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Uptake and Deacylation of Bacterial Lipopolysaccharides by Macrophages from Normal and Endotoxin-Hyporesponsive Mice

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Macrophages are thought to play a central role in the responses of animals to gram-negative bacterial lipopolysaccharides (LPS). Since nothing is known about the metabolism of LPS by these cells, we studied the uptake and deacylation of radiolabeled LPS by thioglycolate-elicited peritoneal macrophages from normal (C3H/HeN) and LPS-hyporesponsive (C3H/HeJ) mice. Macrophages from both kinds of mice took up and deacylated LPS that were added to the culture medium. Opsonization of the LPS with anti-LPS immunoglobulin G antibodies greatly increased LPS uptake; the opsonized LPS also underwent deacylation at rates that were directly related to the amount of cell-associated LPS. An analysis of the fatty acid composition of the cell-associated LPS indicated that the cells have one or more acyloxyacyl hydrolases that remove the non-hydroxylated fatty acids that are normally substituted to the hydroxyl groups of (glucosamine-linked) 3-hydroxytetradecanoate residues in lipid A; we also found evidence for deacylation of 3-hydroxytetradecanoate from the glucosamine backbone. LPS deacylation by macrophages from C3H/HeN and C3H/HeJ mice was qualitatively and quantitatively similar. Nonopsonized LPS are able to stimulate LPS-responsive cells; in these studies we established that animal cells can deacylate nonopsonized LPS, thus raising the possibility that LPS metabolism may play a role in modulating cellular stimulation.

According to much recent evidence (13), the lipopolysaccharides (LPS) of gram-negative bacteria produce many of their effects in vivo by first stimulating host cells. The cells then release mediator compounds that produce alterations such as fever, hypotension, and clotting abnormalities. Although it is known that LPS may stimulate macrophages to release various potential mediators, the molecular basis for the interaction between LPS and these cells is not understood. In one approach to the analysis of this interaction, we studied the uptake and metabolism of LPS by macrophages.

Lipid A is responsible for most of the biological activities of LPS. Lipid A is a glucosamine disaccharide to which phosphates, fatty acids, and a polysaccharide chain are covalently attached. Recent evidence (18, 20, 28) indicates that four 3-hydroxytetradecanoic acid (3-OH-14:0) residues are linked directly to the glucosamine disaccharide of *Salmonella* and *Escherichia coli* lipid A (Fig. 1). Non-hydroxylated fatty acids (principally dodecanoic and tetradecanoic acids) are esterified to the hydroxyl groups of some of these 3-OH-14:0 residues. The glucosamine-linked fatty acids thus may have an acyloxyacyl structure. Although the structural requirements for lipid A activity are not known with certainty, much evidence indicates that 3-OH-14:0 residues attached to glucosamine are necessary for activity; in contrast, the non-hydroxylated fatty acids do not appear to be required for many of the activities of lipid A (6, 16, 23).

We reported recently that human peripheral blood neutrophils have an enzyme(s) that deacylates antibody-opsonized *Salmonella typhimurium* LPS by preferentially removing the non-hydroxylated fatty acids from lipid A (10). In the present study we evaluated the deacylation of lipid A by thioglycolate-elicited murine peritoneal macrophages. Murine macrophages were attractive candidates for study since there are well-characterized strains of mice that, unlike normal mice, do not respond to low doses of LPS (25). Thus, macrophages from LPS-responsive (C3H/HeN) and LPS-hyporesponsive (C3H/HeJ) mice could be compared, testing the hypothesis that the defect in the LPS-hyporesponsive cells alters their inability to deacylate LPS. Preliminary experiments showed that thioglycolate-elicited macrophages took up much more LPS from the culture medium in the absence of specific anti-LPS antibodies than did resident peritoneal macrophages; by using the elicited cells it was possible to compare for the first time the uptake and metabolism of antibody-opsonized and nonopsonized LPS by animal cells.

MATERIALS AND METHODS

LPS preparations. *S. typhimurium* PR122 (*galE nag*$^-$) cells were biosynthetically labeled with [2-^{3}H]acetate and *N*-acetyl-1-[^{14}C]glucosamine (New England Nuclear Corp., Boston, Mass.) in proteose peptone-beef extract broth (10). Two preparations of LPS were used for these studies. To examine the uptake and deacylation of LPS in the absence of specific opsonins, we used LPS that had short and relatively homogeneous polysaccharide chains (rough LPS [R-LPS]). To study the uptake and deacylation of antibody-opsonized LPS, we prepared LPS with long polysaccharide chains (smooth LPS [S-LPS]) that contained O-antigenic determinants recognized by a rabbit antiserum to *S. typhimurium* (14). R-LPS were prepared by growing strain PR122 cells in the absence of D-galactose and extracting the LPS by the method of Galanos et al. (7). The R-LPS were extracted several times with diethyl ether, suspended at a concentration of 1 μg/μl in distilled water that contained 0.1% (vol/vol) triethylamine, and stored at −70°C; 1 μg of R-LPS (0.27 nmol, assuming an M_r of 3,600) contained 66,800 cpm of ^{3}H-fatty acid and 6,600 cpm of [^{14}C]glucosamine. To prepare S-LPS, strain PR122 cells were grown in the presence of 0.05 mM D-galactose, and the LPS were purified from isolated bacterial outer membranes as previously described (15). Af-

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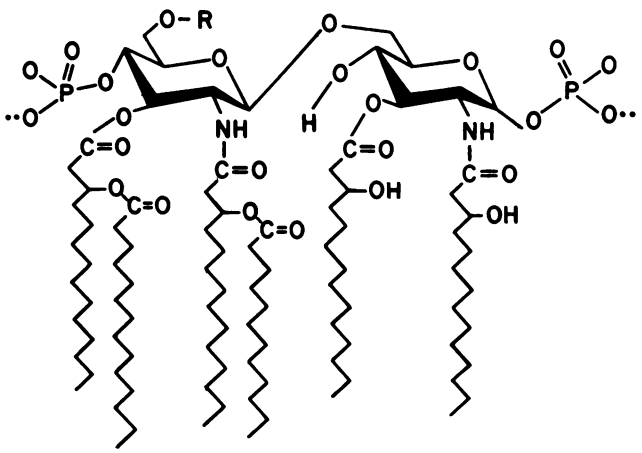

FIG. 1. Structure of *S. typhimurium* lipid A. The structure proposed by Takayama et al. (20) is shown. R indicates the site of attachment of the polysaccharide chain to the 6′ position of the disaccharide. Within a preparation of LPS there is heterogeneity in the degree to which the hydroxyl groups of the 3-O-14:0 residues are substituted with normal fatty acids (C_{12}, C_{14}, and C_{16}) (28). There is also micro-heterogeneity in the phosphate and polysaccharide contents of LPS.

ter several extractions with diethyl ether and extensive dialysis against distilled water, the S-LPS were stored in aliquots at −70°C. The amount of S-LPS in the preparation was estimated from the specific activities of the LPS fatty acids, as determined by high-pressure liquid chromatography (HPLC) (see below). An average of six fatty acids per molecule and an average M_r of 7,000 were assumed; 1 μg (0.14 nmol) had 16,000 cpm of ^{3}H-fatty acid and 2,400 cpm of [^{14}C]glucosamine.

Mouse macrophages. Mice that were 4 to 5 weeks old were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. (C3H/HeN mice), or from Jackson Laboratories, Bar Harbor, Maine (C3H/HeJ mice), and were stored in the animal facility of the University of Texas Health Science Center, Dallas. The mice were injected intraperitoneally with 2.0 ml of sterile Brewer thioglycolate broth (Difco Laboratories, Detroit, Mich.), and peritoneal cells were harvested 5 days later (24). In each experiment, C3H/HeN and C3H/HeJ mice of comparable ages (within 1 week) were used; all experiments were performed when the mice were 5 to 12 weeks old. Peritoneal cells from three or four mice were pooled, washed twice with Hanks buffered salt solution (M.A. Bioproducts, Walkersville, Md.), and suspended in RPMI 1640 medium (M.A. Bioproducts) that contained 2 mM L-glutamine, 1% (vol/vol) fetal bovine serum, 200 U of penicillin G per ml, and 4 μg of gentamicin per ml. Differential counts of cells stained with Wright-Giemsa stain showed that more than 95% of the cells were macrophages, and examination by a fluorescein diacetate-ethidium bromide method (21) revealed that more than 80% of the cells were viable. The cells were counted, adjusted to a concentration of approximately 1.5×10^6 viable cells per ml, and added in 1-ml portions to tissue culture wells (diameter, 35 mm; Costar, Cambridge, Mass.). After preliminary incubation for 3 to 4 h at 37°C under 5% CO_2, the adherent cells were washed twice with RPMI 1640 medium and then incubated again in RPMI 1640 medium that contained LPS in addition to the additives described above. To allow the cells to take up LPS by antibody-dependent phagocytosis, S-LPS were added to cells in the presence of immunoglobulin G (IgG) prepared from a rabbit antiserum to *S. typhimurium* (14). At the end of the incubation period the cells were washed twice with RPMI 1640 medium and examined for viability by using trypan blue exclusion; in the experiments reported here, more than 95% of the adherent cells remained viable at the time that they were harvested. The cells were scraped from the plastic wells into 1.0 ml of 0.05% (vol/vol) Triton X-100 and stored at −70°C. After the cells were thawed at 4°C, samples were removed to determine the amounts of cell-associated radioactive LPS (100 μl) and chloroform-extractable ^{3}H-fatty acids (500 μl) as previously described (10). An additional 150-μl portion was used to measure the amount of cellular DNA in each well (see below). LPS were incubated in culture medium without cells to control for nonspecific adsorption to the culture wells and for spontaneous deacylation of the LPS.

In other experiments, cells were incubated with LPS for 14 to 18 h (load), washed twice with warm RPMI 1640 medium containing 1% fetal bovine serum, and incubated (chase) in complete RPMI 1640 medium that did not contain LPS. The cells were harvested at sequential intervals as described above. The rate of LPS deacylation was defined as the increment in the amount of LPS-derived ^{3}H-fatty acids that became chloroform soluble during the first 30 h of the chase (nanomoles per milligram of cell DNA per 30 h).

Radioactivity measurements. Scintillation counting was performed with a model 2425 counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The amount of LPS in a sample was determined by relating its ^{14}C radioactivity to the specific activity (counts per minute per microgram) of the [^{14}C]glucosamine in the LPS. Determination of the ^{3}H radioactivity in samples included corrections for background events, as well as channel spillover (typically, there was 39% spillover from the ^{14}C to ^{3}H channels); for the samples tested there was little variability in quenching (tested by using an external standard), and a constant quench correction was assumed. The method used to calculate the amount of ^{3}H-fatty acid released from cell-associated LPS included a correction for the presence of small amounts of acylated LPS in the chloroform extracts; with the preparations of double-labeled LPS used, approximately 3 to 5% of the ^{14}C-LPS appeared in chloroform, regardless of the incubation conditions. The following formula was used for this correction: corrected ^{3}H-fatty acid counts per minute = (^{3}H counts per minute − background and spillover counts per minute) − (^{14}C counts per minute × K), where K is the ratio of ^{3}H counts per minute to ^{14}C counts per minute in the LPS used for the experiment. The average specific activity of the ^{3}H-fatty acids in the LPS was used to convert ^{3}H counts per minute to nanomoles of fatty acid.

Lipid analyses. Cells were scraped from culture wells into 0.9% NaCl that contained 50 mM EDTA and then extracted with chloroform-methanol (1). Butylated hydroxytoluene (0.005%, vol/vol) was added to the extraction mixtures. The distribution of LPS-derived ^{3}H-fatty acids in the chloroform phase was determined by two-dimensional thin-layer chromatography (TLC) on Silica Gel G plates (Analtech); the solvent used for the first dimension was petroleum ether-diethyl ether-acetic acid (70:30:2) (solvent 1), and the second dimension was run in chloroform-methanol-water (60:35:16) (solvent 2). To quantitate the fatty acid composition of the partially deacylated LPS, the methanol-water phase from the chloroform-methanol extraction was dried under N_2, and the fatty acids were hydrolyzed with acid (4 N HCl, 100°C, 90 min) and then with base (4 N NaOH, 100°C, 30 min). The hydrolysis mixture was brought to pH 4 with glacial acetic

acid or HCl, and the fatty acids were extracted into diethyl ether. The hydrolyzed fatty acids were examined by one-dimensional TLC on Silica Gel G, using solvent 1, or they were derivatized with α,*p*-dibromoacetophenone for analysis by reverse-phase HPLC (10). The amount of each LPS-derived fatty acid was determined by reference to linear plots (absorbance versus nanomoles of authentic standard) for each fatty acid, and the amount of radioactivity in each peak was determined by counting the column effluent as previously described (10). The mean specific activities of the fatty acids in R-LPS and S-LPS preparations were 29,200 and 19,000 cpm/nmol, respectively.

DNA measurement. To determine the numbers of cells in the cultures, we measured the DNA contents of cell homogenates by the diphenylamine method (3), using calf thymus DNA (a generous gift from Michael Norgard, University of Texas Health Science Center, Dallas) as the standard. The results of the assay were linear (DNA content versus number of cells); 10^6 cells contained approximately 10 μg of DNA. In some experiments, the amount of DNA increased or decreased by as much as 30% over the 48- to 72-h incubation period. A decrease in the amount of cell DNA was assumed to represent cell loss (cytotoxicity was usually seen only when C3H/HeN cells were incubated with higher concentrations of LPS [24], and the calculation for the amount of cell-associated LPS (micrograms of LPS per milligram of cell DNA) was adjusted accordingly. An increase in the amount of cell DNA over time was assumed to represent cell proliferation; when this change occurred during the chase period of an experiment, the amount of cell DNA at the end of the load period was used for calculating the amount of cell-associated LPS throughout the experiment.

Miscellaneous methods. The method of Markwell (12) was used to measure protein concentration. Prostaglandin E_2 (PGE_2) levels in culture supernatants were determined by a radioimmunoassay (2).

RESULTS

PGE_2 measurements. To confirm the phenotype of the mice in each shipment used for the experiments, we measured the amounts of PGE_2 that were released into the culture medium by macrophages which were incubated with LPS. Typical results are shown in Table 1; as previously reported (26), LPS induced PGE_2 secretion by responsive (C3H/HeN) macrophages, whereas hyporesponsive (C3H/HeJ) macrophages did not secrete PGE_2 when they were incubated with LPS. In contrast, cells from both strains released PGE_2 when LPS were taken up by antibody-dependent phagocytosis (17).

Uptake and deacylation of R-LPS. Plastic-adherent macrophages slowly took up R-LPS from the culture medium over time (Fig. 2A). After incubation for 20 h, approximately 10 to 15% of the R-LPS in the medium was cell

TABLE 1. PGE_2 concentrations in macrophage supernatants[a]

Mouse strain	PGE_2 concn (pg/ml)		
	No LPS	LPS	LPS + IgG
C3H/HeN	69 ± 6	1,121 ± 100	2,241 ± 241
C3H/HeJ	70 ± 16	63 ± 9	821 ± 104

[a] Macrophages (1.5×10^6 cells per well) were incubated without LPS, with R-LPS (1.5 μg/ml), or with S-LPS (0.4 μg/ml) plus anti-*S. typhimurium* IgG (40 μg/ml) for 18 h. The culture supernatants (2 ml) were removed, frozen at −70°C, and subsequently thawed for the PGE_2 assay. The values are means ± 1 standard deviation of three determinations.

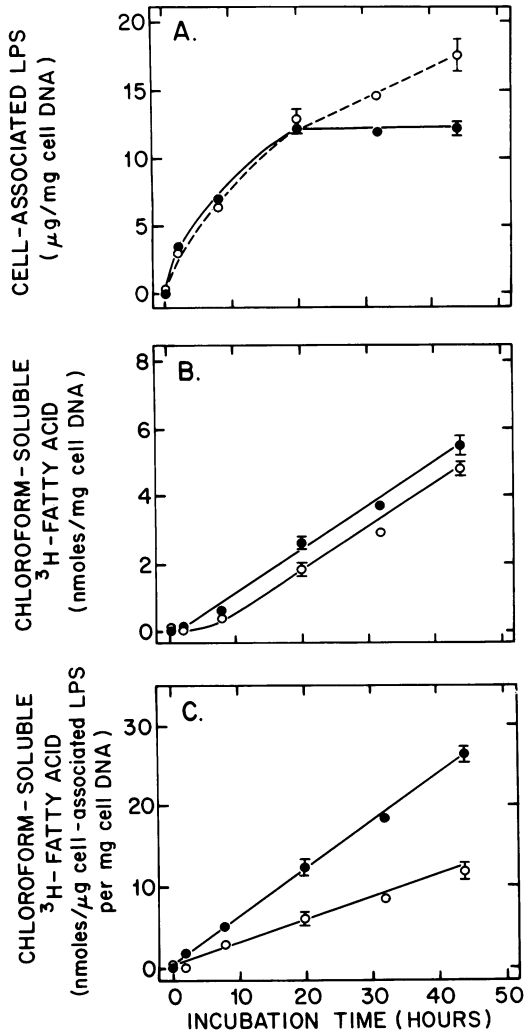

FIG. 2. Uptake and deacylation of R-LPS by macrophages. R-LPS (1.5 μg/ml) were incubated with 1.5×10^6 macrophages (0.015 mg of cell DNA). Symbols: ○ and ●, data obtained by using C3H/HeJ and C3H/HeN macrophages, respectively. Each point represents the mean of three determinations; brackets indicating ±1 standard deviation are shown for representative points. (A) Amount of cell-associated LPS at each time. (B) Data indicating that there was a linear increase over time in the amount of LPS-derived ^{3}H-fatty acid that was chloroform soluble. (C) Amount of chloroform-soluble (deacylated) ^{3}H-fatty acid adjusted for both cell number and the amount of cell-associated LPS at each time point.

associated. The amount of LPS-derived ^{3}H-fatty acid that was chloroform extractable also increased over time (Fig. 2B); approximately 11% (C3H/HeJ mice) or 16% (C3H/HeN mice) of the cell-associated ^{3}H-LPS was deacylated after 20 h of incubation. When the chloroform-soluble ^{3}H-fatty acid content of the cells was corrected for the amount of cell-associated LPS and for cell number (DNA content), the cells appeared to deacylate LPS continuously (Fig. 2C). The C3H/HeJ cells took up more LPS than the C3H/HeN cells did, particularly after prolonged incubation times, and this difference in the amount of cell-associated LPS accounted for much of the apparent difference in the deacylating abilities of the two strains.

As Fig. 3 shows, uptake and deacylation of R-LPS were directly related to the LPS concentration in the medium.

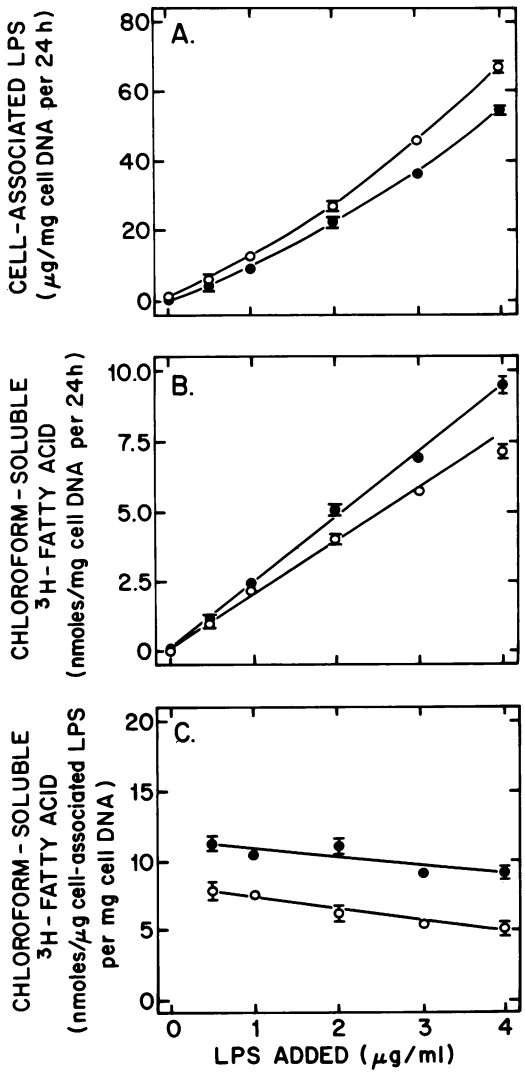

FIG. 3. Uptake and deacylation of R-LPS: effect of LPS concentration. Approximately 1.5 × 10^6 cells were incubated with different amounts of R-LPS for 20 h. Symbols: ○ and ●, data obtained by using C3H/HeJ and C3H/HeN macrophages, respectively. The amount of LPS that was taken up and deacylated increased as the amount of LPS in the culture medium was increased. C3H/HeJ cells took up more LPS and deacylated slightly less LPS than C3H/HeN cells at each LPS concentration.

Again, there was a consistent difference between cells from the two mouse strains; over the range of LPS concentrations studied, the C3H/HeJ cells had more cell-associated LPS (17% of the LPS in the medium, compared with 11% for C3H/HeN cells) and deacylated slightly less LPS than the C3H/HeN cells. The fraction of the cell-associated LPS that underwent deacylation decreased over the range of LPS concentrations used (from 17.4 to 9.1% [C3H/HeJ cells] and from 20.2 to 13.2% [C3H/HeN cells]).

Figure 4 shows that the relationship between LPS uptake and cell density was much steeper for C3H/HeJ cells than for C3H/HeN cells. In contrast, the amounts of ^{3}H-fatty acid that accumulated in the cells of the two mouse strains were similar over the range of cell densities studied. Thus, the tendency for C3H/HeJ cells to deacylate a smaller fraction of the cell-associated LPS was enhanced at higher cell densities.

Antibody-mediated uptake of S-LPS. High concentrations of nonopsonized LPS are toxic to LPS-responsive macrophages (24). This observation was confirmed in this study (data not shown) and limited our ability to study the metabolism of larger amounts of cell-associated R-LPS. Thus, we turned to S-LPS, which we were able to opsonize for uptake by macrophages by using an anti-LPS IgG. In agreement with the data presented above, the macrophages slowly accumulated nonopsonized S-LPS from the media, yet after 20 h only 2% of the labeled S-LPS had become cell associated. When anti-*S. typhimurium* IgG was added, much greater uptake of the S-LPS was found; with 40 μg of IgG per ml approximately 60% of the LPS in the medium became cell associated (Fig. 5A). The amount of LPS-derived ^{3}H-fatty acid that was chloroform extractable increased in parallel with LPS uptake (Fig. 5B). Finally, when the chloroform-soluble ^{3}H-fatty acid content of the cells at each time point was corrected for both the amount of cell-associated LPS and cell number, the apparent deacylation of the

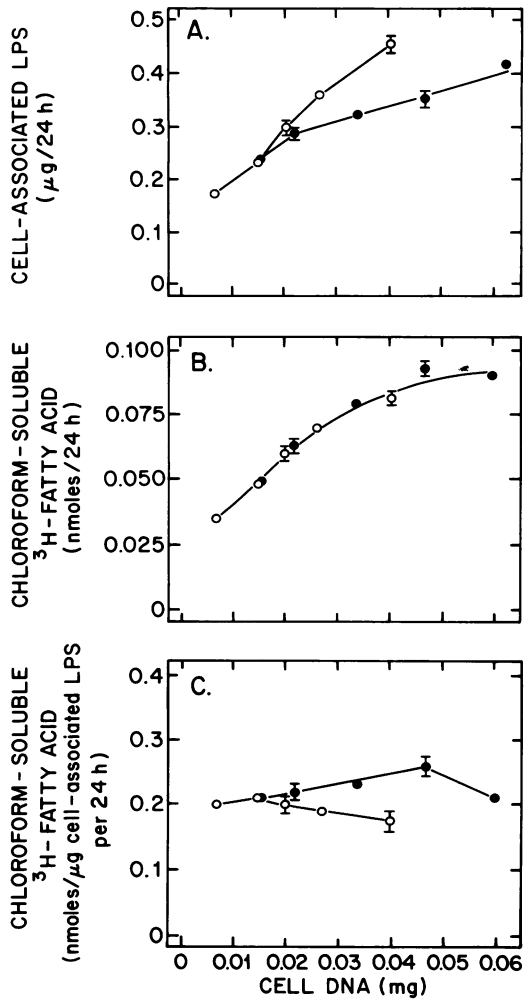

FIG. 4. Uptake and deacylation of R-LPS: effect of cell number. Different numbers of plastic-adherent macrophages were incubated with R-LPS (1.5 μg/ml) for 20 h. At higher cell densities (above 0.015 mg of cell DNA or approximately 1.5 × 10^6 cells per well), C3H/HeJ cells (○) took up more LPS than C3H/HeN cells (●). Although the amounts of LPS that were deacylated (B) were similar for the two kinds of cells, at higher cell densities the C3H/HeN cells deacylated a larger fraction of the cell-associated LPS (C).

LPS by the cells was related inversely to the amount of opsonizing antibody added (and thus to the amount of LPS that was cell associated) (Fig. 5C). In other words, the fraction of the cell-associated ^{3}H-LPS that became chloroform soluble decreased from approximately 21% (no antibody) to 10% (40 μg of IgG per ml).

Load-chase experiments. Control experiments showed that a small fraction (approximately 2 to 5%) of the ^{3}H-fatty acids in the double-labeled LPS was spontaneously released into the culture medium during a 72-h incubation period. Moreover, the cells were able to take up free fatty acids from the medium. In addition, estimates of the rate of LPS deacylation were complicated if LPS uptake and deacylation took place concurrently. Accordingly, cells were allowed to take up LPS for 14 to 18 h, washed extensively, and reincubated

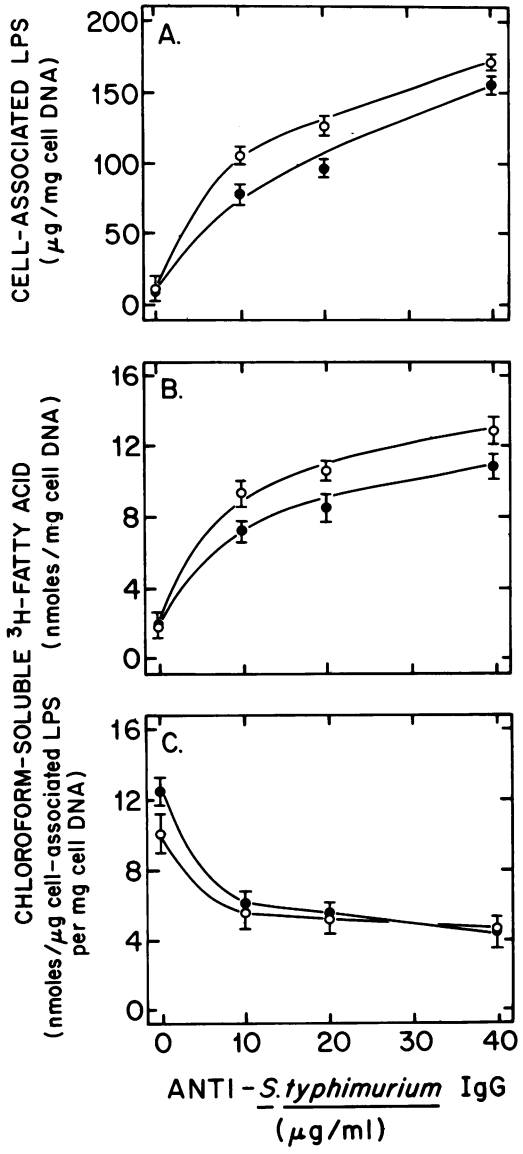

FIG. 5. Uptake and deacylation of S-LPS by macrophages: effect of anti-LPS IgG. S-LPS (0.4 μg/ml) were added to wells containing approximately 1.5 × 10^6 macrophages and different amounts of anti-*S. typhimurium* IgG. The incubation period was 20 h. Symbols: ○ and ●, data obtained by using C3H/HeJ and C3H/HeN macrophages, respectively.

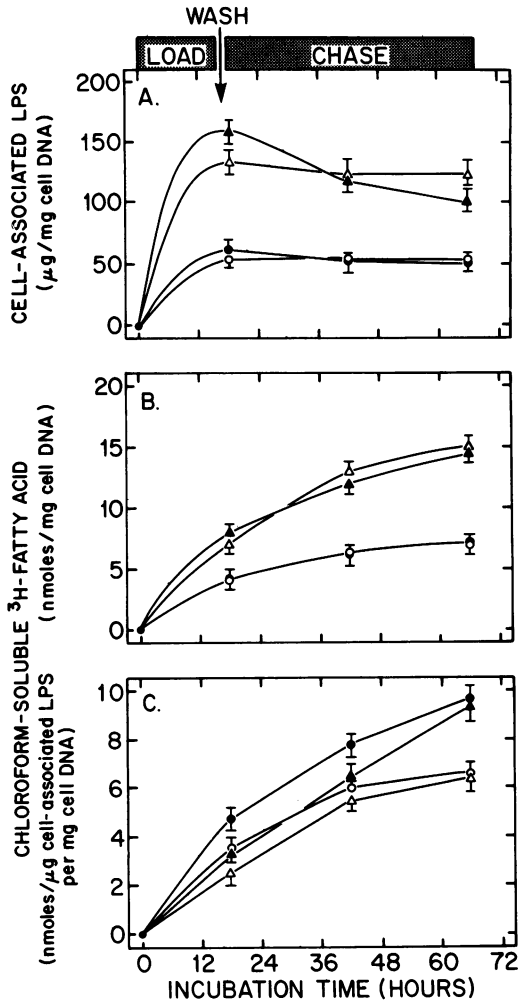

FIG. 6. Load-chase analysis of LPS deacylation. S-LPS (0.4 μg/ml) were added to wells that contained approximately 1.5 × 10^6 adherent macrophages and either low (5 μg/ml) or high (40 μg/ml) concentrations of anti-LPS IgG. After an incubation period of 18 h (load), the cells were washed and either harvested or reincubated in medium that did not contain LPS (chase). The data points indicate means ± 1 standard deviation of three determinations. Symbols: ○ and △, C3H/HeJ macrophages; ● and ▲, C3H/HeN macrophages. ○ and ●, wells that had low IgG concentrations; △ and ▲, wells that had high IgG concentrations.

(chased) in fresh medium that did not contain LPS. Data from a representative experiment are shown in Fig. 6. Two results are noteworthy. First, some LPS dissociated from the cells during the chase period. Thus, the amount of cell-associated LPS at a given time was the net result of uptake and release of LPS by the cells. In 7 of 10 experiments in which nonopsonized R-LPS were studied, the C3H/HeN cells lost a greater fraction of the cell-associated LPS into the medium during the chase period (the average loss of LPS was 21 ± 5% for C3H/HeN cells and 16 ± 5% for C3H/HeJ cells). This difference was of borderline statistical significance ($P < 0.1$, Wilcoxon test for pair differences). Second, as Fig. 6B shows, deacylation of ^{3}H-fatty acids from LPS occurred during the chase. When the amount of chloroform-soluble ^{3}H-fatty acid at each time point was adjusted for the number of cells and the amount of cell-associated LPS, it was again apparent that deacylation occurred throughout the chase period (Fig. 6C).

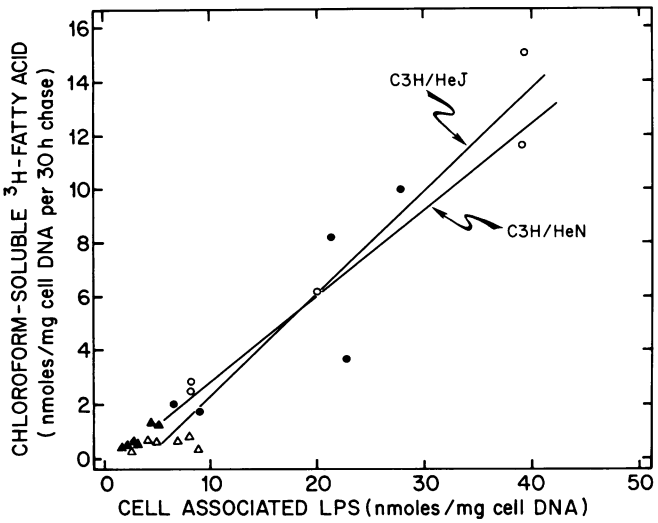

FIG. 7. Relationship between uptake and deacylation of LPS. The rate of LPS deacylation is plotted against the amount of LPS that was cell associated at the beginning of the chase period. The data were taken from five separate experiments in which macrophages from C3H/HeJ mice (○ and △) and C3H/HeN mice (● and ▲) were compared; data for both nonopsonized R-LPSs (△ and ▲) and opsonized S-LPSs (○ and ●) are shown. For this comparison the amount of cell-associated LPS is expressed in nanomoles; 1 nmol was 3.7 and 7.1 μg of R-LPS and S-LPS, respectively. The lines of best fit are shown for the data from each mouse strain.

To evaluate the relationship between substrate (R-LPS or S-LPS) concentration and deacylation rate, the increment of chloroform-soluble ^{3}H-fatty acid that occurred during the first 30 h of the chase period was plotted according to the amount of LPS that was cell associated at the beginning of the chase (Fig. 7). The results of this analysis suggested that the rate of deacylation was substrate limited and possibly independent of the uptake mechanism. Macrophages from C3H/HeN and C3H/HeJ mice were quite similar in the ability to deacylate both opsonized and nonopsonized LPS.

Fatty acid composition of the partially deacylated LPS. When macrophages that had taken up LPS were extracted with chloroform-methanol, the partially deacylated LPS remained at the interface and in the methanol-water phase. After these LPS were hydrolyzed as described above, more than 85% of the ^{3}H counts and less than 10% of the ^{14}C counts were recovered in the organic phase, in agreement with the assumed locations of ^{3}H and ^{14}C in fatty acids and polysaccharides, respectively. The hydrolysis mixtures were studied first by one-dimensional TLC, which separated 3-OH-14:0 (R_f, 0.3) from non-hydroxylated fatty acids (oleic acid standard; R_f, 0.8) (10). As Table 2 shows, the LPS that were recovered from cells after a 24-h incubation period were modestly depleted in non-hydroxylated fatty acids. With incubation for an additional 48 h (chase period), the percentage of the LPS-derived fatty acids that comigrated with the non-hydroxylated fatty acid standard decreased even further. Similar results were obtained with macrophages from both kinds of mice and with both R-LPS and antibody-opsonized S-LPS. These results are consistent with the conclusion that non-hydroxylated fatty acids were removed from the LPS during incubation with the cells.

The location of the non-hydroxylated fatty acids in *Salmonella* lipid A has been determined by Takayama et al. (20); tetradecanoic acid is thought to be substituted principally to the hydroxyl group of the 3-OH-14:0 that is joined by ester linkage to the 3′ position on the glucosamine backbone, whereas dodecanoic acid and (when present) hexadecanoic acid are substituted to amide-linked 3-OH-14:0 at positions 2′ and 2, respectively (20). As Table 3 shows, analysis of the fatty acid composition of deacylated S-LPS by using HPLC indicated that the LPS that had been partially deacylated by either C3H/HeN or C3H/HeJ cells were enriched in 3-OH-14:0 and depleted in dodecanoic, tetradecanoic, and hexadecanoic acids. These results exclude a major differ-

TABLE 2. Analysis of cell-associated LPS by TLC[a]

Prepn	Incubation time with cells (h)	Mouse strain	Radioactivity in spots (% of recovered cpm)		
			Origin	3-OH-14:0	Non-hydroxylated fatty acids[b]
S-LPS	0[c]		4.3 ± 0.6	67.0 ± 0.4	27.9 ± 0.2
	24	C3H/HeN	4.8 ± 2.0	70.1 ± 0.6	23.4 ± 0.1
		C3H/HeJ	3.9 ± 0.5	72.9 ± 0.4	22.5 ± 0.2
	72	C3H/HeN	4.5 ± 0.1	85.2 ± 0.4	9.5 ± 0.1
		C3H/HeJ	3.7 ± 0.1	84.6 ± 0.4	11.1 ± 0.5
R-LPS	0[c]		5.9 ± 1.0	69.6 ± 0.5	23.1 ± 0.2
	24	C3H/HeN	4.2 ± 1.4	73.0 ± 0.2	20.5 ± 0.5
		C3H/HeJ	3.9 ± 2.1	73.9 ± 0.4	20.0 ± 0.2
	72	C3H/HeN	6.4 ± 0.7	81.5 ± 0.5	11.2 ± 0.1
		C3H/HeJ	4.4 ± 0.6	80.9 ± 0.1	13.1 ± 0.4

[a] Plastic-adherent macrophages were allowed to take up S-LPS (opsonized with IgG) or R-LPS (no added IgG) for 24 h, washed, and either harvested (24-h incubation time) or reincubated in medium without LPS for an additional 48 h (72-h incubation time). LPS that were incubated for 72 h in medium without cells served as controls. The cells and the control LPS were extracted with chloroform-methanol, the LPS remaining at the interface and in the methanol-water phase were hydrolyzed (see text), and the ^{3}H-fatty acids were extracted into diethyl ether. One-dimensional TLC was performed with solvent 1; 87.4 ± 4.4% (mean ± standard deviation; n = 20) of the added ^{3}H counts were recovered when the individual spots were scraped and counted. The values are means ± 1 standard deviation of three analyses.

[b] Oleic acid was the standard used.

[c] Incubated in medium without cells for 72 h and then extracted with chloroform-methanol in parallel with the LPS-cell mixtures.

TABLE 3. Analysis of cell-associated LPS by high-pressure liquid chromatography[a]

Incubation time with cells (h)	Mouse strain	^{3}H-fatty acid radioactivity (% of recovered cpm)			
		3-OH-14:0	12:0	14:0	16:0
0		54.6	21.9	17.8	6.5
24	C3H/HeN	63.9	21.2	11.6	3.3
	C3H/HeJ	65.8	19.1	11.4	3.6
72	C3H/HeN	77.7	14.3	5.4	2.1
	C3H/HeJ	78.1	13.0	7.1	1.6

[a] Plastic-adherent macrophages that had taken up S-LPS were washed after incubation for 24 h and either harvested (24-h incubation time) or reincubated in medium without LPS for an additional 48 h (72-h incubation time). LPS that had not been incubated served as the control. The ^{3}H-fatty acids were hydrolyzed from the chloroform-insoluble fraction of the cells or from control LPS, derivatized (87 ± 4% recovery), and analyzed by HPLC. The samples contained 5,000 to 10,000 cpm of ^{3}H-fatty acids, and more than 85% of the ^{3}H counts were recovered from the column. Each data point is the average of two determinations.

TABLE 4. Location of LPS-derived ^{3}H radioactivity in macrophage lipid fractions[a]

Cells incubated with ^{3}H-radio-labeled:	Mouse strain	Recovery of ^{3}H (% of recovered cpm) in the following fractions:				
		Phosphatidyl-inositol + phosphatidyl-choline	Phosphatidyl-ethanol-amine	3-OH-14:0	Normal fatty acids	Neutral lipids
S-LPS	C3H/HeN	77.8	9.6	3.7	2.1	4.1
	C3H/HeJ	78.7	11.1	2.7	2.1	0.8
R-LPS	C3H/HeN	63.3	6.5	13.1	5.8	5.5
	C3H/HeJ	56.4	14.1	11.8	6.6	1.2
3-OH-14:0	C3H/HeN	86.0	9.3	1.2	1.5	1.2

[a] Macrophages (approximately 1.5 × 10^6 cells per well) were incubated in medium that contained either double-labeled LPS (S-LPS plus IgG or R-LPS) or ^{3}H-labeled 3-OH-14:0 (hydrolyzed from LPS and separated from non-hydroxylated fatty acids by TLC). After incubation for 48 h, the cells were extracted with chloroform-methanol, and the chloroform phase was analyzed by two-dimensional TLC (see text); 30% of the ^{3}H-labeled 3-OH-14:0 in the medium was taken up by the cells (approximately 17 nmol of fatty acid taken up per mg of cell DNA). More than 85% of the added ^{3}H counts were recovered from the TLC plates. The results are means of three determinations; standard deviations were less than 10% of the means.

ence in the degree to which the two kinds of cells remove the individual fatty acids from lipid A.

Intracellular fate of LPS-derived fatty acids. Chloroform extracts of macrophages that had deacylated labeled LPS or taken up ^{3}H-labeled 3-OH-14:0 from the culture medium were studied by two-dimensional TLC. The locations of the ^{3}H-fatty acids on the plates were detected by fluorography (Fig. 8) and by counting the radioactivity in spots that were scraped from the plates (Table 4). The ^{3}H counts comigrated with various cellular lipid fractions, principally phospholipids. A larger fraction of the ^{3}H counts comigrated with 3-OH-14:0 when nonopsonized R-LPS were deacylated by cells than when opsonized S-LPS were deacylated or when ^{3}H-labeled 3-OH-14:0 was taken up by the cells. These results indicate that the cells are able to cleave 3-OH-14:0 from lipid A and suggest that the intracellular fate of 3-OH-14:0 that is taken up from the medium may differ from the fate of 3-OH-14:0 that is deacylated from LPS. The recovery of more ^{3}H-labeled 3-OH-14:0 from nonopsonized LPS than from opsonized LPS may reflect the fact that a greater fraction of the ^{3}H-fatty acids was deacylated from nonopsonized LPS (i.e., as shown in Fig. 5, the extent of deacylation was inversely related to the amount of cell-associated LPS).

Deacylation of LPS by cell homogenates. Whole-cell homogenates were prepared by suspending washed peritoneal macrophages in homogenization buffer (0.34 M sucrose, 1 mM EDTA, 1 mM potassium phosphate, pH 7.2) and disrupting the cells with a Dounce homogenizer. Low-speed centrifugation (200 × g, 10 min) pelleted nuclei and unbroken cells, and further centrifugation of the low-speed supernatant (8,000 × g, 15 min) produced a granule pellet that was enriched in LPS-deacylating activity. Deacylation was studied by incubating double-labeled LPS with macrophage

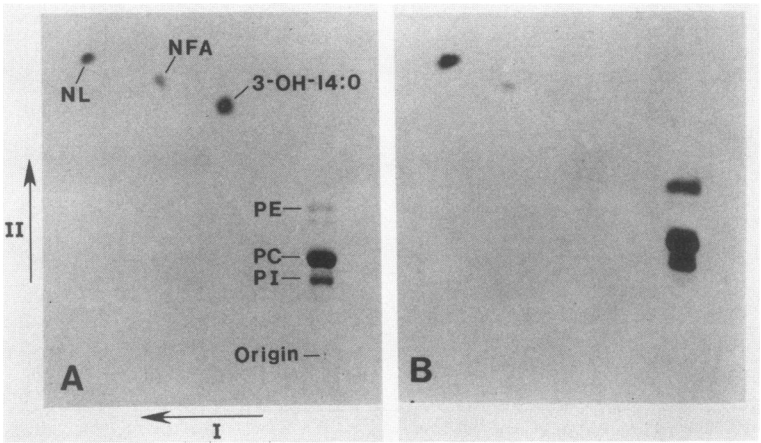

FIG. 8. Two-dimensional TLC analysis of LPS-derived ^{3}H radioactivity in cellular lipid fractions. Two-dimensional TLC was performed as described in the text on chloroform extracts of C3H/HeN cells that had deacylated nonopsonized R-LPS (A) or opsonized S-LPS (B). Approximately 2,500 ^{3}H cpm was loaded per plate. The plates were sprayed with En^{3}Hance (New England Nuclear Corp.) and exposed to Kodak SB-5 film at −70°C. The labels indicate the locations of the nonradioactive lipids on the plates (visualized with iodine vapor before fluorography). The lipids were identified by using authentic standards. NL, Neutral lipids; NFA, normal fatty acids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; I, solvent 1; II, solvent 2.

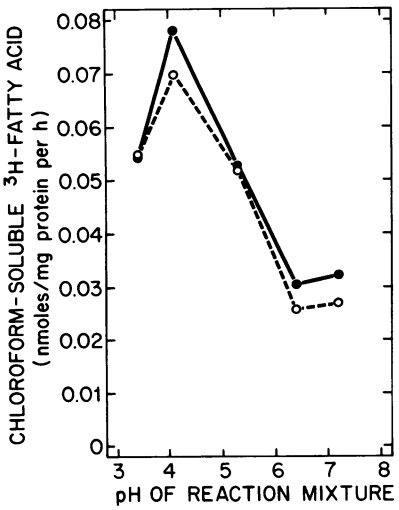

FIG. 9. pH optimum of LPS deacylation by macrophage granules. Granules (30 μg) from C3H/HeN (●) and C3H/HeJ (○) macrophages were incubated with double-labeled LPS for 12 h at 37°C as described in the text. The reaction mixtures were buffered with Tris-citrate or Tris-maleate (10 mM). Each data point represents the mean of two determinations; the experiment was repeated with similar results. The data were corrected for the amount of ^{3}H-fatty acid that was deacylated from LPS in the absence of granules at each pH.

granules in a mixture that contained 1 mg of fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) per ml, 0.2% (vol/vol) Triton X-100, 0.9% (vol/vol) NaCl, and 10 mM Tris-citrate buffer (pH 4.3). After incubation at 37°C for 6 to 24 h, the mixtures were extracted with chloroform-methanol, and the radioactivity in the chloroform phase was counted. Deacylation proceeded linearly with respect to time (to 24 h) and the amount of granule protein (15 to 75 μg). One-dimensional TLC of the LPS-derived, chloroform-soluble ^{3}H-fatty acids showed that approximately 80% of the chloroform-soluble ^{3}H counts comigrated with the non-hydroxylated fatty acid standard. The pH optimum for deacylation by granules from both strains of mice was approximately 4.2 (Fig. 9), and the reaction showed substrate saturability. The apparent K_m values for deacylation (derived from Lineweaver-Burke plots) were 0.30 and 0.20 μM for C3H/HeN and C3H/HeJ granules, respectively, (data not shown). The V_{max} values were 0.28 and 0.24 nmol of fatty acid released per mg of protein per h for C3H/HeN and C3H/HeJ granules, respectively.

DISCUSSION

Our results indicate that murine macrophages take up both opsonized and nonopsonized LPS from their growth medium, deacylate some of the fatty acids from lipid A, and reutilize these fatty acids for cellular lipid synthesis. Moreover, our data suggest strongly that the major deacylating activity is the hydrolysis of the acyloxyacyl bonds that attach non-hydroxylated fatty acids to the hydroxyl groups of glucosamine-linked 3-OH-14:0 residues.

Our first aim was to characterize the uptake of LPS by macrophages in culture. Since previous studies had shown that human neutrophils could take up and deacylate antibody-opsonized LPS (10), major attention was given to the uptake of nonopsonized LPS, which are able to stimulate responsive macrophages. In our long-term cultures, the amounts of cell-associated nonopsonized R-LPS and S-LPS increased for approximately 20 h and then reached a plateau in the LPS-responsive cells. In agreement with previous workers (8, 11, 19), we found that cells from LPS-hyporesponsive mice do not lack the ability to take up LPS; indeed, in 11 of 13 experiments more nonopsonized LPS was associated with C3H/HeJ macrophages than with C3H/HeN cells. Moreover, the difference in the amounts of nonopsonized LPS that were associated with the two kinds of macrophages increased with prolonged incubation (Fig. 2) and larger numbers of cells (Fig. 4). In studies not described here we found that neither preincubation of macrophages with LPS nor buffering the culture medium with 50 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) (pH 7.4) consistently altered the amount of LPS that became associated with the cells. A possible insight into the difference in the amount of cell-associated LPS did emerge from the load-chase experiments. As has been reported for macrophages that take up intact bacteria (5), we found that the cells released some of the cell-associated LPS into the culture medium over time. Moreover, in most experiments the C3H/HeN cells lost more nonopsonized LPS than the C3H/HeJ cells; the fatty acid composition of the cell-associated LPS that were released into the medium was similar to that of complete (i.e., fully acylated) LPS (data not shown). Thus, it is possible that a difference in the adsorption-desorption cycling (4) of acylated LPS may explain the tendency for more LPS to be found in association with C3H/HeJ cells.

Both opsonized and nonopsonized LPS were deacylated by macrophages in culture. Two results suggest that the principal step in the deacylation of lipid A by murine macrophages was acyloxyacyl hydrolysis. First, the cell-associated LPS underwent a time-dependent depletion in non-hydroxylated fatty acid content, so that the ratio of 3-OH-14:0 to non-hydroxylated fatty acids in the LPS increased from approximately 2.5 to 8. Second, cell homogenates contained enzymes that carried out acyloxyacyl hydrolysis. Although structural heterogeneity in the LPS probes precluded precise localization of the site(s) of deacylation to specific acyloxyacyl linkages, the data in Table 4 indicate that each type of non-hydroxylated fatty acid was hydrolyzed from lipid A. Thus, by extrapolation from the previously reported structure (20, 28), it is likely that fatty acids were cleaved from both O- and N-linked acyloxyacyl residues. It is important to note that a small fraction of the deacylated ^{3}H-fatty acids was recovered as ^{3}H-labeled 3-OH-14:0, indicating that the cells were able to deacylate this fatty acid from the glucosamine backbone. The precise magnitude of 3-OH-14:0 deacylation is uncertain; if it is assumed that the cells reuse this fatty acid slowly, the observed maximum of 11 to 13% of deacylated ^{3}H-fatty acids (or approximately 3% of the total LPS fatty acids) may be a reasonable estimate. The development of lipid A probes that are radiolabeled only in the 3-OH-14:0 residues should permit more accurate estimates of 3-OH-14:0 deacylation.

Accurate investigation of the biological activities of deacylated LPS will require separation of the different reaction products, a feat that is not technically possible at this time. Other tests of the role of acyloxyacyl hydrolysis in modulating the biologial activity of LPS are more feasible. In this study we examined the hypothesis that macrophages from LPS-hyporesponsive mice are defective in the ability to deacylate lipid A. The importance of this issue is indicated by observations which suggest (i) that the non-hydroxylated fatty acid component is not necessary for many biological activities of lipid A (6, 16, 23) (i.e., that the products of acyloxyacyl hydrolysis should be bioactive) and (ii) that

animal cells may be able to modify lipid A to an active form. Truffa-Bachi et al. (22) found that lymphocytes from normal (LPS-responsive) mice were able to modify LPS to a low-molecular-weight form that stimulated lymphocytes from C3H/HeJ (LPS-hyporesponsive) mice. More recently, Vogel et al. (23) found that a precursor form of *Salmonella* lipid A stimulated C3H/HeJ lymphocytes and macrophages; this precursor resembles complete lipid A but lacks the non-hydroxylated fatty acids. These workers suggested that the LPS-hyporesponsive mice, which have a defect that has been mapped to a single gene (LPS^d) on chromosome 4 (27), might lack an enzyme that removes the non-hydroxylated fatty acids from lipid A. Using different assay conditions, Raetz and co-workers found that lipid X (2,3-diacylglucosamine 1-phosphate) stimulated lymphocytes from LPS-responsive mice but not lymphocytes from C3H/HeJ mice (16). These authors presented evidence that the 3-OH-14:0 residue in position 3 of lipid X (position 3 or 3′ of lipid A) was essential for biological activity and hypothesized that normal lymphocytes (but not LPS-hyporesponsive lymphocytes) were able to transfer this fatty acid to a cellular target protein. Since macrophages from C3H/HeJ mice are also hyporesponsive to LPS, we tested these hypotheses in this study by comparing the deacylation of lipid A by macrophages from normal (C3H/HeN) and LPS-hyporesponsive (C3H/HeJ) mice.

As discussed above, C3H/HeJ and C3H/HeN macrophages differed in net uptake of LPS. In contrast, deacylation of LPS by the two kinds of macrophages was qualitatively and quantitatively similar. Our results exclude the possibility that the LPS-hyporesponsive cells lack an acyloxyacyl hydrolase(s), and it seems unlikely that there are major differences in the abilities of the two kinds of cells to remove the individual non-hydroxylated fatty acids from lipid A. It is more difficult to be certain that a normally small but critical fraction of the enzyme is absent in a particular cellular location (e.g., the plasma membrane) in the LPS-hyporesponsive cells. We also note that our results were dominated by the major enzymatic reaction found (acyloxyacyl hydrolysis) and that it is possible that the LPS-hyporesponsive cells are deficient in an additional deacylating enzyme that might be detected by using lipid A probes that are radiolabeled only in the 3-OH-14:0 residues. Finally, it should be emphasized that the results of our experiments do not exclude the possibility that lipid A deacylation may contribute to some of the biological activities of lipid A; the defect in the LPS-hyporesponsive cells might involve a different step in the activation sequence.

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