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Modulation of Endotoxin-Induced Monokine Release in Human Monocytes by Lipid A Partial Structures That Inhibit Binding of ^{125}I-Lipopolysaccharide

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We have previously shown that the synthetic tetraacyl precursor Ia (compound 406, LA-14-PP, or lipid IVa) was not able to induce the production of tumor necrosis factor, interleukin-1, and interleukin-6 in human monocytes but strongly antagonized lipopolysaccharide (LPS)-induced formation of these monokines. This inhibition was detectable at the level of mRNA production. To achieve a better understanding of molecular basis of this inhibition, we investigated whether lipid A precursor Ia (LA-14-PP), *Escherichia coli*-type lipid A (LA-15-PP), *Chromobacterium violaceum*-type lipid A (LA-22-PP), and synthetic lipid A partial structures and analogs (LA-23-PP, LA-24-PP, and PE-4) were able to influence the binding of ^{125}I-LPS to human monocytes and compared this inhibitory activity with the agonistic and antagonistic action in the induction of monokines in human monocytes. ^{125}I-LPS (20 ng per well) was added to human monocytes in the presence or absence of unlabeled rough Re mutant-derived LPS (Re-LPS) or lipid A compounds, and specific LPS binding was determined after 7 h. This binding was found to be dependent on CD14 as shown by the use of an anti-CD14 monoclonal antibody. Compound LA-14-PP was found to inhibit the binding of ^{125}I-LPS to the cells in a similar dose-response to that of unlabeled LPS. This shows that the inhibitory capacity on LPS binding does not correlate with the monokine-inducing capacity because Re-LPS is active in inducing tumor necrosis factor, interleukin-1, and interleukin-6, while LA-14-PP is not. The strong capacity of LA-14-PP to inhibit ^{125}I-LPS binding, however, correlates with the strong inhibitory capacity of this compound on LPS-induced monokine production. Compounds LA-15-PP, LA-23-PP, and LA-24-PP were active in the inhibition of ^{125}I-LPS binding but were 5- to 10-fold weaker than Re-LPS and LA-14-PP. Of all lipid A structures tested, compound LA-22-PP expressed the weakest inhibitory capacity on LPS binding. These compounds showed again that the activity of binding inhibition does not correlate with the monokine-inducing capacity. We assume that the inhibitory effects of lipid A partial structures on LPS-induced monokine production have their origin in a competitive inhibition between LPS and the lipid A partial structures for the same binding site on the cell membrane.

Lipopolysaccharide (LPS), also known as endotoxin, from the outer leaflet of the outer membrane of gram-negative bacteria is considered to be responsible for the induction of pathophysiological reactions of an infected host (25, 27). It is known today that the pathophysiological effects of endotoxin are not due to a direct action of LPS but are elicited by various mediators including tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) and, furthermore, that monocytes or macrophages are the major source of these mediators.

LPS consists of a hydrophilic polysaccharide and a covalently linked hydrophobic lipid part, termed lipid A. Lipid A represents the endotoxic principle of LPS, i.e., the biological effects of LPS are reproduced by free lipid A (2, 8). The successful chemical synthesis of lipid A and corresponding partial structures of LPS, such as *Escherichia coli*-type lipid A (compound 506, LA-15-PP) and precursor Ia (compound 406, LA-14-PP, or lipid IVa) has provided the experimental basis to investigate the bioactive regions of LPS with regard to structure-activity relationships (2, 8, 12, 19, 25). A structure-dependent hierarchy of LPS and LPS partial structures with respect to their IL-1-, IL-6-, and TNF-inducing capacity was demonstrated, showing that the hexaacyl type lipid A LA-15-PP is able to induce monokine production by human monocytes, while the tetraacyl compound LA-14-PP exhibited no inducing capacity (4, 22). The synthetic LA-14-PP became of particular interest since our studies as well as those of others showed that LA-14-PP was able to block LPS-induced monokine production in human monocytes in a specific manner (5, 16, 21, 36, 37). Therefore, this compound may be able to prevent the fatal endotoxin-induced reactions of an infected host. To achieve a better understanding of the molecular basis of this inhibition, we have investigated whether LA-14-PP and other synthetic lipid A partial structures (LA-15-PP, LA-22-PP, LA-23-PP, LA-24-PP, and PE-4) are able to influence the binding of LPS to monocytes or macrophages. Previously, we have described binding experiments using murine macrophage-like J774.1 cells (15). However, since it is known that the biological activities of lipid A partial structures are different in mice and humans (e.g., LA-14-PP is still toxic in mice and able to induce monokines as well as B-lymphocyte proliferation [6, 11], whereas it is not active on human monocytes in vitro [4, 22]), these experiments had to be extended to human monocytes. The results of such experiments are described in the present

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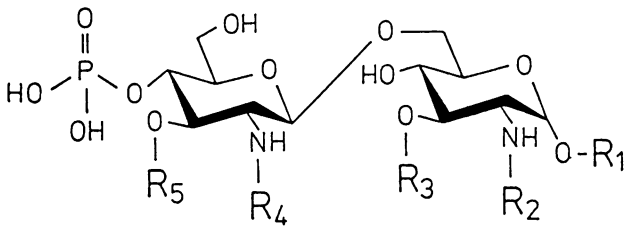

Compound	Nature of					No of acylresidues
	R_5	R_4	R_3	R_2	R_1	
LA-15-PP (comp. 506)	C_{14}-O-C_{14}	C_{14}-O-C_{12}	C_{14}-OH	C_{14}-OH	$PO(OH)_2$	6
LA-22-PP	C_{14}-OH	C_{14}-O-C_{14}	C_{14}-OH	C_{14}-O-C_{14}	$PO(OH)_2$	6
LA-14-PP (comp. 406)	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	$PO(OH)_2$	4
LA-24-PP	C_{10}	C_{14}-OH	C_{10}	C_{14}-OH	$PO(OH)_2$	4
LA-23-PP	C_{10}	C_{10}	C_{10}	C_{10}	$PO(OH)_2$	4
PE-4	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	$(CH_2)_2$-O-$PO(OH)_2$	4

FIG. 1. Chemical structure of the synthetic lipid A and analogs used.

article. When comparing the inhibition of binding of ^{125}I-LPS with the inhibition of biological activity, we found that both LA-14-PP as well as its phosphonooxyethyl analog PE-4 are the best tested inhibitors of LPS binding to human monocytes as well as preventers of monokine production tested here. Taken together, our results provide strong evidence for the concept that inhibition of LPS-cell interaction by LA-14-PP and other lipid A partial structures is based on a competitive inhibition of a specific LPS-binding protein of the responder cell.

MATERIALS AND METHODS

LPS, synthetic lipid A, precursor Ia, and lipid A analogs. LPS of *Salmonella friedenau* and *Salmonella minnesota* R595 core-defective deep rough Re mutant (Re-LPS) were prepared with the phenol-water method (38) and the phenol-chloroform-petroleum ether method (9), respectively, purified by repeated ultracentrifugation, and converted to the uniform sodium salt after electrodialysis as reported previously (7). Such preparations contain less than 0.1% of protein and nucleic acid as determined by chemical analysis.

Synthetic hexaacyl *E. coli*-type lipid A, LA-15-PP (or compound 506), is composed of a bisphosphorylated glucosamine disaccharide with six acyl residues and structurally corresponds to free lipid A prepared from LPS of the *E. coli* Re-mutant strain F515. It was synthesized as described previously (12). Synthetic LA-22-PP is a hexaacylated lipid A but differs from LA-15-PP in the acylation pattern as indicated in Fig. 1.

Synthetic tetraacyl lipid A precursor Ia (compound 406 or LA-14-PP) represents a partial structure of LA-15-PP lacking dodecanoic and tetradecanoic acids. LA-14-PP was synthesized as described elsewhere (13). Synthetic LA-23-PP and LA-24-PP are tetraacyl lipid A partial structures which differ from LA-14-PP in the pattern of acylation as indicated in Fig. 1. Preparation PE-4 is a synthetic analog of LA-14-PP having an oxyethyl-linked (-O-CH_2-CH_2-) phosphoryl group in position 1 of the reducing glucosaminyl residue (GlcN I). This phosphonooxyethyl analog of LA-14-PP was synthesized as described previously (17, 18).

All synthetic compounds were stored in aliquots at 1 mg/ml at 4°C. The chemical structures of the compounds are given in Fig. 1.

Isolation of human PBMC and monocytes. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation (35) with Ficoll-Paque (Pharmacia, Uppsala, Sweden). Monocytes were isolated from PBMC by elutriation in a J2-21 M/E Elutriator (Beckmann, München, Germany) as described elsewhere (5). PBMC were suspended in Hanks' balanced salt solution supplemented with 0.1% bovine serum albumin and loaded

into the elutriation chamber at 500 × g at 4°C and a flow rate of 25 ml/min using a total volume of 200 ml. After enhancing the flow rate stepwise to 44 ml/min, the rotor was stopped and the monocytes were eluted from the separation chamber at a flow rate of 47 ml/min. This procedure yielded a population of cells consisting of more than 95% monocytes as determined by light scatter and CD14 expression in a Cytofluorograf (System 50H; Ortho Diagnostic System, Inc., Westwood, Mass.).

Induction of IL-1 and IL-6 in PBMC. PBMC at 4×10^6/ml were stimulated at a volume of 200 μl in U-form microtiter plates (Greiner, Nürtingen, Germany) in the absence of serum in RPMI 1640 containing antibiotics and the appropriate cytokine inducer. In costimulatory experiments, PBMC were treated for 30 min with the first stimulus (LA-14-PP, LA-24-PP, or PE-4), and then the second stimulus (LPS or LA-15-PP) was added. After incubation for 12 h, supernatants were harvested and analyzed for cytokine activity immediately or stored at −75°C.

IL-1 proliferation assay with human fibroblasts. Human dermal fibroblasts were used for IL-1 detection as described previously (23). Fibroblasts isolated from human foreskin were grown in permanent culture in 80-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) in Dulbecco modified Eagle medium (Biochrom KG, Berlin, Germany) containing 10% fetal calf serum (FCS) (Biochrom KG), L-glutamine, and antibiotics (7.5% CO_2, 37°C) at a concentration of 4×10^4 cells per ml and subcultured every 7 days. For the IL-1 assay, fibroblasts were harvested by trypsin-EDTA (0.5%-0.2%; Biochrom KG) treatment after 7 days of culture. Cultures containing 100 μl medium of a cell suspension adjusted to 5×10^4 cells per ml were incubated in flat-bottom microtiter plates (Greiner). After 24 h, the medium was replaced by an equal volume of fresh Dulbecco modified Eagle medium containing L-glutamine, antibiotics, and 10% FCS. Test samples (50 μl per well) were added and further diluted in five steps (1:3). A standard of recombinant human IL-1β (kindly provided by Glaxo/Biogen S.A., Geneva, Switzerland) containing 100 IU/ml was used as a positive control. After an incubation time of 96 h, the number of cells was determined by using the crystal violet method (5). The A_{550} of the stained and lysed cells was measured with a microplate reader (MR 700; Dynatech, Denkendorf, Germany). IL-1 activity in the samples was calculated by using a standard of recombinant IL-1β and by comparing the dilutions giving 30% proliferation by probit analysis (10). The results are given in units of IL-1 per milliliter.

IL-6 proliferation assay with B9.9-3A4 cells. IL-6 activity was determined by using an IL-6-dependent B9.9-3A4 cell proliferation assay. B9.9-3A4 cells, derived from a murine hybridoma cell line with IL-6-dependent proliferation, were originally established by L. Aarden (Central Laboratory, Blood Transfusion Service, Amsterdam, The Netherlands) (1) and cultured in RPMI 1640 containing antibiotics, 10% FCS, 5×10^{-5} M of 2-mercaptoethanol, and 50 U of human IL-6 per ml. The assay was carried out in 96-well flat-bottom microtiter plates with a 200-μl volume per well and 10^4 B9.9-3A4 cells per well. Test samples (25 μl per well) were added to 100 μl of culture medium and further diluted in seven steps (1:6). A standard preparation of IL-6 giving 150 IU/ml was used as a positive control. After incubation (37°C, 5% CO_2, 48 h), 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (5 mg/ml in phosphate-buffered saline [PBS], pH 7.4) was added to each well, and cells were further incubated for 3 h (37°C, 5% CO_2). The MTT reaction was stopped by adding 100 μl of acidified isopropanol (0.04 N HCl in isopropanol) per well, and MTT formazan was dissolved by vigorous mixing with a stirring machine. The A_{550} was measured with a microplate reader (MR 700, Dynatech). IL-6 activity in the samples was determined by using a standard of recombinant human IL-6 with a specific activity of 10^7 U/mg (from Genzyme Fine Chemicals, Boston, Mass.) and by comparing the dilutions giving 50% proliferation by probit analysis (10). The results are given in units of IL-6 per milliliter.

Preparation of ^{125}I-LPS. Radiolabeled ^{125}I-LPS was prepared by the method described by Ulevitch (34) with some modifications. In brief, *S. minnesota* R595 LPS was coupled to *p*-OH methylbenzimidate by incubation in 50 mM borate buffer (pH 8.0) for 18 h at 37°C. *p*-OH methylbenzimidate-coupled LPS was radiolabeled with 1 mCi of Na ^{125}I (Amersham Buchler, Braunschweig, Germany) per mg of LPS in a solid-phase reaction using IODO-BEADS (Pierce, Rockford, Ill.). One mole of methylbenzimidate was introduced per about 1,000 mol of Re-LPS (estimated molecular weight, 2,800). The radiolabeled LPS had a specific activity of 1.1 to 2.8 μCi/μg (1 to 2 mol of ^{125}I-LPS per 1,000 mol of LPS). The biological activity of LPS was not significantly altered by the radiolabeling procedure as measured by the induction of monokines in human PBMC. Retention of the biological activity indicates that the structure of R595 LPS was not substantially affected by the radiolabeling procedure, which could be expected if, for example, the ester linkages in the lipid A were influenced. However, since only 1 per 1,000 molecules was labeled, we cannot exclude that an actually labeled ^{125}I-LPS molecule has a different biological activity than an unlabeled LPS after the labeling procedure.

Binding assay for ^{125}I-LPS. Binding of ^{125}I-LPS was performed by a method described elsewhere (15). In brief, monocytes (7×10^5) were incubated in 1 ml of RPMI 1640 medium plus 10% FCS in 24-well Costar tissue culture plates (Technomara, Fernwald, Germany) overnight at 37°C, in 5% CO_2. After the plates were washed three times with PBS, 280 μl of ice-cold Hanks' balanced salt solution with 5% FCS and 0.1% sodium azide was added to the adherent cells. The plates were put on ice, and 10 μl of unlabeled LPS, another unlabeled competitor, or anti-CD14 monoclonal antibody (clone MEM18; kindly provided by V. Bazil, Prague, Czechoslovakia) was added, followed by the addition of 10 μl of ^{125}I-LPS (2 μg/ml). After gentle shaking, the cells were incubated for 7 h on ice in a refrigerator. Binding was terminated by washing the cells two times with ice-cold Hanks' balanced salt solution containing 5% FCS and 0.1% sodium azide and two times with ice-cold PBS. The cells were lysed by the addition of 500 μl of 0.1 N NaOH, and the radioactivity of the solubilized cells was measured in a gamma counter. Specific binding was defined as the differences between radioactivity of samples incubated with excess (25-fold) unlabeled LPS (nonspecific binding) and the radioactivity measured in the absence of unlabeled ligand (total binding).

RESULTS

Induction of monokine production by LPS, lipid A, and lipid A analogs. The dose-response relationship of LPS, synthetic lipid A, and synthetic lipid A analogs in the induction of IL-1 and IL-6 in human PBMC was investigated. The cells were stimulated for 12 h with concentrations of the different compounds ranging from 0.01 to 1,000 ng/ml, and the amount of monokines released into the culture supernatants was determined. The results show that LPS,

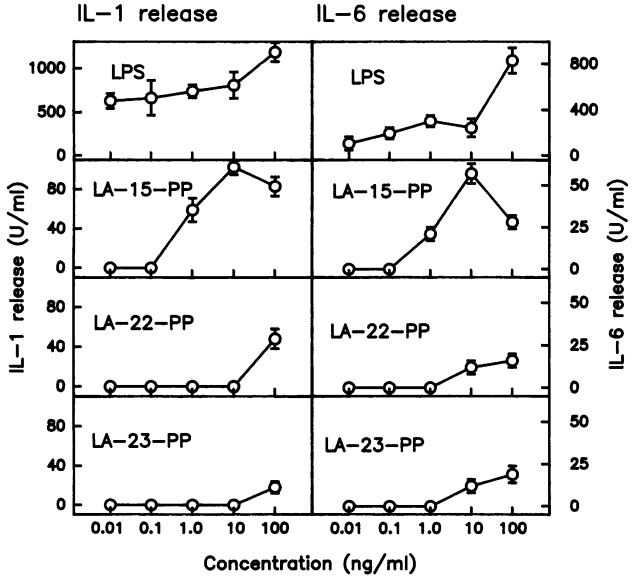

FIG. 2. Induction of IL-1 and IL-6 by LPS, lipid A, and lipid A partial structures in human PBMC. Human PBMC (4×10^6/ml) were stimulated with LPS, synthetic lipid A (LA-15-PP), or synthetic lipid A partial structures (LA-22-PP, LA-23-PP) at concentrations ranging from 0.01 to 100 ng/ml. After an incubation period of 12 h, the supernatants were harvested and tested for the presence of biologically active IL-1 and IL-6 as described in Materials and Methods. The results are given in units of IL-1 or IL-6 per milliliter. Each value represents the mean ± standard deviation of triplicate cultures.

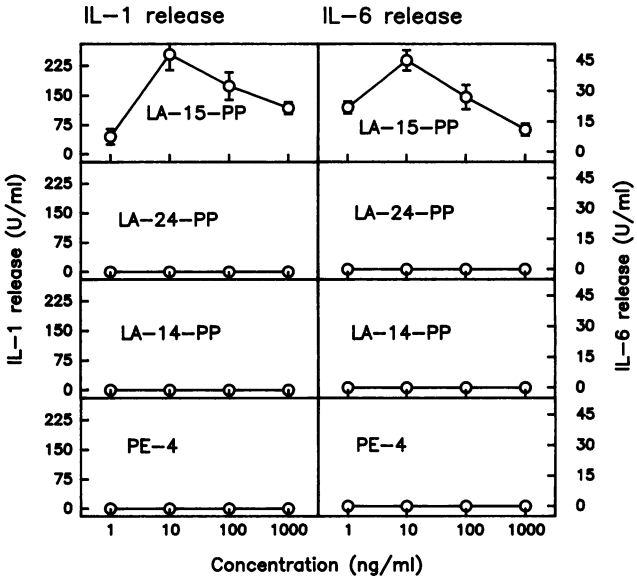

FIG. 3. Induction of IL-1 and IL-6 by lipid A and lipid A partial structures in human PBMC. Human PBMC (4×10^6/ml) were stimulated with LPS, synthetic lipid A (LA-15-PP), or synthetic lipid A partial structures (LA-24-PP, LA-14-PP, or PE-4) at concentrations ranging from 1.0 to 1,000 ng/ml. For further details, see the legend to Fig. 2.

LA-15-PP, and LA-22-PP as well as LA-23-PP were able to induce IL-1 and IL-6 production in monocytes (Fig. 2). The best stimulatory response was found on induction with LPS. In this experiment, an amount as small as 0.01 ng of LPS per ml was sufficient to induce monokine release, whereas the minimal concentration of LA-15-PP for monocyte induction was 1.0 ng/ml. LA-22-PP and LA-23-PP were even less active: 100 or 10 ng/ml was necessary to induce IL-1 or IL-6, respectively. A comparison of the dose-response of the different compounds further indicates that there is also a different maximal release of monokines after activation, with optimal release after stimulation with LPS, followed by that with LA-15-PP. Compounds LA-22-PP and LA-23-PP both express similarly low inducing capacities. When testing synthetic tetraacyl lipid A partial structures (LA-24-PP or LA-14-PP) or the synthetic analog PE-4, it was found that they were not able to induce monokine production in human PBMC even at a concentration of 1,000 ng/ml (Fig. 3).

Inhibitory effects of LA-14-PP, LA-24-PP, and PE-4 on monokine production. Experiments were performed to investigate the inhibitory effects of noninducing synthetic tetraacyl compounds on monokine production. In the first experiments, the inhibitory effect of precusor Ia (LA-14-PP) on LPS-, LA-15-PP-, or LA-22-PP-induced monokine production was analyzed. Cells were first treated for 30 min with LA-14-PP (250 ng/ml) and then stimulated with different concentrations of LPS, LA-15-PP, or LA-22-PP. The results presented in Fig. 4 show that 250 ng of LA-14-PP per ml is able to block monokine production induced by LA-15-PP or LA-22-PP at every concentration tested (up to 100 ng/ml). In contrast, LPS-induced monokine production was abolished only up to a ratio of 1 ng of LPS per ml to 250 ng of LA-14-PP per ml. At concentrations of 10 ng of LPS per ml or higher, only a partial inhibition by LA-14-PP was observed. In addition, the inhibitory capacities of compounds LA-24-PP and PE-4 were compared with that of LA-14-PP. Human PBMC were preincubated with LA-14-PP, LA-24-PP, or PE-4 at a concentration of 250 ng/ml, and after 30 min, the cells were stimulated with different amounts of LPS. A comparison of the effect of LA-24-PP with that of LA-14-PP reveals that, as expected (Fig. 4), LA-14-PP abolished IL-1 release after stimulation of the cells with LPS at concentrations of up to 1.0 ng/ml (Fig. 5). On the other hand, LA-24-PP was found to have a lower inhibitory activity, blocking only at an LPS concentration of 0.1 ng/ml (Fig. 5). At higher LPS concentrations (10 or 100 ng/ml), LA-24-PP even enhanced IL-1 release. Compound PE-4 exhibited an inhibitory effect quite similar to that of compound LA-14-PP (Fig. 6).

Inhibition of binding of ^{125}I-LPS by synthetic lipid A and lipid A analogs. Before carrying out the competitive binding experiments, we determined the time kinetics of the specific binding of ^{125}I-LPS to human monocytes. The specific binding increased time dependently and reached a steady state by 7 h (data not shown). Specific binding of ^{125}I-LPS to monocytes was linear, with cell numbers between 3×10^5 and 8×10^5 per well (data not shown). Accordingly, we routinely plated cells at 7×10^5 per well and incubated them with ^{125}I-LPS for 7 h in the competitive binding studies. In subsequent experiments, the inhibitory capacity of Re-LPS and of different synthetic lipid A structures was determined with regard to the binding of ^{125}I-LPS to human monocytes. Cells were preincubated with unlabeled Re-LPS or unlabeled synthetic lipid A and analogs, and then the binding of ^{125}I-LPS was analyzed. The best inhibition of binding was found with compound LA-14-PP, its analog PE-4 having a competitive effect similar to that of unlabeled Re-LPS (Fig. 7A and E). In contrast, preparations LA-15-PP and LA-23-

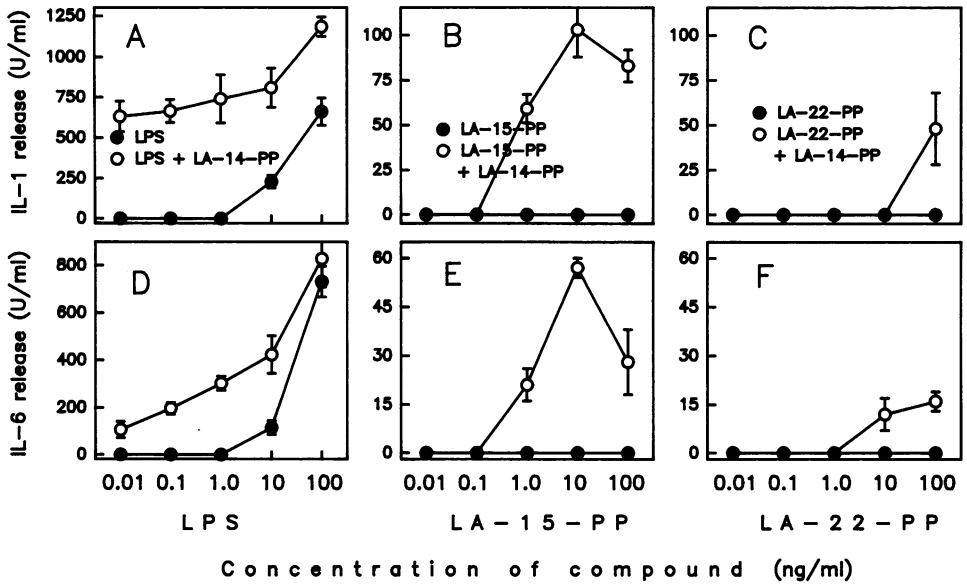

FIG. 4. Inhibition of LPS-, lipid A-, or lipid A partial structure-induced monokine production by LA-14-PP. PBMC (4 × 10^6/ml) were preincubated in the presence of LA-14-PP (250 ng/ml). After 1 h, LPS (A and D), LA-15-PP (B and E), or LA-22-PP (C and F) was added at different concentrations as indicated. After 12 h of incubation, the supernatants were harvested and assayed for IL-1 (A to C) and IL-6 (D to F) activity in bioassays. The results are expressed as units of IL-1 or IL-6 per milliliter, respectively. Each value represents the mean ± standard deviation of three independent cultures.

PP, possessing monokine-inducing capacity, and compound LA-24-PP, lacking monokine-inducing activity, were found to inhibit the binding of ^{125}I-LPS to monocytes to a lower degree (Fig. 7A, C, and D). Compound LA-22-PP expressed the lowest inhibitory activity of ^{125}I-LPS binding to cells (Fig. 7B). These data indicate that, at least with these special structures, there is only a partial correlation between the capacity of inducing monokine production in human PBMC by these compounds and the inhibition of binding of ^{125}I-LPS.

To characterize the binding structure which reacts with ^{125}I-LPS, the binding assay was performed in the presence of an anti-CD14 monoclonal antibody (clone MEM18). As shown in Table 1, this monoclonal antibody blocks the binding of ^{125}I-LPS to human monocytes.

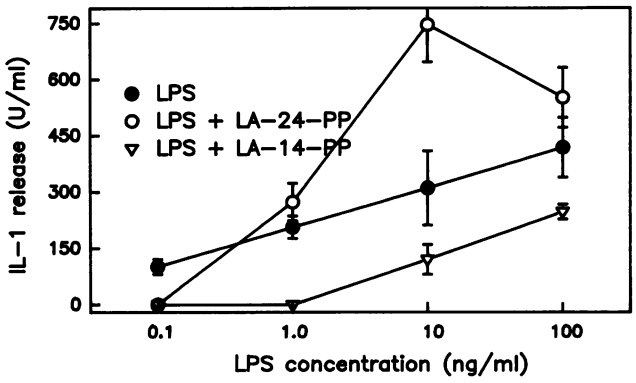

FIG. 5. Inhibition of LPS-induced IL-1 production by LA-14-PP or LA-24-PP. PBMC (4 × 10^6/ml) were preincubated for 1 h in the presence of LA-14-PP or LA-24-PP at 250 ng/ml. After 12 h of stimulation with LPS at different concentrations, the supernatants were harvested and assayed for IL-1 activity in bioassays. The results are expressed as units of IL-1 per milliliter. Each value represents the mean ± standard deviation of three independent cultures.

DISCUSSION

This article describes the results of experiments concerning the structural dependency of two LPS properties, i.e., binding to monocytes and induction of peptide mediators of inflammatory reactions. Table 2 summarizes the experimental results. As previously demonstrated, full monokine-inducing activity is expressed by a molecule having two gluco-configurated hexosamine residues, two phosphoryl groups, and six fatty acids as is present in *E. coli* lipid A or compound LA-15-PP (26). Lipid A partial structures deficient in one of these elements are less active or even nonactive in inducing monokines in human monocytes. For instance, the tetraacylated lipid A precursor Ia (LA-14-PP) is

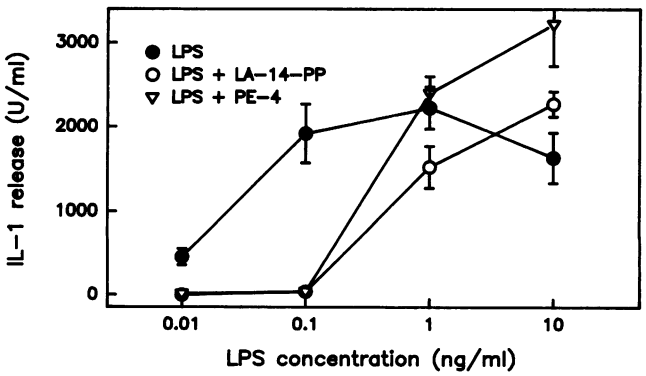

FIG. 6. Inhibition of LPS-induced IL-1 production by LA-14-PP or PE-4. PBMC (4 × 10^6/ml) were preincubated for 1 h in the presence of LA-14-PP or PE-4 at 5 ng/ml. For further details, see the legend to Fig. 5.

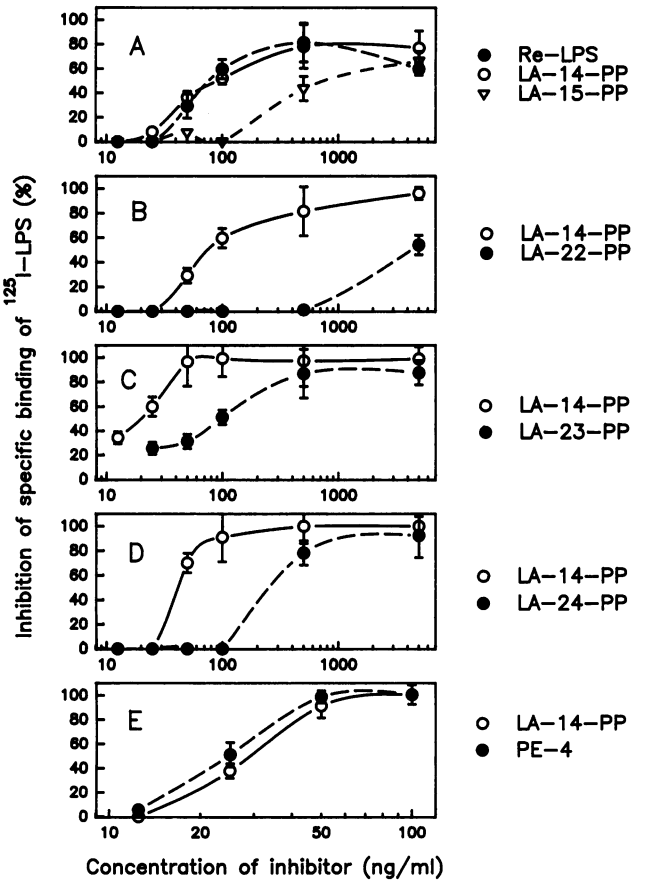

FIG. 7. Inhibition of specific binding of ^{125}I-LPS to human monocytes by synthetic lipid A and related partial structures. Human monocytes (7×10^5 per well) were incubated with ^{125}I-LPS in the presence or absence of unlabeled LPS, lipid A, or related partial structures at different concentrations as indicated. After incubation for 7 h on ice, the cells were washed four times and lysed by the addition of NaOH, and the radioactivity was measured with a gamma counter. The results are expressed as percent inhibition of specific binding of ^{125}I-LPS to the cells. Each value represents the mean ± standard deviation of three independent wells.

TABLE 1. Inhibition of ^{125}I-LPS binding by Re-LPS and anti-CD14 monoclonal antibody (MEM18)

Antagonist	^{125}I-LPS binding (cpm/well)[a]
None	1,129 ± 15
Re-LPS	
5,000 ng/ml	173 ± 50
500 ng/ml	189 ± 20
50 ng/ml	787 ± 28
5 ng/ml	1,132 ± 36
MEM18	
1:80	189 ± 18
1:320	244 ± 7

[a] Mean ± standard deviation of triplicate wells.

inactive in inducing IL-1, IL-6, and TNF release in human monocytes (4, 16, 22, 36). In addition to LA-15-PP and LA-14-PP, we have now tested several other new synthetic lipid A partial structures and investigated the inhibition of binding of ^{125}I-LPS to gain better insight into the structure-activity relationship and the mechanism of action of these structures, respectively.

Compounds LA-15-PP and LA-22-PP do not differ in the number of acyl residues but show a different distribution pattern (Fig. 1). Although this seems to be only a slight modification, our results show that LA-22-PP has a markedly lower monokine-inducing activity than LA-15-PP (Fig. 2). The reduced biological activity might be caused by a reduced affinity to a receptor(s), since LA-15-PP has a much stronger inhibitory effect on the binding of ^{125}I-LPS to monocytes than LA-22-PP does (Fig. 7). Thus, asymmetrically acylated LA-15-PP is more active than symmetrically acylated LA-22-PP, indicating that a different distribution of lipid A fatty acids results in a marked alteration of the biological activity. As already described for LPS-induced monokine production (5, 16, 21, 33, 36, 37), the biological activity of LA-15-PP as well as LA-22-PP can be inhibited by precusor Ia (LA-14-PP) (Fig. 4). The inhibitory activity of 250 ng of LA-14-PP per ml can be observed even in the presence of 100 ng of LA-15-PP or LA-22-PP per ml; this is in contrast to its inhibitory effect on LPS-induced monokine production, which can be totally blocked at a concentration as low as 1.0 ng of LPS per ml (Fig. 4). This again reflects the lower affinity of LA-15-PP and LA-22-PP to its receptor(s) in comparison with that of LPS.

The fact that compound LA-23-PP is able to induce monokines, although to a small extent, was not expected. Compound LA-23-PP represents, like LA-14-PP, a tetraacylated lipid A partial structure (Fig. 1). In contrast to LA-14-PP, it contains four residues of nonhydroxylated decanoic acid (10:0).

In addition to LA-23-PP, two other analogs of LA-14-PP, i.e., LA-24-PP and PE-4, were investigated regarding their activity. LA-24-PP carries 2 mol of nonhydroxylated decanoic acid in addition to two hydroxylated acyl residues resulted in a compound which was, like LA-14-PP, not active in inducing monokines in human monocytes (Fig. 3). However, the inhibitory activity of LA-24-PP is much less pronounced than that of LA-14-PP on LPS-induced IL-1 production (Fig. 5). This low inhibitory bioactivity is accompanied by a low capacity to inhibit the binding of ^{125}I-LPS (Fig. 7). In this respect, compound LA-24-PP is comparable to LA-15-PP and LA-23-PP.

Compound PE-4 differs from LA-14-PP in containing an α-oxyethyl(-O-CH_2-CH_2-)-linked phosphoryl group in position 1 of the reducing glucosamine residue of lipid A. From the biological point of view, the α-glycosyl phosphate group has been found to be of special significance for the biological activity of lipid A or LPS, since its absence resulted in a dramatic reduction of biological activity (26, 28). Our results indicate that the introduction of the oxyethyl linkage does not cause a considerable alteration in the activity of the compound. This was found not only with respect to the bioactivity but also when the inhibition of binding of ^{125}I-LPS to monocytes was investigated. This finding is of special importance. This novel lipid A analog can be more easily synthesized and purified and is more stable than the regular synthetic compound LA-14-PP. Furthermore, such stable α-phosphonooxyethyl glycosides make synthetic strategies more flexible and extend the possibilities to create new compounds for laboratory and clinical application.

In conclusion, the data presented here and elsewhere (reviewed in reference 26) suggest that the biological activity of lipid A and lipid A partial structures depends on the phosphorylation and acylation pattern of the hexosamine

TABLE 2. Summary of the activities of synthetic lipid A and related partial structures

Compound	No. of acyl residues	Acylation pattern R5/R4, R3/R2[a]	Induction of monokines[b]	Inhibition of monokine release[b]	Inhibition of binding of ^{125}I-LPS[b]
LA-15-PP (compound 506)	6	$C_{14}OC_{14}/C_{14}OC_{12}$, $C_{14}OH/C_{14}OH$	++	ND[b]	++
LA-22-PP	6	$C_{14}OH/C_{14}OC_{14}$, $C_{14}OH/C_{14}OC_{14}$	+	ND	+
LA-14-PP (compound 406)	4	$C_{14}OH/C_{14}OH$, $C_{14}OH/C_{14}OH$	−	+++	+++
LA-24-PP	4	$C_{10}/C_{14}OH$, $C_{10}/C_{14}OH$	−	+	++
LA-23-PP	4	C_{10}/C_{10}, C_{10}/C_{10}	+	ND	++
PE-4	4	$C_{14}OH/C_{14}OH$, $C_{14}OH/C_{14}OH$	−	+++	+++

[a] Positions of residues; see Fig. 1.
[b] Symbols indicate induction or inhibition ranging from none (−) to very strong (+++).
[c] ND, not determined.

disaccharide. Maximal monokine-inducing activity is displayed by the bisphosphorylated lipid A possessing six acyl residues, which structurally corresponds to *E. coli*-type lipid A (LA-15-PP; Fig. 1). Partial structures lacking one of these components, structures containing different constituents, or structures with a different pattern of constituents are either less or not active in inducing monokines. Although the α-glycosyl phosphate group is an important constituent for the expression of biological activity of lipid A, an introduction of an oxyethyl linkage has no considerable effect on activity. Surprisingly, we found discrepancies between the biological activity of the lipid A partial structures tested and the activity of these components to inhibit the binding of ^{125}I-LPS. If one assumes that this inhibition is of a competitive nature, there should be a correlation between inhibition of binding and affinity to a given binding structure (or receptor). This would mean that high-affinity binding of lipid A partial structures to monocytes should be associated with an optimal induction of monokine production. Our results show that this is not the case.

A discrepancy between competition in a ^{125}I-LPS binding assay and the induction of TNF and IL-6 by various lipid A partial structures has already been described in mouse macrophage-like J774.1 cells (15). These experiments lead to the conclusion that the hexosamine backbone with its two phosphoryl groups is involved in the specific binding of lipid A to a receptor, whereas the number, type, and distribution of fatty acids are critical for the induction of cytokine production (15). However, the lipid A partial structures (except PE-4) used in our experiments all constitute the same hexosamine backbone with two phosphoryl groups and have only a different acylation pattern. Therefore, only the different acylation pattern can account for the outcome of the experiment presented here. However, one should consider that lipids like LPS or lipid A are amphiphiles in aqueous solutions and form supramolecular structures (32). Certainly, the physical state of these aggregates depends on experimental conditions, which differ in our bioassays and binding assays. Therefore, one simple explanation for the different outcomes of the biological assays and the binding assays may be the different conformations of the compounds under the different incubation conditions, resulting in a variation in binding affinity. This situation becomes more complex because LPS and lipid A will also interact with binding proteins that are present in a soluble form in the serum (3, 24, 31), proteins which are known to be involved in the biological and binding activities of LPS. In view of the fact that induction of IL-1 and IL-6 was carried out in the absence of serum, while the binding experiments had to be done in the presence of serum because of experimental conditions, some control experiments were performed (data not given) that proved the effect of serum on IL-1 and IL-6 induction. We have found that also in the presence of serum, LA-15-PP but not LA-14-PP is able to induce monokine production and, furthermore, that LA-14-PP blocks LPS-induced monokine production. We, therefore, assume that the structure-activity requirements of LPS and lipid A are not affected by serum supplementation. On the other hand, several different binding structures for the binding of LPS to macrophages or monocytes have been described. CD14 is one of the LPS-binding proteins on the surface of monocytes which is involved in LPS-mediated induction of monokines (14, 29, 30, 39). As shown in Table 1, the binding of ^{125}I-LPS can be totally blocked by a monoclonal antibody against CD14 (MEM18), and, furthermore, anti-CD14 is able to abrogate LPS-induced monokine production (data not given). This indicates the central role of this antigen during binding of LPS to human monocytes and during induction of monokine production. Nevertheless, although binding of ^{125}I-LPS is CD14 dependent, one may assume that the different structure-activity requirements for binding and induction of monokines may reflect different additional binding elements in both assays. However, our experiments performed in human monocytes provide no information towards a characterization of such a binding structure, which is also involved with CD14 in our bioassays and binding assays.

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