Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6671–6675, November 1983
Medical Sciences

Enzymatic deacylation of the lipid A moiety of *Salmonella typhimurium* lipopolysaccharides by human neutrophils

(endotoxin/glycolipid metabolism/hydroxylated fatty acids)

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Communicated by Jean D. Wilson, July 21, 1983

ABSTRACT **Lipid A, the toxic moiety of Gram-negative bacterial lipopolysaccharides (endotoxins), is a glucosamine disaccharide to which fatty acid and phosphate residues are covalently attached. Recent studies of *Salmonella* lipid A indicate that 3-hydroxytetradecanoic acid (3-OH-14:0) residues are directly linked to the glucosamine backbone and that nonhydroxylated fatty acids (principally dodecanoic and tetradecanoic acids) are esterified to the hydroxyl groups of some of the 3-OH-14:0 molecules. We report here that the granule fraction of human neutrophils contains one or more enzymes that partially deacylate *Salmonella typhimurium* lipid A by removing the nonhydroxylated fatty acids, leaving almost all of the 3-OH-14:0 residues linked to glucosamine. The available evidence suggests that similar reactions also occur in living neutrophils that ingest lipopolysaccharides by antibody-dependent phagocytosis.**

Gram-negative bacterial lipopolysaccharides (LPS; endotoxins) are complex molecules that, when injected into animals, can produce such toxic effects as fever, hypotension, coagulation abnormalities, and death. Much is known about the chemical structure of LPS and the biological activities that LPS exhibit in various experimental systems. In contrast, no information is available about the metabolism of LPS by animals, although it seems likely that such metabolism should influence the biological activities of LPS. We here report the deacylation of LPS by human peripheral blood neutrophils (PBN), cells that normally ingest and kill Gram-negative bacteria.

Salmonella LPS have three major regions, the O polysaccharide, the R-core oligosaccharide, and lipid A. The lipid A region is responsible for most of the biological activities of the LPS (1). Recent evidence indicates that the D-glucosamine disaccharide backbone of lipid A is substituted by four 3-hydroxytetradecanoic acid (3-OH-14:0) residues, two in amide linkage and two in ester linkage to the disaccharide (Fig. 1). Dodecanoic, tetradecanoic, hexadecanoic, and 2-hydroxytetradecanoic acids may in turn be attached to the hydroxyl groups of the glucosamine-linked 3-OH-14:0 residues (2). Because the fatty acids (in particular, the 3-OH-14:0 residues attached to glucosamine) are thought to be essential for lipid A to have biological activity (1, 4–6), it seemed reasonable to expect that the metabolism of LPS by host cells might involve cleavage of some or all of the fatty acids from lipid A. To detect the deacylation of lipid A, we prepared LPS that had distinct radioactive labels in the carbohydrate (^{14}C) and fatty acid (^{3}H) moieties. Removal of the fatty acids from LPS could then be measured by the appearance of free ^{3}H-labeled fatty acids, but not ^{14}C-labeled polysaccharides, in chloroform/methanol extracts of cells that had ingested purified LPS. Using this approach, we found that human neutrophils contain an enzyme or enzymes that preferentially cleave nonhydroxylated fatty acids from lipid A.

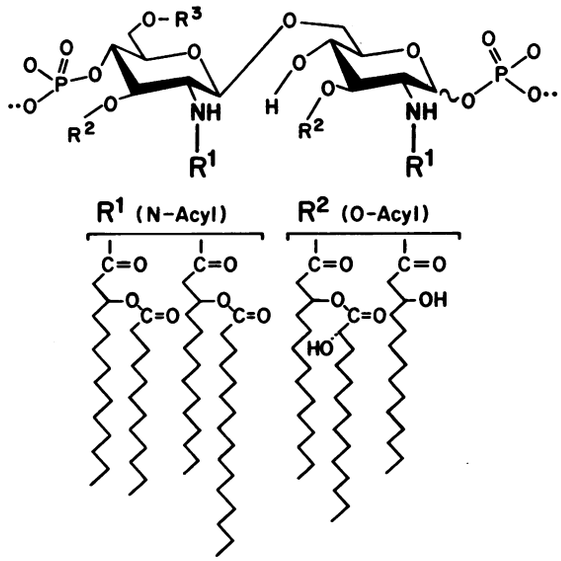

FIG. 1 Structure of *Salmonella* lipid A, modified slightly from that proposed by Wollenweber *et al.* (2). R^1 and R^2 indicate the sites of *N*- and O-linked fatty acids, respectively, and R^3 shows the site at which the polysaccharide chain is attached (3). Recent evidence indicates that the amide-linked fatty acyl moieties (R^1) have an acyl-D-3-oxyacyl structure, with nonhydroxylated fatty acids attached to the hydroxyl groups of most of the *N*-linked 3-OH-14:0 residues. The ester-linked 3-OH-14:0 residues (R^2) are often substituted by a nonhydroxylated (usually tetradecanoic) or hydroxylated (possibly 2-hydroxytetradecanoic) fatty acid (2).

METHODS

Salmonella typhimurium PR122 cells [deficient in glucosamine deaminase and UDP-glucose-4-epimerase (7)] were grown in proteose peptone-beef extract broth as described (7) or in M9 medium (8) containing L-methionine (2 mM), L-histidine (2 mM), DL-tryptophan (2 mM), and glucose [0.4% (wt/vol)]. Cells were biosynthetically labeled with [2-^{3}H]acetate and *N*-acetyl-[1-^{14}C]glucosamine (New England Nuclear). This *Salmonella* mutant incorporates GlcNAc into the R core and lipid A regions of LPS. Exogenous acetate is almost entirely incorporated into fatty acids. To prepare labeled smooth LPS, D-galactose (0.1 mM) was added to the medium; outer membranes were prepared from the labeled cells, and LPS were extracted from the

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Abbreviations: LPS, lipopolysaccharide(s); PBN, peripheral blood neutrophil(s); 3-OH-14:0, 3-hydroxytetradecanoic acid.

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membranes with 45% (wt/vol) phenol and dialyzed against distilled water as described (7). To prepare labeled rough LPS, galactose was omitted from the growth medium, and LPS were extracted from the labeled cells with phenol/chloroform/petroleum ether, 2:5:8 (vol/vol) (9). Both smooth and rough LPS were extracted several times with diethyl ether to remove residual labeled lipid, which was shown to be absent by TLC and HPLC. The amount of LPS in the preparations of smooth LPS was estimated by an O-antigen immunoassay (10); the amount of rough LPS was determined by weighing the dry preparations before they were suspended in distilled water containing triethylamine (1 μl/ml). Both smooth and rough LPS were stored in aliquots at −70°C.

Heparinized whole blood was obtained from normal human volunteers (in the laboratories of J. Cuthbert and P. Lipsky, this institution) and sedimented over sodium diatrizoate/Ficoll cushions (Isolymph, Teva, Jerusalem) to remove mononuclear cells (11). We harvested the PBN from the Ficoll pellets by suspending the cells in 3.5% dextran (Sigma; M_r 70,300) in 0.9% NaCl and allowing the erythrocytes to settle at 1 × g for 1 hr. The supernatant was removed and centrifuged (400 × g, 10 min), residual erythrocytes were removed from the leukocyte pellet by lysis with 0.87% NH_4Cl, and the leukocytes were washed and suspended in RPMI 1640 (M. A. Bioproducts, Walkersville, MD), to which 2 mM L-glutamine had been added. The cell preparations contained ≈95% granulocytes and <5% mononuclear cells; when tested with the fluorescein diacetate/ethidium bromide method (12), >90% of the cells were viable.

Fatty acids were hydrolyzed from LPS under N_2 with 4 M HCl (100°C, 90 min); the mixture was cooled and then alkalinized with 4 M NaOH, heated again (100°C, 30 min), and acidified with acetic acid (to pH 4) prior to extraction of the fatty acids with chloroform/methanol, 2:1 (vol/vol). Fatty acids were derivatized with α-,p-dibromoacetophenone in the presence of crown ether in acetonitrile (13); >90% derivatization was achieved for each preparation. Analysis of the derivatized fatty acids was performed on a Waters Associates μBondapak column with a Waters Associates WISP 710B sample loader, a model 441 UV detector, and a Waters Associates recorder. A solvent mixture of methanol/water, 88:12 (vol/vol) was used. Derivatized nonradioactive standards were used to generate linear dose–response plots from which the molar amounts of the radioactive fatty acids were determined. Heptadecanoic acid was added to all samples (internal standard). The radioactivity in each peak was measured by collecting the column effluent (in 0.5-min fractions) into scintillation vials, drying the fractions, and adding scintillant for counting the radioactivity. Authentic DL-3-OH-14:0 was obtained from Applied Science Laboratories (State College, PA).

Protein was measured by the method of Markwell *et al.* (14).

RESULTS

Antibody-Dependent Uptake of LPS by Human Neutrophils. When labeled smooth LPS were incubated with PBN at 37°C in the presence of anti-LPS IgG, the cells rapidly took up LPS from the medium. Almost all of the cellular uptake of the opsonized LPS occurred in the first 40–60 min of incubation (Fig. 2*A*), and the amount of LPS that was associated with the washed cells remained relatively constant over the next 3 hr. Uptake of the ^3H and ^{14}C occurred in parallel (data not shown). No uptake of the LPS by the cells was observed at 4°C, and <1% of the added LPS became cell-associated at either temperature in the absence of anti-*S. typhimurium* IgG.

Deacylation of Cell-Associated LPS. Neutrophils that had been incubated with radiolabeled LPS for various time periods at 37°C were washed with ice-cold 0.9% NaCl and then extracted with chloroform/methanol. As is shown in Fig. 2*B*, the percentage of the total cell-associated ^3H that was extractable into chloroform increased over the period of incubation; at no time did significant amounts of ^{14}C appear in the chloroform extracts. These findings suggested that ^3H-labeled fatty acids were being cleaved from the LPS.

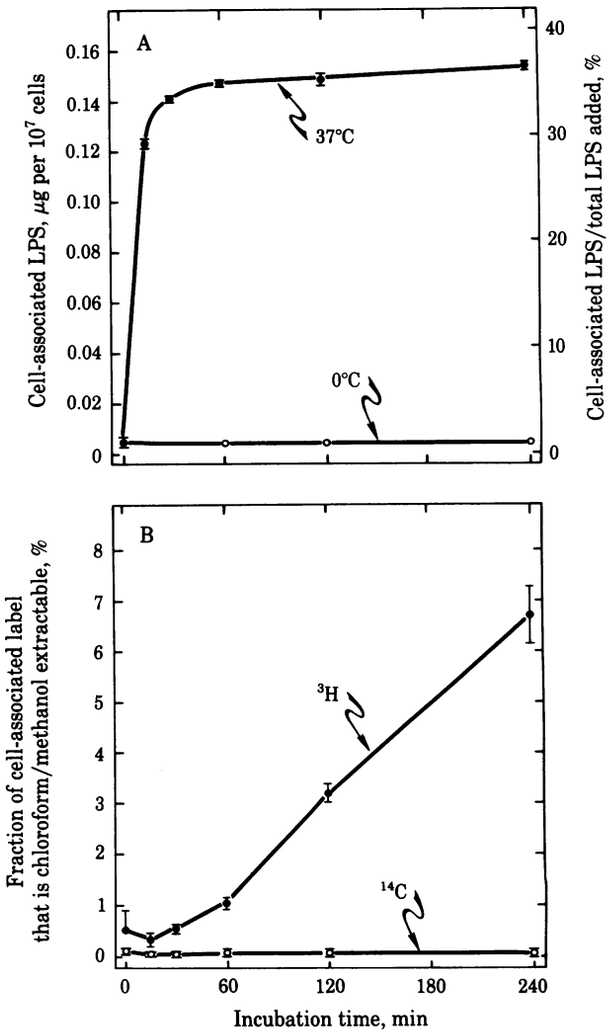

FIG. 2. Uptake and degradation of LPS by human PBN. Freshly isolated PBN (1.5 × 10^7), 0.5 μg of smooth LPS (19,600 cpm of ^3H and 9,100 cpm of ^{14}C), 2.5% normal human serum, 15 mM sodium acetate, and 4 μg of rabbit anti-*S. typhimurium* IgG (10) were incubated in a total volume of 1.0 ml of RPMI 1640 in polystyrene tubes. Incubations were carried out at 37°C in a shaking water bath or at 0°C in an ice bath. At sequential intervals the mixtures were chilled and centrifuged (500 × g, 5 min) and the pellets were washed twice with ice-cold saline. The washed cells were digested overnight at room temperature in 0.4 ml of a solution that contained 0.08% $NaDodSO_4$, 0.08 mM EDTA, and 0.4 M NaOH. The digestion mixtures were brought to neutral pH with acetic acid prior to mixing with 10 ml of Budget-Solve (Research Products International, Mount Prospect, IL) and counting the radioactivity. At each time point, washed cells were also extracted with chloroform/methanol (15) and the radioactivity in the chloroform phase was counted. Data points represent the mean ± SD of three determinations.

Deacylation of LPS by the Neutrophil Granule Fraction. To study the early stages of LPS degradation we prepared granules from disrupted neutrophils and tested the ability of the granule contents to release chloroform-extractable ^3H-labeled fatty acids from double-labeled LPS. The granule fraction was prepared by differential centrifugation of PBN homogenates (16) and stored

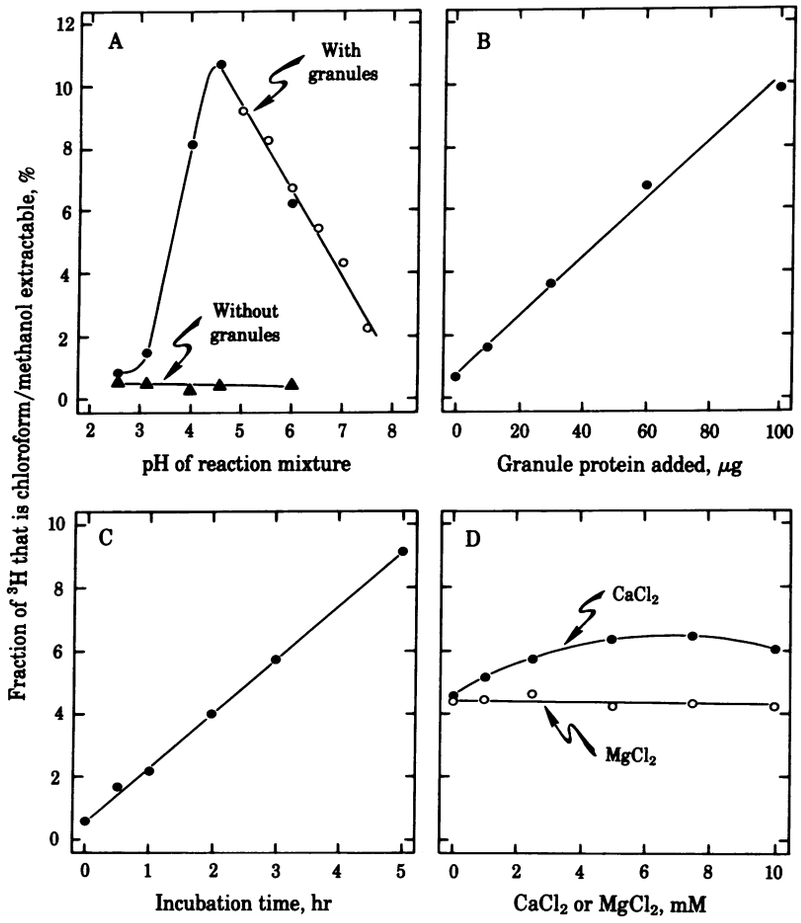

FIG. 3. LPS deacylation by the PBN granule fraction. Double-labeled smooth LPS (1.0 μg containing 39,300 cpm of ^3H and 18,200 cpm of ^{14}C) were incubated with PBN granules at 37°C for 4 hr or as otherwise indicated. Reaction mixtures also contained Triton X-100 [0.1% (vol/vol)], $CaCl_2$ (5 mM or as otherwise indicated), NaCl [0.5% (wt/vol)], and bovine serum albumin [0.1% (wt/vol)]. After incubation for the desired time at 37°C in a shaking water bath, the mixtures were chilled, further acidified with 5 μl of glacial acetic acid, and extracted twice with chloroform/methanol. The chloroform extracts were dried and their radioactivity was counted. Two buffer systems were used for the experiment shown in *A*: 10 mM citrate/NaOH (solid symbols) and 10 mM Tris maleate (open symbols). In the experiments shown in *B–D*, the buffer was 10 mM Tris citrate at pH 4.3. In the experiments shown in *A*, *C*, and *D*, the amount of granule protein was 70 μg per reaction mixture. Each point represents the average of two to four determinations.

at −70°C. The granule fraction sedimented at 8,200 × *g* and was enriched, relative to the 8,200 × *g* supernatant, in peroxidase (17) and LPS deacylating activities. As is shown in Fig. 3, LPS deacylation by the granules had a pH optimum of 4.3 (Fig. 3*A*) and was directly related to the amount of granule protein added (Fig. 3*B*). The reaction was linear over time (Fig. 3*C*) and was stimulated ≈1.5-fold by added $CaCl_2$; $MgCl_2$ had no effect (Fig. 3*D*). Less than 0.5% of the ^{14}C cpm in the mixtures was extractable into chloroform. The deacylating activity was increased approximately 3- to 5-fold by the addition of Triton X-100 (0.1%) to the reaction mixtures. Treatment of the PBN granules at 100°C for 10 min completely abolished their ability to deacylate LPS.

The PBN granule-deacylating activity showed substrate (LPS) saturability. When neutrophil granules from three different individuals were tested by using rough LPS ($M_r \approx 3{,}600$) the mean (±SD) apparent K_m for LPS was 0.63 ± 0.06 μM, and the mean apparent V_{max} was 0.75 ± 0.21 nmol of fatty acid released per mg of protein per hour.

Analysis of the Reaction Products. Essentially all of the chloroform-insoluble radioactivity (both ^3H and ^{14}C) in the rough LPS-granule incubation mixtures could be recovered from the proteinaceous material that collected at the interface between the chloroform and the methanol/water phases. These radioactive labels comigrated with untreated labeled rough LPS as a single peak on $NaDodSO_4$/polyacrylamide tube gels (7) (data not shown), indicating that the labels remained chemically linked after deacylation had taken place. The interface contained a mixture of undegraded and partially deacylated LPS, which we call LPS intermediate, or LPS^I. Sequential hydrolysis of this interface material with acid (4 M HCl, 100°C, 90 min) and base (4 M NaOH, 100°C, 30 min) liberated >98% of the ^3H in the interface into a chloroform-soluble form. Approximately 90% of the ^{14}C in the interface could be recovered in the methanol/water phase of the post-hydrolysis chloroform/methanol extraction.

We first analyzed the fatty acid composition of the original LPS and the reaction products by one-dimensional TLC. As is shown in Table 1, 68% of the ^3H-labeled fatty acids in the hydrolysate of the original LPS comigrated with 3-OH-14:0,

Table 1. Analysis of reaction products by TLC

Compound	Incubation time, hr	cpm in spot, % of recovered cpm		
		Origin	3-OH-14:0	NFA*
Original LPS†	0	4.4	68.2	26.6
Reaction products				
Chloroform-soluble	4	7.8	5.0	86.4
	72	5.4	7.1	86.7
LPS^I†	4	4.8	74.8	21.3
	72	5.3	86.8	7.8

Double-labeled rough LPS (9.0 μg containing 261,000 cpm of ^3H and 2,800 cpm of ^{14}C) were incubated with PBN granules (200 μg of protein) at pH 4.3 and 37°C for the indicated time periods and the reaction products were analyzed. After incubation for 4 hr, 10% of the ^3H in the LPS was extractable into chloroform, whereas after incubation for 72 hr, 23% of the ^3H was chloroform-extractable. One-dimensional TLC was performed by using petroleum ether/diethyl ether/acetic acid, 70:30:1 (vol/vol), on silica gel G plates. Individual spots were scraped from the plates and the radioactivity was counted; recovery of the added ^3H was >85%. Each value is the average of three or four measurements.

* Nonhydroxylated fatty acids (oleic acid standard).

† Hydrolyzed with HCl and then with NaOH at 100°C.

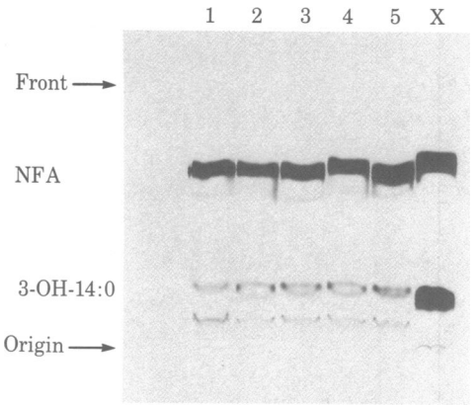

FIG. 4. Analysis of the chloroform-soluble reaction product by TLC. The positions of authentic standards are shown: NFA (nonhydroxylated fatty acids) corresponds to oleic acid. After chromatography was completed, the plate was sprayed with EN^{3}HANCE (New England Nuclear) and exposed to Kodak SB-5 film at −70°C. Lanes 1–5 contain chloroform-soluble reaction product that had been treated with 0.5 M NaOH at 56°C for 0, 15, 30, 60, and 120 min, respectively, and then reextracted into chloroform. Lane X contains chloroform-extracted ^{3}H-labeled fatty acids from LPS that had been treated with 0.5 M NaOH for 20 min at 56°C.

whereas 27% comigrated with oleic acid; these estimates of the relative amounts of hydroxylated and nonhydroxylated fatty acids are similar to those previously reported for *Salmonella* LPS (2, 4). In contrast, 86% of the chloroform-extractable ^{3}H-labeled fatty acids from LPS/PBN granule mixtures comigrated with oleic acid. The distribution of the fatty acids in the hydrolysate of the LPS^I was consistent with the removal of some nonhydroxylated fatty acids during the LPS-granule reaction; prolonged incubation, so that a maximum of 23% of the ^{3}H-labeled fatty acids was cleaved from the LPS, produced further enrichment of the LPS in 3-OH-14:0.

The nonhydroxylated fatty acids in *Salmonella* LPS are thought to be attached to the hydroxyl group of a (glucosamine-linked) 3-hydroxytetradecanoic acid (ref. 2; Fig. 1). Acyloxyacyl residues (containing one hydroxylated and one nonhydroxylated fatty acid), if cleaved directly from the glucosamine, might migrate on TLC as a nonhydroxylated fatty acid, because the polar hydroxyl group would not be exposed. This possibility was excluded by the experiment shown in Fig. 4, in which the LPS-granule product was treated with NaOH for as long as 2 hr; no hydroxylated fatty acid was released. Thus, the chloroform-soluble reaction product appeared to be one or more nonhydroxylated fatty acids with a small amount of hydroxylated fatty acid.

The fatty acid composition of LPS and the two LPS-granule reaction products was further analyzed by HPLC. The specific activities of the major LPS fatty acids were similar (≈27,500 cpm/nmol); only a minor amount of hexadecanoic acid was present in the LPS, and its specific activity was lower (16,000 cpm/nmol). As is shown in Table 2 and in Fig. 5*A*, the original LPS had a typical fatty acid composition, with ≈67% of the ^{3}H-labeled fatty acids appearing in the 3-OH-14:0 peak. The composition of LPS^I was enriched in 3-OH-14:0 and depleted in 12:0 and 14:0 (Fig. 5*B*). Analysis of the chloroform-soluble reaction product showed that the material that comigrated with oleic acid on TLC was principally 12:0 and 14:0 (Fig. 5*C*).

Table 2. Analysis of reaction products by HPLC

Compound	Recovered fatty acids, mol %			
	3-OH-14:0	12:0	14:0	16:0
Original LPS*	67.1	18.7	10.4	1.1
Reaction products				
Chloroform-soluble†	—	54.5	41.5	0.9
LPS^I*	78.0	9.5	7.4	1.7

The reaction products were studied after incubation of labeled rough LPS with PBN granules for 48–72 hr. Each value is the mean of three or four analyses of samples that contained 5,000–10,000 cpm of ^{3}H-labeled fatty acids.

* Hydrolyzed with HCl and then with NaOH at 100°C.

† These results were obtained from material that was scraped from the nonhydroxylated fatty acid spot on TLC.

Thus, the neutrophil granule enzyme or enzymes appear to remove principally the nonhydroxylated fatty acids from LPS. We have not identified the trace amount of chloroform-soluble reaction product that comigrates with 3-OH-14:0 on TLC. It is possible that this is either 3-OH-14:0 or 2-OH-14:0 (ref. 2; Fig. 1).

Analysis of the Products of LPS Deacylation by Intact Cells. We now examined the fate of the LPS-derived ^{3}H-labeled fatty acids that were deacylated in intact PBN. Chloroform extracts of cells that had degraded LPS were analyzed by two-dimen-

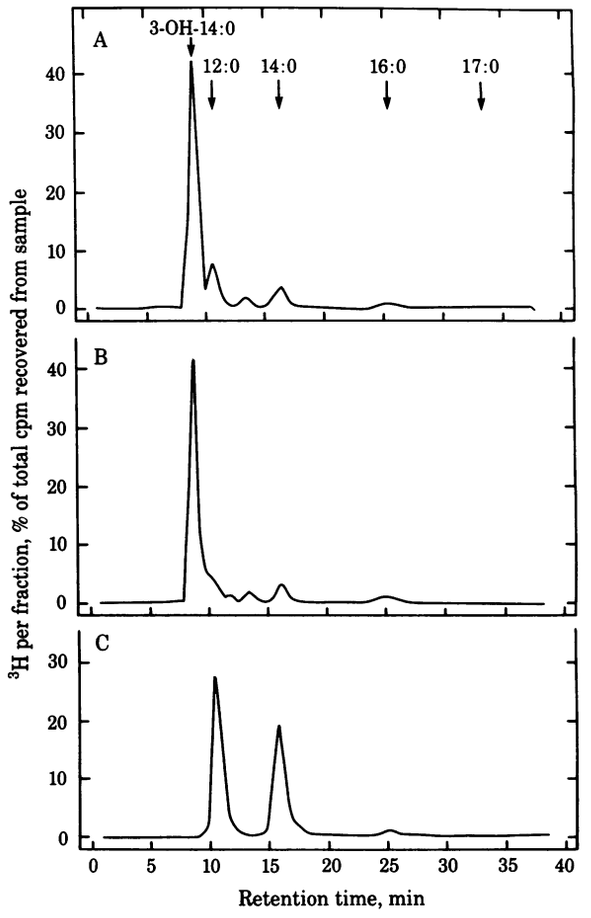

FIG. 5. Analysis of the reaction products by HPLC. Each sample contained 5,000–10,000 cpm of ^{3}H-labeled fatty acids, and >85% of the added radioactivity was recovered from the column. The arrows in *A* indicate the elution positions of the nonradioactive fatty acids. The small peak between 12:0 and 14:0 in the hydrolysates of LPS and LPS^I (*A* and *B*, respectively) is assumed to represent an unsaturated hydrolysis product of 3-OH-14:0 (2); this peak was much larger in the HPLC profiles of LPS-derived fatty acids that were hydrolyzed only with alkali. The chloroform-soluble product (*C*) was scraped from the nonhydroxylated fatty acid spot on TLC plates and extracted into chloroform prior to derivatization; this sample also contained a large mass of PBN-derived nonradioactive fatty acids.

sional TLC [chloroform/methanol/water, 120:70:16 (vol/vol), first dimension; petroleum ether/diethyl ether/glacial acetic acid, 80:20:1 (vol/vol), second dimension]. In separate experiments utilizing PBN from two donors (four TLC analyses) the ^{3}H radioactivity (mean ± SD) comigrated with fatty acids (13.5% ± 0.5%), phospholipids (40.0% ± 1.4%), and neutral lipids (44.0% ± 1.2%). Thus, the LPS-derived ^{3}H-labeled fatty acids seemed to have been reutilized for neutrophil lipid synthesis. We also treated the chloroform-extracted lipids with acid and base at 100°C as described above, extracted the lipids with chloroform/methanol, and analyzed the fatty acids by using one-dimensional TLC. Essentially all of the saponified ^{3}H-labeled fatty acids comigrated with an oleic acid standard; only trace amounts of ^{3}H comigrated with 3-OH-14:0. Thus, it appeared either that the nonhydroxylated fatty acids were being cleaved almost exclusively from lipid A or that the 3-OH-14:0 residues were being cleaved, modified by the cells, and then reutilized.

DISCUSSION

These experiments have shown that the granule (lysosome-containing) fraction of human PBN contains an enzyme or enzymes that partially deacylate the lipid A moiety of *S. typhimurium* LPS. The principal activity removes nonhydroxylated fatty acids from lipid A; because these fatty acids are normally esterified to the hydroxyl groups of D-3-OH-14:0 residues, this activity is consistent with that of an acyl-D-3-oxyacyl hydrolase.

There has been surprisingly little experimental work on lipid A deacylation in the past. It is known that *Dictyostelium discoideum* (slime mold), which utilizes Gram-negative bacteria as a major foodstuff, contains enzymes that remove nonhydroxylated (18) and hydroxylated (19) fatty acids from lipid A. In experiments not described here, we have used different substrates (LPS, LPS^I, and LPS that were treated with mild alkali to remove esterified fatty acids) to search for additional PBN granule-deacylating activity over the pH range 3.5–7.5. Further deacylation, with removal of 3-OH-14:0 residues from lipid A, occurred much more slowly under the conditions studied. Thus, our data suggest that acyl-D-3-oxyacyl hydrolysis is the major deacylating activity present in the PBN granule fraction; its pH optimum (4.3) and cofractionation with peroxidase (data not shown) suggest that the enzyme or enzymes are present in lysosomes.

As suggested by the experiments of Gimber and Rafter (20), partial deacylation of LPS is also carried out by intact neutrophils. In our studies the LPS were taken up by human PBN in an antibody- and temperature-dependent process. Although the evidence suggests that the lipid A deacylating activity observed in the PBN granule fraction also occurs in the intact cell, it should be noted that the nature of the reaction products that arise in intact PBN is uncertain. We were able to find only traces of hydroxylated fatty acid after hydrolysis of the ^{3}H-labeled cellular lipids. This finding is consistent with the idea that large amounts of 3-OH-14:0 are not removed from the LPS by the intracellular deacylating activity; yet it is also possible that the 3-OH-14:0 residues may be elongated (21) or otherwise modified prior to their reutilization as components of cellular lipids. Incorporation of the LPS-derived fatty acids into neutrophil lipids is in keeping with previous evidence that the fatty acids in bacterial phospholipids may be hydrolyzed and incorporated into granulocyte lipids (16, 22).

Accurate tests of the biological significance of LPS deacylation by PBN will require purification of the specific hydrolase(s) and detailed chemical analysis of the partially deacylated lipid A to exclude other modifications that might influence activity. Because glucosamine-linked hydroxylated fatty acids are thought to be necessary for lipid A to have biological activity (1, 6), it is interesting that the enzymatic activity described here leaves the 3-OH-14:0 residues attached to the glucosamine disaccharide. In fact, by cleaving the acyloxyacyl linkages, the PBN enzyme(s) may expose hydroxyl groups that were previously unexposed, and it is thus possible that the partially deacylated LPS are more polar (and perhaps more membrane-active) molecules than the original LPS. In other words, partial deacylation might actually enhance one or more of the biological activities of the LPS. If this hypothesis is correct, further deacylation [or another process, such as dephosphorylation (4)] would be required for detoxification. It seems likely that the study of LPS-degrading enzymes and their products will bring new understanding of the mechanisms of endotoxin activity and inactivation.

Drs. Jennifer Cuthbert and Peter Lipsky provided blood for isolating neutrophils, Drs. Kenneth Chien and James Willerson generously allowed us to use their HPLC equipment, and Drs. Timothy Sullivan, Leon Eidels, Dennis McGarry, David Bass, Kenneth Chien, and John Dietschy gave much helpful advice. Aena Kim taught us how to perform HPLC, Pat Ladd prepared the illustrations, Carol Hendry typed the manuscript, and Marva Griffith provided outstanding technical support. This work was funded by Grant 18188 from the National Institute of Allergy and Infectious Diseases.

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