulating factor and interferon produced upon challenge with Escherichia coli LPS 3 days later and also mitigated toxic manifestations, as evidenced by a marked increase in the 50% lethal dose. Like the early study, we induced by wild-type (toxic) LPS, MPL-induced tolerance was characterized by an accompanying elevation in the number of bone marrow-derived macrophage progenitor cells and by an alteration in bone marrow cell sizing profiles. These results indicate that MPL is effective in inducing a state of LPS-hyporesponsiveness without the toxic side effects of endotoxin and that the structural component(s) necessary for induction of early-phase endotoxin tolerance is contained within MPL.

Endotoxin, the lipopolysaccharide (LPS) derived from cell walls of gram-negative bacteria, has been shown to induce many of the harmful manifestations seen in patients with gram-negative sepsis. Among these are pyrogenic activity, hypoglycemia, and changes in blood clotting and in blood pressure which can result in shock or death (reviewed in reference 1). However, some of the effects of endotoxin are beneficial, i.e., immunogenicity, protection against lethal irradiation, immune adjuvant effects, and enhancement of nonspecific resistance to infection or to toxic doses of endotoxin (reviewed in reference 2). Because humans are very sensitive to the toxic effects of endotoxin, therapeutic approaches which involve the use of endotoxin to induce beneficial effects have been limited. The toxic activity of the endotoxin molecule has been shown to reside in the lipid A portion of the LPS molecule which consists of a phosphorylated glucosamine disaccharide unit substituted with ester- and amide-linked long-chain fatty acids. Recent advances in chemical modification techniques and chromatography have allowed the isolation, purification, and characterization of nontoxic and toxic lipid A analogs from polysaccharide-deficient, heptoseless Re mutants of Salmonella typhimurium or Salmonella minnesota (9, 11, 12, 16, 17). A monophosphoryl lipid A (MPL) derivative has recently been described (17), purified (9), and demonstrated to be at least 1,000 times less toxic or pyrogenic for rabbits than either the parent endotoxin or diphasporolys phoryl lipid A (15, 17).

In this study, we focused on one of the beneficial effects of endotoxin, the induction of a state of hyporesponsiveness to LPS, which is referred to as endotoxin tolerance. Briefly stated, endotoxin tolerance can be induced after an initial, sublethal exposure to LPS, which renders experimental animals or people refractory to subsequent LPS challenge. Endotoxin tolerance has been divided temporally into two phases. Early-phase endotoxin tolerance occurs within the first few days after exposure to LPS, is transient, and is not O-antigen specific. Late-phase tolerance is related to the production of anti-O-specific antibodies, occurs several weeks after the initial exposure to LPS, and persists for several weeks (3). Recent work by Williams et al. (19) and Madonna and Vogel (6) has provided insights into the cellular mechanisms which underlie the induction of early-phase endotoxin tolerance. Williams et al. demonstrated the involvement of lymphoid cell types derived principally from the peritoneal cavity and spleen for the abrogation of early endotoxin tolerance. Madonna and Vogel found that the induction and maintenance of early endotoxin tolerance is associated with a marked increase in bone marrow-derived macrophage progenitor cells (macrophage-CFU [M-CFU]). This suggested that early endotoxin tolerance might be related to an accumulation of immature monocyctic precursors in the bone marrow which could, in turn, result in reduced availability of mature (and more LPS-responsive) monocyctic cells in the periphery.

The primary goal of this work was to assess the efficacy of the nontoxic, MPL derivative to induce a state of early endotoxin tolerance to a fully toxic preparation of LPS. The data presented herein demonstrate that MPL derived from S. typhimurium, like intact LPS, induces the production of soluble factors, such as colony-stimulating factor (CSF) and interferon (IFN), without the toxic effects associated with intact LPS or lipid A. In addition, administration of the nontoxic MPL also induced a state of refractoriness to challenge with wild-type LPS (derived from Escherichia coli K235) which was equivalent to that induced by the toxic homologous preparation of LPS. Last, a concomitant increase in bone marrow-derived macrophage progenitor cells was observed in response to MPL. The data suggest that...
MPL is an effective tolerogen and contains the structural component(s) necessary for induction of early-phase endotoxin tolerance without the induction of endotoxic manifestations.

MATERIALS AND METHODS

Mice. Outbred female HSD(ICR)BR mice (ICR) were obtained from Harlan Sprague Dawley, Indianapolis, Ind. C3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine. All mice were housed in cages with Micro-Isolator unit tops (Lab Products, Rockville, Md.) in a negative-pressure Horsefall Isolator unit (Hazeltex Systems Inc., Aberdeen, Md.). All mice were used at 6 to 8 weeks of age and were allowed access to food and acid water ad libitum.

Reagents. Detoxified endotoxin (MPL, lot 375) from an S. typhimurium Re mutant was obtained from Ribi Immunolochem Research, Inc., Hamilton, Mont. The MPL, 1 mg per vial, was solubilized by the addition of 0.5 ml of sterile distilled water, followed by swirling the suspension in a 65 to 70°C water bath for 10 to 20 s and by sonication for 10 to 20 s. After three to four cycles of heating and sonication, the solution clarified to slight opalescence. This solution was added 0.5 ml of 3.6% NaCl to give a final stock solution of MPL (1 mg/ml) which was subjected to one additional cycle of heating and sonication.

Protein-free, phenol-water-extracted E. coli K235 LPS was prepared by the method of McIntire et al. (5). This LPS preparation was sonicated in pyrogen-free saline at a final concentration of 1 mg/ml.

Measurement of CSF activity in serum. Serum was tested for CSF activity in a bone marrow colony assay in semisolid agar as described previously (19). Briefly, serum was obtained from pooled blood collected from the orbital plexus of mice 6 h after the injection of saline, LPS, or MPL. Various dilutions of the serum were then made in six-well tissue culture plates (Costar, Cambridge, Mass.). Bone marrow cells were obtained from the femurs and tibias of C3H/HeJ mice and were further processed by density gradient centrifugation in lymphocyte separation medium (Litton Bionetics, Charlestone, S.C.) to enrich for mononuclear cells. The cells were then collected from the gradient interface and were enumerated (model ZM; Coulter Electronics Ltd., England). The bone marrow cells were then diluted to a final concentration of $10^8$ cells per ml in RPMI 1640 medium supplemented with antibiotics, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, glutamine, 15% fetal calf serum, and 0.3% molten agar. Then, 1 ml of the cell suspension in agar was added to each of the wells which contained 0.2 ml of the serum dilution, swirled to allow mixing, and allowed to solidify. Cultures were then incubated at 37°C (5% CO_2) for 7 days at which time bone marrow colonies (>50 cells per colony) were enumerated with an inverted microscope. Cytochemical staining of colonies revealed that >90% of the colonies were of macrophage lineage. Serum CSF activity is expressed as CFU per milliliter of serum.

Measurement of IFN activity in serum. The level of IFN in serum samples collected 2 h after intravenous (i.v.) administration of saline, LPS, or MPL was measured by antiviral activity (13) which tests the ability of the serum to protect murine L929 fibroblasts from infection with vesicular stomatitis virus (Indiana strain). The details of this assay are described elsewhere (18).

Determination of LD_{50}. Mice (20 mice per group) were injected intraperitoneally (i.p.) with either saline (control), E. coli K235 LPS (25 μg per mouse), or MPL (100 μg per mouse) on day 0. On day 3, five mice from each group were injected with E. coli LPS in 0.5 ml of pyrogen-free saline at the following concentrations: saline-treated mice, 1,500, 750, 375, or 187.5 μg of LPS; LPS- and MPL-treated mice, 3,000, 1,500, 750, or 375 μg of LPS. Deaths were recorded for 72 h. The 50% lethal dose (LD_{50}) for each experiment was calculated by the method of Reed and Muench (10).

Determination of the number of macrophage progenitor cells M-CFU in the bone marrow. The number of macrophage progenitor cells per femur was determined as described elsewhere (6). Briefly, groups of five mice were injected i.p. with saline, E. coli LPS, or various doses of MPL as indicated. After 3 days, the mice from each group were killed, and five femurs per group (one femur per mouse) were homogenized to obtain a bone marrow cell suspension. A portion of the cell suspension was used to obtain a nucleated cell count and cell sizing profile with a Coulter Counter model ZM and Coulter Channelyzer C1000.

The number of macrophage progenitor cells (M-CFU), adjusted for the total nucleated cell count from each cell suspension, was then determined by using a double-layer, semisolid agar colony assay (4). Although the single-layer colony assay is adequate for the assessment of CSF levels in serum (7-day incubation period), the double-layer assay is preferred for enumeration of progenitor cells because it is nutritionally enriched to accommodate the longer (10 day) incubation period and provides some protection against desiccation. Approximately 7,000 U of partially purified, macrophage-specific CSF (CSF-1) was contained in the bottom layer. The upper layer contained $5 \times 10^4$ bone marrow cells from each group. Duplicate cultures were incubated for 10 days at 37°C (5% CO_2), and colonies (>50 cells) were counted.

RESULTS

Induction of serum CSF by MPL. Previous studies have revealed that injection of endotoxin into mice was followed by a rapid rise of serum CSF which reached a maximum level at approximately 6 h postinjection (7). To test whether the nontoxic lipid A derivative, MPL, was also able to induce this manifestation of endotoxin responsiveness, groups of five mice were injected i.v. with various doses of MPL. At 6 h after injection, the mice were bled, and the serum was subsequently tested for the presence of CSF. Serum CSF was induced over a wide dose range (1.0 to 100 μg per mouse) and was induced optimally at 50 μg of MPL per mouse (Fig. 1A). Additionally, MPL induced a similar dose-dependent response in mice injected i.p. (data not shown).

Induction of early endotoxin tolerance by MPL to challenge with intact E. coli K235 LPS. In two previous reports in which CSF was used as an indicator of in vivo LPS responsiveness, it was found that early endotoxin tolerance was optimal 3 to 4 days after initial exposure to either a homologous or heterologous preparation of LPS (6, 19). To test whether an initial exposure to MPL would also render animals refractory to LPS, we injected groups of mice with various concentrations of MPL, and 3 days later we challenged these same mice with E. coli LPS (25 μg per mouse). At 6 h after challenge, blood was collected, and the serum was assayed for CSF. The results show that increasing the dose of MPL given on day 0 reduced significantly the serum CSF response induced upon challenge with 25 μg of E. coli LPS on day 3 (Fig. 1B). This level of hyporesponsiveness...
FIG. 1. MPL induction of serum CSF and induction of early tolerance to *E. coli* LPS. (A) ICR mice were injected with either saline, *E. coli* K235 LPS (25 µg per mouse), or MPL (1 to 100 µg per mouse) i.v. The mice were bled 6 h later, and sera were collected, pooled, and assayed for CSF activity. (B) Mice were injected on day 0 with either saline, *E. coli* LPS (25 µg per mouse), or the indicated concentration of MPL (1 to 100 µg per mouse). On day 3, mice were challenged with *E. coli* K235 LPS (25 µg per mouse), and 6 h later sera were collected and assayed for CSF activity. The results represent the arithmetic mean standard deviation of duplicate samples of serum pools from three separate experiments in which results from five mice per treatment per experiment were pooled. The treatment designations in panel B indicate the treatments which were administered on days 0 and 3, respectively.

was equivalent to that seen when *E. coli* LPS was used as the tolerogen (Fig. 1B).

Another indication of in vivo LPS responsiveness is the induction of serum IFN which usually peaks 2 to 3 h after i.v. administration of intact LPS or lipid A (20). The level of serum IFN induced by MPL was comparable to that stimulated by *E. coli* LPS (Fig. 2); however, the tolerance induced by MPL for IFN was less striking than the tolerance induced by the intact LPS for IFN or the MPL-induced tolerance for CSF (Fig. 1B).

The effect of initial MPL exposure on LPS-induced LD50 was also determined. It was found that prior exposure of mice to an initial dose of MPL (100 µg per mouse) significantly increased the LD50 to *E. coli* LPS approximately
fivefold (1,156 μg of LPS in mice with MPL-induced tolerance versus 258 μg of LPS in control, saline-treated mice). This increase was found to be comparable to that induced by E. coli LPS (972 μg). Additionally, it was found that prior exposure of MPL on day 0 essentially eliminated LPS-induced symptoms of endotoxicity after LPS challenge on day 3 (i.e., ruffled fur, conjunctival discharge, and diarrhea).

MPL-induced changes in the number of bone marrow-derived macrophage progenitor cells. Previous studies have shown that injection of E. coli LPS on day 0 greatly increased the number of CSF-responsive macrophage progenitor cells (M-CFU) by day 3 after injection (the optimal day for early endotoxin tolerance). To test the efficacy of MPL to increase bone marrow M-CFU, groups of five mice were injected with saline, E. coli LPS (25 μg per mouse), or various concentrations of MPL, and on day 3 after injection, bone marrow cells from the femurs of each group were enumerated and cultured in an excess of CSF-1. This factor has been shown to be specific for cells of macrophage lineage and to give rise exclusively to macrophage colonies in culture (14). The results show that MPL increased the number of M-CFU in the bone marrow in a dose-dependent fashion (Fig. 3). When mice were given 100 μg of MPL per mouse, the level of M-CFU per femur was equal to that induced by 25 μg of E. coli LPS.

These findings were further supported by changes in bone marrow cell sizing profiles obtained with a Coulter Channelyzer. Previous work showed that injection of E. coli LPS on day 0 resulted in an increase in size and cell number of the Coulter Channelyzer profile peak 2 which was found to contain all of the macrophage progenitor activity (6). Bone marrow cell suspensions from groups of five mice injected 3 days earlier with saline, E. coli LPS, or MPL were compared by using a Coulter Channelyzer. As can be seen in Fig. 4, like LPS, the injection of MPL induced a marked reduction in the relative number of bone marrow cells in peak 1 (20% of total) and a compensatory increase in the number of cells within peak 2 (80%). This was accompanied by an increase in cell size in peak 2. As was previously observed with E. coli LPS, this change in cell sizing profile induced by MPL
populations of mice the following days after LPS.

DISCUSSION

Early-phase endotoxin tolerance occurs within the first few days after LPS exposure, fades within a week, and is inducible with serologically unrelated species of LPS (3). By following the early endotoxin tolerance protocol set forth by Williams et al. (19), we established an early tolerance system by the injection of mice with 25 μg of E. coli K235 LPS (6). By using CSF as an initial indicator of in vivo LPS responsiveness, it was found that maximal hyporesponsiveness to a challenge injection occurred 3 to 4 days after the initial injection. It was shown in that study that the acquisition and maintenance of the tolerant state coincided temporally with an increase in the number of macrophage progenitor cells in the bone marrow. Further, density gradient separation and cell sizing profiles confirmed that the bone marrow cell populations of mice with MPL-induced tolerance exhibited an increase in cell size and in the number of macrophage precursors.

By using a modification of this early endotoxin tolerance system, we replaced the toxic, wild-type E. coli LPS used on day 0 in our previous study with a nontoxic, MPL derivative to assess its ability to induce early tolerance. Unlike initial exposure to wild-type LPS, MPL did not induce symptoms of endotoxicity, i.e., ruffled fur, conjunctival discharge, or diarrhea, even at 100 μg per mouse. Despite the absence of these overt manifestations of endotoxicity, MPL was shown to induce both serum CSF and IFN to levels induced by wild-type LPS. Next, it was found that injection of MPL on day 0 reduced the LPS-induced serum CSF (and to a lesser extent, IFN) upon challenge with wild-type E. coli LPS 3 days later. MPL was also shown to increase greatly the LD₅₀ to challenge injection with E. coli LPS and effectively reduce overt symptoms of endotoxicity upon challenge. As seen in

FIG. 3. Effect of a tolerance-inducing injection of MPL on the number of macrophage precursors in the bone marrow. ICR mice were injected on day 0 with either saline, E. coli LPS (25 μg per mouse), or MPL (at the indicated doses), and 3 days later, the bone marrow cells were collected and pooled (5 mice per treatment group). Then, 5 × 10⁶ cells from each cell suspension were plated in duplicate in an excess of partially purified CSF-1 and incubated for 10 days, and the number of M-CFU was determined. The results represent the mean standard deviation of duplicate determinations.

FIG. 4. Effect of tolerance-inducing doses of MPL on bone marrow cell sizing profiles. ICR mice were injected on day 0 with either saline, E. coli LPS (25 μg per mouse), or MPL (at the indicated doses) i.p. and killed 3 days later. By using a Coulter Channelizer, cell sizing profiles were obtained from suspensions of bone marrow cells (five mice per group). The arrow indicates the initial position of peak 2 in mice injected with saline on day 0. The administration of 25 μg of MPL gives a cell sizing profile which is superimposable to 50 μg of MPL (data not shown).
the LPS-induced tolerance system used previously (6), MPL administration greatly increased the number of macrophage progenitor cells in the bone marrow, and this increase in M-CFU coincided with an increase in cell size and cell number of Coulter Channelizer profile peak 2. Taken collectively, these results indicate that MPL possesses the necessary structural component(s) required to induce a state of early endotoxin tolerance. Most importantly, MPL fails to induce the initial toxic reactions typically observed with wild-type endotoxin or diphosphoryl lipid A preparations.

An intriguing aspect of this study is that tolerance, as assessed by IFN production, was less affected by MPL than other manifestations of LPS responsiveness are. One possible implication is that IFN may be less involved in the induction of the toxic effects of LPS than other serum factors, such as serum CSF. In this regard, previous work by Moore et al. (8) supports a role for CSF as an immunoregulatory agent capable of rendering macrophages more sensitive to LPS in vitro as assessed by increased production of IFN and interleukin-1.

The major obstacle which has limited the clinical application of endotoxin for its beneficial effects has been its toxic side effects. Chemical modification of the endotoxin molecule has resulted in a nontoxic, MPL which, because of its greatly reduced capacity to cause endotoxicy, holds great potential for clinical utilization.

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LITERATURE CITED