INFECTION AND IMMUNITY, Nov. 1986, p. 465–471
0019-9567/86/110465-07$02.00/0

Vol. 54, No. 2

Inability of Pyrogenic, Purified *Bordetella pertussis* Lipid A to Induce Interleukin-1 Release by Human Monocytes

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Received 7 April 1986/Accepted 12 August 1986

Free lipid A of *Bordetella pertussis*, *Neisseria meningitidis*, and *Escherichia coli* lipopolysaccharide (LPS) was prepared by hydrolysis in acetate buffer (pH 4.5); in addition, lipid A from *B. pertussis* and *E. coli* was prepared by hydrolysis in mineral acid (HCl). The precipitates obtained were purified by extraction methods in toluene-methanol and are referred to as crude lipid A. Purified lipid A from *N. meningitidis* and *B. pertussis* was obtained by extraction in a mixture of chloroform-methanol-water-triethylamine. The different preparations were tested for their pyrogenicity (endogenous pyrogen; EP) and their capacity to trigger the release of interleukin-1 (IL-1; previously known as lymphocyte-activating factor; LAF) by human monocytes. Crude lipid A from *E. coli* and *N. meningitidis* were both IL-1 inducers. Crude *B. pertussis* lipid A (acetate buffer; pH 4.5), which contains a β-1-6-linked D-glucosamine disaccharide, two phosphoryl groups, and five fatty acids, was pyrogenic and an IL-1 inducer (EP^+/LAF^+); but crude *B. pertussis* lipid A (0.25 N HCl), which lacked the glycosidic phosphoryl group, was 1,000-fold less pyrogenic than the diphosphorylated lipid A, yet it retained its IL-1-inducing capacity (EP^-/LAF^+). Purified *N. meningitidis* lipid A was not an inducer of IL-1 release and purified *B. pertussis* lipid A exhibited identical pyrogenicity as the parent LPS but was devoid of any IL-1-release inducing capacity (EP^+/LAF^-). These results demonstrate that for some endotoxins, purified lipid A is unable to induce IL-1 release by human monocytes; however, it is pyrogenic, supporting the hypothesis that IL-1 and EP are induced by different determinants on the LPS molecule.

Interleukin-1 (IL-1), previously known as lymphocyte-activating factor (LAF), is a key mediator of the immune response (for a review, see reference 8). This mediator can be released by monocytes and macrophages on stimulation by bacterial lipopolysaccharide (LPS) (18). IL-1 also regulates nonimmunological responses such as fever (8) and has been suggested by several investigators (13, 20) to be identical to endogenous pyrogen (EP), the causative agent of LPS-induced fever. This conclusion was based on the fact that EP and LAF (IL-1) activities are copurified and share common physical characteristics (8). It was reported by Damais et al. (7), however, that certain Muramyl dipeptide (MDP) derivatives that were not pyrogenic were LAF (IL-1) inducers, demonstrating a dissociation between these two activities. In this study, we observed a similar dissociation of these activities with LPS derivatives. Most of the biological activities of endotoxins have been ascribed to the lipid A moiety. The chemical structure of lipid A of members of the family *Enterobacteriaceae* has been elucidated from a detailed investigation of salmonellae and *Escherichia coli*, and synthetic lipid A and various lipid A analogs have become available (6, 14, 15). From a consideration of previously published data, it can be concluded that lethal toxicity, B-lymphocyte mitogenicity, induction of the Shwartzman phenomenon, and pyrogenicity are mediated by the lipid A moiety of the LPS molecule. This moiety contains a β-1,6-linked D-glucosamine disaccharide bearing phosphoryl groups in positions 1 and 4′ and ester- and amide-bound fatty acids (11). These data demonstrate that the isolated lipid A moiety of some LPS molecules, in accordance with results of other studies, is responsible for B-cell mitogenicity and EP induction but is unable to induce IL-1 release by human monocytes.

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MATERIALS AND METHODS

Endotoxins and derived fragments. *Neisseria meningitidis* A85 LPS, kindly donated by D. Schulz (Institut Mérieux, Lyon, France), and *Bordetella pertussis* LPS were extracted by the procedure described by Westphal et al. (21) and purified as described previously (12). *E. coli* Re mutant F515, extracted by the procedure described by Westphal et al. (21), was kindly donated by H. Brade (Forschungsinstitut-Borstel, Borstel, Federal Republic of Germany). A *B. pertussis* crude lipid A preparation isolated by hydrolysis with 0.25 N HCl for 30 min at 100°C was purified as described previously (12). Lipid A isolated from *B. pertussis* and *N. meningitidis* LPS by hydrolysis at 100°C for 1 h in sodium acetate buffer (10 mM, pH 4.5) in the presence of 1% sodium dodecyl sulfate (SDS) was purified as follows. SDS was extracted with acidified ethanol after lyophilization, and the material was dissolved in water and centrifuged (150,000 × *g* for 1 h) (the absence of SDS in lipid A was assessed with labeled SDS). The lipid A component was extracted from the pellet in a mixture of acidified toluene-methanol (9:1) and is referred to as *B. pertussis* and *N. meningitidis* crude lipid A. Purified lipid A was obtained by filtration in a mixture of chloroform-methanol-water-triethylamine (30:14:2.5:0.1) (M. Caroff, A. Tacken, and S. Szabo, submitted for publication). The absence of LPS or polysaccharide contamination in purified lipid A preparations was ascertained by thin-layer chromatography (TLC; precoated TLC aluminum sheet, silica gel 60; E. Merck AG, Darmstadt, Federal Republic of Germany; solvent, chloroform-methanol-water-triethylamine) and was confirmed by the absence of heptose

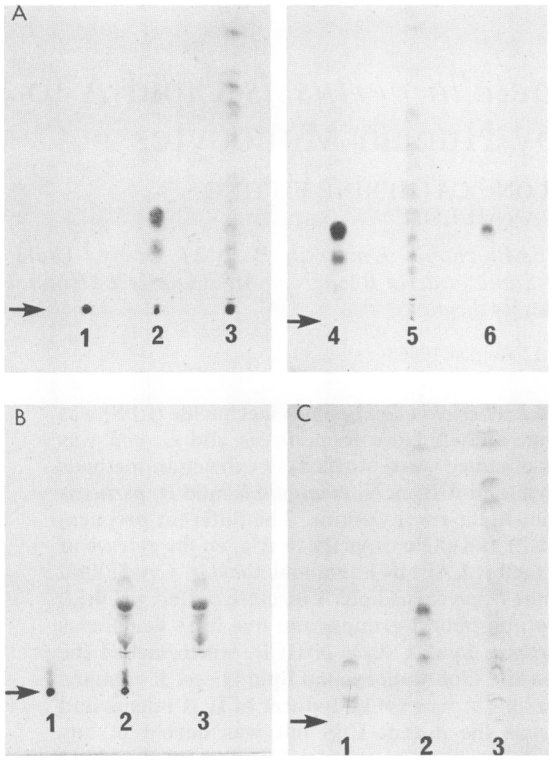

FIG. 1. TLC of LPS and lipid A preparations from different gram-negative bacteria. (A) *B. pertussis*. Lane 1, LPS; lane 2, crude lipid A liberated by hydrolysis of LPS in acetate buffer (pH 4.5); lane 3, crude lipid A liberated from LPS by 0.25 N HCl hydrolysis; lane 4, purified lipid A (acetate buffer [pH 4.5]); lane 5, purified lipid A (0.25 N HCl); lane 6, homogenous purified lipid A (T2) (see text). (B) *N. meningitidis*. Lanes 1, 2, and 3 correspond, respectively, to LPS, crude lipid A liberated from LPS by hydrolysis in acetate buffer (pH 4.5), and purified lipid A (acetate buffer [pH 4.5]). (C) *E. coli*. Lanes 1, 2, and 3 correspond, respectively, to LPS, crude lipid A liberated by hydrolysis of LPS in acetate buffer (pH 4.5), and crude lipid A liberated by hydrolysis of LPS in 0.1 N HCl. Arrows correspond to origin.

residues, as determined by gas-liquid chromatography. The lipid A purified from *B. pertussis* analyzed by TLC contained two compounds (T1, T2) which were separated by preparative TLC. *E. coli* lipid A obtained after hydrolysis of LPS at 100°C for 2 h in sodium acetate buffer (10 mM, pH 4.5) or for 1 h in 0.1 N HCl was centrifuged at 150,000 × *g* for 1 h; the washed pellets are referred to as *E. coli* crude lipid A. Analytical data (glucosamine, phosphorus, and fatty acid content) for *B. pertussis* lipid A was determined as described previously (12) by using colorimetric methods.

IL-1 induction and LAF (IL-1) assay. Human mononuclear adherent cells (5×10^5 nonspecific esterase-stained [NSE^+] cells per well) cultured in RPMI 1640 medium without serum were incubated for 24 h in the presence or absence of IL-1 inducers. LAF (IL-1) activity was determined by [^{3}H]thymidine uptake by C3H/HeJ mice thymocytes in the presence of a suboptimal dose of concanavalin A (0.075 μg per assay) as described previously (12). The standard deviation did not exceed 15%. All experiments were performed with at least five different donors.

Mitogenicity. Stimulation of BALB/c mouse splenocytes was carried out as described previously (5). Briefly, 5×10^6 cells per well (96-well microplates; Nunc, Roskilde, Denmark) were cultured in RPMI 1640 medium (supplemented with antibiotics and 2% fetal calf serum) for 48 h at 37°C in an atmosphere of 7% CO_2–93% air. Seven hours before harvesting, 0.25 μCi of [^{3}H]thymidine was added per well. The standard deviation did not exceed 15%.

Pyrogenicity. Pyrogenicity was measured by standard procedures, using New Zealand albino rabbits (weight, 2.5 to 2.7 kg) in groups of three for each substance to be tested. Each animal received intravenously 1 ml of saline containing the substance to be tested per kg. Rectal temperatures were monitored continuously for either 3 or 6 h, and the maximal increase of temperature was recorded for each animal, as described previously (2).

RESULTS

Crude and purified lipid A preparations isolated from *B. pertussis*, *N. meningitidis*, and *E. coli* LPS. Lipid A was isolated by standard procedures (i.e., cleavage of the 2-keto-3-deoxyoctonic acid [KDO] ketosidic bond and centrifugation, followed by washing of the pellet), leading to lipid A preparations contaminated by LPS that was degraded to various degrees. Crude lipid A preparations from *B. pertussis* endotoxin contained about 5% LPS, according to analysis by SDS-polyacrylamide gel electrophoresis. Lipid A preparations from *B. pertussis* and *N. meningitidis* LPS devoid of LPS contaminants were obtained only when the standard procedure was followed by filtration of the lipid A in a chloroform-methanol-water-triethylamine mixture (Fig. 1). We further observed that mineral acid hydrolysis produced greater lipid A degradation than hydrolysis with sodium acetate buffer (pH 4.5), as was expected.

B. pertussis lipid A obtained by hydrolysis of the LPS in sodium acetate buffer (pH 4.5) contained two compounds which were separated by preparative TLC. The major compound (T2) contained 1.08 μEq of phosphorus, 1.00 μEq of glucosamine, and 2.60 μEq of fatty acids per mg (the lipid preparation obtained before preparative TLC contained 1.14 μEq of phosphorus, 1.13 μEq of glucosamine, and 2.96 μEq of fatty acids per mg). The purified lipid A preparation isolated after mineral hydrolysis of LPS (0.25 N HCl, 30 min, 100°C) contained 0.64 μEq of phosphorus, 1.17 μEq of glucosamine, and 2.8 μEq of fatty acids per mg. Based on the *B. pertussis* lipid A structure (4), the purified lipid A preparation, as well as compound T2 obtained after cleavage of the KDO ketosidic bond by acetate buffer (pH 4.5), contains a glucosamine disaccharide with two phosphates in positions 1 and 4′ and fatty acids, whereas the purified lipid A obtained by mineral hydrolysis (0.25 N HCl) contains a mixture of fragments derived from the original structure; all of the compounds lost the glycosidic phosphate and some of the acid-labile fatty acids. In the case of *N. meningitidis*, (Fig. 1B) and *E. coli* (Fig. 1C), lipid A preparations obtained after centrifugation were contaminated with LPS that was degraded to various degrees. The LPS contamination was removed from the *N. meningitidis* preparation by the same procedure as described above for *B. pertussis*.

IL-1 activity in the supernatants of human adherent cells stimulated with crude lipid A preparations. The crude lipid A preparations isolated from *E. coli* and *B. pertussis* induced secretion of LAF (IL-1) to levels similar to those observed with native LPS (Fig. 2). The activity of the different lipid A preparations tested was not dependent on the acidic conditions used to release the lipid A (acetate buffer [pH 4.5], 0.1 N HCl, or 0.25 N HCl). The LPS of *Salmonella minnesota* Re mutant R595 and *E. coli* F515, the inner core of which is composed of only two KDO acid residues (3, 19), was also able to induce IL-1 secretion.

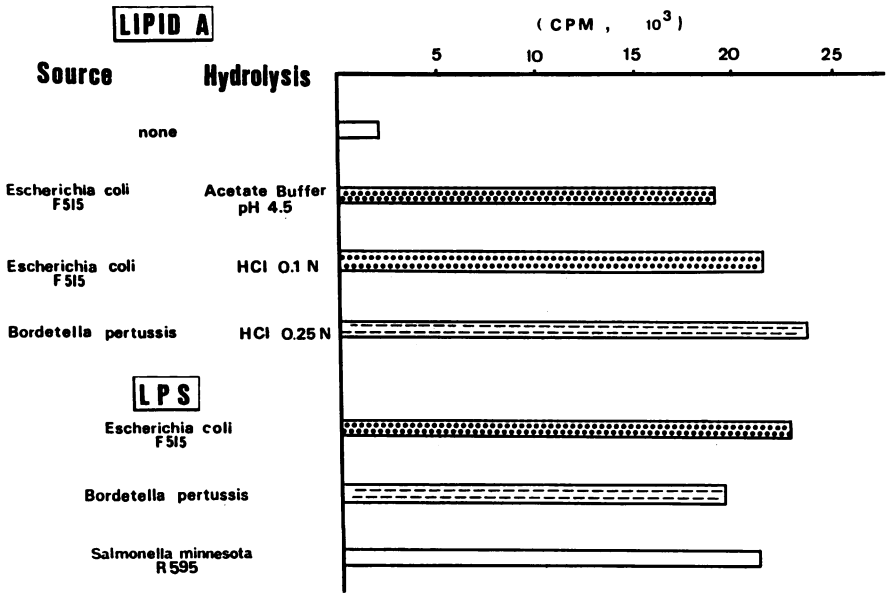

FIG. 2. IL-1 activity in culture supernatants (1/10) of human mononuclear adherent cells stimulated with LPS (10 μg per assay) or crude lipid A (10 μg per assay) from different gram-negative bacteria. LPS and crude lipid A were suspended in RPMI 1640 medium and sonicated for the time required to obtain an apparently homogeneous dispersion. The IL-1 activity was assayed by measuring the [^{3}H]thymidine incorporation by C3H/HeJ thymocytes in the presence of 0.075 μg of concanavalin A. Results are expressed as the mean counts per minute of triplicate cultures (standard deviations did not exceed 15%). The values shown are those of an experiment representative of five separate experiments performed with monocytes from different donors.

IL-1 activity in the supernatants of human adherent cells stimulated with purified lipid A preparations. Purified lipid A isolated from *B. pertussis* (Fig. 3A and B) or *N. meningitidis* (Fig. 3C), which was devoid of LPS degraded to various degrees, was unable to induce IL-1 release. When the *N. meningitidis* lipid A preparation was converted to its more soluble triethylamine salt form, the inability to induce IL-1 release remained unchanged (Fig. 3C). Similar results were observed with *B. pertussis* lipid A (Fig. 3B). Conversion of native LPS, or crude lipid A, to the triethylamine salt form did not change their IL-1-inducing capacities, thereby excluding a negative effect of triethylamine on the soluble purified lipid A (data not shown). The lack of IL-1 induction by pure *B. pertussis* lipid A was not due to an increased production of prostaglandins, because similar results were observed when the cultures were performed in the presence of indomethacin (Table 1). The IL-1 released by human monocytes stimulated by 10 μg of *B. pertussis* LPS per assay was variable among the 17 donors tested but significant, whereas the IL-1 activity released by monocytes stimulated by 10 μg of pure lipid A per assay remained very low (Fig. 4). IL-1 activity, measured in cell lysates from monocytes stimulated by 10 μg of pure lipid A per assay, reached higher levels than activity measured in supernatants obtained from the same monocytes. However, the results for intracellular IL-1 were more variable than for released IL-1 (Table 2).

Mitogenicity of the crude and purified lipid A preparations. The mitogenic activities of the crude and purified lipid A preparations isolated from *B. pertussis* and *N. meningitidis* on mouse splenocytes are shown in Fig. 5. Both crude and purified lipid A preparations elicited a similar dose-dependent mitogenic response which was maximal with 10 μg per assay.

Pyrogenicity of different *B. pertussis* lipid A preparations. The purified diphosphorylated lipid A preparation isolated from *B. pertussis* (acetate buffer [pH 4.5], 1% SDS, 1 h, 100°C) induced a fever response which was similar to that obtained with intact LPS (Fig. 6). In Table 3 it is shown that the pyrogenicity of the different lipid A preparations was very different and varied according to the hydrolysis method used to liberate the lipid A. Thus, crude lipid A (which is a LAF [IL-1] inducer) or purified lipid A (which is not a LAF [IL-1] inducer) obtained after mild acid hydrolysis and containing two phosphate groups attached to the glucosamine disaccharide were as pyrogenic as native LPS. The minimal dose leading to a pyrogenic effect for all three compounds was 0.002 μg/kg. Crude and purified preparations of lipid A obtained after mineral acid hydrolysis (0.25 N HCl), which lead to potent and impotent monophosphorylated lipid A LAF inducers, respectively (Fig. 3B), were almost 1,000-fold less pyrogenic than diphosphorylated lipid A (Table 3).

DISCUSSION

In this study we compared the capacity of different lipid A preparations to induce IL-1 release by human monocytes in vitro (assessed by the classical comitogenic assay on C3H/HeJ thymocytes [LAF assay]) and to induce an in vivo endotoxin fever response (pyrogenicity was measured by the conventional rabbit test by recording continuously the rectal temperature for 6 h after intravenous injection). Crude lipid A isolated from *N. meningitidis*, *E. coli*, and *B. pertussis* led to similar levels of IL-1 release, whereas purified preparations from *N. meningitidis* and *B. pertussis* (devoid of any heptose residues [core sugars] as assessed by gas-liquid chromatography) were poor LAF (IL-1) inducers. It is noteworthy that at the tested dose of the crude *B. pertussis* lipid A preparation (10 μg per assay), the amount of LPS was below 0.5 μg per assay, a dose which by itself was not able to induce IL-1 release; this result suggests a potentiating effect of lipid A on LPS-induced IL-1 release.

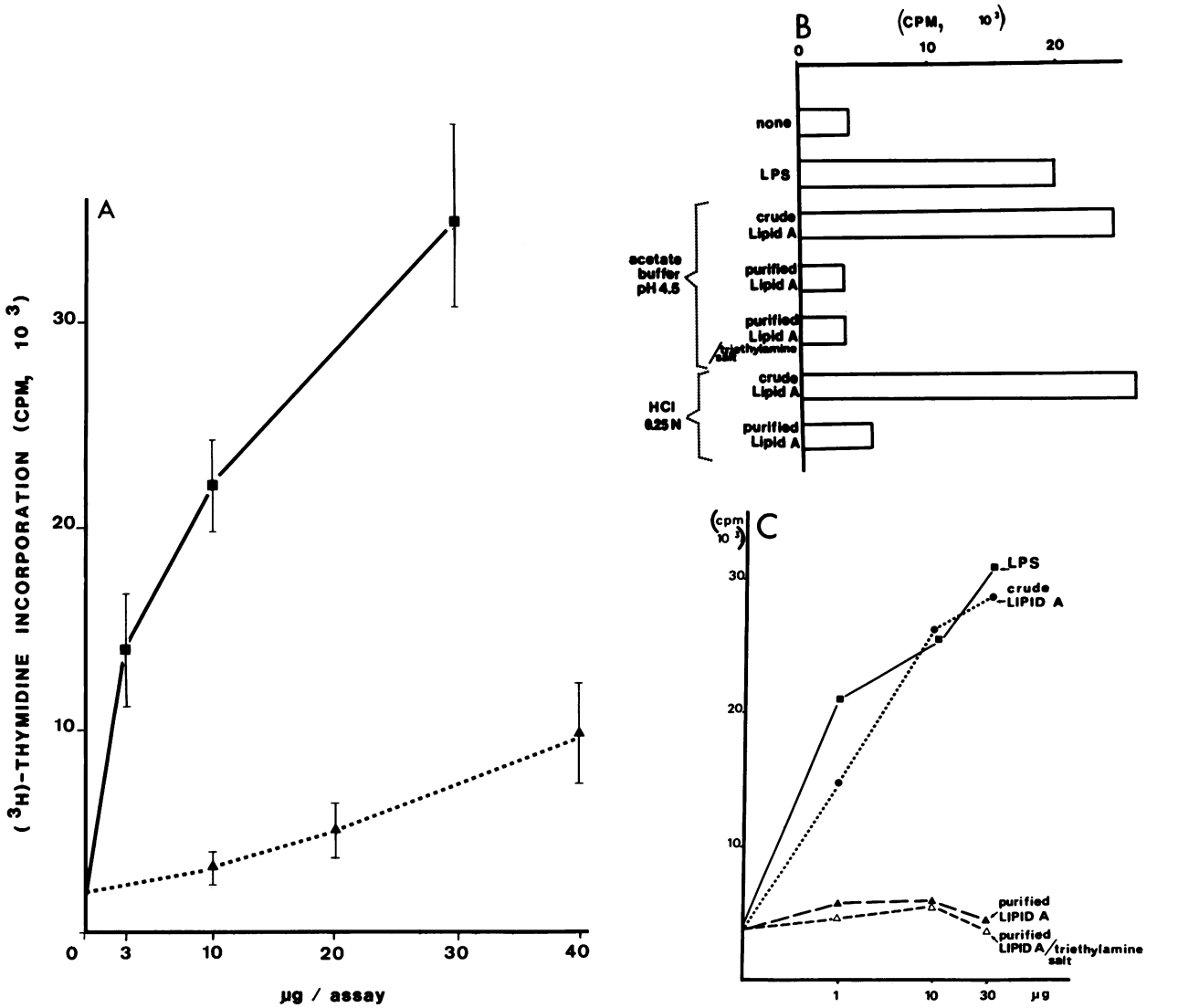

FIG. 3. (A) IL-1 activity in culture supernatants (1/10) of human monocytes stimulated by increasing amounts of *B. pertussis* LPS (■) (3 to 30 µg per assay) and purified lipid A (▲) (10 to 40 µg per assay) liberated by hydrolysis of LPS in acetate buffer (pH 4.5, 1 h, 100°C). (B) IL-1 activity in culture supernatants (1/10) of human monocytes stimulated by 30 µg (per assay) of *B. pertussis* LPS, crude lipid A and untreated purified lipid A (acetate buffer [pH 4.5], 1 h, 100°C) or pure lipid A converted to its triethylamine salt form, and crude and purified lipid A liberated by hydrolysis of LPS in HCl (0.25 N, 30 min, 100°C). (C) IL-1 activity in culture supernatants (1/10) of human monocytes stimulated with increasing amounts (1 to 30 µg per assay) of *N. meningitidis* LPS and crude and purified lipid A liberated by hydrolysis of LPS in sodium acetate buffer (pH 4.5, 1 h, 100°C). Values shown in each panel are from experiments representative of five separate experiments performed with monocytes from different donors.

Because it has been reported that the triethylammonium salt form of LPS is highly pyrogenic and that the pyrogenicity decreases with decreasing solubility (19), the purified lipid A preparations devoid of IL-1-inducing capacity were converted into their more soluble triethylammonium forms. The solubilization of the preparations did not affect the lack of inducing capacity of purified lipid A. The incapacity of pure *B. pertussis* lipid A preparation to induce IL-1 release was not due to an increased production of prostaglandins known to inhibit IL-1 synthesis and thymocyte proliferation. Indeed, monocyte cultures stimulated by pure lipid A in the presence or absence of indomethacin gave similar results. The lack of IL-1-inducing capacity of pure lipid A also was not due to a denaturing effect of the solvants used to purify the lipid A, because LPS treated under the same conditions was as active as untreated LPS (data not shown). It is noteworthy that pure lipid A, despite its impotency to induce the release of IL-1, was able to induce intracellular IL-1 synthesis measured in cell lysates. This IL-1 synthesis was variable among donors, however.

Loppnow et al. (17) observed that synthetic lipid A (10 µg per assay) constructed according to *E. coli* lipid A structure induced IL-1 release by human mononuclear cells cultured for 72 h. They also reported that the IL-1-stimulating capacity of synthetic lipid A derivatives is optimally expressed by hexaacyl compounds, whereas tetraacyl precursors are inactive. The phosphorylation pattern of the molecule has a profound modulating effect on the IL-1-inducing activity of synthetic *E. coli* lipid A. The pure *B. pertussis* lipid A used in our study contained five fatty acids, and the low IL-1-inducing capacity of the molecule might be related to its low fatty acid content. However, we observed that polymyxin B,

TABLE 1. Induction of IL-1 release by human monocytes stimulated by *B. pertussis* endotoxin and its derived pure lipid A in the presence of indomethacin

Inducers (10 μg/assay)	Supernatant dilutions	[^{3}H]thymidine incorporation (cpm ± SD) with or without indomethacin (1 μg/ml)[a]	
		−	+
None	1:40	2,005 ± 100	1,935 ± 41
	1:10	1,757 ± 329	1,929 ± 255
LPS	1:40	4,781 ± 506	6,997 ± 1,409
	1:10	11,987 ± 2,748	21,983 ± 1,177
Lipid A	1:40	1,983 ± 591	2,687 ± 1,082
	1:10	1,734 ± 328	1,999 ± 307

[a] [^{3}H]thymidine incorporation by C3H/HeJ thymocytes. Values shown are representative of three separate experiments performed with supernatants from monocytes from different donors.

which is known to inhibit lipid A-mediated activities, did not inhibit IL-1 induction by *B. pertussis* LPS, whereas it totally inhibited that induced by *E. coli* LPS (5a). Therefore, it can be concluded that lipid A is not equally involved in IL-1 induction by LPS from all gram-negative bacteria.

Despite their inability to induce IL-1 release, *N. meningitidis*- and *B. pertussis*-purified lipid A preparations were still mitogenic for mouse splenocytes. This response was dose dependent, occurred to similar degrees with both crude and purified preparations, and was maximal at 10 μg. Because *S. minnesota* Re mutant R595 LPS consists of only lipid A and KDO residues (3) and is able to induce IL-1 release, the presence of KDO may be required for IL-1 induction. This hypothesis was corroborated by our observation that hep-

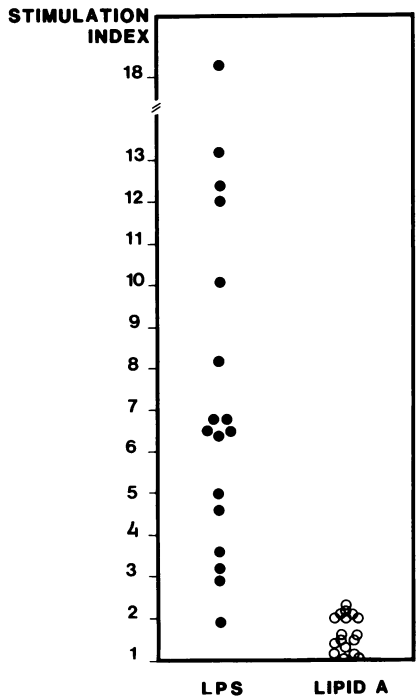

FIG. 4. IL-1 secretion by human monocytes induced by 10 μg of native *B. pertussis* LPS and purified lipid A per assay. Each symbol represents the results obtained with monocytes from one individual donor among the 17 tested. Results are expressed as stimulation index (SI): SI = (cpm in the presence of supernatants [1/10] from monocytes cultured with LPS or lipid A)/(cpm in the presence of supernatants [1/10] from unstimulated cultured monocytes).

TABLE 2. IL-1 synthesis by human monocytes stimulated by *B. pertussis* LPS and its derived pure lipid A

Expt	Inducers	IL-1 activity[a]	
		Extracellular	Intracellular
1	None	1,561 ± 152	5,871 ± 856
	LPS		
	1 μg	1,435 ± 154	23,545 ± 1,729
	10 μg	10,085 ± 1,944	48,337 ± 6,607
	Lipid A		
	1 μg	875 ± 90	6,030 ± 612
	10 μg	903 ± 221	7,099 ± 2,185
2	None	1,463 ± 381	938 ± 252
	LPS, 10 μg	18,187 ± 377	11,245 ± 1,659
	Lipid A, 10 μg	1,494 ± 395	9,565 ± 690
3	None	2,285 ± 782	2,582 ± 756
	LPS, 10 μg	30,135 ± 3,731	33,175 ± 3,167
	Lipid A, 10 μg	4,735 ± 706	21,728 ± 1,222

[a] IL-1 synthesis was assessed by measuring the IL-1 activity detected in the cell lysates. After the culture period, the supernatants of human monocytes isolated from three different donors were harvested and assayed for IL-1 activity (extracellular IL-1); 0.5 ml of fresh medium was added to the adherent cells, and the culture plates were frozen; after freezing and thawing 3 times, the supernatants were collected and centrifuged before they were tested for IL-1 activity (intracellular IL-1).

tose-heptose-KDO trisaccharide alone, which was isolated from salmonella LPS, induced IL-1 secretion (16). The KDO monosaccharide itself, however, was insufficient to induce IL-1 secretion.

Aside from results of our previous study (12), in which we indicated that LPS contamination of *B. pertussis* lipid A could be responsible for the observed IL-1 induction, the only report of isolated lipid A-mediated induction of IL-1 secretion that we are aware of is that of Charon et al. (6). Charon et al. (6) compared IL-1 secretion by rabbit monocytes induced with various synthetic lipid A analogs complexed with bovine serum albumin with IL-1 secretion induced by *B. pertussis* lipid A complexed with bovine serum albumin. Their crude lipid A preparation, however, contained 1.4% neutral sugars, i.e., 10% contamination with variously degraded LPS, and the bovine serum albumin alone strongly stimulated IL-1 secretion. According to our previous results (16) and those reported by others (17), we suggest that the lipid A region is not the only active moiety of the LPS molecule for IL-1 induction by all endotoxins. Because much higher amounts of isolated polysaccharide and lipid A moieties are required to induce IL-1 than those needed for native LPS, we suggest that both polysaccharide and lipid A regions are required within the LPS molecule to induce IL-1 synthesis and release. The inner core region, contains the negatively charged KDO molecules and is the first determinant involved in ligand-receptor interactions; the surrounding residues promote attachment because of hydrophobic interactions.

In this study we have shown, in agreement with results of many other studies, that the pyrogenicity elicited by LPS is mediated by determinants residing in lipid A. Purified lipid A obtained from *B. pertussis* after mild hydrolysis with acetate buffer (pH 4.5) was as pyrogenic as native LPS, with each exhibiting a threshold pyrogenic dose of 0.002 μg/kg. As observed by others (1, 19), the presence of a glycosidically linked phosphate group appears necessary for the expression of toxic and of EP functions. Crude lipid A obtained by mineral hydrolysis (0.25 N HCl) is devoid of glycosidically linked phosphate (in addition to other LPS constituents) and

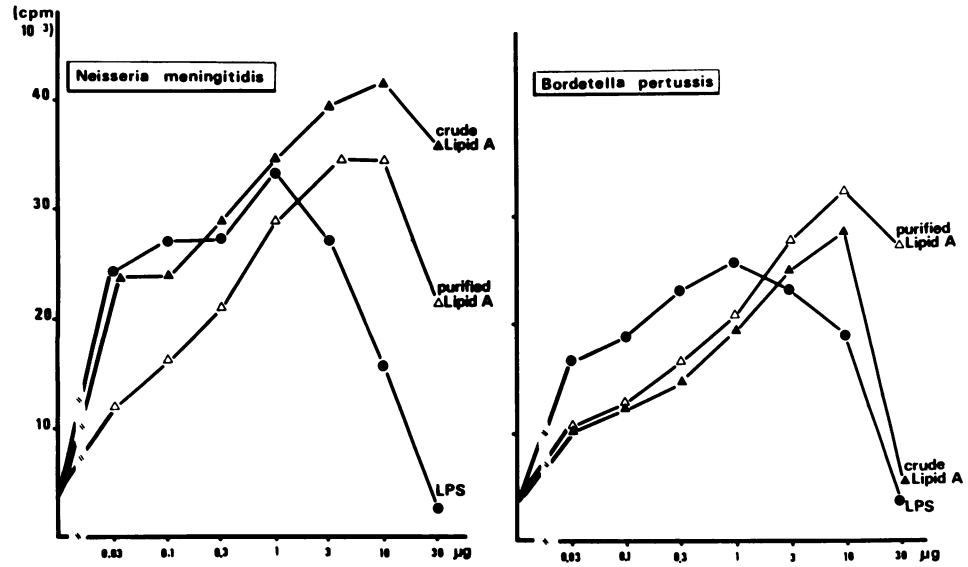

FIG. 5. Mitogenic dose response induced by LPS and crude and purified lipid A (acetate buffer [pH 4.5]) from either *N. meningitidis* or *B. pertussis*. Stimulation of BALB/c mouse splenocytes (2×10^5 cells per well) was assessed by the addition of 0.25 μCi of [^{3}H]thymidine 7 h prior to the end of a 48-h culture.

is 1,000 times less pyrogenic than native LPS. It should be noted that the same hydrolysis of the glycosidically linked phosphate in *B. pertussis* crude lipid A did not diminish its effect on IL-1 induction. Similarly, crude diphosphorylated or monophosphorylated lipid A from *E. coli* (obtained after acidic hydrolysis in acetate buffer [pH 4.5] or 0.1 N HCl, respectively) were potent IL-1 inducers. However, from our results it cannot be concluded that the acid-labile phosphate group is the determinant responsible for the induction of EP, because it is possible that the phosphate group is required to maintain particular conformation of the lipid A which allows the exposure of another determinant(s). In support of this concept is the observation that lipid A derived from *Chromobacterium violaceum*, which lacks the C-1 phosphoryl group, is a potent pyrogen (19). Moreover, in the case of *B. pertussis* lipid A, the loss of pyrogenicity correlates not only with the loss of the glycosidic phosphate but also with the partial release of one extremely labile ester-linked fatty acid (β-hydroxydecanoic acid) (M. Caroff, unpublished data). Expression of the local Shwartzman phenomenon has been shown to depend on the presence of nonhydroxylated fatty acids (19), and it may be that a full complement of lipid A fatty acids is necessary for a maximal expression of pyrogenicity.

In conclusion, we have shown that IL-1 and EP induction do not involve the same structural determinants on the LPS molecule and that LAF and EP are not necessarily induced simultaneously. Moreover, it has recently been reported that

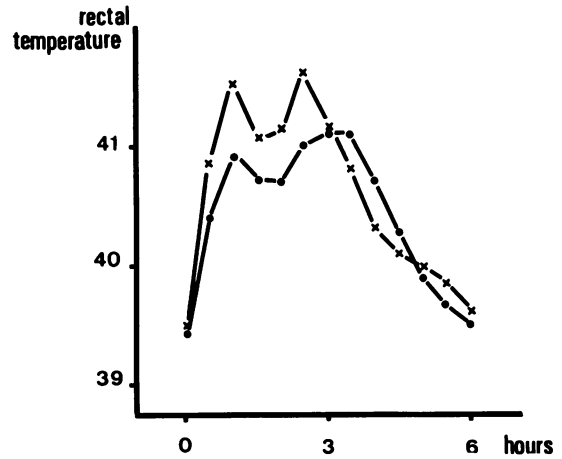

FIG. 6. Pyrogenicity of *B. pertussis* LPS and purified lipid A (acetate buffer [pH 4.5]). New Zealand albino rabbits received intravenous injections of 0.4 μg of *B. pertussis* LPS per kg (×) or 0.4 μg of lipid A per kg (●). Fever responses were recorded for 6 h after injection.

TABLE 3. Pyrogenicity of different *B. pertussis* lipid A preparations

Prepn	Injected doses (μg/kg)	Sum of ΔT[a]	Dose (μg/kg) giving ΣΔT = 1°15
LPS	0.0016	0.95	0.002 (r = 0.998)
	0.008	2.95	
	0.04	5.45	
Crude lipid A (acetate buffer; pH 4.5)	0.0005	0.95	0.002 (r = 0.978)
	0.005	0.95	
	0.05	2.75	
	0.5	4.55	
Crude lipid A (0.25 N HCl)	0.05	0.40	1.75 (r = 0.909)
	0.5	0.95	
	5.5	5.80	
Purified lipid A (acetate buffer; pH 4.5)	0.016	1.15	0.0023 (r = 0.936)
	0.008	1.65	
	0.04	4.0	

[a] A group of three New Zealand rabbits (2.5 to 2.7 kg) of the same sex received intravenously the amount of each substance to be tested suspended in 1 ml of saline per kg. Rectal temperatures were recorded continuously for 3 h, and the maximal increase in temperature (ΔT) was recorded for each animal. Results are expressed as the sum (Σ) of the ΔT values for the three rabbits. r, Correlation coefficient.

molecules different from IL-1, such as tumor necrosis factor (TNF) and alpha interferon (IFNα) (9, 10), which can be also secreted by LPS-stimulated macrophages, may also induce an in vivo fever response. Because neither TNF nor IFNα possess mitogenic activity on thymocytes, it can be suggested that some lipid A preparations may simultaneously induce LAF and TNF or IFNα and others may stimulate only TNF or IFNα.

ACKNOWLEDGMENTS

This study was supported by Contrat de Recherche Externe 843004 from the Institut National de la Santé et de la Recherche Médicale. We thank J. Cristofari and colleagues (Centre National de Transfusion Sanguine, Paris) for providing the blood samples, H. Brade (Forschungsinstitut Borstel) for providing *E. coli* and *S. minnesota* LPS, and D. Schulz (Institut Mérieux, Marcy l'Etoile) for providing *B. pertussis* and *N. meningitidis* LPS and for testing the pyrogenicity of our samples. We thank Paul Siefert for help with translation.

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