INFECTION AND IMMUNITY, June 1993, p. 2325–2333
0019-9567/93/062325-09$02.00/0

Vol. 61, No. 6

Dissociation of Lipopolysaccharide (LPS)-Inducible Gene Expression in Murine Macrophages Pretreated with Smooth LPS versus Monophosphoryl Lipid A

BETH E. HENRICSON,[1] CARL L. MANTHEY,[1] PIN YU PERERA,[1] THOMAS A. HAMILTON,[2] AND STEFANIE N. VOGEL[1]*

Department of Microbiology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814,[1] and Department of Immunology, Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195[2]

Received 28 December 1992/Accepted 17 March 1993

Lipopolysaccharide (LPS) and the nontoxic derivative of lipid A, monophosphoryl lipid A (MPL), were employed to assess the relationship between expression of LPS-inducible inflammatory genes and the induction of tolerance to LPS in murine macrophages. Both LPS and MPL induced expression (as assessed by increased steady-state mRNA levels) of a panel of seven "early" inflammatory genes including the tumor necrosis factor alpha (TNF-α), interleukin-1β, type 2 TNF receptor (TNFR-2), IP-10, D3, D8, and D2 genes (the last four represent LPS-inducible early genes whose functions remain unknown). In addition, LPS and MPL were both capable of inducing tolerance to LPS. The two stimuli differed in the relative concentration required to induce various outcome measures, with LPS being 100- to 1,000-fold more potent on a mass concentration basis. Characterization of the tolerant state identified three distinct categories of responsiveness. Two genes (IP-10 and D8) exhibited strong desensitization in macrophages pretreated with tolerance-inducing concentrations of either LPS or MPL. In macrophages rendered tolerant by pretreatment with LPS or MPL, a second group of inducible mRNAs (TNF-α, interleukin-1β, and D3) showed moderate suppression of response to secondary stimulation by LPS. The third category of inducible genes (TNFR-2 and D2) showed increased expression in macrophages pretreated with tolerance-inducing concentrations of either LPS or MPL. All of the LPS-inducible genes examined exhibited modest superinduction with less than tolerance-inducing concentrations of either stimulus, suggesting a priming effect of these adjuvants at low concentration. The differential behavior of the members of this panel of endotoxin-responsive genes thus offers insight into molecular events associated with acquisition of transient tolerance to LPS.

The physiologic effects of bacterial endotoxin on humans are pleiotropic and may produce toxicity so severe that endotoxic shock following gram-negative sepsis results in an estimated 70,000 deaths annually in the United States (3). In vivo, endotoxin, or its most toxic fraction, lipopolysaccharide (LPS), acts principally on macrophages to elaborate a cascade of effector molecules known as cytokines, which mediate many of the toxic symptoms associated with LPS (reviewed in references 24 and 41). However, a single sublethal injection of LPS can result in a transient state of hyporesponsiveness to subsequent LPS challenge which has been referred to as early endotoxin tolerance (13, 19–21). In cell transfer studies, Freudenberg et al. (7) demonstrated that the macrophage plays a central role not only in LPS-induced toxicity but also in the induction of tolerance. Vogel et al. (43) showed that combined administration of recombinant interleukin-1β (IL-1β) and recombinant tumor necrosis factor alpha (TNF-α) could induce in mice an LPS-hyporesponsive state that mimicked tolerance, suggesting a role for these two cytokines in the induction of tolerance. Subsequently, it was reported that administration of either recombinant IL-1 receptor antagonist (14) or anti-TNF antibodies (12a, 40) partially mitigated the induction of tolerance by LPS in mice. Not only is tolerance inducible by LPS and its toxic substructure, lipid A, but also by the nontoxic derivative of lipid A, monophosphoryl lipid A (MPL); however, significantly higher concentrations of MPL than LPS or lipid A are required to induce an equivalent degree of refractoriness in vivo.

In vitro models for studying endotoxin tolerance in macrophages have been developed recently (25, 39, 44). An 18- to 48-h exposure of murine or human macrophages or a monocytic cell line to low concentrations of LPS renders the cells refractory to further LPS treatment, although pretreated cells respond normally to non-LPS stimuli to secrete TNF. Using adherent peritoneal exudate macrophages in this system, we found that MPL, like LPS, could induce tolerance to LPS challenge in vitro, but much higher concentrations of MPL than LPS were required to elicit a comparably refractory state (15).

Recently, six LPS-inducible genes (designated D2, D3, D5, D7, D8, and C7 genes) were cloned by differential screening of a cDNA library created from LPS-treated C57BL/6J mouse peritoneal exudate macrophages (34). These genes were all strongly induced by 3 h of LPS stimulation in the presence of cycloheximide and were therefore classified as immediate early genes. The C7 gene has been identified as the murine homolog of IP-10, first identified as a gamma interferon-inducible gene (28), the product of which is a member of the platelet factor 4 chemokine family of small chemotactic and proinflammatory molecules (18). The D5 gene was identified as the IL-1β gene (29), the D3 gene appears to be a member of the alpha interferon-inducible family of genes previously referred to as 202-204 (5), and the D7 gene has recently been identified as

* Corresponding author.

the gene which encodes the p75 TNF receptor (TNFR-2) (8, 35, 37). The availability of these probes presented a unique opportunity to investigate responses to endotoxin in vitro, at the level of gene induction. In this study, we utilized this panel of six LPS-inducible genes, plus the gene for TNF-α, to explore in vitro induction of tolerance to LPS as compared with induction by its nontoxic derivative, MPL (31).

(This work was carried out by B. E. Henricson in partial fulfillment of the requirements for the Ph.D. degree from the Uniformed Services University of the Health Sciences, 1992.)

MATERIALS AND METHODS

Mice. Five- to six-week-old female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and were used within 1 week of receipt. Mice were housed in a laminar flow hood and were maintained under 12-h light and dark cycles until use. Mice were fed standard laboratory chow and acid water ad libitum. The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* [National Research Council DHEW publication no. (NIH) 85-23].

Reagents. Protein-free (<0.008%), phenol-water-extracted *Escherichia coli* K235 LPS was prepared by the method of McIntire et al. (26). *Salmonella minnesota* MPL, isolated from deep rough chemotype R595, was purchased from Ribi ImmunoChem Research, Inc. (Hamilton, Mont.) (31). Stock solutions of LPS and MPL were prepared at 1 mg/ml in normal saline containing 0.2% triethylamine and were sonicated vigorously and heated to 65°C alternately to increase solubility. Stock solutions were maintained at −20°C until use.

TNF assay. TNF activity in serum samples was determined by a standard cytotoxicity assay using actinomycin D-treated fibroblasts (15, 42). The lower and upper limits of sensitivity of the assay are 80 U/ml (i.e., 4U/50 μl) and 40,960 U/ml (i.e., 2,048 U/50 μl), respectively (42).

Macrophage isolation and culture. Peritoneal exudate macrophages were isolated by peritoneal lavage with ice-cold sterile physiologic saline 4 days after intraperitoneal injection of 3 ml of sterile 3% thioglycolate broth (23). Cells were resuspended in RPMI 1640 supplemented with glutamine, penicillin, streptomycin, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), sodium bicarbonate, and 2% heat-inactivated fetal calf serum and were cultured at 10^7 cells per plate in 60-mm-diameter tissue culture dishes (Costar, Cambridge, Mass.) at 37°C and 6% CO_2 overnight prior to treatment. Nonadherent cells were removed by washing twice with fresh medium 20 to 24 h after plating, and the remaining adherent cells were cultured as indicated. To determine responsiveness of freshly isolated adherent macrophages to LPS derivatives, cells were pretreated with medium or the LPS derivatives for 4 h (or the indicated time) at 37°C in 6% CO_2 prior to sampling of supernatant fluids for TNF bioassay and harvest of cellular RNA for Northern (RNA) blot analysis.

The induction of in vitro tolerance was essentially identical to that originally described by Virca et al. (39). Adherent macrophages were first cultured identically as for the production of TNF. For tolerance induction experiments, macrophage cultures were pretreated for 20 h with medium or with various concentrations of LPS or MPL. Cells were then washed twice with fresh medium and treated with medium only or 10 ng of LPS per ml. Cultures were incubated for an additional 4 h prior to isolation of RNA.

Isolation of mRNA. Macrophages were lysed at the indicated times post-LPS and -MPL stimulation with 4 M guanidine isothiocyanate. Total cellular RNA was isolated by the cesium chloride gradient centrifugation method of Chirgwin et al. (4). Northern blot analysis was carried out by the method of Maniatis et al. (22) as follows. Five micrograms of RNA for each experimental condition was subjected to 1% formaldehyde-agarose gel electrophoresis, and total RNA was transferred to Nytran filters (Schleicher & Schuell, Inc., Keene, N.H.) by means of capillary action in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNA was cross-linked to filters, and the filters were hybridized overnight with ^{32}P-labeled cDNA probes after a 4-h prehybridization at 42°C. Probes utilized for these experiments were as follows: an 1,100-bp segment of the cDNA for TNF-α (30), kindly provided by Bruce Beutler (Howard Hughes Medical Institute, Dallas, Tex.); a 750-bp cDNA probe specific for murine β-actin (38), provided by Michael Prystowsky (University of Pennsylvania, Philadelphia); a cDNA encoding bases 275 to 1329 for IL-1β (29); a 450-base cDNA segment for murine IP-10 (28); a specific cDNA probe for the p 75 TNF receptor (TNFR-2); and cDNA probes for three other LPS-inducible genes (i.e., D2, D3, and D8) first described by Tannenbaum et al. (34) as strongly inducible by LPS within 3 h (35). After hybridization, filters were washed twice at room temperature for 20 min in 2× SSC containing 0.1% sodium dodecyl sulfate and once at 65°C for 15 min in 0.1× SSC containing 0.1% sodium dodecyl sulfate. Northern blot filters were exposed to Kodak XAR-5 film with intensifier screens for 4 to 24 h at −70°C and subsequently were used to expose PhosphorImager screens. Exposed PhosphorImager screens were analyzed by using PhosphorImager software in a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Exposed X-ray film was analyzed with a scanning densitometer (Xerox, Corp., Rochester, N.Y.) and NIH-Image software kindly donated by Wayne Rasband, National Institutes of Health, Bethesda, Md. Between successive hybridizations, filters were stripped by boiling in water for 5 min. The relative expression for each gene was internally normalized to the expression of the β-actin gene by calculating the gene-to-β-actin gene expression ratio for every lane on the Northern blot, and this normalization controlled for lane-to-lane loading differences. Neither LPS nor MPL induced any significant modulation of β-actin-specific, steady-state mRNA levels over the incubation periods examined in these studies (data not shown). For the dose-response and in vitro tolerance experiments, the normalized data were then converted to a percentage of the steady-state level of the gene following stimulation by 10 ng of LPS per ml for 4 h. For time course experiments, data were converted to a percentage of maximal expression induced by 1,000 ng of LPS per ml at 12 h. Statistical analyses of the data were carried out by least squared means comparison of the log-transformed data, using IBM format SAS software, by Lawrence Douglass (statistician) and Jian-Zheng Zhou (analyst), University of Maryland, College Park.

RESULTS

Induction of early gene expression by LPS and MPL. In preliminary studies, it was determined that 1 μg of MPL per ml could induce murine macrophages to express high levels of each of the LPS-inducible genes under consideration,

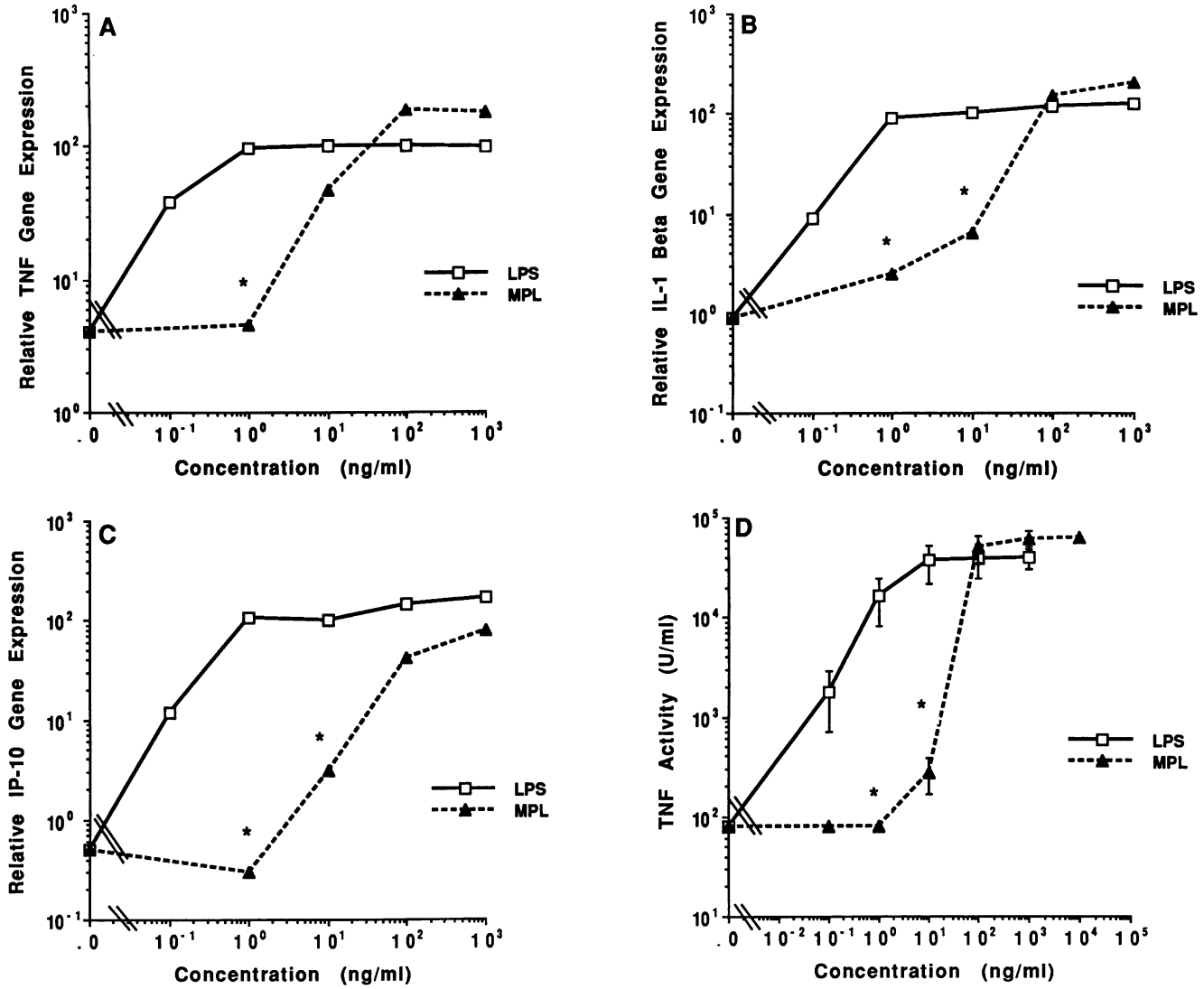

FIG. 1. LPS- and MPL-induced dose responses for TNF-α (A), IL-1β (B), and IP-10 (C) mRNA and TNF activity (D) at 4 h of stimulation by LPS or MPL. Values in panels A to C are mean values from three or four separate experiments. Data were normalized for β-actin and then expressed as a percentage of the maximum LPS response induced by 10 ng/ml at 4 h (100%) as described in Materials and Methods. Asterisks indicate MPL-induced values significantly different from LPS-induced values, as determined by least squared means comparison of the log-transformed data. (D) Comparison of secreted TNF activity dose responses for LPS and MPL. TNF activity was measured in supernatants of macrophages that were used to generate Northern blot data for panels A to C. The data represent the geometric means ± standard errors of the means for three or four separate experiments.

viz., the TNF-α gene, as well as the IP-10, IL-1β, TNFR-2, D3, D8, and D2 genes (34). These studies were extended to determine the relative concentration dependence for MPL- and LPS-induced gene expression. For the TNF-α, IL-1β, and IP-10 genes (Fig. 1A to C, respectively) and the D3 and D8 genes (data not shown), maximal steady-state mRNA was induced at 1 ng of smooth LPS per ml. However, MPL-induced gene expression for all these genes required ~100- to 300-fold-higher concentrations. MPL was also ~100-fold less potent than LPS in inducing TNF activity (Fig. 1D).

The D2 and TNFR-2 genes (35) exhibited a second dose-response pattern. They showed relatively high constitutive expression, ~40% of the level routinely induced by 10 ng of LPS per ml after 4 h of stimulation (Fig. 2). Although both LPS and MPL stimulated increases in D2 and TNFR-2 steady-state mRNA, the relative increases above background were modest (ca. two- to fivefold) when compared with all other genes examined in the panel. For these two genes, MPL proved to be ~100- to 1,000-fold less potent than LPS in the induction of steady-state mRNA. Finally, the induction of TNFR-2 mRNA was different from that of all other genes examined in that it peaked sharply and declined with increasing concentrations of either LPS or MPL.

Time course for induction of LPS-inducible genes. The panel of LPS-inducible genes was also examined for differences in the time course patterns when macrophages were stimulated by an excess (1,000 ng/ml) of either LPS or MPL. TNF-α (Fig. 3A) and IL-1β (data not shown) mRNAs were induced maximally by LPS within 1 to 2 h after stimulation (1 h for TNF-α and 2 h for IL-1β) and were reduced to near

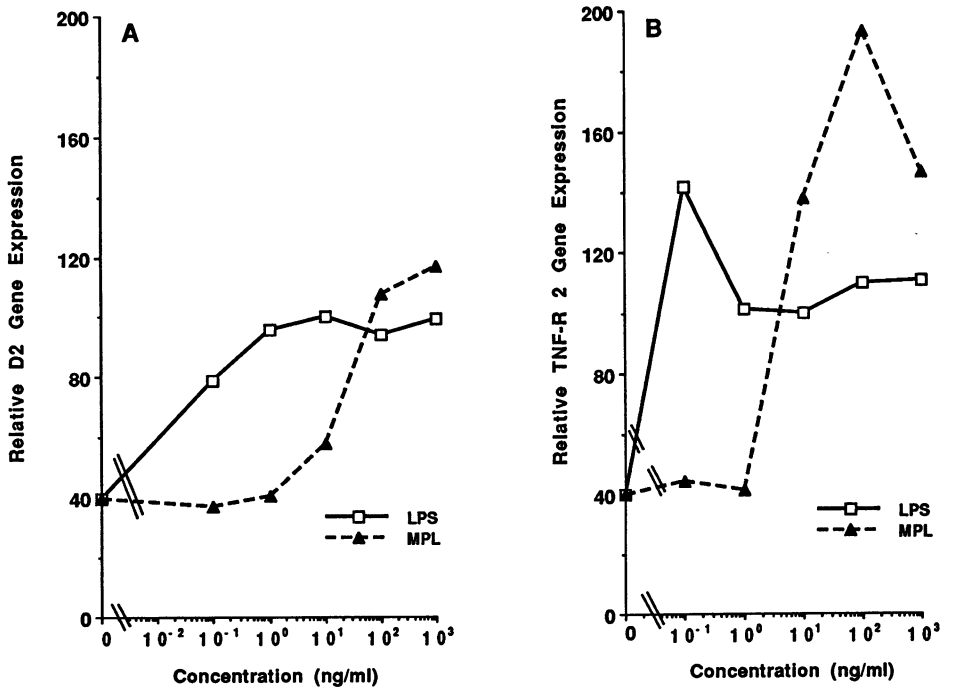

FIG. 2. LPS- and MPL-induced dose-response curves for D2 (A) and TNFR-2 (B) mRNA expression 4 h after stimulation by LPS or MPL. See the legend to Figure 1A to C for further details.

background levels within 12 h. For TNF-α, peak expression induced by MPL was slightly delayed and, for both TNFα and IL-1β, it was more sustained than that induced by LPS, even though the maximal levels of expression were found to be approximately equivalent. The constitutively expressed genes, the TNFR-2 (Fig. 3B) and D2 (data not shown) genes, exhibited peak expression typically at 2 h after LPS stimulation. As was observed for TNF-α and IL-1β steady-state RNA, MPL-induced gene expression was somewhat more protracted than LPS-induced expression of the TNFR-2 and D2 genes.

In contrast to the kinetic differences described for LPS- versus MPL-induced TNF-α, IL-1β, TNFR-2, and D2 steady-state mRNA, no such differences were observed between MPL- and LPS-induced D8 (Fig. 3C), IP-10 (data not shown), and D3 (data not shown) steady-state mRNA. Maximal expression of both D8 and IP-10 occurred 4 h poststimulation; however, IP-10 mRNA levels declined to ~20% relative gene expression over 12 h, whereas D8 expression (Fig. 3C) declined more gradually. Maximal D3 gene expression occurred somewhat later and remained elevated at 8 to 12 h following stimulation by either LPS or MPL. The kinetic behavior of these genes is consistent with previous observations (11, 34).

Gene expression in macrophages pretreated with LPS or MPL and stimulated by LPS challenge. We have demonstrated previously that significantly more MPL than LPS is required in vivo and in vitro to induce a state of tolerance (13, 19–21). To examine the ability of pretreated macrophages to express genes in response to a second LPS challenge, adherent peritoneal exudate macrophages were first exposed to medium only or the indicated concentrations of LPS or MPL for 20 h. Cultures were then washed and restimulated with 10 ng of LPS per ml for 4 h prior to RNA isolation. The 10-ng/ml challenge concentration of LPS was based on the maximal LPS gene expression data presented in Fig. 1 and 2 and our finding that a 10-ng/ml LPS challenge allows for reliable discrimination between LPS-responsive and -tolerant macrophages in vitro, as indicated by suppression of TNF secretion (15). LPS challenge of cultures pretreated with LPS or MPL resulted in TNF-α, IL-1β, and D3 gene induction (Fig. 4) which exhibited the following general features. At concentrations of LPS or MPL below those which render cells LPS hyporesponsive in vitro (e.g., <1 ng/ml and <100 ng/ml, respectively, for LPS and MPL), a priming effect in response to challenge was observed. This is indicated by a moderate superinduction of gene expression induced by LPS challenge above that observed for LPS challenge of medium-pretreated cells (dotted line). Although the increase "above the line" was least pronounced for LPS-induced IL-1β gene expression, this trend was observed for LPS-induced TNF and D3 gene expression, as well as for all three genes stimulated with sub-tolerance-inducing concentrations of MPL. However, upon exposure to concentrations shown previously to induce tolerance in vitro (15), LPS-pretreated macrophages responded to LPS challenge with a moderate decrease in IL-1β, TNF-α, and D3 gene expression (e.g., 40 to 60% of control levels), as did MPL-pretreated cells at higher pretreatment concentrations (>100 ng/ml).

In contrast to the moderate suppression of TNF-α, IL-1β, and D3 gene expression after challenge that was induced by pretreatment of macrophages with tolerance-inducing doses of LPS and MPL, postchallenge IP-10 and D8 gene expression was reduced profoundly (to near background levels) (Fig. 5). At less than tolerance-inducing concentrations of LPS or MPL, superinduction of IP-10 and D8 mRNA expression was also observed, although, like LPS-induced IL-1β gene expression, this was minimal for LPS-induced D8 gene expression.

For the TNFR-2 and D2 genes (Fig. 6) there was no concentration-dependent suppression of steady-state mRNA

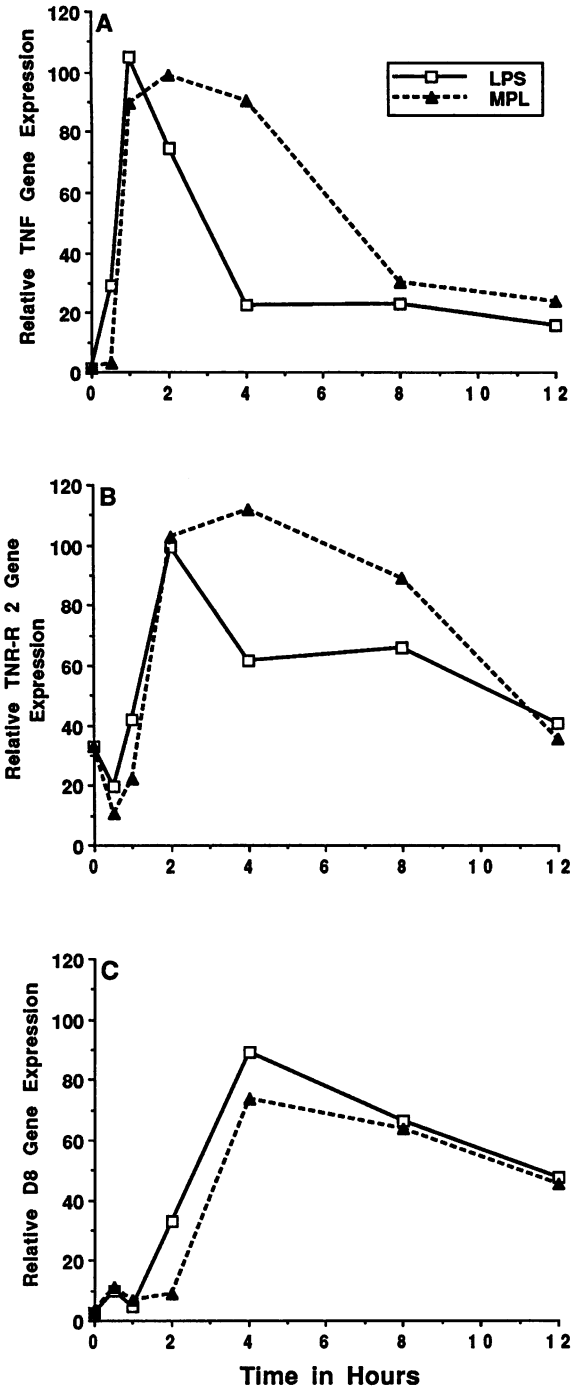

FIG. 3. Comparison of time course of TNF-α (A), TNFR-2 (B), and D8 (C) mRNA expression over 12 h of stimulation by LPS or MPL. Results are least squared means from three to five separate experiments. Data were normalized for β-actin and then expressed as a percentage of the maximum response induced by 1,000 ng of LPS per ml (100%) as described in Materials and Methods.

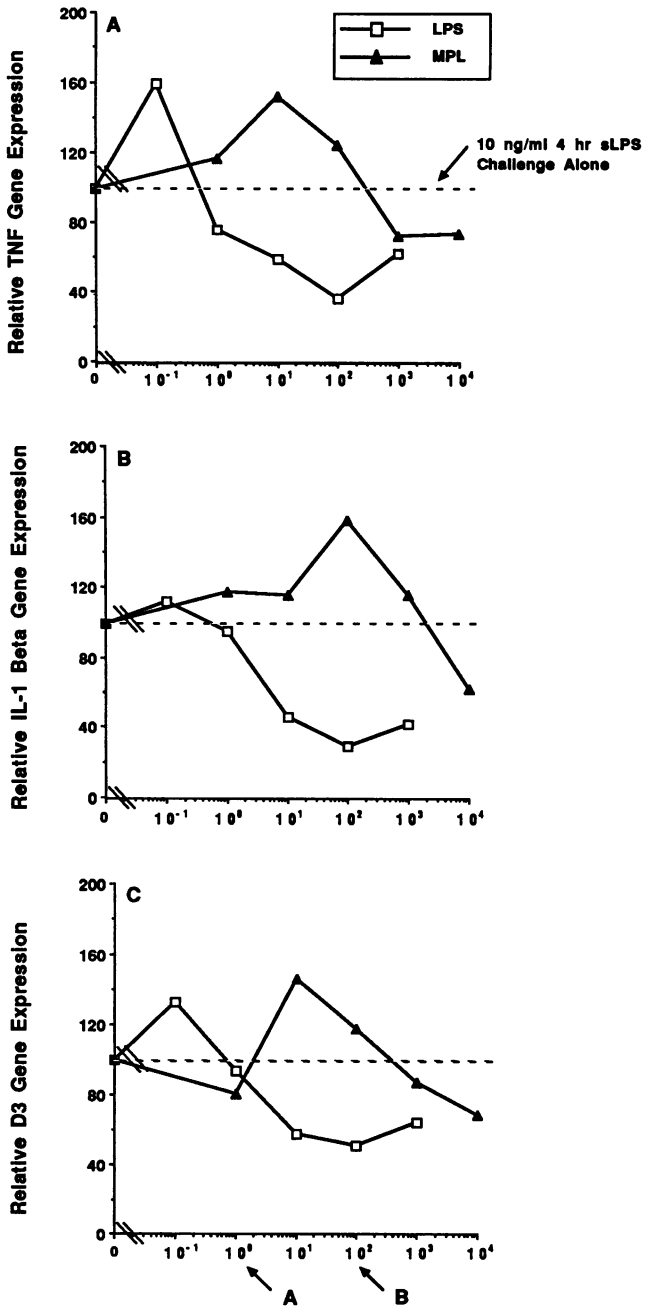

FIG. 4. Comparison of TNF-α (A), IL-1β (B), and D3 (C) gene expression 4 h after challenge (10 ng of LPS per ml) of macrophages pretreated for 20 h with the indicated concentrations of LPS or MPL. Results are least squared means from four separate experiments. Gene expression was normalized to the expression of β-actin and then expressed as a percentage of the expression induced by cells pretreated for 20 h with medium and then challenged with 10 ng of *E. coli* K235 LPS per ml (100%) (dotted line). Arrows under the *x* axis, labeled A and B, represent concentrations of LPS and MPL, respectively, for which minimal in vitro tolerance was induced, as measured by secreted TNF activity.

expression upon LPS challenge of either LPS- or MPL-pretreated macrophages. Pretreatment with LPS or MPL resulted only in a modest superinduction of TNFR-2 and D2 mRNA expression, and no differences were observed between LPS- and MPL-pretreated cultures for these two

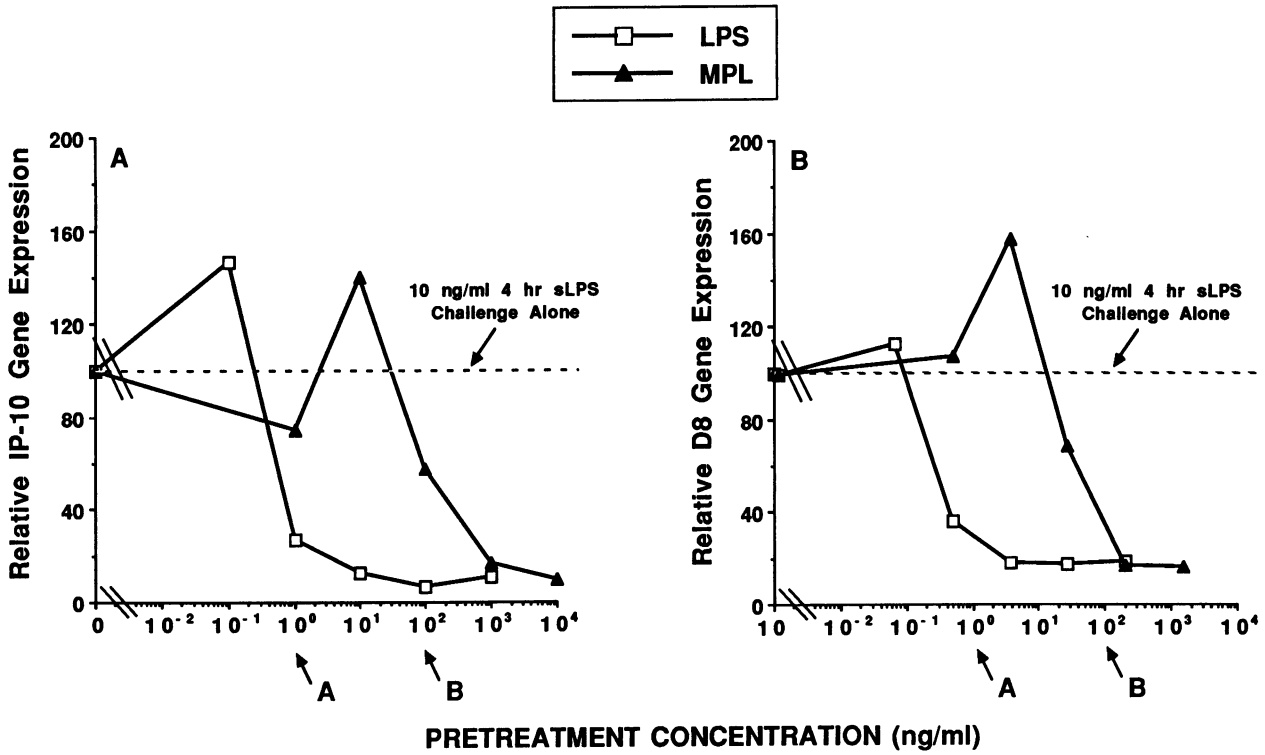

FIG. 5. Comparison of IP-10 (A) and D8 (B) gene expression 4 h after challenge (10 ng of LPS per ml) of macrophages pretreated for 20 h with the indicated concentrations of LPS or MPL. See the legend to Fig. 4 for further details.

genes. When compared directly with LPS-induced IP-10 gene expression, which was strongly suppressed by LPS pretreatment, both TNFR-2 and D2 gene expression were maximal at concentrations of LPS pretreatment that completely suppressed IP-10 (compare Fig. 5 and 6).

DISCUSSION

The immunostimulant MPL is currently under consideration for its potential as a therapeutic agent for treatment of endotoxic shock (17), for prophylaxis against postoperative wound infections (2), and as an immune adjuvant capable of increasing antigen-specific immune responses to vaccines (16). MPL is attractive for all of these uses since it is substantially nontoxic as compared with LPS yet retains many of the beneficial effects attributed to endotoxin. One of the beneficial effects that has been studied in detail is the induction of early endotoxin tolerance (19). Although MPL induces a state of tolerance in vivo equivalent to that induced by LPS, ca. eightfold more MPL is required (13).

The basis of MPL's lack of toxicity has been attributed to its induction of lower levels of toxic symptom-inducing cytokines, such as TNF, IL-6, interferon (13), and IL-1α (data not shown). These findings are extended here by examination of the capacity of MPL to induce early gene expression as compared with LPS and characterization of the effects of LPS versus MPL pretreatment on gene expression induced upon LPS challenge. Although originally cloned based on their capacity to be activated transcriptionally by LPS, the induction of subsets of these LPS-inducible genes by other agents has since been characterized (11, 18, 28, 29, 33–36). For example, the IP-10, D3, and D8 genes might be classified as broadly inducible, as evidenced by their capacity to be stimulated not only by LPS but also by interferons in macrophages and by platelet-derived growth factor in fibroblasts (36). The D2 and TNFR-2 genes have been grouped previously as a subset of LPS-inducible genes which are selectively expressed upon exposure of macrophages to phorbol myristic acid (34).

Similarities in the expression patterns of certain genes may provide insights into the molecular mechanisms which underlie the induction of tolerance. While LPS and MPL differed in their ability to induce both expression of the genes studied and the state of tolerance in terms of the relative mass concentrations required, the endpoints achieved were essentially indistinguishable.

Pretreatment of macrophages with a broad concentration range of LPS or MPL followed by an LPS challenge results in three general groupings of genes: TNF-α, IL-1β, and D3 (e.g., the expression of all three is superinduced at sub-tolerance-inducing concentrations, and is, at best, only modestly inhibited by pretreatment in the tolerance-inducing range; IP-10 and D8 (e.g., the expression of these two genes is superinduced at sub-tolerance-inducing concentrations but profoundly suppressed in response to tolerance-inducing concentrations); and, finally, TNFR-2 and D2 (e.g., superinduction of these two genes was observed over the entire range of LPS or MPL pretreatment concentrations tested). The moderate suppression of TNF-α steady-state mRNA during tolerance, coupled with profound suppression of TNF secretion, is consistent with the finding that during tolerance a 26-kDa TNF precursor accumulates in LPS-refractory murine macrophages, indicating posttranslational regulation of TNF secretion (12, 39, 44, 45). Superinduction of gene expression upon challenge initiated by pretreatment of macrophages with LPS or MPL may reflect a true "priming" of

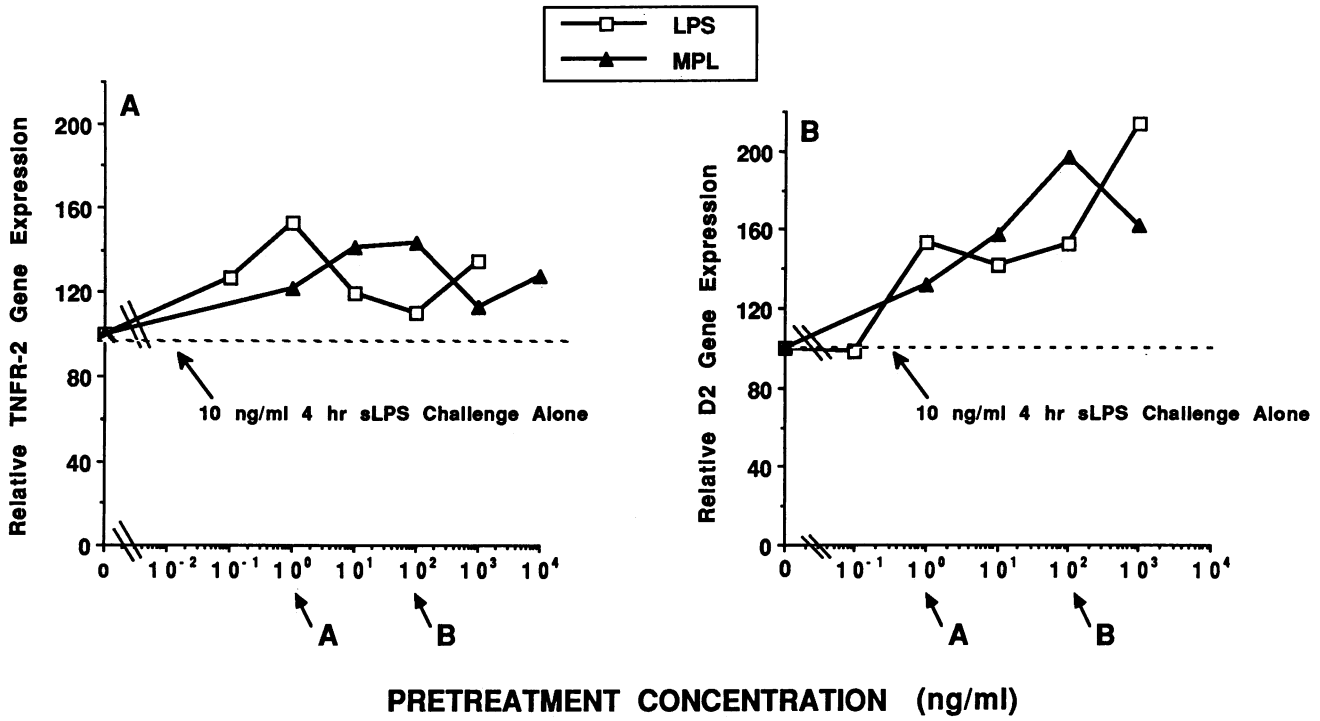

FIG. 6. Comparison of TNFR-2 (A) and D2 (B) gene expression 4 h after challenge (10 ng of LPS per ml) of macrophages pretreated for 20 h with the indicated concentrations of LPS or MPL. See the legend to Fig. 4 for further details.

macrophage responsiveness to LPS. Priming of macrophages by LPS or MPL may provide a mechanism by which macrophages increase their capacity for nonspecific resistance to infection. Macrophages exposed to low levels of LPS (or MPL) might then be more able to contain spreading focal infection, but exposure to higher levels of LPS would induce tolerance and thereby help to minimize deleterious toxic responses.

As IP-10 has been identified as a member of a family of potent chemotactic and proinflammatory intercrine proteins (18, 27), one could postulate that a profound suppression of IP-10 message levels (Fig. 5A) in tolerant macrophages might strikingly inhibit the proinflammatory/chemotactic response to LPS upon challenge. The two groups of genes examined in the experiments of Fig. 4 and 5 differed in the degree to which the expression of steady-state mRNA was suppressed during tolerance. Functions for the products of both the IP-10 and the D8 genes have not yet been identified. Nevertheless, the profound nature of down-regulation of steady-state mRNA levels for D8 and IP-10 during tolerance may imply their essential participation in toxicity. It remains to be determined if lowered steady-state mRNA results from inhibition of transcription, although it has been shown that the decrease in TNF-α mRNA after LPS challenge is not due to a decrease in mRNA half-life (39). It has been hypothesized that LPS stimulates the expression of transcriptional "silencers" during tolerance (9), leading to the decline of steady-state TNF-α mRNA levels, because during tolerance, the induction of nuclear transcription factors utilized in LPS-stimulated cytokine gene transcription, such as NF-κB, is not disrupted (9, 10).

Expression of the TNFR-2 and D2 gene products is correlated with the acquisition or maintenance of tolerance, since they are superinduced after LPS challenge in pretreated macrophages under conditions in which expression of all other genes tested was suppressed. That TNFR-2 induction is not inhibited during tolerance may be related to the finding that, in T cells, the two different types of TNF receptors serve different functions: TNFR-1 signals for initiation of cytotoxic effects, while TNFR-2 signals for stimulation of proliferation of thymocytes and cytotoxic T cells (37). Although mature end-stage macrophages proliferate little, and initially down-regulate the surface expression of TNFR-1 upon induction of tolerance (6), an increase in the expression of the TNFR-2 receptor type may allow the macrophage to respond in some autocrine fashion to TNF that serves a different function than in T cells. Alternately, if TNFR-2 is shed from the surface of the macrophage, or serves an intracellular function, it might act to mitigate a toxic response to TNF. By virtue of its ability to bind circulating or intracellular TNF, it may dampen toxic effects during tolerance. The functional identity of the protein product encoded by the D2 gene remains obscure. The correlation between superinduction of D2 and/or refractoriness to LPS in tolerant macrophages suggests that it may, like TNFR-2, contribute to mechanisms of tolerance induction or maintenance.

ACKNOWLEDGMENTS

We thank Ranney McNally for technical assistance during this study.

This research was supported by Public Health Service grant AI-18797, Uniformed Services University of the Health Sciences protocol RO7338 (to S.N.V), and Public Health Service grant CA39621 (to T.A.H).

REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NFκB transcription factor. Science **242**:540–546.

2. **Beatty, J. D., J. A. Rudbach, M. Smith, T. Ulrich, A. Mison, K. B. VonEschen, B. G. Beatty, and J. Esteban.** 1991. Monophosphoryl lipid A as prophylaxis for surgical wound infection: a phase I/II study. Newer Aspects of the Adjuvant Action of Lipid A and Its Analogs, Airlie, Va., 6 to 9 April 1991.
3. **Centers for Disease Control.** 1990. Increase in national hospital discharge survey rates for septicemia—United States 1979–1987. Morbid. Mortal. Weekly Rep. **39:**31–34.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
5. **Choubey, D., J. Snoddy, V. Chaturvedi, E. Toniato, G. Opdenakker, A. Thakur, H. Samanta, D. A. Engel, and P. Lengyel.** 1989. Interferons as gene activators. J. Biol. Chem. **264:**17182–17187.
6. **Ding, A., E. Sanchez, S. Srimal, and C. F. Nathan.** 1989. Macrophages rapidly internalize their tumor necrosis factor receptors in response to bacterial lipopolysaccharide. J. Biol. Chem. **264:**3924–3939.
7. **Freudenberg, M., D. Keppler, and C. Galanos.** 1987. Induction of tolerance to lipopolysaccharide (LPS)–D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. Infect. Immun. **56:**1352–1357.
8. **Gehr, G., R. Gentz, M. Brockhaus, H. Loetscher, and W. Lesslauer.** 1992. Both tumor necrosis factor receptor types mediate proliferative signals in human mononuclear cell activation. J. Immunol. **149:**911–917.
9. **Haas, J. G., P. A. Baeuerle, G. Riethmuller, and H. W. L. Ziegler-Heitbrock.** 1990. Molecular mechanisms in down-regulation of tumor necrosis factor expression. Proc. Natl. Acad. Sci. USA **87:**9563–9567.
10. **Haas, J. G., C. Thiel, K. Blomer, E. H. Weiss, G. Riethmuller, and H. W. L. Ziegler-Heitbrock.** 1989. Down regulation of tumor necrosis factor expression in the human Mono-Mac-6 cell line by lipopolysaccharide. J. Leukocyte Biol. **46:**11–14.
11. **Hamilton, T. A., N. Bredon, Y. Ohmori, and C. S. Tannenbaum.** 1989. IFN-γ and IFN-β independently stimulate the expression of lipopolysaccharide-inducible genes in murine peritoneal exudate macrophages. J. Immunol. **142:**2325–2331.
12. **Han, J., T. Brown, and B. Beutler.** 1990. Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. J. Exp. Med. **171:**465–475.

12a. **Henricson, B. E.** 1992. Analysis of the cellular and molecular mechanisms which underlie sensitivity to bacterial endotoxin and early endotoxin tolerance. Ph.D. dissertation. Uniformed Services University of the Health Sciences, Bethesda, Md.

13. **Henricson, B. E., W. R. Benjamin, and S. N. Vogel.** 1990. Differential cytokine induction by doses of lipopolysaccharide and monophosphoryl lipid A that result in equivalent early endotoxin tolerance. Infect. Immun. **58:**2429–2437.
14. **Henricson, B. E., R. Neta, and S. N. Vogel.** 1991. An interleukin-1 receptor antagonist blocks lipopolysaccharide-induced colony-stimulating factor production and early endotoxin tolerance. Infect. Immun. **59:**1188–1191.
15. **Henricson, B. E., P. Y. Perera, N. Qureshi, K. Takayama, and S. N. Vogel.** 1992. *Rhodopseudomonas sphaeroides* lipid A derivatives block in vitro induction of tumor necrosis factor and endotoxin tolerance by smooth lipopolysaccharide and monophosphoryl lipid A. Infect. Immun. **60:**4285–4290.
16. **Hiernaux, J. R., P. W. Stashak, J. L. Cantrell, J. A. Rudbach, and P. J. Baker.** 1989. Immunomodulatory activity of monophosphoryl lipid A in C3H/HeJ and C3H/HeSnJ mice. Infect. Immun. **57:**1483–1490.
17. **Ivy, R. E.** 1991. Prevention of septic shock using MPL for immune stimulation. New advances in the treatment of endotoxemia and sepsis, Philadelphia, Pa., 6 and 7 May 1991.
18. **Luster, A. D., J. D. Unkeless, and J. V. Ravetch.** 1985. γ-interferon transcriptionally regulates an early response gene containing homology to platelet proteins. Nature (London) **315:**672–675.
19. **Madonna, G. S., J. Peterson, E. E. Ribi, and S. N. Vogel.** 1986. Early-phase endotoxin tolerance: induction by a detoxified lipid A derivative, monophosphoryl lipid A. Infect. Immun. **52:**6–11.
20. **Madonna, G. S., and S. N. Vogel.** 1985. Early endotoxin tolerance is associated with alterations in bone marrow-derived macrophage precursor pools. J. Immunol. **135:**3763–3771.
21. **Madonna, G. S., and S. N. Vogel.** 1986. Induction of early-phase endotoxin tolerance in athymic (nude) mice, B-cell-deficient (*xid*) mice, and splenectomized mice. Infect. Immun. **53:**707–710.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Manthey, C. L., M. E. Brandes, P. Y. Perera, and S. N. Vogel.** 1992. Taxol increases steady-state levels of lipopolysaccharide-inducible genes and protein-tyrosine phosphorylation in murine macrophages. J. Immunol. **149:**2459–2465.
24. **Manthey, C. L., and S. N. Vogel.** 1992. The role of cytokines in host responses to endotoxin. Rev. Med. Microbiol. **3:**72–79.
25. **Mathison, J. C., E. Wolfson, and R. J. Ulevitch.** 1988. Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. J. Clin. Invest. **81:**1925–1937.
26. **McIntire, F. C., H. W. Sievert, G. H. Barlow, R. A. Finley, and W. Y. Lee.** 1967. Chemical, physical, and biological properties of a lipopolysaccharide from *Escherichia coli* K235. Biochemistry **6:**2363–2372.
27. **Narumi, S., and T. A. Hamilton.** 1991. Inducible expression of murine IP-10 mRNA varies with the state of macrophage inflammatory activity. J. Immunol. **146:**3038–3044.
28. **Ohmori, Y., and T. A. Hamilton.** 1990. A macrophage LPS-induced early gene encodes the murine homologue of IP-10. Biochem. Biophys. Res. Commun. **168:**1261–1267.
29. **Ohmori, Y., G. Strassman, and T. A. Hamilton.** 1990. cAMP differentially regulates expression of mRNA encoding IL-1α and IL-1β in murine peritoneal exudate macrophages. J Immunol. **145:**3333–3339.
30. **Pennica, D., J. S. Hayflick, T. S. Bringman, M. A. Palladino, and D. V. Goeddel.** 1985. Cloning and expression in *Escherichia coli* of the cDNA for murine tumor necrosis factor. Proc. Natl. Acad. Sci. USA **82:**6060–6064.
31. **Ribi, E. E.** 1984. Beneficial modification of the endotoxin molecule. J. Biol. Response Modif. **3:**1–9.
32. **Salkowski, C. A., and S. N. Vogel.** 1992. IFN-γ mediates increased glucocorticoid receptor expression in murine macrophages. J. Immunol. **148:**2770–2777.
33. **Tannenbaum, C. S., and T. A. Hamilton.** 1989. Lipopolysaccharide-induced gene expression in murine peritoneal macrophages is selectively suppressed by agents that elevate intracellular cAMP. J. Immunol. **142:**1274–1280.
34. **Tannenbaum, C. S., T. J. Koerner, M. M. Jansen, and T. A. Hamilton.** 1988. Characterization of lipopolysaccharide-induced macrophage gene expression. J. Immunol. **140:**3640–3645.
35. **Tannenbaum, C. S., J. Major, and T. A. Hamilton.** Unpublished data.
36. **Tannenbaum, C. S., J. Major, E. Poptic, P. E. DiCorleto, and T. A. Hamilton.** 1989. Lipopolysaccharide-inducible macrophage early genes are induced in Balb/c 3T3 cells by platelet-derived growth factor. J. Biol. Chem. **264:**4052–4057.
37. **Tartaglia, L. A., R. F. Weber, I. S. Figari, C. Reynolds, M. A. Palladino, and D. V. Goeddel.** 1991. The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc. Natl. Acad. Sci. USA **88:**9292–9296.
38. **Tokunaga, K., H. Taniguchi, K. Shimizu, and S. Sakiyama.** 1986. Nucleotide sequence of a full-length cDNA for mouse cytoskeletal β-actin mRNA. Nucleic Acids Res. **14:**2829–2832.
39. **Virca, G. D., S. Y. Kim, K. B. Glaser, and R. J. Ulevitch.** 1989. Lipopolysaccharide induces hyporesponsiveness to its own action in RAW 264.7 cells. J. Biol. Chem. **264:**21951–21956.
40. **Vogel, S. N., and E. A. Havell.** 1990. Differential inhibition of lipopolysaccharide-induced phenomena by anti-tumor necrosis factor alpha antibody. Infect. Immun. **58:**2397–2400.
41. **Vogel, S. N., and M. M. Hogan.** 1990. The role of cytokines in endotoxin-mediated responses, p. 238–258. *In* J. J. Oppenheim and E. Shevach (ed.), Immunophysiology: role of cells and

cytokines. Immunity and inflammation. Oxford University Press, New York.
42. **Vogel, S. N., and M. M. Hogan.** 1991. Measurement of TNF α/β, p. 6.10.1–6.10.5. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology. Green Publishing and Wiley-Interscience, New York.
43. **Vogel, S. N., E. Kaufman, M. Tate, and R. Neta.** 1988. Recombinant interleukin-1α and recombinant tumor necrosis factor alpha synergize in vivo to induce early endotoxin tolerance and associated hematopoetic changes. Infect. Immun. **56:**2650–2657.
44. **Zuckerman, S. H., G. F. Evans, and L. D. Butler.** 1991. Endotoxin tolerance: independent regulation of interleukin-1 and tumor necrosis factor expression. Infect. Immun. **59:**2774–2780.
45. **Zuckerman, S. H., G. F. Evans, Y. M. Snyder, and W. D. Roeder.** 1989. Endotoxin-macrophage interaction: post-translational regulation of tumor necrosis factor expression. J. Immunol. **143:**1223–1277.