Inhibition of Endotoxin-Induced Interleukin-6 Production by Synthetic Lipid A Partial Structures in Human Peripheral Blood Mononuclear Cells

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The effect of two synthetic lipid A partial structures, compound 406 (or LA-14-PP, identical in structure to the lipid A precursor, known as Ia or Iva) and compound 401 (lipid X), on the in vitro modulation of endotoxin (lipopolysaccharide)-induced interleukin-6 production by human blood mononuclear cells was investigated. Lipopolysaccharide of Salmonella abortus equi and synthetic Escherichia coli-type lipid A (compound 506, or LA-15-PP) had potent interleukin-6-inducing capacities. The maximum release of interleukin-6 was found after stimulation with 1 to 10 ng of lipopolysaccharide or 10 to 100 ng of synthetic E. coli-type lipid A per ml. Both synthetic lipid A partial structures (compounds 406 and 401) failed to induce interleukin-6 release. However, they inhibited lipopolysaccharide- or lipid A-induced interleukin-6 production in a dose-dependent manner. Inhibition was found not only in mononuclear cells but also in purified monocytes and was not due to a shift in the kinetics of cytokine production. Suppression was manifested in the early stage of interleukin-6 production. Inhibition was also found in the presence of recombinant gamma interferon, indicating that compound 406 and recombinant gamma interferon act in different, independent pathways. Our data, therefore, indicate that the inhibition of interleukin-6 production by lipid A partial structures may help elucidate the mechanism of interaction of the lipid A component of lipopolysaccharide with immune cells in the inflammatory reaction during gram-negative infection.

Cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), play an important role in inflammatory reactions and immunological functions induced by bacterial lipopolysaccharide (LPS) (endotoxin) during infection with gram-negative bacteria (4, 8). The multiple inflammatory activities of these cytokines in the pathogenesis of certain diseases have been reported (2, 18, 22). The symptoms observed during gram-negative infection, such as fever, acute-phase response, leukocytosis, and septic shock, are considered to be due mainly to the diverse activities of these cytokines released from immune cells after exposure to LPS and other bacterial products (8, 42, 44). Recently, IL-6 has also been implicated in the pathophysiology of bacterial infections (16, 20, 46). Clinically, elevated levels of IL-6 in various biological fluids have been found in patients with infectious diseases such as meningococcal meningitis and septic shock (8, 13, 22, 24). It was shown that high levels of IL-6 in serum coexisted with TNF, IL-1, and LPS in the systemic circulation during the initial phase of bacterial septic shock and were directly associated with a fatal outcome (44). It was also reported that clinical signs of endotoxemia were correlated with high levels of IL-6 in serum when TNF production was selectively inhibited (48). Moreover, it was shown that anti-IL-6 monoclonal antibody pretreatment of mice subsequently challenged intraperitoneally with lethal doses of Escherichia coli or intravenously with lethal doses of TNF protected mice from death caused by these treatments (12). Although the precise role of IL-6 remains largely unknown, accumulated evidence strongly indicates that IL-6 may be an important factor in the development of the lethal shock caused by the combination of TNF and IL-1 induced by bacterial LPS (38). Therefore, the abnormal expression and dysregulation of IL-6 in certain disorders may be typical features of this cytokine directly related to pathogenesis (20, 46).

Two lipid A partial structures, lipid A precursor Ia and lipid X, were initially reported to be less active in vivo and in vitro than other bioactive lipid A preparations in endotoxic reactions, including pyrogenicity, local Shwartzman reaction, lethal toxicity in mice, mitogenicity for mouse B lymphocytes, and complement activation (3, 7, 14, 26). Subsequently, both synthetic substances were used in vitro with human peripheral blood mononuclear cells (PBMC) and monocytes to evaluate their cytokine-inducing capacities. We found that compound 406 (synthetic lipid A precursor Ia) and compound 401 (synthetic lipid X) were inactive in the induction of IL-1 and TNF release (10, 29, 32) but that they could block LPS- or lipid A-induced production of cytokines such as IL-1 and TNF (31, 45). Similar results were found when natural lipid A partial structures, such as lipid IVa, were used (27). The observations that both lipid A partial structures largely lacked cytokine-inducing capacities in human monocytes but could modulate cytokine release can be regarded as a potential step forward in the use of such derivatives as immunomodulators in vivo.

Despite the fact that IL-6 is regulated, at least in part, independently of IL-1 and TNF (48) and is the most important monokine released during endotoxic reactions, it is necessary to investigate the effect of compounds 406 and 401 on LPS-induced IL-6 production. In the present study, we demonstrate that both compounds 406 and 401 are also

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inactive in stimulating IL-6 release by human PBMC or monocytes. Apart from their suppressive activities on IL-1 and TNF production (27, 31, 40, 45), synthetic lipid A partial structures also inhibit IL-6 release induced by wild-type LPS and synthetic lipid A. Monocytes may be one of the target cells influenced by lipid A partial structures in the production of IL-6 induced by LPS and lipid A. On the basis of the biochemical and immunological properties of synthetic lipid A partial structures, we postulate that these structures prevent the effective action of LPS required for the induction of IL-6 in immune cells and have the potential to be used for the modulation of abnormal immunological reactions in certain diseases (11).

**MATERIALS AND METHODS**

LPS, synthetic lipid A, and partial structures. LPS was extracted from *Salmonella abortus equi* purified by the phenol-chloroform-ethanol method as described by Galanos et al. (15). Compound 506 (synthetic hexaacyl *E. coli*-type lipid A) was synthesized as described previously (25), is composed of a bisphosphorylated glucosamine residue and five acyl residues, and corresponds structurally to free lipid A prepared from LPS of the *E. coli* Re mutant strain F515 (25). Compound 406 (synthetic tetraacyl lipid A precursor Ia) represents a partial structure of compound 506 lacking dodecanoic and tetradecanoic acids and was synthesized as described previously (14). Compound 401 (synthetic lipid X), representing a 1-phosphorylated glucosamine residue carrying two molecules of (R)-3-hydroxytetradecanoic acids, was synthesized as described previously (28). It was solubilized as described elsewhere (29). In brief, the dry substance was wetted with ethanol (10 μl/mg of compound 401) and sonicated for 1 min in an ultrasound water bath, ensuring that the temperature was below 10°C. During further sonification, 1 ml of pyrogen-free phosphate-buffered saline (PBS) was added dropwise. Sonication was continued until a slightly opalescent solution was obtained; this solution was filtered (0.45-μm-pore-size filter) and kept at 4°C. The other substances were treated and stored in the same way. The chemical structures of the synthetic compounds are shown in Fig. 1.

**Cytokines and antibodies.** Human recombinant IL-6 (rIL-6) exhibiting a specific activity of 10^7 U/mg of protein was purchased from Genzyme Fine Chemicals (Boston, Mass.). Human recombinant gamma interferon (rIFN-γ) was kindly provided by Karl Thoma GmbH (Biberach, Germany); the specific activity was 4 × 10^7 U/mg of protein. A mouse monoclonal antibody against human IL-6 was kindly provided by F. di Padova (Sandoz AG, Basel, Switzerland). A rabbit polyclonal antiserum against human IL-6 was purchased from Genzyme Fine Chemicals.

**Biological reagents.** *Mycobacterium bovis* BCG was provided by Institut Mérieux GmbH (Leimen, Germany). *Staphylococcus aureus* Cowan I (Pansorbin) was purchased from Calbiochem GmbH (Frankfurt am Main, Germany).

**PBMC separation and preparation of monocytes.** Samples of blood from various healthy donors were collected in heparin at 10 U/ml. PBMC were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation as described previously (6). After three washes with cold Hanks solution at 4°C, the PBMC were suspended in serum-free RPMI 1640 medium supplemented with 100 U of penicillin and 100 μg of streptomycin per ml (RPMI 1640-M). For the preparation of monocytes, PBMC at 2 × 10^6/ml were placed in a plastic petri dish and incubated at 37°C in 5% CO₂ in air for 60 min. Non-adherent cells were discarded by extensive washing. Adherent monocytes were removed after the addition of 5 ml of cold EDTA solution and incubation at 4°C for 30 min. Cells thus prepared normally yielded approximately 90% monocytes, as determined by nonspecific α-naphthylacetate esterase staining (43). During preparation, cells were not in contact with other serum, except for autologous plasma.

**Induction of IL-6 release.** PBMC at 4 × 10^6/ml or monocytes at 2 × 10^6/ml in serum-free RPMI 1640-M were incubated in U-form microtiter plates (Greiner, Nürtigen, Germany) at 200 μl per well. LPS or compound 506, 406, or 401 at different concentrations was added. In coinubation experiments, compound 406 or 401 was added to the cell cultures simultaneously with LPS, compound 506, or other stimuli. Cells were usually incubated at 37°C in 5% CO₂ for 20 h. Supernatants from each well were harvested after the plates were centrifuged for 10 min. For determination of the kinetics of IL-6 release, supernatants were harvested at different times after stimulation. For assessment of the effect of compound 406 or 401 on IL-6 release induced by BCG or *S. aureus* Cowan 1, PBMC were stimulated with BCG (0.01 μl/ml) or *S. aureus* Cowan 1 (1:5,000 dilution) together with different amounts of compound 406 or 401 for 20 h. All supernatants were analyzed at once for IL-6 activity or stored at –20°C before being assayed.

**Bioassay for IL-6 activity.** IL-6 activity was determined by using a IL-6-dependent B9.9-3A4 cell proliferation assay but with methylthiazol tetrazolium bromide (MTT) for detection (1, 35). The assay was carried out in 96-well flat-bottom microtiter plates at 200 μl per well. The number of B9.9-3A4 cells in each well was 10^4. The samples were titrated at various dilutions in triplicate. A standard preparation of rIL-6 (150 U/ml) was used as a positive control. After incubation at 37°C in 5% CO₂ for 56 h, 10 μl of MTT solution (5 mg/ml in PBS [pH 7.4]) was added to each well. Cells were cultured for another 3 h at 37°C. The MTT reaction was stopped by the addition of 100 μl of acidified isopropanol (0.04 N HCl in isopropanol). The dark blue formazan products were dissolved by vigorous mixing with a microplate shaker and measured at 550 nm with a microplate enzyme-linked immunosorbent assay (ELISA) reader (MR 700; Dynatech, Denkendorf, Germany). IL-6 activity was calculated by probit analysis as described elsewhere for IL-2 (17).

**Sandwich ELISA for IL-6.** The method used for the detection of IL-6 was based on a detection method described for IL-2 (5), with slight modifications. In brief, Microtest III plates were coated with 100 μl of mouse monoconal anti-IL-6 antibody per ml and incubated at 4°C overnight, and unspecific binding was blocked by the addition of 150 μl of 1% bovine serum albumin in PBS per well as a blocking solution. Samples and a standard preparation of rIL-6 were diluted in sample buffer (1% bovine serum albumin and 1% EDTA in PBS). Wells containing sample buffer only were used as negative controls. The plates were incubated at room temperature for 4 h. A rabbit polyclonal anti-IL-6 antibody serving as a detecting antibody was added to each well, and the plates were incubated for another 2 h. Between each step, the wells were washed three times with washing buffer (0.1% Tween 20 in PBS). The reaction was developed with peroxidase-conjugated donkey anti-rabbit immunoglobulin G antibody (Dianova, Hamburg, Germany). The A₅₀ was measured with a microplate ELISA reader. The amounts of...
IL-6 molecules were calculated by comparison with the defined standard preparation of rIL-6.

**Statistical analysis.** All experiments were performed with three different donors. Samples were tested in triplicate. Each point in the figures represents the mean number for three wells. The significance of differences between groups was calculated by Student's t test. A P value of 0.05 was used as the limit of statistical significance.

**RESULTS**

**IL-6 activity induced by S. abortus equi LPS and compounds 506, 406, and 401.** In preliminary experiments, PBMC from several healthy donors were stimulated to produce IL-6 by different concentrations of *S. abortus equi* LPS or compound 506, 406, or 401. *S. abortus equi* LPS could induce high amounts of IL-6 activity in supernatants (Fig. 2). The minimal concentration at which we could detect IL-6 release was 10 pg of *S. abortus equi* LPS per ml. In comparison with *S. abortus equi* LPS, compound 506 was less active in the induction of IL-6 in PBMC (Fig. 2). The dose-response curves indicated that the maximal release of IL-6 in PBMC was found after stimulation with 1 to 10 ng of *S. abortus equi* LPS or 10 to 100 ng of compound 506 per ml and that the amount of IL-6 produced after LPS stimulation was about four- or fivefold higher than that produced after compound 506 stimulation. In contrast, compound 406 did not induce IL-6 release from PBMC, even when high concentrations, up to 2 μg/ml, were used (Fig. 2). Similar results were found for compound 401 (data not shown). In subsequent experiments, 1 ng of *S. abortus equi* LPS or 100 ng of compound 506 per ml was used routinely.
Effect of compound 406 on *S. abortus equi* LPS-induced IL-6 release. PBMC from six individual donors were stimulated with *S. abortus equi* LPS (1 ng/ml) in the presence or absence of compound 406 (1,000 ng/ml). PBMC stimulated with *S. abortus equi* LPS showed a strong release of IL-6 after 20 h of incubation (Table 1). Control cultures without a stimulus showed mostly negative values, except for those for two donors showing a spontaneous release of IL-6 at low levels. Similarly, PBMC incubated with compound 406 also did not release IL-6. However, although not bioactive by itself, compound 406 significantly inhibited *S. abortus equi* LPS-induced IL-6 release in all six experiments. The inhibition ranged from 90 to 99%. In addition, we found by trypan blue staining that compound 406 at concentrations of up to 2 µg/ml had no demonstrable effect on the survival of either *S. abortus equi* LPS-stimulated or nonstimulated PBMC after 20 h of culturing (data not shown).

Dose-dependent inhibition of IL-6 release by lipid A partial structures. PBMC were stimulated with *S. abortus equi* LPS (1 ng/ml) and cultured in the presence of various concentrations of compound 406 or 401. Typical results (Fig. 3) showed that both compounds 406 and 401 exerted a defined dose-dependent inhibition. Although as little as 0.1 ng of compound 406 per ml exerted inhibition, 100 to 1,000 ng of the substance per ml was necessary to abolish nearly totally LPS-induced IL-6 release. However, compound 401 exerted relatively less inhibition at analogous doses. Inhibition was exerted not only in PBMC but also in purified monocytes (data not shown).

**Inhibition by compound 406 of IL-6 release, as measured by an ELISA.** As described above, compound 406 inhibited the release of bioactive IL-6 by *S. abortus equi* LPS-stimulated PBMC or monocytes. We further investigated whether not only bioactive IL-6 but also immunoreactive IL-6 peptide, secreted by LPS-activated cells, was influenced by the addition of compound 406. Supernatants from the same donor were analyzed by a sandwich ELISA, and the results were compared with the results from the bioassay. The release of immunoreactive IL-6 peptide was also inhibited by the addition of compound 406 in a dose-dependent manner (Fig. 4). The decrease in the biological activity of IL-6 in the supernatants paralleled the reduction in the IL-6 peptide content. This result indicates that the inhibition was due to a blocking of IL-6 peptide release from IL-6-producing cells.

**Effect of compound 406 on IL-6 release induced by compound 506.** According to our previously reported results, compound 506 represents the least complex lipid A structure capable of inducing IL-1 and TNF release by human monocytes (10, 31, 32). As described here, this component also has IL-6-inducing capacity in PBMC and monocytes. To determine whether compound 406 could also exert an inhibitory effect on IL-6 release induced by compound 506, we incubated PBMC in the presence of different concentrations of compound 406 and simultaneously stimulated them with 100 ng of compound 506 per ml. Compound 406 also suppressed IL-6 release induced by compound 506 in a concentration-dependent manner (Fig. 5). This suppression was not due to the toxicity of compound 406 (data not shown).

**Kinetics of IL-6 release and inhibition of IL-6 release by lipid A partial structures.** The experiments described above
demonstrate a significant reduction in *S. abortus equi* LPS- or compound 506-induced IL-6 release in the presence of compound 406 or 401. However, we needed to ascertain whether this effect was due to a shift in the peak of IL-6 secretion. Therefore, kinetics studies were done with PBMC or monocytes and with compounds 406 and 401. Typical results of experiments with two donors are shown in Fig. 6. IL-6 activity appeared in the supernatants after 6 h of exposure of PBMC to *S. abortus equi* LPS, reached a peak level at 28 h, and slowly declined up to 40 h. When cells were stimulated with LPS in the presence of compound 406 or 401, the results clearly indicated that the inhibitory effects of the partial structures were not due to a delay in the kinetics of IL-6 release. Again, the results showed that compound 406 had stronger suppressive activity than compound 401.

**Kinetics of the effect of compound 406 on the stimulation of PBMC by S. abortus equi LPS.** In the next series of experiments, we tested at what time during culturing the addition of compound 406 or 401 was required to inhibit IL-6 release. Compound 406 (1 μg/ml) or 401 (1 μg/ml) was added to PBMC cultures together with *S. abortus equi* LPS or compound 506 at the initiation of culturing and at 2, 4, 8, 16, and 20 h of culturing. In all cases, supernatants were harvested after 24 h of culturing. Maximum suppression was observed when compound 406 was added at the beginning of culturing (0 h) (Fig. 7). When compound 406 was added later, the extent of suppression decreased, but even at 4 h after LPS stimulation, compound 406 still had significant blocking activity. No further significant suppression was observed when compound 406 was added 8 h or later after the beginning of culturing. These results indicate that compound 406-induced inhibitory activity takes place at an early step in the activation of cytokine release.

**Effect of compound 406 on IL-6 production induced by different IL-6 inducers.** An experiment was done to investigate the specificity of the inhibitory activity of compound 406. PBMC from two healthy donors were cultured with 1 μg of compound 406 per ml in the presence of BCG (0.01 μl/ml) or *S. aureus* Cowan 1 (1:5,000 dilution). Cells stimulated with *S. abortus equi* LPS in the presence or absence of

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**FIG. 3.** Dose-dependent inhibition of IL-6 release by synthetic lipid A partial structures, compounds 406 and 401. PBMC were stimulated with *S. abortus equi* LPS (1 ng/ml) in the presence or absence of different concentrations of compound 406 (○) or 401 (●). For further details, see the legend to Fig. 2.

**FIG. 4.** Inhibition of release of IL-6 peptides by compound 406, as measured by a sandwich ELISA (A) in comparison with an IL-6 bioassay (B). For further details, see the legend to Fig. 2.
compound 406 served as controls. For the various IL-6 inducers used, IL-6 release induced by BCG or *S. aureus* Cowan 1 was not influenced, whereas IL-6 release induced by *S. abortus equi* LPS was blocked by the addition of compound 406 (Table 2).

**Modulation by rIFN-γ of lipid A partial structure-mediated IL-6 inhibition.** We tested whether rIFN-γ was capable of counteracting the inhibitory action of lipid A partial structures on LPS-induced IL-6 release. PBMC were stimulated with *S. abortus equi* LPS (1 ng/ml) together with different amounts of compound 406 or 401. Human rIFN-γ (100 U/ml) was added simultaneously. Cells stimulated with LPS plus rIFN-γ produced higher amounts of IL-6 than did those stimulated with LPS alone, whereas compound 406 or 401 was not able to induce IL-6 release, even in the presence of rIFN-γ (Table 3). When the modulating effect of rIFN-γ on lipid A partial structure-mediated inhibition was investigated, we found suppression by compound 406 or 401 of LPS-induced IL-6 release in the presence or absence of rIFN-γ, as well as enhancement of LPS-induced IL-6 release by rIFN-γ in the presence of compound 406 or 401.

**DISCUSSION**

LPS from the cell wall of gram-negative bacteria exerts profound inflammatory effects on mammalian physiological systems. Several of these effects, such as fever, acute-phase response, and septic shock, are mediated by the LPS-induced release of inflammatory cytokines such as IL-1, IL-6, and TNF. Structurally, LPS isolated from smooth organisms consists of three regions, of which the lipid A component represents the endotoxically active center. The biological effects of LPS are reproduced by free lipid A (37). Studies with natural or synthetic analogs of lipid A have demonstrated relationships between the structures of lipid A and their cytokine-inducing capacities (10, 31, 32). Recently,
we and others found modulating activities of defined structures of lipid A for cytokine production induced by LPS in vitro, suggesting that inactive lipid A partial structures may serve as antagonists against LPS-mediated inflammatory reactions in vivo (11, 19, 27, 36, 45). In the present study, we focused our experimental approaches on the immunostimulatory effects of LPS and synthetic lipid A on the induction of IL-6 and its regulation at the cellular level by synthetic lipid A partial structures, including compounds 406 and 401.

LPS and synthetic E. coli-type lipid A (compound 506) were potent inducers of IL-6 release in PBMC and monocytes. The production of IL-6, as determined by IL-6-dependent B9.9-3A4 cell proliferation, was detectable at S. abortus equi LPS concentrations as low as 10 µg/ml. Although compound 506 was active in inducing the production of IL-6, it was not as potent as S. abortus equi LPS. In terms of dose-response relationships (Fig. 2), the IL-6-inducing capacity of compound 506 was significantly lower than that of naturally occurring LPS. Further insight into the structural requirements was gained by the finding that compounds 406 and 401 were not able to induce the production of IL-6 in PBMC and monocytes under defined experimental conditions, even when higher concentrations (2 µg/ml) of both lipid A partial structures were used with PBMC from different donors. These results are consistent with those in our previous reports, in which it was shown that compounds 406 and 401 were unable to induce the release of IL-1 and TNF and that the minimal structure of lipid A needed to induce monokine release was bisphosphorylated and hexaacylated lipid A (10, 11, 31, 32). We here further confirmed that both compounds 406 and 401 also had no stimulatory activity in the induction of IL-6 in human PBMC and monocytes in vitro.

Table 2. Effect of compound 406 on IL-6 release stimulated by different IL-6 inducers in PBMC

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Compound 406 (1 µg/ml)</th>
<th>Results* of exp: 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td>IL-6 (U/ml)</td>
<td>Inhibition (%)</td>
<td>IL-6 (U/ml)</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>LPS</td>
<td>-</td>
<td>16,934</td>
<td>0</td>
<td>24,370</td>
<td>30</td>
</tr>
<tr>
<td>BCG</td>
<td>-</td>
<td>8,295</td>
<td>-8.8</td>
<td>5,865</td>
<td>7,188</td>
</tr>
<tr>
<td>S. aureus Cowan 1</td>
<td>+</td>
<td>20,609</td>
<td>-17.9</td>
<td>34,308</td>
<td>32,715</td>
</tr>
</tbody>
</table>

* PBMC were stimulated with S. abortus equi LPS (1 ng/ml), BCG (0.01 µg/ml), or S. aureus Cowan 1 (1:5,000 dilution) alone or in the presence of compound 406 for 20 h. Standard deviations were less than 20%.

Table 3. Modulation by rIL-1β of lipid A partial structure-mediated IL-6 inhibition

<table>
<thead>
<tr>
<th>Substance (ng/ml)</th>
<th>IL-6 (U/ml)* in exp:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without rIL-1β at 100 U/ml</td>
<td>With rIL-1β at 100 U/ml</td>
<td>Without rIL-1β at 100 U/ml</td>
<td>With rIL-1β at 100 U/ml</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>18,710</td>
<td>28,157</td>
<td>20,704</td>
<td>33,787</td>
<td></td>
</tr>
<tr>
<td>Compound 406 (1,000)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Compound 401 (1,000)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LPS + compound 406 (0.1)</td>
<td>13,324</td>
<td>23,918</td>
<td>19,665</td>
<td>26,212</td>
<td></td>
</tr>
<tr>
<td>LPS + compound 406 (100)</td>
<td>165</td>
<td>1,113</td>
<td>4,855</td>
<td>5,639</td>
<td></td>
</tr>
<tr>
<td>LPS + compound 401 (0.1)</td>
<td>17,931</td>
<td>28,512</td>
<td>25,147</td>
<td>38,103</td>
<td></td>
</tr>
<tr>
<td>LPS + compound 401 (100)</td>
<td>5,099</td>
<td>9,922</td>
<td>5,653</td>
<td>6,743</td>
<td></td>
</tr>
</tbody>
</table>

* PBMC were stimulated with S. abortus equi LPS (1 ng/ml) alone or together with compound 406 or 401. rIL-1β was added simultaneously. Each value represents the mean of triplicates. Standard deviations were less than 20%.
partial structures and LPS or lipid A, experiments were performed to investigate the role of compounds 406 and 401 as antagonists in the activation of PBMC and monocytes by LPS. Our interest was our finding that both lipid A partial structures inhibited the production of IL-6 from human cells activated by S. abortus equi LPS or compound 506. The degree of inhibition was related to the dose of lipid A partial structures used. Interestingly, we found much stronger inhibitory activity of compound 406 than of compound 401 in cytokine release. This phenomenon might be related to the structural compositions of compounds 406 and 401, because compound 401 differs from compound 406 by the presence of one instead of two phosphorylated glucosamine residues, so that it carries two acyl residues as compared with four in compound 406. The effect of compound 401 was further confirmed not only as the suppression of LPS-induced TNF formation (30) but also as the blocking of LPS-induced expression of monocyte procagulant activity (29) and other LPS-related reactions (19). In our experiments, the diminished IL-6 bioactivities in the culture supernatants after the addition of compound 406 were correlated with the decreased quantities of IL-6 molecules in the supernatants, as measured by a sandwich ELISA. This result implies that the inhibition of IL-6 release mediated by lipid A partial structures was indeed caused by a change in the release of IL-6 peptide molecules. Further investigations of the kinetics of LPS-induced IL-6 production indicated that both compounds 406 and 401 not only delayed the onset of IL-6 release but also reduced the maximal amount of biologically active IL-6 secreted by PBMC as well as monocytes.

Although a number of studies have reported the suppression of cytokine release and of the secretion of prostaglandin E₂ and prostacyclin by lipid A partial structures as well as deacylated LPS in PBMC and vascular endothelial cells (27, 31, 36, 45), the mechanism by which lipid A partial structures exert their inhibitory action is still not clear. Recently, it was reported that different LPS-binding sites that recognize different substructures or spatial configurations of the lipid A moiety of LPS are present on the surface of mouse macrophages (34, 39, 47). At present, it is not possible to determine from our data whether the suppression of IL-6 release by lipid A partial structures is mediated through a competitive mechanism or a noncompetitive mechanism. It should be noted that the inhibition of IL-6 release by treatment with compound 406 appears at an early stage. The release of IL-6 induced by BCG and S. aureus Cowan 1, which may use different pathways for inducing IL-6 release, was not influenced by the addition of compound 406 (Table 2). These results agree well with the previous findings that Rhodobacter capsulatus LPS, which largely lacks cytokine-inducing capacity, abolished the induction of IL-1 and TNF by other LPSs but did not alter the induction of cytokines by gram-positive Streptococcus epidermidis (33). Furthermore, evidence provided by Kovach et al. (27) indicated that the specific inhibition by lipid A partial structure IVa (natural sources) of the accumulation of TNF mRNA and the release of TNF induced by Salmonella typhimurium LPS may be part of a competitive mechanism, perhaps at the receptor level. In favor of suppression via a specific mechanism, Riede et al. (36) found that deacylated LPS could also specifically block a variety of endothelial cell responses to LPS, supporting the hypothesis that LPS partial structures and LPS interact with a common target molecule or in endothelial cells. These data, together with those of our present investigations, strongly suggest that synthetic or naturally occurring poorly acylated LPS or lipid A partial structures are unable to mediate LPS-induced toxic reactions but can antagonize LPS-induced inflammatory reactions specificaly by a competitive receptor-mediated phenomenon.

IFN-γ, produced by T cells upon stimulation, has many immunomodulatory properties, including enhancement of LPS-induced IL-1 and TNF release by cultured macrophages (9) and augmentation of TNF cytotoxicity in vivo (41). Recently, evidence indicating the pathophysiological role of IFN-γ in the Shwartzman phenomenon and other endotoxin-related pathological phenomena has accumulated (23). Elevated concentrations of IFN-γ in serum have indeed been found in children with severe infectious purpura (18) and in adults with gram-negative septicemia (8). It was reported that mice pretreated with IFN-γ had dramatic increases in serum TNF during endotoxemia (21). Pretreatment with an anti-IFN-γ monoclonal antibody before LPS challenge significantly reduced the mortality of mice from endotoxic shock, indicating the direct involvement of IFN-γ in the pathology of septic shock (21). Because of the synergistic effects of IFN-γ on LPS-mediated reactions in vitro as well as in vivo (9, 41), we performed experiments to investigate whether IFN-γ could counteract the inhibitory effects of synthetic lipid A partial structures on LPS- or compound 506-induced IL-6 production. The results demonstrated that the synergism of LPS with IFN-γ in the induction of IL-6 release from PBMC was significantly inhibited by the addition of synthetic lipid A partial structures at high concentrations in coinubcation experiments. However, IFN-γ still stimulated LPS-induced IL-6 release in the presence of low concentrations of compound 406 or 401 (Table 3). On the basis of these results, we presume that IFN-γ acts via a pathway which is independent of compound 406 or 401. Therefore, the antagonistic function of synthetic lipid A partial structures involves not only suppression of LPS-mediated effects but also counteraction of the synergistic effects of IFN-γ with LPS in the induction of IL-6.

Further work is required to resolve the detailed mechanism(s) of the suppressive action of synthetic lipid A partial structures on the molecular level as well as on the subpopulation of cells involved in the inhibition of IL-6 production. With regard to the inflammatory activities of IL-1, IL-6, IL-12, and TNF production and during granuloma formation, the cooperation of compounds 406 and 401 can be considered potent immunomodulators able to prevent the LPS-mediated production of these cytokines in vivo. Therefore, the use of these bacterial LPS antagonists may represent a new approach in the reduction of inflammatory reactions caused by systemic endotoxins.

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REFERENCES

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