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# Cytokine Response by Monocytes and Macrophages to Free and Lipoprotein-Bound Lipopolysaccharide

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**Recent evidence suggests that bacterial lipopolysaccharide binds to serum lipoproteins in vitro and in vivo and that lipopolysaccharide in the form that is bound to lipoprotein is less biologically active in several experimental models. In order to study the mechanism of this apparent detoxification, we compared the ability of free and lipoprotein-bound lipopolysaccharide from *Escherichia coli* O18 to stimulate interleukin-1, interleukin-6, and tumor necrosis factor from elicited murine peritoneal macrophages and circulating human monocytes. Lipopolysaccharide bound to lipoprotein was 20- to 1,000-fold less active than the unbound form in inducing the release of each cytokine. We also studied the binding of each form of lipopolysaccharide to the macrophage surface. Lipopolysaccharide complexed to lipoprotein was unable to compete for the binding of radiolabeled heterologous lipopolysaccharide to murine macrophages, and radiolabeled lipopolysaccharide-lipoprotein complexes bound poorly compared with molar equivalents of free lipopolysaccharide. Our experiments suggest that in the process of binding to lipoproteins, lipopolysaccharide may be rendered less toxic through a mechanism of decreased ability to induce monocytes and macrophages to release cytokines, perhaps because of an altered interaction at the cell surface.**

The role that bacterial endotoxin (lipopolysaccharide [LPS]) plays in the pathogenesis of severe gram-negative infections has been studied for over 50 years. The majority of these investigations have utilized LPS that has been chemically extracted and then suspended in aqueous buffers. Over the last decade, however, it has been shown that LPS binds to lipoproteins (LP) in serum and plasma (15, 16, 28, 29) and that the resulting LPS-LP complex is much less active than unbound LPS in numerous assays of biological activity, including LPS-induced fever (16, 28), neutropenia (28), thromobocytopenia (29), complement activation (28), rate of development of hypotension (12), and death in adrenalectomized mice (28). Other studies have shown that the formation of LPS-LP complexes also occurs in vivo (16) and that LPS contained in membrane fragments binds to LP in a similar manner (16).

There is considerable evidence that the binding of LPS to LP is altered in inflammatory serum. Tobias et al. (22–25) have reported that the binding of LPS from rough mutant *Salmonella minnesota* Re 595 to LP is slower in inflammatory serum than in control serum. The delay is due to the formation of an intermediate complex of the LPS with a glycoprotein with a molecular weight of 60,000 called LPS-binding protein. Moreau and Skarnes, however, suggested in 1973 that LPS derived from smooth organisms incubated in tolerant rabbit serum underwent rapid transformation into a less toxic complex that contained LP (13). We have confirmed that two other LPS derived from smooth organisms, *Escherichia coli* O113 and *Salmonella typhimurium*, bind much more rapidly to LP in sera prepared from rabbits made tolerant to LPS (32) or in serum prepared from blood drawn after a single injection of interleukin-1 (IL-1) (33) than in normal serum. These experiments suggest that there are inducible humoral mechanisms that may function to decrease the bioactivity of smooth LPS by modulating LPS-LP binding (13, 18, 32, 33).

Although two studies have indicated that the structure of LPS within the LPS-LP complex remains intact (15, 28), there is little known about the mechanism(s) of the relative decrease in biological activity of the bound form. We previously reported that LPS-LP is less potent than unbound LPS in stimulating rabbit peritoneal macrophages to produce IL-1 (33). These results raised several questions concerning the interaction of LPS with macrophages. First, it was unclear whether the decrease in IL-1 secretion represented a decrease in IL-1 production or normal IL-1 production with a decrease in IL-1 release, as has been recently described for LPS contained in liposomes (1). Second, it was unclear whether the secretion of both IL-1α and IL-1β is decreased and whether the release of other cytokines, such as tumor necrosis factor (TNF) and IL-6, is similarly decreased. Third, in our previous report we utilized only elicited peritoneal macrophages from rabbits, and it was not known whether macrophages from different species and different sources would behave in the same manner. Since injected LPS that is converted to the LPS-LP form is cleared from the bloodstream relatively slowly, with a half-life of 12 h (12), a relevant question was whether the LPS-LP could stimulate circulating monocytes. A final question was whether the LPS that is bound to LP is less able to bind to the macrophage surface.

In order to address these questions and in light of recent reports suggesting that cytokines such as IL-1, TNF, and IL-6 may play a direct role in the pathogenesis of endotoxic shock (2, 3, 26, 31), we studied the interaction of the two forms of LPS with murine peritoneal macrophages and human circulating monocytes. We initially studied the release of IL-1, TNF, and IL-6 in response to LPS and purified LPS-LP. These findings confirmed that LPS-LP was less able to stimulate release of each cytokine and that the

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decrease in IL-1 resulted from a decrease in production rather than a block in secretion. We then employed a competitive-binding assay with radiolabeled LPS from *Neisseria meningitidis* to estimate the relative binding of each form of LPS to the macrophage surface (6). We found that LPS-LP cannot compete with *N. meningitidis* LPS for binding to macrophages, suggesting that its decreased activity may result from an altered interaction with the macrophage surface. Direct-binding experiments confirmed this finding. Our results are complementary to a study published after we had initiated our experiments suggesting that the release of IL-1, TNF, and IL-6 from human monocytes by LPS is diminished by prior incubation of the LPS with human serum and that this inhibition is due to LP (8).

## MATERIALS AND METHODS

**Preparation of LPS.** A culture of *E. coli* O18K− was the kind gift of H. Williams Smith (Houghton Poultry Station). Biosynthetically radiolabeled *E. coli* O18 LPS was prepared by growing the organism in the presence of [$^3$H]acetate followed by hot-phenol extraction according to a modification of the procedure of Rudbach et al. (20) as previously described (32). Briefly, we grew cultures of *E. coli* O18 to an optical density of 0.9 at 540 nm in tryptic soy broth in the presence of 10 mCi of [$^3$H]acetate. The cells were chilled and washed three times in saline, and then the LPS was twice extracted at 65°C by using the hot-phenol method. The resulting preparation was exhaustively dialyzed against water and then treated with DNase, RNase, and protease as described by Romeo et al. (19). The concentration of LPS was estimated by a spectrophotometric *Limulus* lysate gelation assay utilizing an *E. coli* O113 LPS standard containing 10 endotoxin units/ng (lot 20; Associates of Cape Cod, Falmouth, Mass.) (17). A solution adjusted to 1 μg of LPS biological activity per ml contained 6,150 cpm/μg when a 0.4-ml volume was combined with 4 ml of Optifluor scintillation fluid (Packard, Downers Grove, Ill.). In prior experiments using this technique for the intrinsic labeling of LPS, greater than 99% of the radiolabeled LPS was demonstrated to remain in the water phase after a 1:1 ether-water extraction at pH 5, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography resulted in a regularly spaced band pattern typical of LPS. Unlabeled LPS from *E. coli* O18 was prepared in an identical manner from bacterial cells that were grown in the absence of [$^3$H]acetate.

LPS-LP was produced essentially as described by Ulevitch and Johnston (28). Radiolabeled *E. coli* O18 LPS was diluted 1:39 in unlabeled *E. coli* O18 LPS and was then incubated at concentrations of 60 or 250 μg/ml in 4 ml of normal rabbit serum made 20 mM with EDTA for 3 h at 37°C. Sufficient KBr and water was then added to adjust the volume to 10 ml and the density to 1.21 g/ml, and this solution was centrifuged in two 5-ml ultracentrifuge tubes for 48 h at 225,000 × *g*. The top one-third of each tube was then collected. Under these conditions, most of the LPS is found within the lower-density LP fractions at the top of the tube (32). Control unbound LPS was made by incubating and centrifuging identical quantities of radiolabeled LPS in saline made 20 mM with EDTA, followed by collection of the bottom one-third of the tube. Each fraction was then dialyzed exhaustively against pyrogen-free saline and adjusted on the basis of counts per minute to the concentrations indicated. A similar procedure was utilized to prepare a small amount of undiluted [$^3$H]LPS-LP from $^3$H-labeled *N. meningitidis* which was radiolabeled as described below. Control LP not complexed to LPS were made by using the same techniques but adding pyrogen-free water in place of LPS. Preparation of LPS, LPS-LP, and LP were done at the same time in parallel.

Extrinsically radiolabeled LPS from *N. meningitidis* was the kind gift of Martine Caroff (UA Centre National de la Recherche Scientifique 1116, Orsay, France). We used this LPS for competition and direct-binding experiments as previously described in order to achieve the high specific activity necessary to measure binding (10). This LPS had been extrinsically radiolabeled by a modification of the procedure of Watson and Riblet (34). Briefly, 2 mg of the endotoxin was suspended in 500 μl of $NaIO_4$ solution ($2 \times 10^{-2}$ M), and the mixture was kept for 20 min at room temperature. Then 5 μl of ethylene glycol (5 M) was added. After 15 min at room temperature, the suspension was centrifuged at 290,000 × *g*. The pellet was suspended in 100 μl of ice-cold water and treated for 1 h at 4°C with $NaB[^3H]_4$ (2.7 mCi; specific activity, 1 Ci/mmol) in 400 μl of borate buffer (0.05 M; pH 9); after addition of 100 μl of a solution of $NaBH_4$ in water (5 mg/ml), the mixture was kept overnight at 4°C. Five hundred micrograms of $NaBH_4$ was added. After 1 h at 4°C, excess sodium borohydride was destroyed with 5 μl of acetic acid. After centrifugation for 45 min at 290,000 × *g*, the pellet was suspended in water and lyophilized. The specific activity of the endotoxin preparation was 75,000 cpm/μg. A recent observation indicated that the radiolabeled *N. meningitidis* LPS was a mixture of two forms with different ratios of binding to cell membranes. *N. meningitidis* [$^3$H]LPS with a high binding efficiency was obtained following further separation of both forms in an isobutyric acid-ammonia mixture (unpublished data). Either this isolated form or the initial preparation was used in the binding experiments.

**Preparation of human monocytes.** Peripheral blood mononuclear cells were obtained by centrifugation on Ficoll (MSL; Eurobio, Paris, France) of 1:2 diluted heparinized venous blood from healthy adult volunteers. Monocytes were selected by allowing the mononuclear cells to adhere to plastic culture dishes (24 wells; Nunc, Roskilde, Denmark) in the absence of serum (11). In these conditions, more than 85% of the adherent cells were monocytes, as assessed by morphological analysis by phase-contrast microscopy, histochemical staining for nonspecific esterase activity (27), and indirect immunofluorescence staining using anti-lymphocyte antibodies, OKT11 (Ortho Diagnostics, Inc., Raritan, N.J.) and IOBI (Immunotech, Marseille, France).

**Mouse peritoneal macrophages.** BALB/c mice (Institut Pasteur, Paris, France) of either sex, 2 to 3 months old, were injected intraperitoneally with 1.5 ml of thioglycolate medium (Diagnostic Pasteur, Paris, France); 5 days later, the peritoneal cavities were washed twice with 2 ml of RPMI 1640 medium containing 2 IU of heparin per ml. Macrophages were purified from peritoneal exudate cells by surface adherence as previously described (5). As judged by nonspecific esterase staining, more than 85% of the adherent cells were macrophages.

**IL-1 induction and IL-1 assay.** Human mononuclear adherent cells ($5 \times 10^5$ nonspecific esterase-positive cells per well) cultured in RPMI 1640 medium (GIBCO) without serum, supplemented with 100 IU of penicillin per ml and 100 μg of streptomycin per ml were incubated for 24 h in the presence or absence of IL-1 inducers. The culture supernatants were collected and centrifuged at 3,000 × *g* for 15 min. IL-1 activity found in the supernatant will be referred to as

extracellular or released IL-1. The adherent cells were lysed by three freeze-thaw cycles in 0.5 ml of fresh RPMI 1640 medium. The lysates were centrifuged at 3,000 × *g* for 15 min; the IL-1 activity found in the supernatants will be referred to as intracellular IL-1 activity. IL-1 activity was determined by [$^3$H]thymidine uptake by C3H/HeJ mouse thymocytes in the presence of a suboptimal dose of concanavalin A (0.075 μg per well) as described previously (11). The standard deviation of the results from triplicate thymocyte cultures did not exceed 18%. Results are presented as [$^3$H]thymidine incorporation. IL-1α and IL-1β were quantified by using a radioimmunoassay and enzyme-linked immunosorbent assays (Amersham, les Ulis, France, and Cistron Biotechnology, Pine Brook, N.J., respectively).

**TNF assay.** TNF activity was estimated as follows: $3 \times 10^4$ L929 fibroblasts in 0.1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum were cultured overnight in 96-well flat-bottomed Falcon microtiter plates (Becton Dickinson Labware, Oxnard, Calif.). The next day, serial dilutions of test supernatant were added in the presence of actinomycin D at a final concentration of 2 μg/ml. Plates were incubated at 37°C for 18 h; then cells were washed and stained with crystal violet (50 μl; 0.1% in 20% methanol—$H_2O$) for 20 min at room temperature. The microtiter plates were rinsed gently in saline, cells were solubilized in 1% sodium dodecyl sulfate (100 μl), and dye uptake was calculated at $A_{540}$ by using an automated micro-ELISA autoreader (Titertek Multiscan; Flow Laboratories, Inc., McLean, Va.). One unit of TNF activity was defined as the amount required to lyse 50% of L929 target cells. The assay was standardized with a human recombinant TNF-α preparation containing $2.3 \times 10^6$ U/mg of protein, kindly provided by Rhone-Poulenc (Vitry-sur-Seine, France). Titrations were performed in triplicate, and the standard deviations of the means did not exceed 20%. Activity of the assay could be inhibited by a monoclonal anti-TNF-α antibody.

**IL-6 assay.** IL-6 activity was determined by using the factor-dependent 7TD1 mouse-mouse hybridoma, which was the kind gift of J. Van Snick (Brussels, Belgium). IL-6 activity was assessed as described by Van Snick et al. (30) with minor modifications. Briefly, cells (2,000 per well; 96-well microtiter plates) were grown in 200 μl of RPMI 1640 medium supplemented with antibiotics, 2-mercaptoethanol ($5 \times 10^{-5}$ M), and 10% fetal calf serum, in the presence of serial dilutions of supernatants from stimulated macrophages. The potency of the samples was monitored by [$^3$H]thymidine uptake after 4 days of culture. [$^3$H]thymidine (0.25 μCi per well) was added 18 h before the end of the culture. One unit of IL-6 corresponds to half-maximal growth of the hybridoma cells. Recombinant human IL-6 (Genzyme, Boston, Mass.) was used as a positive control. Titrations were performed in duplicate, and the standard deviations of the means did not exceed 20%.

**Direct- and competition binding assays.** Assays were performed as previously described (10). Briefly, plated monocytes or peritoneal macrophages ($8 \times 10^5$ cells per well) were incubated in each experiment with [$^3$H]LPS from *N. meningitidis* alone and 10 μl of normal human serum (final concentration, 4%). The residual binding measured in the presence of a large excess of nonradioactive endotoxin was taken to represent nonspecific binding; specific binding of LPS was defined as the difference between total and nonspecific binding. Since the binding of [$^3$H]LPS increases as a function of time, reaching a maximum after 45 min at 22°C (data not shown), all experiments were performed at 22°C with an incubation period of 45 to 60 min. Direct-binding assays compared binding of [$^3$H]LPS and [$^3$H]LPS-LP. For competition experiments, the cells were incubated either with 0.2 μg of [$^3$H]LPS from *N. meningitidis* alone or with [$^3$H]LPS plus a 10-fold concentration of *N. meningitidis* LPS or *E. coli* O18 LPS or LPS-LP. After washing to remove the unbound material, the [$^3$H]LPS bound to the membrane was solubilized in a sodium dodecyl sulfate-EDTA mixture (1% sodium dodecyl sulfate, 20 mM EDTA). The solubilized radioactive material was then mixed with Instagel (Packard) and measured by liquid scintillation counting. Each experiment was performed in triplicate, and each set of experiments was performed at least three times.

**Mitogenicity.** Stimulation of BALB/c or C3H/HePas mouse splenocytes was carried out as described previously (4). Briefly, $5 \times 10^5$ cells per well (96-well microplates; Nunc) were cultured in RPMI 1640 medium (supplemented with antibiotics and 2% fetal calf serum) in the presence of varied concentrations of *E. coli* O18 LPS or LPS-LP for 48 h at 37°C in an atmosphere of 7% CO–93% air. Seven hours before harvesting, 0.25 μCi of [$^3$H]thymidine was added per well. Thereafter, the cells were harvested on glass-fiber filters with a cell harvester (Skatron). Thymidine incorporation was determined by liquid scintillation counting.

## RESULTS

**Induction of IL-1 by LPS and LPS-LP.** IL-1 activity was found in the cell supernatants, on the cell membranes, and in the cell lysates of elicited murine peritoneal macrophages stimulated with both LPS and LPS-LP. However, LPS was 20- to 100-fold less active in the LPS-LP form than in the unbound form (Fig. 1). Similar results were obtained with resident peritoneal macrophages (data not shown). When human circulating monocytes were studied, there was no IL-1 production or release induced by LPS-LP from human circulating monocytes even at the highest concentration studied (2 μg/ml), indicating a decrease in activity of at least 1,000-fold compared with that with unbound LPS (Fig. 2). Measurement of IL-1α and IL-1β in these cell supernatants and lysates by radioimmunoassay was consistent with the results of the biological assays for each form of IL-1, although the magnitude of the difference was somewhat less (Table 1). In both the human and mouse systems, the addition of free LP and LPS-LP to the LPS-macrophage incubations did not diminish the production or release of IL-1, confirming that serum components are necessary for the formation of LPS-LP (15) and indicating that LP do not directly or indirectly inhibit the stimulation of IL-1 by LPS (data not shown).

**Induction of TNF by LPS and LPS-LP.** The TNF activity in cell supernatants of elicited murine peritoneal macrophages stimulated with LPS-LP was markedly reduced in comparison with that with unbound LPS (Table 2; experiment performed twice). When human circulating monocytes were studied, free LPS induced TNF at concentrations of 2.0 to 200 ng of LPS/ml, whereas there was no TNF detected when the cells were stimulated with LPS-LP (Table 3; experiment repeated five times).

**Induction of IL-6 by LPS and LPS-LP.** LPS-LP induced markedly less IL-6 activity compared with LPS alone from murine peritoneal macrophages (Table 4) and circulating human monocytes (Table 5; experiment repeated three times).

**LPS-macrophage binding.** As described in Materials and Methods, we utilized a competitive-binding assay (10) to evaluate the interaction of LPS and LPS-LP with the murine

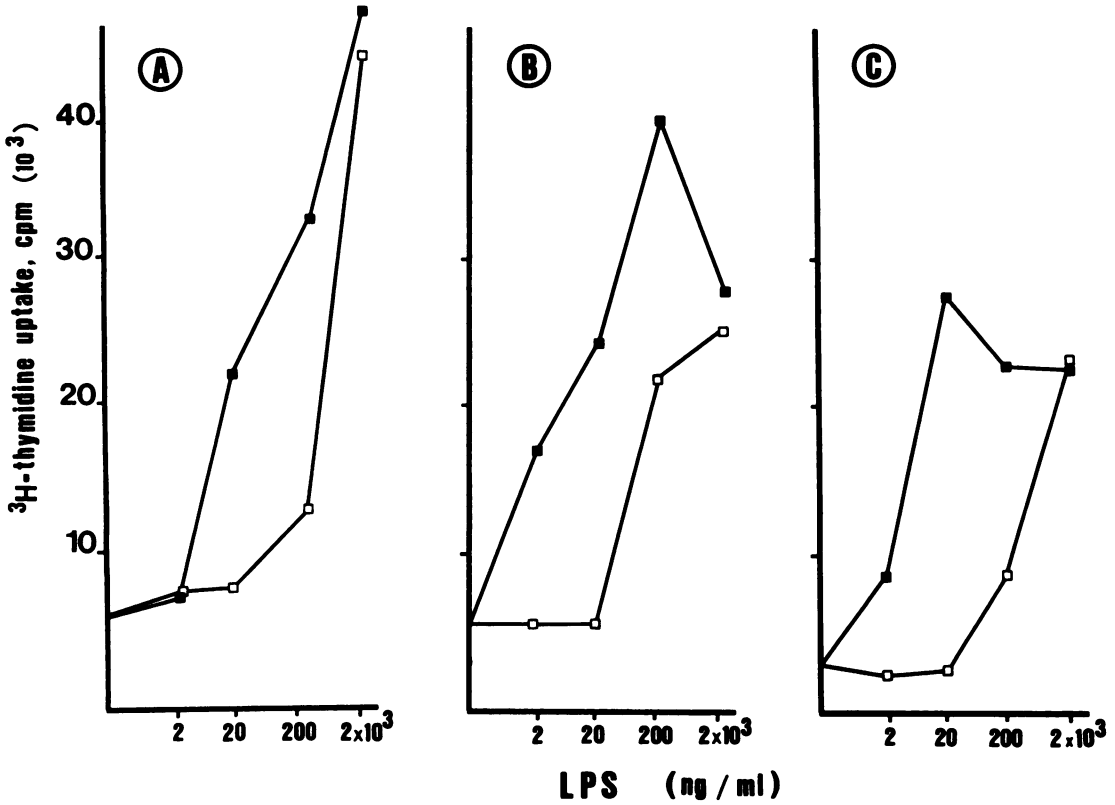


FIG. 1. IL-1 activity in cell supernatants (diluted 1:10) (A), in cell lysates (1:10) (B), or associated with the cell membrane (C) of elicited peritoneal macrophages from BALB/c mice triggered by increasing amounts of free *E. coli* O18 LPS (■) and *E. coli* O18 LPS-LP (□). Results are expressed as [$^3$H]thymidine uptake (counts per minute) by mouse C3H/HeJ thymocytes in the presence of suboptimal doses of concanavalin A and cell supernatants or cell lysates (1:10). Standard deviation did not exceed 18%. This experiment was performed three times with similar results.

macrophage membrane. A 10-fold excess of LPS (unlabeled *N. meningitidis*, *E. coli* O18, and *E. coli* O18 in the LPS-LP form) were incubated with the macrophages, followed by the addition of extrinsically radiolabeled LPS from *N. meningitidis*. The cells were then washed and evaluated for bound counts per minute. Homologous (*N. meningitidis*) and heterologous (*E. coli* O18) LPSs were able to compete for the binding of radiolabeled *N. meningitidis* LPS. In contrast, LPS-LP did not decrease this binding (Table 6). We also directly compared the binding of [$^3$H]LPS and [$^3$H]LPS-LP

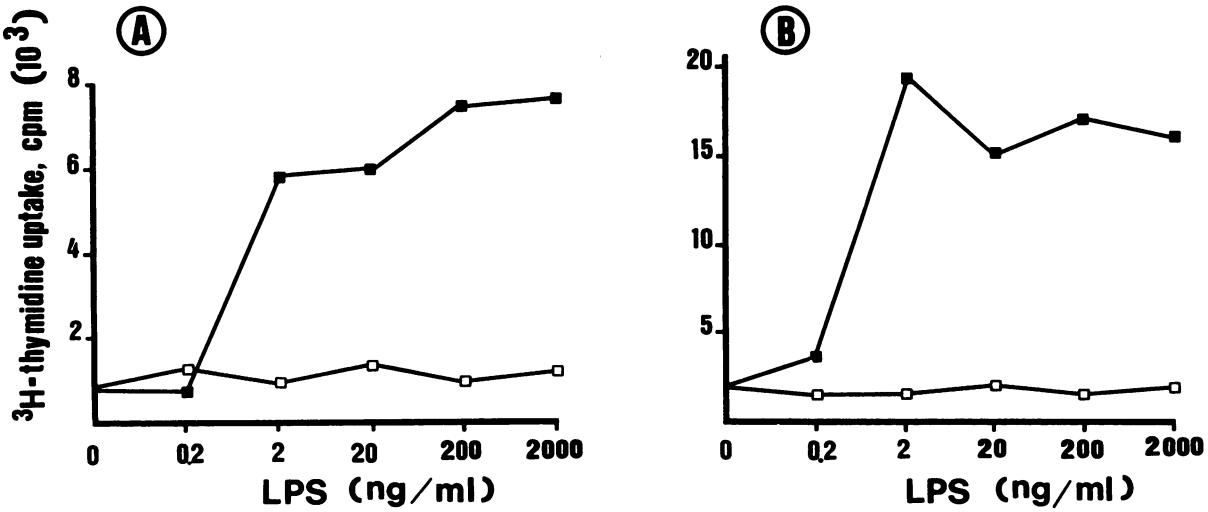


FIG. 2. IL-1 activity in cell supernatants (A) and in cell lysates (B) of human monocytes triggered by increasing amounts of free *E. coli* O18 LPS (■) and *E. coli* O18 LPS-LP (□). Standard deviations did not exceed 18%. This experiment was performed seven times with similar results.

TABLE 1. IL-1α and IL-1β produced by human monocytes triggered by LPS and LPS-LP

| Inducer and concn (ng/ml) | IL-1 concn (pg/ml) | | | |
|---|---|---|---|---|
| | IL-1β | | IL-1α | |
| | Extracellular | Cell-associated | Extracellular | Cell-associated |
| None | 40 | 80 | <170 | 187 |
| LPS | | | | |
| 2 | 2,160 | 2,466 | 408 | 3,966 |
| 20 | 3,373 | 3,575 | 374 | 4,930 |
| LPS-LP | | | | |
| 2 | 150 | 140 | <170 | 374 |
| 20 | 120 | 160 | 204 | 221 |

TABLE 3. TNF activity released by human monocytes

| Inducer and concn (ng/ml) | TNF activity (U) | |
|---|---|---|
| | Donor 1 | Donor 2 |
| None | <1 | 1 |
| LPS | | |
| 2 | ND[a] | 135 |
| 20 | 160 | 125 |
| 200 | 220 | 55 |
| LPS-LP | | |
| 2 | ND | 1.3 |
| 20 | <1 | <1 |
| 200 | <1 | <1 |

[a] ND, Not determined.

from *N. meningitidis* to murine peritoneal macrophages. These experiments confirmed that LPS in the LPS-LP form binds poorly to the macrophage membrane.

**Mitogenic assay.** LPS-LP was slightly less mitogenic than unbound LPS for splenocytes from C3H/HePas mice (Fig. 3A) and had essentially the same mitogenicity for splenocytes from BALB/c mice (Fig. 3B). This experiment was repeated a total of three times with similar results.

## DISCUSSION

Recent studies suggest that cytokines are important in the pathogenesis of endotoxic shock. Although the induction of IL-1, IL-6, and TNF by LPS is well known, the stimulation of these cytokines by LPS-LP has not been directly and systematically addressed. Investigation of the interaction of the LP-bound forms of LPS with macrophages seemed warranted because LPS-LP complexes may be the predominant form which circulates in the bloodstream (12) and because of recent studies which suggest that the binding of LPS to LP is modulated in inflammatory sera (22–25, 32, 33). In addition, a study published after we had initiated our experiments suggested that the release of these cytokines by monocytes is inhibited by LP in human serum (8).

We recently reported that LPS-LP is less able to induce IL-1 activity from peritoneal macrophages compared with free LPS (33). Our present studies extend this finding in several ways. First, LPS-LP was much less active than unbound LPS in stimulating the release of IL-6 and TNF from macrophages. Such a finding was not unexpected for TNF because LPS-LP is apyrogenic even in microgram quantities (16, 28), whereas TNF is a potent pyrogen (7). Second, our previous studies utilized only lapine peritoneal macrophages that were elicited with thioglycolate. Our present data indicate that LPS-LP is similarly less active in stimulating resident and elicited murine macrophages and circulating human monocytes. Third, our data indicate that both IL-1α and IL-1β are diminished in monocyte supernatants stimulated with LPS-LP compared with unbound LPS and that the decrease in extracellular IL-1 from murine macrophages reflects a decrease in production rather than a block in its release. Fourth, we found that *E. coli* O18 LPS-LP was unable to compete for the binding of a heterologous extrinsically radiolabeled LPS to the macrophage surface, whereas molar equivalents of the unbound form of *E. coli* O18 LPS did compete for binding. Direct-binding experiments utilizing LPS and LPS-LP from *N. meningitidis* confirmed that LPS-LP bound poorly to the cell membrane. Finally, in an initial attempt to compare the activation of macrophages with the activation of splenocytes, we found that LPS-LP was only slightly less mitogenic than unbound LPS.

These findings are consistent with the hypothesis that at least one mechanism by which LPS-LP is less toxic in vivo is that it is less able to induce the release of IL-1, IL-6, and TNF. Our findings are complementary to those of Flegel et al., who reported that LPS preincubated in 20% normal human serum was less active in stimulating human monocytes to produce IL-1, IL-6, and TNF compared with

TABLE 2. TNF activity released by mouse peritoneal macrophages

| Inducer and concn (ng/ml) | TNF activity (U) | |
|---|---|---|
| | Expt 1 | Expt 2 |
| None | <5 | <5 |
| LPS | | |
| 2 | <30 | 90 |
| 20 | <30 | 700 |
| 200 | 920 | 2,000 |
| 2,000 | 9,500 | 480 |
| LPS-LP | | |
| 2 | <5 | <5 |
| 20 | <5 | <5 |
| 200 | 84 | <5 |
| 2,000 | 175 | <5 |

TABLE 4. IL-6 activity released by mouse peritoneal macrophages

| Inducer and concn (ng/ml) | IL-6 activity (U/ml) |
|---|---|
| None | 18 |
| LPS | |
| 20 | 1,700 |
| 200 | 1,200 |
| LPS-LP | |
| 20 | 55 |
| 200 | 120 |

TABLE 5. IL-6 released by human monocytes

| Inducer and concn (ng/ml) | IL-6 activity (U/ml) | | |
|---|---|---|---|
| | Donor 1 | Donor 2 | Donor 3 |
| None | <30 | 90 | 83 |
| LPS | | | |
| 2 | ND[a] | ND | 1,750 |
| 20 | 5,400 | ND | 2,200 |
| 200 | 4,650 | 4,500 | 2,000 |
| 2,000 | 2,600 | 1,700 | 2,200 |
| LPS-LP | | | |
| 2 | ND | ND | 68 |
| 20 | 55 | ND | 90 |
| 200 | 30 | 150 | 340 |
| 2,000 | 70 | 560 | 1,300 |

[a] ND, Not determined.

TABLE 6. Inability of LPS-LP to compete for binding or to bind directly to mouse peritoneal macrophages[a]

| Unlabeled material (2 μg) | $^3$H-labeled *N. meningitidis* compound (0.2 μg) | cpm bound | Δcpm[b] |
|---|---|---|---|
| Expt 1 | | | |
| None | LPS | 2,715 ± 978 | |
| *N. meningitidis* LPS | LPS | 630 ± 252 | 2,085 |
| *E. coli* O18 LPS | LPS | 1,584 ± 617 | 1,131 |
| *E. coli* O18 LPS-LP | LPS | 2,750 ± 277 | 0 |
| Expt 2 | | | |
| None | LPS | 5,329 ± 446 | |
| *N. meningitidis* LPS | LPS | 218 ± 15 | 5,111 |
| None | LPS-LP | 92 ± 30 | |
| *N. meningitidis* LPS | LPS-LP | 97 ± 21 | 0 |

[a] Experiments 1 and 2 are representative of six and three different results, respectively. cpm, Counts per minute.
[b] Relative to value for the same experiment with no unlabeled material.

controls (8). The decrease in cytokine release was not seen in delipidated serum but was found in delipidated serum reconstituted with LP prepared by ultracentrifugation. Although the degree of LPS-LP binding was not measured in this study, it seems likely that part or all of the inhibition may have been due to the formation of LPS-LP.

Of interest is a recent study in which macrophages incubated with LPS incorporated into liposomes were shown to release much less IL-1 than free LPS (1). Importantly, the intracellular and membrane-associated IL-1 were not decreased, suggesting that the production of IL-1 was normal but that the release of IL-1 did not occur (1). This was not the case with LPS-LP, in which both production and release were decreased. Our findings parallel our previous findings that gangliosides inhibit the production and release of IL-1 by macrophages (6), presumably by forming LPS-ganglioside complexes (14), which are then less able to stimulate the macrophage at the cell surface.

We hypothesize that LPS-LP is less able to stimulate the macrophage membranes because the LPS component of the complex is less able to interact with the macrophage surface. Such a notion is supported by our binding studies, although we cannot be certain that these two events are related. We have previously shown for different sorts of LPS-ganglioside complexes that the competition for LPS binding and IL-1 production and release are correlated (6). Our studies are consistent with those of Freudenberg and Galanos (9), who reported that there was less LPS internalized into murine macrophages after 24 h of cultivation if the LPS was bound to high-density LP.

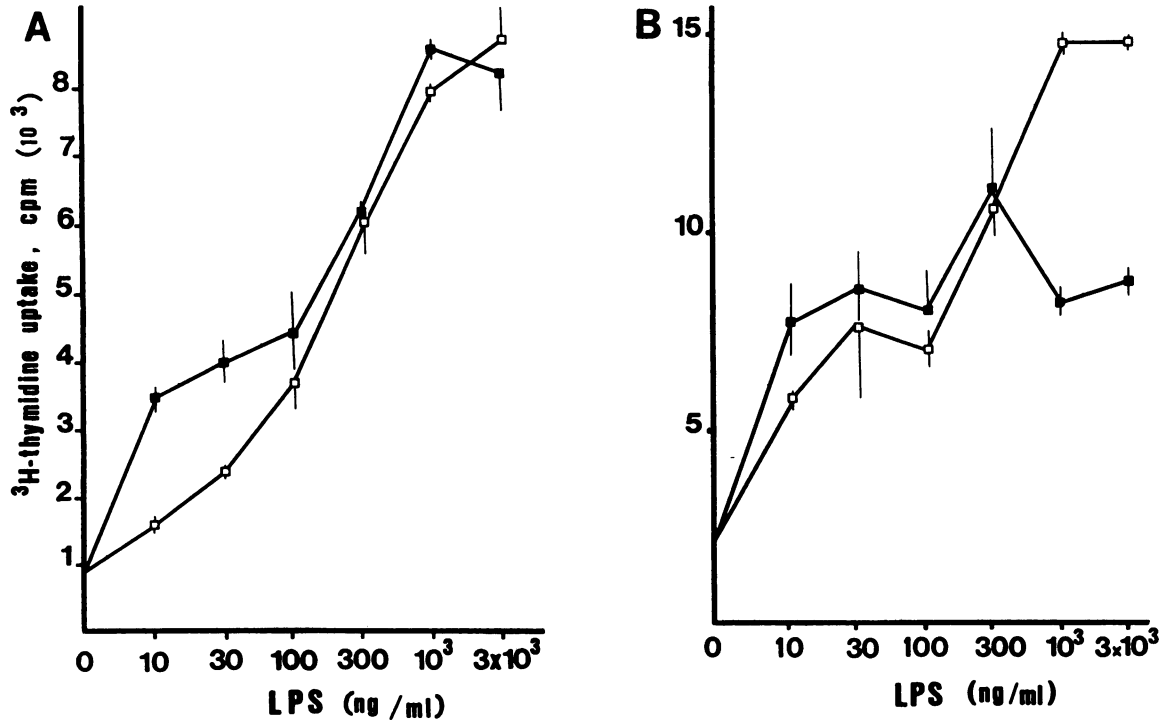


FIG. 3. Mitogenicity of free *E. coli* O18 LPS (■) and *E. coli* O18 LPS-LP (□) on splenocytes from C3H/HePas mice (A) and BALB/c mice (B). This experiment was performed four times with similar results.

Since the physicochemical forms of LPS are a critical determinant of LPS-induced cytokine release, caution needs to be taken in strictly extrapolating our findings to gram-negative infections. In this study, we utilized intrinsically radiolabeled, chemically extracted LPS from *E. coli* O18 and extrinsically radiolabeled chemically extracted LPS from *N. meningitidis* for the binding assays, a procedure which was necessary in order to have a high enough specific activity in order to measure LPS-cell binding. Chemically extracted LPS does not occur naturally and indeed Tesh and Morrison have recently shown that radiolabeled LPS from *E. coli* O111:B4 released from the bacterial surface in the presence of serum has physicochemical and biological properties different from those of chemically extracted LPS (21). On the other hand, Munford et al. reported that LPS released as membrane fragments into culture supernatants interact with LP in a manner similar to that of chemically extracted LPS, providing some reassurance that our findings are not artifactual (16).

Our observation that LPS-LP was only slightly less mitogenic than LPS is curious when contrasted with the marked difference in the two preparations in stimulating macrophages. It is possible that this finding represents the longer incubation time of each preparation with the cells (48 versus 24 h). It may be, however, that the interaction of LPS with lymphocyte membranes involves mechanisms different from those of macrophages. A similar discrepancy between mitogenicity and IL-1-inducing activity has been observed with lipid A (4, 5) and with LPS complexed to gangliosides (unpublished observation). Further work will be needed to address this issue.

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