Deacylation of Lipopolysaccharide in Whole Escherichia coli during Destruction by Cellular and Extracellular Components of a Rabbit Peritoneal Inflammatory Exudate*

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Deacylation of purified lipopolysaccharides (LPS) markedly reduces its toxicity toward mammals. However, the biological significance of LPS deacylation during infection of the mammalian host is uncertain, particularly because the ability of acyloxyacyl hydrolase, the leukocyte enzyme that deacylates purified LPS, to attack LPS residing in the bacterial cell envelope has not been established. We recently showed that the cellular and extracellular components of a rabbit sterile inflammatory exudate are capable of extensive and selective removal of secondary acyl chains from purified LPS. We now report that LPS as a constituent of the bacterial envelope is also subject to deacylation in the same inflammatory setting. Using Escherichia coli LCD25, a strain that exclusively incorporates radiolabeled acetate into fatty acids, we quantitated LPS deacylation as the loss of radiolabeled secondary (laurate and myristate) and primary fatty acids (3-hydroxymyristate) from the LPS backbone. Isolated mononuclear cells and neutrophils removed 50% and 20–30%, respectively, of the secondary acyl chains of the LPS of ingested whole bacteria. When bacteria were killed extracellularly during incubation with asetic fluid, no LPS deacylation occurred. In this setting, the addition of neutrophils had no effect, but addition of mononuclear cells resulted in removal of >40% of the secondary acyl chains by 20 h. Deacylation of LPS was always restricted to the secondary acyl chains. Thus, in an inflammatory exudate, primarily in mononuclear phagocytes, the LPS in whole bacterial envelopes undergoes substantial and selective acyloxyacyl hydrolase-like deacylation, both after phagocytosis of intact bacteria and after uptake of LPS shed from extracellularly killed bacteria. This study demonstrates for the first time that the destruction of Gram-negative bacteria by a mammalian host is not restricted to degradation of phospholipids, protein, and RNA, but also includes extensive deacylation of the envelope LPS.

The prominent role ascribed to LPS in evoking beneficial as well as harmful mammalian host responses to invading Gram-negative bacteria has prompted intense scrutiny of host recognition, detoxification, and elimination of this bacterial product. Underacylated and underphosphorylated biosynthetic precursors and chemically synthesized analogs of lipid A (the endotoxic part of LPS) possess greatly diminished endotoxic activity and can act as antagonists in human cell bioassays (1, 2). Mammalian enzyme systems capable of removing acyl and phosphate groups from lipid A may therefore play an important role in host defense against Gram-negative bacteria and their LPS. Thus far, only one mammalian enzyme, acyloxyacyl hydrolase, has been shown to deacetylate LPS (3, 4).

We recently showed that both the cells and extracellular fluid of a sterile inflammatory exudate actively deacylate purified LPS ex vivo in an acyloxyacyl hydrolase-like fashion (5). It has been much more difficult to analyze LPS breakdown products when the LPS is presented in intact bacteria, because methods for selectively labeling the acyl chains of LPS during their biosynthesis do not exist and because the fatty acids released from LPS by leukocytes are rapidly incorporated into cellular lipids (6). As a result, it is still unknown whether the mammalian host is also capable of degrading and detoxifying LPS in its natural setting, the bacterial outer membrane. This is especially important because whole bacteria can signal mammalian host cells with greater potency than isolated LPS (7) and because introduction of purified LPS and live bacteria into animals yield different cellular distributions and subsequent fates of immunologically detectable LPS (8).

We now describe methods that allow quantitative separation of all of the major classes of radiolabeled bacterial molecules and their degradation products, permitting for the first time a reliable quantitative assessment of LPS deacylation during extracellular and intracellular antibacterial action of the mammalian host. We show that in this inflammatory setting, loss of bacterial viability was not only accompanied by hydrolysis of large portions of the bacterial phospholipids, RNA, and protein (6, 9, 10) but also by extensive deacylation of LPS by an acy-
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**Experimental Procedures**

**Materials**

Sodium [2-14C]acetate (57 mCi/mmol), l-[35S]methionine (1175 Ci/mmole), [2-14C]ursulic acid (2.25 mCi/mmol) were from NEN Life Science Products. Human serum albumin (HSA) was from Armour Pharmaceutical Co. (Kanakee, IL). Oyster glycogen was from U. S. Biochemical Products. Human serum albumin (HSA) was from Armour Pharmaceutical Co. Biological saline, and then subcultured for 20 h at 30 °C at a starting bacteria were harvested by centrifugation at 3000 g, washed once in sterile physiological saline, and then subcultured for 20 h at 30 °C at a starting concentration of 2.4 × 10^7 bacteria/ml. Bacterial LPS and PL were labeled using 0.3 ml (17 μCi/ml) sodium [2-14C]acetate, yielding ~15,000 cpm incorporated/10^6 bacteria. Protein and RNA were labeled using the growth conditions described above, except subcultures were grown with 0.3 ml unlabelled sodium acetate and were supplemented with 5.5 μCi/ml L-[35S]methionine (yielding 700 to 2000 cpmp/10^6 bacteria) or 2 μCi/ml 2-[14C]ursulic acid (yielding 300 cpm/10^6 bacteria). After labeling, the bacteria were sedimented in a Microfuge at 3000 × g and cultured an additional 30 min at 37 °C in an equal volume of fresh modified E medium containing 0.5 ml unlabelled sodium acetate to drive cell-associated radiolabeled precursors into macromolecules. Sterile EF, which was added to 1% (to remove unesterified fatty acids), and the bacteria were harvested by centrifugation at 3000 × g.

**Collection and Separation of Rabbit Leukocytes and Aqueous Fluid (AF)—** Sterile inflammatory peritoneal exudates were collected from New Zealand White rabbits, and cells (PMN and mononuclear cells (MNC)) and AF were isolated as described previously (5). To provide a biological fluid containing opsonins but devoid of antibacterial activity, leukocyte PL (mainly PC, radiolabeled by HEMA-3 stain was from Biochemical Sciences, Inc (Swedesboro, NJ). Cycloheximide was from Sigma. Trichloroacetic acid was from Fisher. CM-Sephadex was from Amersham Pharmacia Biotech. Glass microfibre (GF/C) filters were from Whatman (Clifton, NJ). Aquasol-2 scintillation fluid was from Packard Instrument Co. (Meriden, CT). Silica gel G plates were from Analtech (Newark, DE); reverse-phase KC18 plates were from Whatman. Radiolabeled thin-layer chromatography (TLC) standards, [1-14C]12:0 (58 mCi/mmole), n-9,10-H14:0 (53 Ci/mmole), n-9,10-H16:0, and [1-14C]18:0 (57 mCi/mmole) were all from Amersham Pharmacia Biotech. Unlabeled 18:1, PE, PG, PC, LPE, and LPG standards were from Sigma. [14C]LPS was purified from *Escherichia coli* LCD25 as described previously (11).

**Bacterial Strains and Growth Conditions**

_E. coli_ LCD25, an avrEF, glnA strain of _E. coli_ K12 unable to produce acetate or use acetate as a carbon or energy source, was grown in modified minimal E medium (12) with twice the stated concentrations of salts, glucose, and amino acids as described by Munford et al. (11) with the following modifications. Bacteria were grown overnight at 30 °C in liquid 2 × E medium containing 0.5 ml sodium acetate, sedi-

**Assay Conditions**

Labeled _E. coli_ LCD25 were incubated (final concentration of 1 × 10^9/ml) with 90% (v/v) AF or 50% (v/v) C6d UBAF in HBSS containing 1% HSA. All incubations (200-μl volume) were buffered to pH 7.3–7.4 with 20 mM HEPES. For protein degradation experiments, incubations with leuko-

**Lipid Analysis (see Fig. 1)**

At the indicated times, samples containing bacteria labeled with sodium [2-14C]acetate were extracted according to the procedure of Bligh and Dyer (14). At time = 0, mean partitioning (± S.E.) of the total bacterial radiolabeled material in this step was 74.6 ± 0.8% into chloroform, 23.9 ± 0.8% into the interface (IF), and 1.5 ± 0.2% into H2O/methanol, with essentially complete recovery of cpm.

**A) Phospholipid Degradation—** After growth of the bacteria with [35S]methionine, 68% of the total incorporated cpm were in the PLs. Intact PLs and the radiolabeled products of PL degradation were quantita-

**Table I**

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<th>IF</th>
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<tr>
<td>A</td>
<td>3-OH-14:0</td>
<td>11.2 ± 0.9</td>
<td>≤0.7 ± 0.2</td>
<td>9.9 ± 0.6</td>
</tr>
<tr>
<td>B</td>
<td>3-OH-14:0</td>
<td>1.00</td>
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<td></td>
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<tr>
<td></td>
<td>12:0</td>
<td>0.23</td>
<td>0.24 ± 0.01</td>
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<td>14:0</td>
<td>0.32</td>
<td>0.44 ± 0.01</td>
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*Probably an overestimate due to incomplete resolution from neighboring radiolabelled spots (see Fig. 2A, lanes 4 and 5).* glass microfibre (GFC) filters, and tubes and filtered sediment were washed 3 times with 1.5 ml of 10% ice-cold trichloroacetic acid. Filters were washed once with ice-cold 95% ethanol and air-dried. Precipitates were solubilized with 100 μl of 2% SDS and counted in a Beckman LS6000IC liquid scintillation counter using Aquasol-2 scintillant.

**Protein and RNA Degradation**

To ensure effective resuspension of clumped leukocytes, supernatant fluid (200 μl) was removed from tubes and replaced with 300 μl of divalent cation-poor buffer (HBSS containing 0.1% HSA). Resus-

The amounts of 3-OH-[14C]14:0, [14C]12:0, and [14C]14:0 in these frac-

**Loss of radiolabeled LPS-derived fatty acids (3-OH-14:0, 12:0, and 14:0) from the interface. After release from the IF, these fatty acids accumulated in the chloroform phase as FFAs or fatty acids esterified into host cell lipids (5).**

To measure the fatty acid composition of the LPS collected from the IF, this material was dried under N2 and hydrolyzed with 4 N HCl and
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4 N NaOH as described previously (15). The samples were then acidified to pH 4.0 with acetic acid and extracted by the method of Bligh and Dyer. Of the cpm recovered after hydrolysis of the IF, 95.8 ± 1.2% was recovered after re-extraction. Of these cpm, 92–100% partitioned into the chloroform phase. The LPS-derived FA were then resolved and quantitated using TLC in two steps. First, the hydroxylated (3-OH-14:0) and nonhydroxylated (NFA) (12:0, 14:0) fatty acids were separated by TLC. Second, to resolve 12:0 and 14:0, the NFA from TLC1 were eluted from the silica gel and separated by reverse-phase TLC using KC18-substituted silica gel and solvent system 3 (TLC2, see below). Labeled species on all TLC plates were detected and quantitated by AMBIS-1000 or by autoradiography. Labeled FFA standards (3-OH-14:0 derived from purified 14C-acetate-labeled E. coli LPS after acid/base hydrolysis and 14C-12:0, 14C-16:0, and 14C-18:0 obtained from commercial sources) were run on TLC in parallel to identify labeled species from experimental samples. The total cpm in 3-OH-14:0 in the interface at a given time was calculated as follows: (total cpm in hydrolyzed IF) × (% re-extracted into CHCl3) × (% of this material migrating as 3-OH-14:0 in TLC2).

The total cpm in 12:0 or 14:0 in the interface at a given time was calculated as follows: (total cpm in hydrolyzed IF) × (% re-extracted into CHCl3) × (% of this material migrating as either 12:0 or 14:0 in TLC2).

LPS deacylation is expressed as % loss of each fatty acid from the interface over time, using the values for each acyl species obtained from unincubated bacterial suspensions (time = 0) as 100%.

**TLC**—Three TLC systems were used, as described previously (16). TLC plates were developed for 45–50 min with petroleum ether/diethyl ether/acetic acid (70:30:1; v:v:v) to separate PL and various FA species. Migration of unlabeled 18:1, PE, PG, and triglycerides (at solvent front). Samples were run in parallel with standards: 3-OH-14C-12:0 (purified from TLC3), 3-OH-14C-16:0, and 3-OH-14C-18:0 (at origin), 3-OH-14:0, NFA, and triglycerides (at solvent front). TLC2—Silica gel G plates were developed for 100–110 min with petroleum ether/diethyl ether/acetic acid (70:30:1; v:v:v) to separate various PL and lyso-PL species. Migration of unlabeled 18:1, PE, PG, PC LPE, and LPG standards was determined after detection by iodine vapor staining.

**Elution from Silica G**—After visualization with AMBIS-1000, radioactive spots of silica G were scraped from the plate and eluted by washing once with 200–500 μl of chloroform/methanol/acet/cidic/acid/H2O (55:35:9:4; v:v:v:v) once with an equal volume of chloroform, and once again with chloroform/methanol/acet/cidic/acid/H2O (55:35:9:4; v:v:v:v). Each eluate was then loaded by a 10-min incubation at 42 °C. Recovery of radiolabeled lipids after elution was 83.7 ± 1.5% and was essentially the same for PL and various FA species.

**Uptake of Bacteria by Host Cells**—After a 1-h incubation (see assay conditions), samples containing bacteria and leukocytes were centrifuged at 70–100 × g to separate leukocyte-associated and extracellular bacterial. The leukocyte pellet was washed once with HBSS containing 0.1% HSA and once with HBSS containing 0.1% HSA to remove adherent but nonphagocytosed bacteria. Radioactivity in the pellet, supernatant, and washes were measured by scintillation counting. In addition, cell smears were prepared after 15- and 60-min by cytospin and were stained with HE/K-3.

**Bacterial Viability**—After 1 h at 37 °C, samples were taken, diluted with sterile saline, and plated on nutrient broth agar as described previously (15). Viability was measured as the number of colonies formed after incubation at 37 °C for 18–24 h.

**RESULTS**

**Quantitative Recovery of LPS from E. coli LCD25 and Separation from PL**—To measure deacylation of LPS in E. coli LCD25 during extracellular killing by AF and intracellular killing by inflammatory exudate cells, it was necessary to resolve LPS-derived fatty acids from FA linked to other bacterial macromolecules (principally phospholipids) that were labeled during growth in [14C]acetate-supplemented medium. Acetate-labeled LPS purified from E. coli LCD25 contained mainly three fatty acid species: 3-OH-14:0 (see Fig. 2A, lane 2), 12:0 and 14:0 (Fig. 2B, lane 3) (11); see also Ref. 5. All 3-OH-14:0 and nearly all 12:0 in E. coli is found in the lipid A moiety of LPS, whereas 14:0 in E. coli is divided roughly equally between PL and LPS (17).

Using the extraction procedure of Bligh and Dyer (see Fig. 1), essentially all compounds in E. coli LCD25 that contained labeled 3-OH-14:0 were recovered in the IF between the aqueous and CHCl3 phases (TableIA and Fig. 2A, lanes 3 and 4), and virtually none were recovered in the CHCl3 phase (TableIA and Fig. 2A, lane 5). Since 3-OH-14:0 is unique to the lipid A moiety of bacterial LPS, this finding indicates that nearly all bacterial LPS was recovered in the IF. Similar complete recovery of partially deacylated LPS in the IF was shown recently (5). In contrast, during this extraction procedure, ≥95% PL, FFAs, and other degradation products of PL were recovered in the chloroform phase (18). Although the IF of extracted bacteria contained fatty-acylated compounds other than LPS, acyl groups from these contaminants co-migrated chiefly with 16:0 on TLC (compare lanes 3 and 6 of Fig. 2B). Evidence that these contaminants contained little or no 12:0 and 14:0 is provided by the closely similar relative amounts of 3-OH-14:0, 12:0, and 14:0 in the interface of extracted bacteria and purified LPS (TableIB). Thus, measurement of the loss of 3-OH-14:0, 12:0, and 14:0 from the IF allowed clear assessment of LPS deacylation.

**Determination of PL Hydrolysis**—For analysis of deacylation of phospholipids, we used the chloroform phase of the same extracts of cell suspensions that provided the IF for determination of LPS deacylation. In TLC2, intact PL and lyso-PL remained at the origin of the chromatogram (Fig. 2A, lanes 6–9). The percentage of this radiolabeled material that consisted of intact bacterial PL (PE, PG, or CL) was determined using TLC2. By 20 h in AF (Fig. 2A, lane 7), the labeled species at the origin of TLC2 represented almost exclusively monoacyl catabolates of bacterial PL (PE and PG not shown), whereas after ingestion by PMN (Fig. 2A, lane 8) or MNC (Fig. 2A, lane 9), some of the label at the origin was contained in the phagocyte lysosome PL (not shown). Labeled species that co-migrated with the solvent front also accumulated; these were probably neutral lipids (e.g. triglycerides), reflecting leukocyte incorporation of labeled FA that were released from bacterial lipids, as reported previously (3, 6, 10) and in a recent publication (5).

**Deacylation of LPS and PL during Extracellular Killing of E. coli by AF**—To examine LPS deacylation during extracellular bacterial killing, [14C]acetate-labeled E. coli LCD25 were incu-
bated with 90% AF, resulting in rapid killing of *E. coli* LCD25 (≥97% by 1 h, data not shown). This killing was accompanied by hydrolysis of >95% bacterial PL by 1 h (Fig. 3A). In contrast, no loss of 3-OH-14:0, 12:0, or 14:0 from bacterial LPS was observed even after incubation for 20 h (Fig. 3A).

**Leukocyte-dependent Decaylation of LPS in *E. coli* Killed Extracellularly by AF**—We have shown recently that acyloxyacyl hydrolase activity is much greater in inflammatory exudate leukocytes than in AF (5). Therefore, LPS deacylation may require either uptake of LPS shed from killed bacteria or ingestion of intact bacteria. When bacteria were incubated with inflammatory exudate leukocytes in the presence of AF, bacterial killing and PL hydrolysis took place principally extracellularly, i.e. before ingestion. This was evident from our inability to observe intact bacteria inside phagocytes by light microscopy (not shown). Moreover, the rate and extent of bacterial PL hydrolysis by AF plus phagocytes paralleled much more closely the effects of AF alone than those of PMN and MNC toward ingested bacteria (compare Figs. 3 and 4). The addition of MNC but not of PMN to AF resulted in substantial loss of 12:0 and 14:0 from bacterial LPS (Fig. 3), in a pattern closely similar to that seen when these cells take up purified LPS (5).

![Figure 2](http://www.jbc.org/)

*Fig. 2.* TLC of fractions derived from purified LPS and *E. coli* labeled with [14C]-acetate. *E. coli* LCD25 were labeled with [14C]-acetate during growth as described under “Experimental Procedures.” LPS was prepared from radiolabeled *E. coli* as described previously (11). See Fig. 1 for details of preparation of samples for TLC analysis. A, TLC. Lane 1, purified [14C]12:0 and 3-OH-[14C]14:0 standards; lane 2, hydrolyzed purified LPS; lane 3, hydrolyzed whole bacteria; lane 4, hydrolyzed interface material from extracted bacteria; lane 5, hydrolyzed CHCl3 phase material from extracted bacteria; lane 6, CHCl3 phase from extracted bacteria. CHCl3 phase of extracted cell suspensions after a 20-h incubation of bacteria with AF (lane 7) or with C6-deficient UBAF + PMN (lane 8) or plus MNC (lane 9) is shown. B, TLC. Lane 1, purified [14C]12:0 and [3H]14:0 standards; lane 2, [14C]12:0, -16:0, and -18:0 standards. Total NFA recovered from hydrolyzed purified LPS (lane 3), hydrolyzed bacteria (lane 4), hydrolyzed CHCl3 phase material from extracted bacteria (lane 5), and hydrolyzed interface material from extracted bacteria (lane 6) is shown. Radioactive species in lane 1 of panel B were detected by fluorography; all other samples were detected by proportional argon ionization.

LPS and PL Deacylation during Intracellular Killing by Inflammatory Exudate Leukocytes—Incubation of [14C]-acetate-labeled *E. coli* LCD25 with either PMN or MNC in the presence of C6-deficient UBAF, which lacks antibacterial activity (not shown) but contains complement-derived osonins present in the exudate, resulted in substantial bacterial ingestion. After 1 h, 61% of the radiolabeled bacteria were associated with PMN, which contained an average of 4 bacteria/cell (as judged by light microscopy; not shown). Similarly, MNC took up 65% of bacterial radiolabel after 1 h, with an average of 7 bacteria/cell. Accompanying this uptake, PMN and MNC caused 99 and 86% loss of bacterial colony-forming units within 1 h and up to 70 and 90% hydrolysis of bacterial PL, respectively, within 8–20 h (Fig. 4). PMN caused significant (albeit slower and less extensive) loss of 12:0 and 14:0 from bacterial LPS reaching 20–30% by 20 h (Fig. 4). MNC more rapidly and extensively deacylated LPS, as is shown by the loss of nearly 50% of the 12:0 and 14:0 from the interface by 4 h. No deacylation occurred in C6-deficient UBAF alone (data not shown).

Thus, whereas only MNC effectively deacylated cell-free LPS after extracellular killing of bacteria by inflammatory fluid, PMN as well as MNC caused LPS deacylation after ingestion of intact bacteria. Under all conditions tested, 3-OH-14:0 was quantitatively retained in the interface (Figs. 3 and 4), demonstrating that the acyloxyacyl bonds of lipid A were the sole target of the LPS-deacylating activity manifest during and after killing of *E. coli* by inflammatory exudates.

**Degradation of RNA and Proteins**—The preceding findings show a dramatic difference in the deacylation of PL and LPS during killing by either AF or PMN. To determine whether degradation of bacterial macromolecules under these conditions is generally slow and limited (as for LPS) or more rapid and extensive (as for PL), degradation of biosynthetically labeled bacterial RNA and proteins was determined during extracellular killing of *E. coli* LCD25 by AF or during intracellular killing by inflammatory exudate leukocytes (≥85% PMN). In AF alone, degradation of RNA was maximal at 2 h, reaching 70%, whereas protein degradation reached a plateau of 10% by 1 h (Fig. 5A). Ingestion by exudate leukocytes was accompanied by rapid degradation of RNA that reached its maximum of 65–75% by 2 h, whereas protein degradation reached a plateau of ≤30% by 1 h (Fig. 5B). Thus, like PL, degradation of RNA and proteins in *E. coli* LCD25 is rapid, reaching an early plateau. Degradation of RNA by both cellular and extracellular components of the rabbit exudate was particularly extensive, as has been shown previously in different bacterial species (9, 10, 19). Therefore, deacylation of LPS by AF and PMN is slow and limited relative to degradation of other bacterial components.

![Figure 3](http://www.jbc.org/)

*Fig. 3.* Deacylation of bacterial LPS and PL during incubation of [14C]-acetate-labeled *E. coli* LCD25 with 90% AF alone (A) or in the presence of PMN (B) or MNC (C). Incubations and assays of the time-dependent loss of 12:0 (●), 14:0 (□), and 3-OH-14:0 (○) from LPS and loss of intact bacterial PL (▲) were carried out as described under “Experimental Procedures.” The data shown represent the mean (± S.E.) of at least four separate determinations from at least two separate experiments. Where error bars are not visible, they are smaller than the plot symbols.
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Deacylation of LPS was determined by measuring the loss of the lipid A-specific fatty acids 3-OH-14:0 and 12:0 from the LPS-containing IF between the aqueous and chloroform phases of extracted samples. Because the IF is essentially free of phospholipids (18), deacylation of lipid A was also assessed by measuring the loss of 14:0, a major fatty acyl constituent of lipid A and only a minor component of phospholipids. In the IF of extracts of bacteria, the appearance of material containing radiolabeled species co-migrating with 16:0 (Fig. 2B) may be attributed to the presence of Braun lipoproteins containing labeled acyl chains. The acyl groups in this lipoprotein (75% 16:0 and 18:1) (22) co-migrate with 16:0 and not with any of the fatty acyl groups of lipid A, and therefore do not confound the analysis of LPS deacylation.

During incubation with ascitic fluid, prompt killing of E. coli LCD25 was observed (not shown) in accord with the known antibacterial properties of rabbit inflammatory peritoneal fluid (13). Killing was accompanied by extensive disassembly of the bacteria indicated by complete hydrolysis of PL (Fig. 3), extensive degradation of RNA (Fig. 5), and lack of uptake of visibly intact bacteria by phagocytes (not shown). However, no LPS deacylation was observed (Fig. 3A), despite the ability of ascitic fluid to degrade purified LPS (5). LPS still associated with bacterial remnants or with bacterial outer membrane proteins or phospholipids (23–25) may be less subject to deacylation than purified LPS. Alternatively, because the dose of whole bacteria contained 5–10-fold more LPS than was presented previously in purified form (5), the deacylating capacity of AF may have been exceeded in the current experiments with whole bacteria. In support of this explanation, increasing the amount of purified LPS presented to AF decreased the percentage deacylated.

The addition of PMN during AF-mediated extracellular killing of bacteria did not enhance deacylation (Fig. 3B), similar to the lack of contribution of added PMN to AF-mediated deacylation of purified LPS (5). In contrast, MNC caused progressive deacylation of LPS during AF-mediated extracellular killing of bacteria reaching 40–50% by 20 h (Fig. 3C). Uptake of LPS by the two cell types during extracellular killing was not assessed, but because PMN express at least −5-fold less mCD14 (26) and hence bind purified LPS less effectively than do MNC (5), PMN are also likely to internalize less LPS from extracellularly disassembled bacteria than MNC. However, when intact bacteria were killed intracellularly after ingestion, bacterial LPS was deacylated by both MNC and PMN. As shown by retention of 3-OH-14:0 and loss of NFA in the interface (Fig. 4), this deacylating activity of the cells was acyloxyacyl hydrolase-like. Although ingestion of intact bacteria by PMN and MNC was similar, the rate and extent of LPS deacylation was much greater in MNC (−50% by 4 h versus 20–30% by 20 h in PMN), consistent with a −5-fold higher acyloxyacyl hydrolase activity in these cells (5).

The finding that PMN do partially deacylate LPS of ingested bacteria but do not deacylate purified LPS or LPS shed from bacteria in AF may be explained by avid phagocytosis of intact bacteria by PMN and limited ability to internalize cell-free LPS (5). Thus, the physical presentation of LPS may determine the delivery to different host cells and, therefore, the extent of their participation in deacylation and detoxification of LPS.

We have shown before that phagocytosis of E. coli by rabbit exudate cells is accompanied by extensive degradation of bacterial macromolecules (9, 10). Ingestion of E. coli LCD25 by whole exudate cells (−90% PMN) also triggered extensive degradation of bacterial RNA (Fig. 5) and hydrolysis of most of the bacterial phospholipids by both phagocyte types (Fig. 4). However, only MNC deacylated LPS almost as efficiently as phospholipids (Fig. 4B), consistent with a prominent role of MNC in LPS detoxification.

How LPS is detoxified during an infection in vivo is likely to be influenced prominently by the extent to which the invading bacteria are disassembled by extracellular factors. When invading organisms in the blood stream are relatively fragile and

DISCUSSION

Previous analysis of the fate of LPS of whole bacteria has been hampered until recently by technical difficulties including radiolabeling of LPS to adequate specific activity and separation of LPS and its degradation products from other radiolabeled bacterial constituents. It has been claimed in previous studies that mouse macrophages can slowly degrade LPS of biosynthetically labeled bacteria (20, 21). However, because reaction products could not be identified or chemically characterized, the nature and extent of the process remained undefined.

The introduction of E. coli LCD25 for high specific activity fatty acid radiolabeling with acetate (11) has now made possible reliable detection of lipid A-derived degradation products (FA and partially deacylated LPS) using as few as five bacteria per host cell. Incorporation of acetate by this E. coli mutant is limited to fatty acyl groups of LPS, phospholipids, and lipoproteins. Virtually complete separation of these bacterial products and their constituent fatty acids can be accomplished by the methods used in this study. Our ability to assess the recovery of LPS and the radiological purity of the recovered LPS is based upon the labeling of 3-OH-myristate, a unique and quantitatively constant acyl constituent of lipid A.

Deacylation of LPS was determined by measuring the loss of the lipid A-specific fatty acids 3-OH-14:0 and 12:0 from the LPS-containing IF between the aqueous and chloroform phases of extracted samples. Because the IF is essentially free of phospholipids (18), deacylation of lipid A was also assessed by measuring the loss of 14:0, a major fatty acyl constituent of lipid A and only a minor component of phospholipids. In the IF of extracts of bacteria, the appearance of material containing radiolabeled species co-migrating with 16:0 (Fig. 2B) may be attributed to the presence of Braun lipoproteins containing labeled acyl chains. The acyl groups in this lipoprotein (75% 16:0 and 18:1) (22) co-migrate with 16:0 and not with any of the fatty acyl groups of lipid A, and therefore do not confound the analysis of LPS deacylation.

During incubation with ascitic fluid, prompt killing of E. coli LCD25 was observed (not shown) in accord with the known antibacterial properties of rabbit inflammatory peritoneal fluid (13). Killing was accompanied by extensive disassembly of the bacteria indicated by complete hydrolysis of PL (Fig. 3), extensive degradation of RNA (Fig. 5), and lack of uptake of visibly intact bacteria by phagocytes (not shown). However, no LPS deacylation was observed (Fig. 3A), despite the ability of ascitic fluid to degrade purified LPS (5). LPS still associated with bacterial remnants or with bacterial outer membrane proteins or phospholipids (23–25) may be less subject to deacylation than purified LPS. Alternatively, because the dose of whole bacteria contained 5–10-fold more LPS than was presented previously in purified form (5), the deacylating capacity of AF may have been exceeded in the current experiments with whole bacteria. In support of this explanation, increasing the amount of purified LPS presented to AF decreased the percentage deacylated.2

The addition of PMN during AF-mediated extracellular killing of bacteria did not enhance deacylation (Fig. 3B), similar to the lack of contribution of added PMN to AF-mediated deacylation of purified LPS (5). In contrast, MNC caused progressive deacylation of LPS during AF-mediated extracellular killing of bacteria reaching 40–50% by 20 h (Fig. 3C). Uptake of LPS by the two cell types during extracellular killing was not assessed, but because PMN express at least −5-fold less mCD14 (26) and hence bind purified LPS less effectively than do MNC (5), PMN are also likely to internalize less LPS from extracellularly disassembled bacteria than MNC. However, when intact bacteria were killed intracellularly after ingestion, bacterial LPS was deacylated by both MNC and PMN. As shown by retention of 3-OH-14:0 and loss of NFA in the interface (Fig. 4), this deacylating activity of the cells was acyloxyacyl hydrolase-like. Although ingestion of intact bacteria by PMN and MNC was similar, the rate and extent of LPS deacylation was much greater in MNC (−50% by 4 h versus 20–30% by 20 h in PMN), consistent with a −5-fold higher acyloxyacyl hydrolase activity in these cells (5).

The finding that PMN do partially deacylate LPS of ingested bacteria but do not deacylate purified LPS or LPS shed from bacteria in AF may be explained by avid phagocytosis of intact bacteria by PMN and limited ability to internalize cell-free LPS (5). Thus, the physical presentation of LPS may determine the delivery to different host cells and, therefore, the extent of their participation in deacylation and detoxification of LPS.

We have shown before that phagocytosis of E. coli by rabbit exudate cells is accompanied by extensive degradation of bacterial macromolecules (9, 10). Ingestion of E. coli LCD25 by whole exudate cells (−90% PMN) also triggered extensive degradation of bacterial RNA (Fig. 5) and hydrolysis of most of the bacterial phospholipids by both phagocyte types (Fig. 4). However, only MNC deacylated LPS almost as efficiently as phospholipids (Fig. 4B), consistent with a prominent role of MNC in LPS detoxification.

How LPS is detoxified during an infection in vivo is likely to be influenced prominently by the extent to which the invading bacteria are disassembled by extracellular factors. When invading organisms in the blood stream are relatively fragile and

2 Y. Weinrauch, unpublished observations.
are destroyed extracellularly or when antibiotics are present, neutralization of LPS by plasma (lipo)proteins may be most important in detoxification. Such complexing is viewed as an important step in the delivery of LPS to the liver and its subsequent disposal (24, 27–29). At extravascular sites where lipoprotein-mediated clearance is less prominent, delivery of shed LPS to MNC and subsequent deacylation may be more important. In addition, at localized inflammatory sites (e.g. the peritoneal cavity), cationic proteins such as the bactericidal/permeability-increasing protein (BPI) (13) may both promote ingestion of bacteria (30) leading to deacylation and detoxification by phagocytes and prevent protracted LPS signaling by complexing shed LPS (31). The mobilization of extracellular acyloxyacyl hydrolase at inflammatory sites may contribute further to LPS detoxification, especially when LPS loads are lower than those presented in this study. Moreover, because plasma acyloxyacyl hydrolase levels in the rabbit are markedly elevated in some inflammatory settings (32), extracellular LPS deacylation in vivo may be more prominent than shown here. Inactivation of LPS either by complexing with lipoproteins or by deacylation is not as fast (Refs. 5 and 31 and this study) as the very rapid lipopolysaccharide-binding protein and CD14-mediated LPS signaling and therefore should not interfere with initial activation of host defenses but prevent continued and excessive host responses.

Our findings suggest that the fate of the LPS in Gram-negative bacteria that are resistant to extracellular disassembly (25, 33) will depend on intracellular events after ingestion and particularly upon the participation of MNC. Because PMN greatly outnumber mononuclear phagocytes, the majority of bacterial invaders are initially ingested by PMN. However, after intravenous administration of smooth bacteria to rats, immunologically detectable LPS is found primarily in mononuclear phagocytes (8). The present study shows that mononuclear phagocytes are more effective at LPS deacylation than PMN, suggesting eventual translocation of LPS from PMN to MNC.

Two mechanisms may be proposed for such a transfer. 1) LPS and partially deacylated species may be released from PMN (34) and subsequently taken up by mononuclear phagocytes. 2) Bacteria-laden PMN may become apoptotic (35) and, consequently, subject to ingestion by macrophages (36, 37) in a process leading to dampening of inflammatory responses (38). In summary, deacylation of bacteria-associated or shed LPS over the course of several hours allows initial signaling of host cells and up-regulation of antibacterial defenses but may curb prolonged and potentially self-destructive signaling as LPS remains present at many sites in the host after infection. This study provides the first evidence that mammalian phagocytes in a local inflammatory setting can degrade the endotoxic moiety of LPS when it is presented as a constituent of whole bacteria.

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Deacylation of Lipopolysaccharide in Whole Escherichia coli during Destruction by Cellular and Extracellular Components of a Rabbit Peritoneal Inflammatory Exudate

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