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Biological Activity of Synthetic Phosphonooxyethyl Analogs of Lipid A and Lipid A Partial Structures

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We investigated the biological activity of four new synthetic analogs of lipid A, termed PE-1, PE-2, PE-3, and PE-4. All compounds contain an α-oxyethyl-linked (-O-CH_2-CH_2-) phosphoryl group in position 1 of the reducing glucosaminyl residue (GlcN I) of lipid A. PE-1 is a hexaacylated analog of *Escherichia coli* lipid A (compound 506). PE-2 differs from PE-1 in carrying two myristic acid residues at GlcN I. PE-3 has the same acylation pattern as PE-2, but GlcN I is present in the β anomeric form. Finally, PE-4 represents an analog of tetraacyl precursor Ia (compound 406). Structure-activity relationships of these compounds were determined by measuring their capacity to induce tumor necrosis factor alpha, interleukin 1, and interleukin 6 release by human mononuclear cells and to cause mitogenicity of murine spleen cells. The results show that replacement of the glycosidic phosphoryl residue by a phosphonooxyethyl group had no substantial effect on the biological activity of compounds. However, the anomeric configuration of GlcN I was found to be of great biological relevance, as, in general, the α anomer (PE-2) expressed high activity, and the β anomer (PE-3) expressed low mediator-inducing and mitogenic activity. The absence of the 3-hydroxyl groups within the acyl residues at GlcN I in PE-2 was found to only slightly affect the induction of monokines in human mononuclear cells compared with that of PE-1 or lipid A (506). These stable 1-phosphonooxyethyl analogs of lipid A may be candidates in the development of immunomodulators for the treatment of systemic endotoxicosis.

Lipopolysaccharides (LPS) represent the endotoxic principle of gram-negative bacteria inducing various pathophysiological effects such as fever, hypotension, and disseminated intravascular coagulation, i.e., manifestations of septicemia. The active region of LPS eliciting these severe effects is localized in the lipid A component (27). On the basis of the fact that the monokines interleukin 1 (IL-1), IL-6, and/or tumor necrosis factor (TNF) are involved in the reactions during septicemia, we and others (6, 7, 11, 14, 17, 20, 21, 28, 30, 31) have studied the structure-activity relationship of LPS, free lipid A, and defined partial structures of monokine production in vitro. 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 *Escherichia coli*-type lipid A is a very strong inducer of monokine production in human monocytes, whereas the tetraacyl precursor Ia of lipid A (compound 406) exhibited no inductive capacity (6, 17, 21, 30). This finding indicated that for the induction of monokines by human monocytes, a lipid A structure with more than four fatty acid residues is necessary.

After the effects of LPS and partial structures were analyzed, it was of interest to also investigate whether inactive structures would antagonize, in particular, monokine production by LPS. Thus, we observed that synthetic precursor Ia (compound 406) is able to inhibit effectively the LPS-induced monokine production (7, 20, 30, 31). Similar results were obtained by Kovach et al. (17) using lipid IV_A isolated from *Salmonella typhimurium* and by Takayama et al. (28) having investigated the inhibitory effect of pentaacyl diphosphoryl lipid A isolated from *Rhodopseudomonas sphaeroides* ATCC 17023. Furthermore, also the nonactive LPS of *Rhodobacter capsulatus*, possessing only four acyl residues bound to the lipid A backbone, show inhibitory activity on LPS (e.g., from *Pseudomonas diminuta*)-induced monokine production (23). These results are in line with earlier studies showing that enzymatically prepared tetraacyl LPS inhibits neutrophil-endothelial interactions induced by LPS (24).

We postulate a competitive mechanism to be responsible for the inhibition of LPS-induced monokine production by precursor Ia (7). This mechanism was also assumed by Hampton et al. (14), who showed an inhibition in the binding of LPS to scavenger receptors by lipid IV_A.

The observation that nonactive LPS or lipid A partial structures largely lack cytokine-inducing capacities in human monocytes, but are able to modulate cytokine release, can be regarded as a potential step forward in the use of such derivatives as endotoxin antagonists in vivo. Consequently, in recent studies, partial structures of lipid A have been shown to protect animals from death due to endotoxin (2, 12, 13, 25). In the development of new antagonists, i.e., lipid A partial structures, problems were encountered with the introduction of the α-bound glycosidic phosphates. These difficulties have prompted the synthesis of phosphonooxyethyl analogs (18, 19), which offer the additional advantage that the oxyethylphosphate linkage is more stable than that of the glycosidic phosphate.

In the present paper, we compared the biological activities of four such lipid A analogs, which have an α- or β-oxyethyl-linked (-O-CH_2-CH_2-) phosphoryl group instead of an α-gly-

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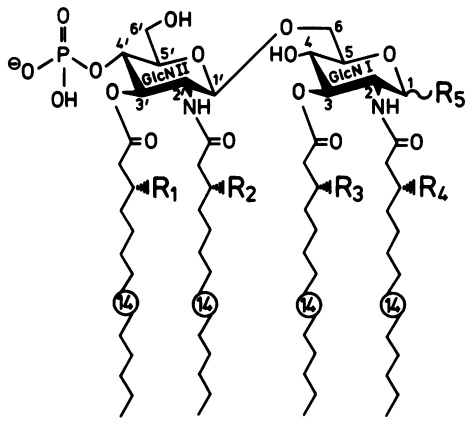

Compound	R_1	R_2	R_3	R_4	R_5
E.coli: Lipid A (compound 506)	14 : 0	12 : 0	OH	OH	α - O - Ⓟ
PE - 1	14 : 0	12 : 0	OH	OH	α - O - $(CH_2)_2$ - O - Ⓟ
PE - 2	14 : 0	12 : 0	H	H	α - O - $(CH_2)_2$ - O - Ⓟ
PE - 3	14 : 0	12 : 0	H	H	β - O - $(CH_2)_2$ - O - Ⓟ
Precursor Ia (compound 406)	OH	OH	OH	OH	α - O - Ⓟ
PE - 4	OH	OH	OH	OH	α - O - $(CH_2)_2$ - O - Ⓟ

FIG. 1. Chemical structures of the synthetic lipid A and analogs used. The numbers in circles indicate the number of carbon atoms in acyl chains.

cosyl phosphate group, with that of synthetic lipid A (compound 506). We will show that the oxyethyl linkage of the phosphate group has only minor influences on the biological activity of synthetic lipid A analogs compared with *E. coli* lipid A. Furthermore, this is the first report showing that the anomeric center of the reducing glucosidic residue of lipid A plays an important role in the expression of endotoxic activity. It will be shown that compounds with a β glycosidically bound phosphonooxyethyl group were, in general, significantly less active than counterparts with an α glycosidically bound phosphonooxyethyl group.

MATERIALS AND METHODS

LPS, synthetic lipid A, precursor Ia, and lipid A analogs. The chemical structures of the compounds used in this study are given in Fig. 1. Synthetic hexaacyl *E. coli*-type lipid A (compound 506 or LA-15-PP) 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 (Fig. 1). It was synthesized as described previously (15). Synthetic tetraacyl lipid A precursor Ia (compound 406 or LA-14-PP) represents a partial structure of compound 506 lacking dodecanoic and tetradecanoic acid (Fig. 1). Compound 406 was synthesized as described elsewhere (16). Compounds PE-1, PE-2, PE-3, and PE-4 carry an oxyethyl-linked (-O-CH_2-CH_2-) phosphoryl group in position 1 of the reducing glucosaminyl residue (GlcN I) of lipid A (Fig. 1). PE-1 is a hexaacylated analog of compound 506. PE-2 differs from PE-1 in having nonhydroxylated fatty acids at GlcN I. PE-3 is structurally identical to PE-2 except that the reducing glucosamine residue of the lipid A backbone (GlcN I) is present in the β anomeric form; PE-4 constitutes an analog of compound 406 (Fig. 1). These phosphonooxyethyl analogs of lipid A were synthesized as described previously (19). All synthetic compounds were stored in aliquots of 1 mg/ml at 4°C.

The synthetic compounds were thoroughly purified prior to the final step of synthesis to remove all the protecting groups by catalytic hydrogenolysis. After deprotection, the target products were purified either by preparative thin-layer chromatography (PE-1, 2, 3, and 4) or medium-pressure column chromatography (compound 506 and 406) on silica gel. The purity of the product was confirmed by silica gel thin-layer chromatography. Besides, mutual contamination of preparations can be absolutely excluded by the present synthetic approaches. LPS was extracted from *Salmonella friedenau* and purified by the phenol-chloroform-ethanol method of Galanos et al. (9). Such preparations contain less than 0.2% protein and nucleic acids, as determined by chemical analysis.

Induction of TNF, IL-1 and IL-6 in hMNC. Human mononuclear cells (hMNC) from healthy donors were isolated by density gradient centrifugation (29) with Ficoll-Paque (Pharmacia, Uppsala, Sweden). After being washed three times, hMNC (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, hMNC were treated for 1 h with the first stimulus (compound 406 or PE-4), after which the second stimulus (LPS) was added. After incubation for 12 h, supernatants were harvested and analyzed for cytokine activity immediately or stored at −20°C.

TNF cytotoxicity assay using L929 cells. TNF-sensitive L929 cells derived from a transformed cell line of a C3H mouse fibrosarcoma (5) were grown in permanent culture in Falcon tissue culture flasks (75 cm^2) with RPMI 1640 containing antibiotics and 10% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany) (30 ml per flask at 1.6×10^4 cells per ml) and subcultured every 3 days. For the TNF assay, L929 cells derived from 3-day-old cultures were harvested by trypsin treatment and adjusted to a concentration of 10^6 cells per ml in RPMI 1640 containing antibiotics and 5% FCS. Test samples (50 μl per well) were added to this medium (100 μl per well) and further diluted in five steps (1:3) in flat-bottom microtiter plates (Becton Dickinson, Heidelberg, Germany). Actinomycin D (50 μl per well) at a concentration of 4 μg/ml and the cell suspension (50 μl) were added. After incubation for 19 h (5% CO_2, 37°C), the supernatants were discarded, and the plates were washed with lukewarm water, which had previously been shown not to affect the L929 cell assay. The remaining cells were fixed (3 min) with formaldehyde (3%), the plates were washed again, and the cells stained with crystal violet for 10 min. After being washed, the stained cells were lysed in 0.5% sodium dodecyl sulfate. A_{550} was measured with a microplate reader (MR 700; Dynatech, Denkendorf, Germany). TNF activity in the samples was determined by using a standard preparation of recombinant human TNF (kindly provided by BASF/Knoll AG, Ludwigshafen, Germany) containing 40 IU/ml, for comparison of the dilutions giving a cytotoxicity of 50% by

probit analysis (10). The results are given in units of TNF activity per milliliter.

IL-1 proliferation assay using human fibroblasts. Human dermal fibroblasts were used for IL-1 detection as described previously (22). Dermal fibroblasts isolated from human foreskin were grown in permanent culture in 80-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium (Biochrom KG) containing 10% 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 and 0.2%, respectively; Biochrom KG) treatment after 7 days of culture. Cultures containing 100 μl of 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 DMEM containing L-glutamine, antibiotics, and 10% FCS. Test samples (33 μl per well) were added and further diluted in five steps (1:4). A standard of recombinant human IL-1β (kindly provided by Glaxo/Biogen S.A., Geneva, Switzerland) containing 100 IU/ml was used as positive control. After an incubation time of 96 h, the number of cells was determined by the crystal violet method as described above for TNF. IL-1 activity in the samples was calculated by using a standard of recombinant IL-1β and comparing the dilutions which gave a proliferation of 30% by probit analysis (10). The results are given in units of IL-1 activity per milliliter.

IL-6 proliferation assay using 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, originally established by L. Aarden (Central Laboratory, Blood Transfusion Service, Amsterdam, The Netherlands) (1), were cultured in RPMI 1640 containing antibiotics, 10% FCS, 5×10^{-5} M 2-mercaptoethanol, and 50 U of human IL-6 per ml. The assay was carried out in 96-well flat-bottom microtiter plates in a 200 μl-volume per well with 10^4 B9.9-3A4 cells per well. Test samples (33 μl per well) were added to 100 μl of culture medium and further diluted in seven steps (1:4). A standard preparation of IL-6 giving 150 IU/ml was used as 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 per well of acidified isopropanol (0.04 N HCl in isopropanol), and formazan was dissolved by vigorous mixing with a stirring machine. A_{550} was measured with a microplate reader (MR 700; Dynatech). IL-6 activity in the samples was determined by using a standard preparation of IL-6 (150 IU/ml), and comparing the dilutions which gave a proliferation of 50% by probit analysis (10). The results are given in units of IL-6 activity per milliliter.

Stimulation of murine spleen cells. Murine spleen cells were isolated and cultured as described elsewhere (4). In brief, mice were subjected to cervical dislocation, and the spleen was removed and teased with forceps in ice-cold RPMI 1640 medium containing antibiotics. Single cells were separated from tissue fragments by filtration through a stainless steel mesh and washed three times with RPMI 1640 medium. Spleen cells (2×10^6/ml) were cultured in a total volume of 150 ml in RPMI 1640 medium supplemented with 5% heat-inactivated (56°C, 30 min) fresh human serum in round-bottom microtiter plates. After 3 days of culture, the cells were pulsed with [^{3}H]thymidine ([^{3}H]TdR) for the last 5 h of culture, harvested on glass fiber filters, and counted in a liquid scintillation spectrometer. The results are expressed in counts per minute per culture.

Pyrogen assay. Pyrogen assays were performed as described elsewhere (26). Briefly, fever responses of rabbits were measured rectally with thermistor probes connected to a recording temperature-measuring device. Pyrogenicity was quantitated by determining the minimal pyrogenic dose causing a 0.6°C rise in temperature 3 h after intravenous injection, defined as MDP-3.

RESULTS

Induction and modulation of monokine production in human mononuclear cells by phosphonooxyethyl analogs of lipid A. In the first set of experiments, we tested the capacity of phosphonooxyethyl analogs of lipid A (PE-1, PE-2, PE-3, and PE-4) to induce IL-1, IL-6, and TNF in hMNC and compared their active doses with those of synthetic *E. coli* lipid A (compound 506) and synthetic precursor Ia (compound 406). First, hMNC were stimulated with different concentrations of compound 506, 406, PE-1, PE-2, PE-3, or PE-4. After 12 h of incubation, the supernatants were harvested and tested for IL-1, IL-6, and TNF activity. When the response of hMNC to compound 506 was compared with the response of the cells to preparation PE-1, it was found that a substitution of the α-glycosyl phosphate group with a α-phosphonooxyethyl group resulted in a somewhat similar dose response curve (Fig. 2A, C, and E): maximum IL-1, IL-6, and TNF release was found at 10 ng/ml, and the response was slightly diminished at higher concentrations. The amount of IL-1, IL-6, and TNF activity was somewhat higher after stimulation with compound PE-1. Stimulation of monokine production by PE-2 (PE-2 differs from PE-1 by having nonhydroxylated tetradecanoic acids at GlcN I) showed a maximal response at 10 to 100 ng/ml (Fig. 2B, D, and F). However, the amount of monokines released after stimulation with PE-2 is lower than that with 506 or PE-1. PE-3 (the β anomeric counterpart of PE-2) was significantly less active than PE-2, requiring at least 1,000 ng/ml for stimulation of monokine production (Fig. 2B, D, and F). Finally, we found that PE-4 (the phosphonooxyethyl analog of compound 406) is, like 406, not active in all doses tested in inducing monokine production (Fig. 2B, D, and F).

This last result raised the question of whether preparation PE-4, like compound 406, is able to inhibit LPS-induced monokine production by hMNC. To test this, hMNC were first incubated for 1 h in the absence or presence of PE-4 or compound 406 at a concentration of 5, 50, or 1,000 ng/ml. Thereafter, the cells were stimulated with different amounts of LPS as indicated in Fig. 3, and the IL-1, IL-6, and TNF activity in the culture supernatants was assayed after a period of 12 h. The results show that PE-4 behaved similarly to compound 406 in modulating LPS-induced monokine production (Fig. 3): LPS-induced TNF release was suppressed by compound 406 or PE-4 at a concentration of 50 ng/ml or higher. The same results were found when the effects of compound 406 or PE-4 were investigated with regard to IL-1 and IL-6 release. Different effects were obtained when low amounts (5 ng/ml) of compound 406 or PE-4 were used during preincubation: stimulation of monokine production with 0.01 or 0.1 ng of LPS per ml was inhibited by these synthetic compounds at all concentrations (Fig. 3A, C, and E). However, preincubation with compound PE-4 or compound 406 at a concentration of 5 ng/ml

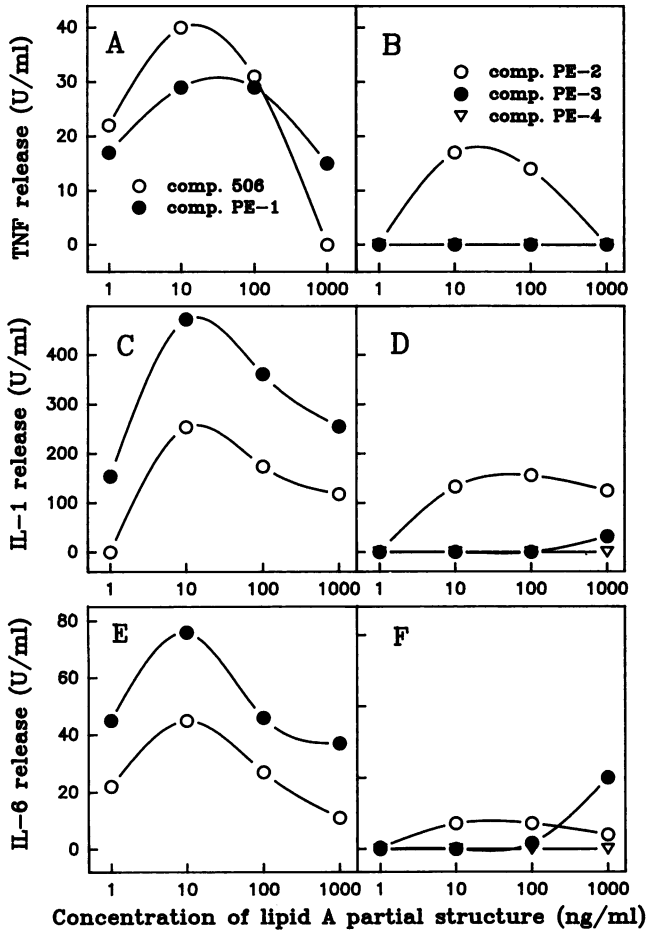

FIG. 2. Induction of monokines in hMNC by different synthetic lipid A's and analogs. hMNC (4×10^6/ml) were stimulated with various synthetic lipid A's or analogs as indicated. After 12 h of incubation, the supernatants were harvested and assayed for TNF, IL-1, and IL-6 activity in bioassays. The results are expressed as units of TNF, IL-1, or IL-6 per ml, respectively. Each value represents the mean of three independent cultures. Standard deviation was less than 15%.

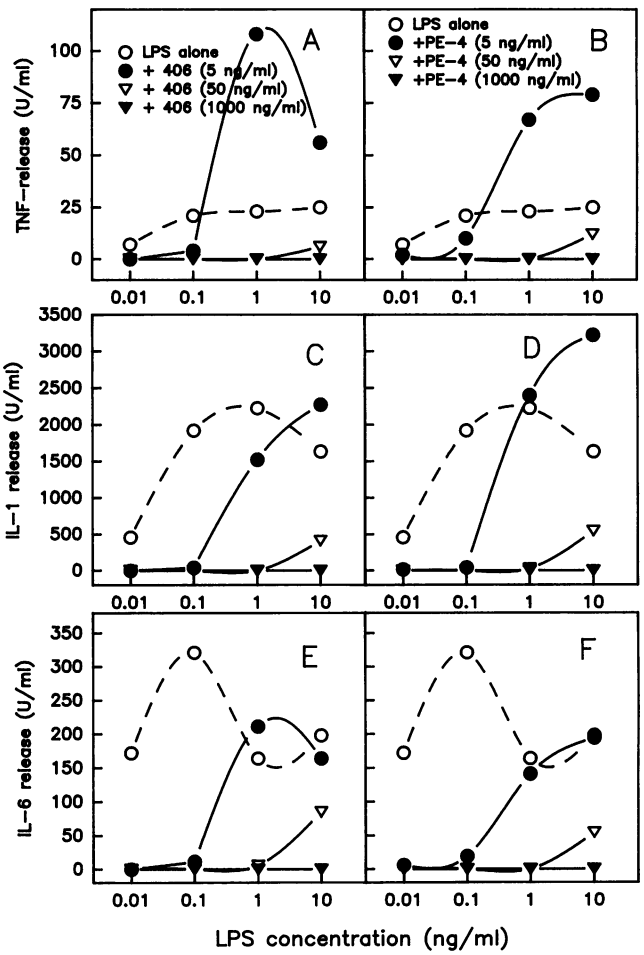

FIG. 3. Inhibition of LPS-induced monokine production by compound 406 or PE-4. hMNC (4×10^6/ml) were preincubated in the presence of different amounts of compound 406 or PE-4 as indicated. After 1 h, LPS was added at different concentrations. For further details, see the legend to Fig. 2.

and stimulation with LPS at a concentration of greater than 0.1 ng/ml, i.e., 1 or 10 ng/ml, did not result in an inhibition of monokine production. When TNF production was investigated under these conditions, a marked enhancement was seen with both compounds 406 and PE-4 (Fig. 3A and B).

Stimulation of mouse spleen cells by phosphonooxyethyl analogs of lipid A. These experiments were done to investigate the induction of DNA synthesis of mouse spleen cells by different phosphonooxyethyl analogs of lipid A and to compare the effects with the activity of compounds 506 and 406. Murine spleen cells were stimulated with different amounts of compounds PE-1, PE-2, PE-3, PE-4, 406, or 506, and the [^{3}H]TdR incorporation was determined after 3 days of culture. No stimulatory activity was detectable by these compounds at concentrations up to 0.1 μg/ml, but all compounds showed a stimulatory activity when a dose of 1 μg/ml or more was used (Fig. 4). When the activity of compound 506 was compared with that of the PE series, it was found that preparations PE-1 and PE-2 are as active as compound 506. However, stimulation with PE-3, the β anomeric isomer of PE-2, resulted in a response of the cells which was reduced to about 50% compared with that induced by compounds 506, PE-1, and PE-2 (Fig. 4A).

When comparing the responses of murine spleen cells to compounds PE-4 and 406, we observed an approximately 2-fold higher [^{3}H]TdR incorporation after stimulation with PE-4 (Fig. 4B). PE-4 is almost as active as compound 506, whereas compound 406 is less active than 506 (Fig. 4A and B).

Pyrogenicity of phosphonooxyethyl analogs. The fever-inducing capacity of synthetic compounds PE-1, PE-2, PE-3, 406, and 506 in rabbits was analyzed. Compounds 506 and PE-1 expressed comparable pyrogenicity, their MPD-3 being 0.1 μg/kg of body weight. Compound PE-2 was slightly less active (MPD-3, 1.0 μg/kg), whereas compound PE-3 had significantly lower activity; with a dose of 10 μg/kg, only a monophasic fever peaking 1 h after injection was observed, indicating that the MPD-3 was equal to or higher than 1,000 μg/kg. Compounds PE-4 and 406 were of comparable activity, the minimal pyrogenic dose of the latter having been estimated as 100 μg/kg (8).

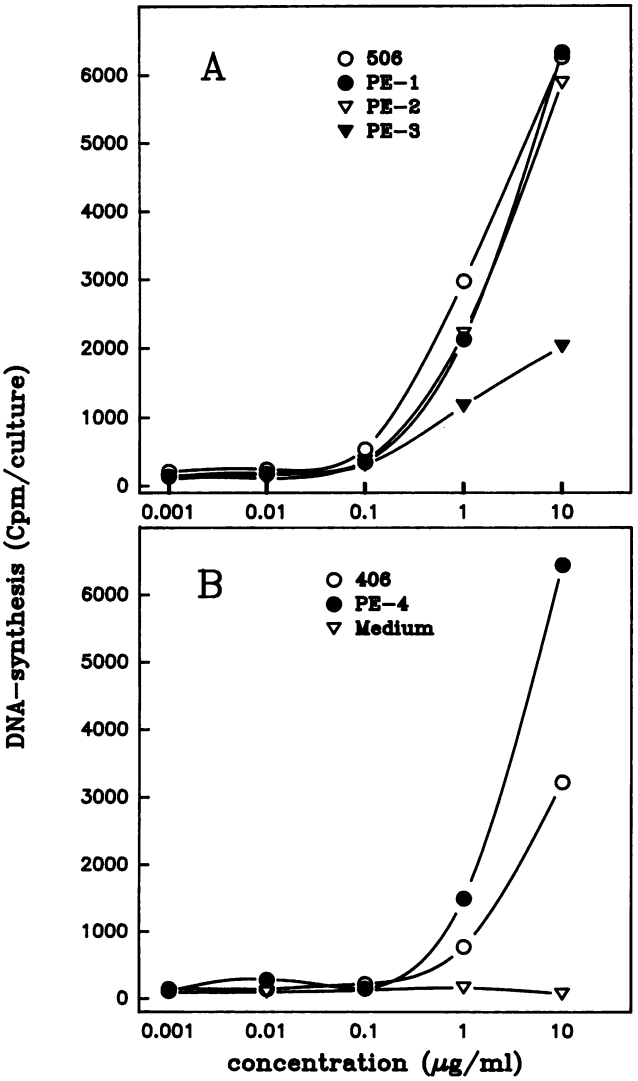

FIG. 4. Mitogenicity of synthetic lipid A and analogs in murine spleen cells. Murine spleen cells (2×10^6/ml) were stimulated by various synthetic lipid A's and analogs as indicated. After 3 days of culture the cells were pulsed with [^{3}H]TdR for the last 5 h. The DNA synthesis of the cells is expressed as counts per minute per culture. Each value represents the mean of triplicate culture; the standard deviation was less than 10%.

DISCUSSION

Structure-activity relationships of lipid A, lipid A partial structures, and analogs have shown that the bioactivity of these compounds depends on both the pattern of acylation and phosphorylation (for a review, see reference 27). In view of this role of phosphate, it was of great interest to investigate the biological activity of four novel lipid A analogs (PE-1, PE-2, PE-3, and PE-4) in which the α-phosphate is not directly bound to GlcN I but linked over an α- or β-oxyethyl (-O-CH_2-CH_2-) bridge to GlcN I of the lipid A backbone.

Our results show that replacement of the glycosyl phosphate by a phosphoric ester in a phosphonooxyethyl glycoside resulted in no significant reduction of the biological activity of synthetic lipid A or analogs: compound PE-1 had even a slightly higher biological activity than compound 506 in the induction of monokines in hMNC. It expressed more-comparable mitogenic activity in murine spleen cells and pyrogenicity in rabbits (Fig. 2 and 4). Similar conclusions were reached in comparing the anti-tumor reactivity of these synthetic compounds (18, 19).

Thus, replacement of the α-glycosyl phosphate by an α-phosphonooxyethyl group does not cause loss of bioactivity. However, when comparing the endotoxic activity of the α anomeric PE-2 with that of the β anomeric PE-3 isomer, it became obvious that α-orientation is important for the expression of biological activity. This result provides, for the first time, evidence that the anomeric form of GlcN I is of importance in endotoxicity as determined by monokine release, B-cell mitogenicity, and pyrogenicity.

We have previously shown that phosphoryl groups play an important role in the epitope specificity of anti-lipid A antibodies (3). It will be of great interest to analyze whether bisphosphoryl lipid A-recognizing antibodies react equally well with (e.g.) compounds 506 and PE-1 or 406 and PE-4 or whether, in this case, serological differences are seen.

Of particular importance is our present finding that, like compound 406, compound PE-4 inhibits LPS-induced monokine production. Since the mechanism of this inhibition is believed to be based on a competitive inhibition of LPS binding, this result indicates that recognition of LPS or partial structures by the cellular receptor(s) involved is not influenced by replacement of the α-glycosidic phosphoryl by the α-glycoside phosphonooxyethyl group.

Our results show that there is a discrepancy in the response of compound 406 or PE-4 in human and murine or rabbit assays: whereas PE-4 and compound 406 were pyrogenic and mitogenic in rabbit and mice assays, respectively, both compounds were antagonists in the human monokine-inducing assays performed. However, murine cells (11) and cat, dog, guinea pig, pig, and rabbit cells (our own unpublished observations) also produce monokines in vitro in response to compound 406. Therefore, the discrepancy is due to the different species used in the assays rather than to the different assay systems.

The results presented in this paper justify the hope that, based on the architectural principle of the readily accessible structure PE-4, new and potent analogs which inhibit endotoxin effects and which may prove to be of therapeutic value in endotoxemia and related shock states may be developed.

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