

Host Cell Phospholipids Are Trafficked to and Then Modified by *Chlamydia trachomatis*

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There is little information on the trafficking of eukaryotic lipids from a host cell to either the cytoplasmic membrane or the vacuolar membrane surrounding intracellular pathogens. Purified *Chlamydia trachomatis*, an obligate intracellular bacterial parasite, contains several eukaryotic glycerophospholipids, yet attempts to demonstrate transfer of these lipids to the chlamydial cell membrane have not been successful. In this report, we demonstrate that eukaryotic glycerophospholipids are trafficked from the host cell to *C. trachomatis*. Phospholipid trafficking was assessed by monitoring the incorporation of radiolabelled isoleucine, a precursor of *C. trachomatis* specific branched-chain fatty acids, into host-derived glycerophospholipids and by monitoring the transfer of host phosphatidylserine to chlamydiae and its subsequent decarboxylation to form phosphatidylethanolamine. Phospholipid trafficking to chlamydiae was unaffected by brefeldin A, an inhibitor of Golgi function. Furthermore, no changes in trafficking were observed when *C. trachomatis* was grown in a mutant cell line with a nonfunctional, nonspecific phospholipid transfer protein. Host glycerophospholipids are modified by *C. trachomatis*, such that a host-synthesized straight-chain fatty acid is replaced with a chlamydia-synthesized branched-chain fatty acid. We also demonstrate that despite the acquisition of host-derived phospholipids, *C. trachomatis* is capable of de novo synthesis of phospholipids typically synthesized by prokaryotic cells. Our results provide novel information on chlamydial phospholipid metabolism and eukaryotic cell lipid trafficking, and they increase our understanding of the evolutionary steps leading to the establishment of an intimate metabolic association between an obligate intracellular bacterial parasite and a eukaryotic host cell.

Chlamydiae are obligate intracellular eubacterial parasites capable of infecting a wide range of eukaryotic host cells. Four species are currently recognized: *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Chlamydia pecorum* (8, 11, 39). *C. trachomatis* is one of the most prevalent sexually transmitted pathogens and is a leading cause of preventable blindness in developing countries (7). *C. pneumoniae* causes acute respiratory disease, including pneumonia, bronchitis, sinusitis, and pharyngitis (29). It has recently been implicated as a potential risk factor in coronary disease (9). Despite their clinical importance, little information is available on the biology of chlamydiae and their interaction with their eukaryotic host. This is due to difficulties in studying chlamydial metabolism, including a current inability to grow chlamydiae in a host-free system, the limited metabolic activity of purified chlamydiae, and the lack of a genetic transfer system.

All members of the genus *Chlamydia* have evolved a unique biphasic life cycle consisting of two morphologically and functionally distinct cell types (39, 49). The infectious process is initiated by a small, dormant cell type, termed the elementary body (EB). Following internalization, EBs differentiate to a larger, metabolically active cell type, termed the reticulate body (RB). RBs replicate within a membrane-bound vacuole, referred to as the chlamydial inclusion. By 16 to 20 h after infection, while some RBs are still replicating, others have begun the process of differentiation back into infectious EBs. Combined RB growth and differentiation continues until approximately 48 to 72 h after infection, when the population

contains predominantly EBs. Host cell lysis results in the release of EBs to begin a new infection cycle.

Data gathered on several intracellular pathogens suggests that pathogens which replicate within vacuoles selectively modify them to create unique intravacuolar environments amenable to growth (48). The chlamydial inclusions do not contain protein markers characteristic of the endoplasmic reticulum, Golgi apparatus, or late endosomes, nor do they acidify or fuse with lysosomes (21, 39, 41, 50). These results indicate that the chlamydial vacuole displays minimal interaction with the endocytic-lysosomal pathway. Recently, however, Hackstadt et al. (12, 13) have used fluorescent probes, designed to analyze lipid trafficking, to show that chlamydiae do interrupt an exocytic pathway, intercepting vesicles derived from the Golgi apparatus. Movement of a fluorescent sphingomyelin (SM) analog from the Golgi apparatus to the cell membrane or wall of *C. trachomatis* was observed. Interruption of this exocytic pathway with brefeldin A was observed to block the transfer of the probe from the Golgi apparatus to the chlamydiae. In spite of blocking the trafficking of SM to chlamydial membranes, brefeldin A had little or no effect on chlamydial growth. This result suggests that while SM trafficking does occur, it is not essential for parasite development.

In addition to SM, purified chlamydiae contain glycerophospholipids normally associated with eukaryotic cells (e.g., phosphatidylcholine [PC] and phosphatidylinositol [PI] (40), suggesting that trafficking of these lipids from the host to the chlamydiae occurs. However, incorporation of fluorescently labelled PC and PI into chlamydiae is not observed (12). Additionally, it is unknown whether the remaining lipids identified in the chlamydial membrane (phosphatidylethanolamine [PE], phosphatidylglycerol [PG], phosphatidylserine [PS], and cardiolipin [CL]), typically synthesized by both prokaryotic and

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eukaryotic cells, are biosynthesized de novo by the chlamydiae or salvaged from the host cell.

Eukaryotic phospholipid synthesis occurs primarily in the endoplasmic reticulum (PC, PE, PS, PI), the Golgi apparatus (SM), and mitochondria (PG, CL) (55). Multiple mechanisms exist to transfer the lipids from their sites of synthesis to other host organelles. These mechanisms include vesicle-mediated transfer, lipid transfer proteins, and direct fusion of organellar membranes (54). Transfer of SM from the Golgi apparatus to intracellular chlamydiae is consistent with the bacterium intercepting normal trafficking within the *trans*-Golgi network, as proposed by Hackstadt et al. (13). However, since most eukaryotic phospholipids are not synthesized in the Golgi apparatus, it is not clear whether other host phospholipids potentially trafficked to the chlamydiae would use this pathway or whether the chlamydial cells intercept lipid flow between other host organelles.

Differentiating a mixture of lipids isolated from infected host cells into prokaryote- and eukaryote-synthesized lipids would normally be difficult, if not impossible; however, the presence of branched-chain fatty acids in chlamydiae (as opposed to straight-chain fatty acids in eukaryotic lipids) circumvents this problem. Branched-chain fatty acids are synthesized by several species of prokaryotes (26). Their absence in eukaryotic cells allows lipids specifically synthesized or modified by chlamydiae to be identified.

We initiated a series of experiments designed to address several aspects of host and chlamydia glycerophospholipid biosynthesis and transfer. Our results indicate that *C. trachomatis* is able to synthesize phospholipids normally made by prokaryotes (PE, PG, and PS), but that trafficking of eukaryotic lipids (PI, PC, CL, and cholesterol) from the host cell to *C. trachomatis* also occurs.

MATERIALS AND METHODS

Materials. [$1\text{-}^3\text{H}$]ethanolamine (18 Ci/mmol) was obtained from Amersham Life Sciences. [$\text{methyl-}^3\text{H}$]choline (81 Ci/mmol), [$1,2\text{-}^{14}\text{C}$]acetic acid (52 mCi/mmol), [$1,3\text{-}^{14}\text{C}$]glycerol (43 mCi/mmol), D-[$U\text{-}^{14}\text{C}$]glucose (251 mCi/mmol), L-[$U\text{-}^{14}\text{C}$]serine (150 mCi/mmol), L-[$U\text{-}^{14}\text{C}$]isoleucine (342 mCi/mmol), L-[$U\text{-}^{14}\text{C}$]valine (283 mCi/mmol), L-[$U\text{-}^{14}\text{C}$]leucine (350 mCi/mmol), [$1\text{-}^{14}\text{C}$]palmitic acid (57 mCi/mmol), [$1\text{-}^{14}\text{C}$]oleic acid (50 mCi/mmol), and [$1\text{-}^{14}\text{C}$]myristic acid (55 mCi/mmol) were obtained from New England Nuclear, Dupont Canada, Inc. Brefeldin A, monensin, *Naja mossaibica* phospholipase A_2 (PLA $_2$), and phospholipid and cholesterol standards were obtained from Sigma.

***C. trachomatis* strains and propagation.** *C. trachomatis* L2/434/Bu was used throughout this study and was grown as previously described (51). Unless otherwise indicated, 1 μg of cycloheximide per ml was present in the postinfection growth medium. The various cell lines were infected with *C. trachomatis* at a multiplicity of infection of 3 to 5 infection-forming units per cell. Mock-infected (MI) host cell cultures were treated in an identical manner to infected cells, except that chlamydiae were not added.

Cell lines and culture conditions. The wild-type HeLa 229 cells were obtained from R. Brunham, University of Manitoba, Winnipeg, Manitoba, Canada. The nonspecific lipid transfer protein (nsLTP)-deficient cell line CHO K1 cell line (ZR78) (53) was provided by R. Theuringer, Merck Research laboratories, Rahway, N.J. The thermolabile CDP-choline synthetase CHO K1 cell line (strain 58) (6) was provided by D. E. Vance, University of Alberta, Edmonton, Alberta, Canada. The above cell lines were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The cytidine-requiring, CTP synthetase-deficient CHO K1 cell line (CR-2) (27) was provided by M. Meuth, Eccles Institute of Human Genetics, University of Utah, Salt Lake City, Utah. CR-2 cells were grown in Dulbecco's modified Eagle's medium containing 7.5% fetal bovine serum, 300 μM proline, 10 μM thymidine, and 20 mM cytidine. Where noted, delipidated serum was substituted for fetal bovine serum in the growth medium for CR-2. Delipidated fetal bovine serum was prepared as previously described (19).

Extraction and purification of lipids. For radiolabelling of phospholipids, MI or *C. trachomatis*-infected cells were incubated with a given radiolabelled precursor (3 $\mu\text{Ci}/5\text{-cm}$ dish) beginning at 20 h p.i. unless otherwise noted. At 6 h later, the cell layer was washed once with ice-cold phosphate-buffered saline and scraped into 2 ml of methanol-water (1:1 [vol/vol]). Lipids were extracted as previously described (17). Hydrolysis of oleic acid-radiolabelled phospholipids was examined by labelling mammalian cells with oleic acid (1 $\mu\text{Ci}/5\text{-cm}$ dish) for

18 h. The cell monolayer was washed with PBS, fresh medium was added, and the cells were either MI or infected with *C. trachomatis*. At 30 h later, the medium was removed and fatty acids were extracted from it as described previously (17). Trafficking of host-derived PS to chlamydiae was monitored by using HeLa cells prelabelled with [^{14}C]serine for 18 h. Following this labelling period, the [^{14}C]serine-containing medium was removed, the monolayer was extensively washed, then the cells were either MI or infected with *C. trachomatis*, fresh medium was added, and the cultures were incubated for a further 44 h. The lipids were extracted and separated by two-dimensional thin-layer chromatography (TLC), and the radioactivity associated with the PS, PE, and SM spots was determined by scintillation counting (see below). Localization of branched-chain fatty acids on host-derived PC was determined by using HeLa cells infected with *C. trachomatis* and radiolabelled with [^{14}C]isoleucine for an entire growth cycle (40 h). At the end of the labelling period, lipids were extracted and separated by two-dimensional TLC. Following visualization with iodine vapor, the PC spot was scraped from the plate, extracted, and treated with *N. mossaibica* PLA $_2$ for 2 h at 37°C. Following extraction, the PLA $_2$ -treated PC sample was analyzed by TLC.

Lipids were separated by a two-dimensional TLC procedure, which gave good separation of all the major phospholipids (17, 19). The organic phase containing the extracted lipids was dried under a stream of N_2 and resuspended in 100 μl of chloroform-methanol (2:1, vol/vol). A 50- μl aliquot, with appropriate standards, was placed on a 10- by 10-cm thin-layer Silica Gel 60 plate (Whatman) that had been previously treated with 0.4 M boric acid. The plates were developed in the first dimension with chloroform-methanol-water-ammonium hydroxide (70:30:3:2) and in the second dimension with chloroform-methanol-water (65:35:5). Individual lipids were visualized with iodine vapor. Areas corresponding to individual lipids were removed and placed in vials containing Universol scintillation fluor (ICN Biomedicals, Inc.) for quantitation. The results were standardized based on the number of milligrams of protein per dish. Protein was assayed by the method of Lowry et al. (31) with bovine serum albumin as the standard.

For determination of the lipid composition of purified chlamydiae, highly purified RBs and EBs were prepared from infected HeLa cell suspension culture by density gradient centrifugation (2). RBs were harvested at 22 h p.i., while EBs were harvested at 44 h p.i. The lipids were extracted as described above. The phospholipids were quantitated by analysis of phospholipid phosphorus (43) after separation on two-dimensional thin-layer plates. The presence of cholesterol and cholesteryl glucosides was assessed by a TLC procedure described by Haque et al. (15). The lipids on the developed plate were visualized by spraying with 0.2% orcinol in 2 N sulfuric acid followed by heating at 120°C. The cholesterol levels were estimated by an enzymatic assay with a Sigma Cholesterolin 20 kit. Fatty acid analysis and quantitation were conducted by Microcheck, Inc., Northfield Falls, Vt.

RESULTS

Fatty acid and lipid composition of *C. trachomatis*. The fatty acid composition of MI HeLa cells, *C. trachomatis* L2/434/Bu (L2)-infected HeLa cells (5, 10, 24, and 48 h p.i.), and highly purified *C. trachomatis* L2 RBs and EBs was determined (Fig. 1). Analysis of the fatty acid composition of *C. trachomatis*-infected HeLa cells revealed an increase over time in the amount of branched-chain fatty acids ($C_{15:0}$ BA) and a concomitant decrease in the amounts of oleic acid ($C_{18:1}$) and, to a lesser extent, palmitoleic acid ($C_{16:1}$) (Fig. 1A). These results verify that branched-chain fatty acids are synthesized by *C. trachomatis* and further suggest that some host-derived straight-chain unsaturated fatty acids have been replaced by chlamydial branched-chain fatty acids. The amount of myristic acid ($C_{14:0}$), a common constituent of bacterial lipopolysaccharide, also increased over the course of the chlamydial infection. Identical trends were revealed following analysis of purified *C. trachomatis* RBs and EBs (Fig. 1B).

We also examined the lipid compositions of MI and 24-h and 48-h-infected HeLa cells and purified *C. trachomatis* RBs and EBs (Table 1). No major differences were evident between MI and *C. trachomatis*-infected HeLa cells, suggesting that infection of HeLa cells with chlamydiae does not result in any major perturbations in the glycerophospholipid composition or content in the eukaryotic host cell. The level of PG is elevated in infected cells, presumably reflecting the higher PG content of *C. trachomatis*. Purified *C. trachomatis* RBs and EBs contained lipids normally associated with prokaryotic cells (PE, PG, PS, and CL) as well as lipids typically found in eukaryotic

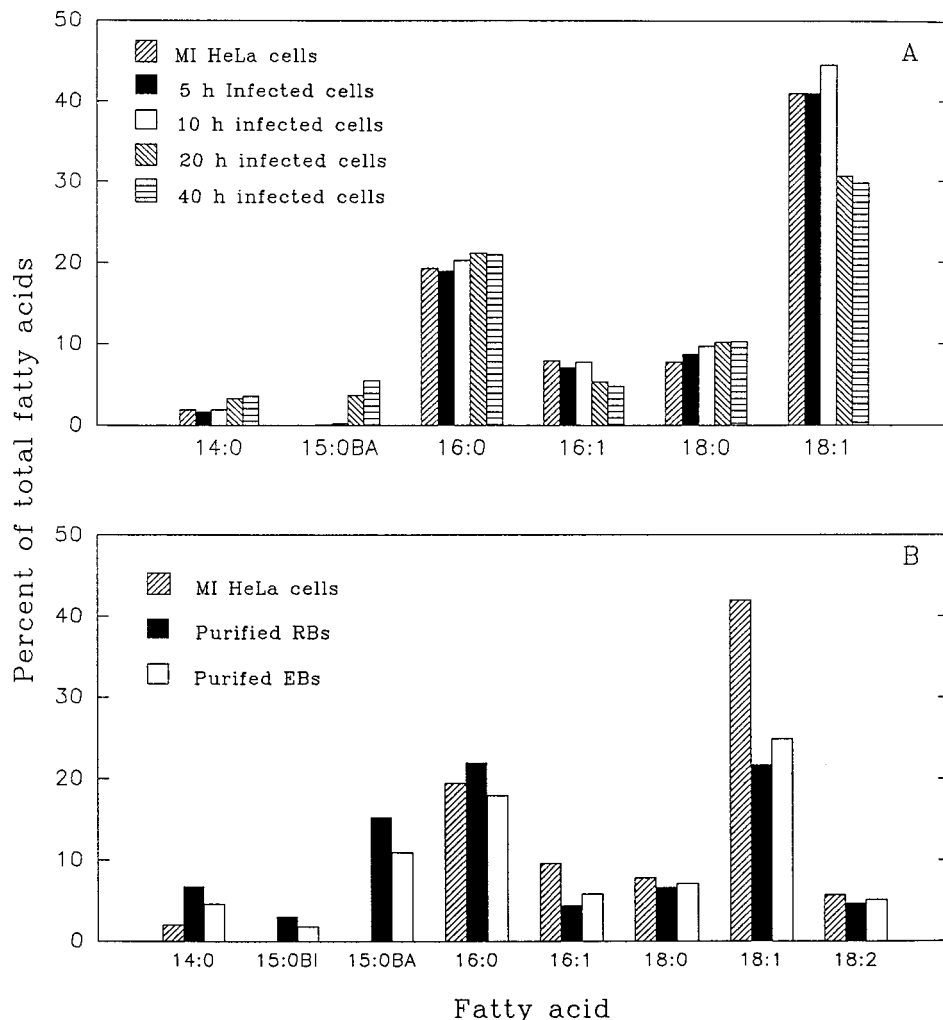


FIG. 1. Fatty acid composition of *C. trachomatis*-infected HeLa cells (A) and purified *C. trachomatis* RBs and EBs (B). Lipids were extracted from MI or *C. trachomatis*-infected HeLa cells or purified *C. trachomatis* RBs or EBs and subjected to fatty acid analysis. The amount of each fatty acid is expressed as a percentage of the total fatty acid content of a given sample. Abbreviations: BA, branched chain anteiso fatty acid; BI, branched chain iso fatty acid.

cells (PC, PI, cholesterol, and SM). PC, PI, SM, and cholesterol were found at similar concentrations in both MI cells and purified *C. trachomatis* cells. Their presence in *C. trachomatis* cells suggests that trafficking of several host-derived lipids to chlamydiae occurs. Several lipids normally synthesized by prokaryotic cells are present at higher concentrations in purified *C. trachomatis* RBs and EBs (PE and PG) than in MI HeLa cells, potentially suggesting *de novo* synthesis of these lipids by chlamydiae.

Effect of *C. trachomatis* infection on host cell phospholipid synthesis. The apparent trafficking of phospholipids from the host cell to *C. trachomatis* and the potential replacement of host-derived fatty acids by chlamydial branched-chain fatty acids prompted us to investigate in greater detail the phospholipid biosynthetic activity which occurs during infection of a eukaryotic cell by chlamydiae. Initial experiments centered on characterizing the changes in phospholipid synthesis or turnover in the host cell in response to a chlamydial infection. HeLa cells were used as the host cells. The HeLa cells were infected with *C. trachomatis*, and a given radiolabelled precursor was added 20 h p.i. Total lipids from MI and *C. trachomatis*-infected cells were extracted 6 h later and separated by

TLC. The labels used included typical phospholipid precursors: ethanolamine, choline, acetate, and glycerol.

The incorporation of ethanolamine, choline, acetate, or glycerol into phospholipids was essentially identical in MI and *C. trachomatis*-infected cells, indicating that host phospholipid synthesis did not increase or decrease following infection with chlamydiae (data not shown).

***C. trachomatis* replication is not dependent on host glycerophospholipid synthesis.** Since increased host phospholipid synthesis is not necessary for chlamydial replication, it was of interest to determine to what extent host phospholipid synthesis could be inhibited without affecting *C. trachomatis* growth. To address this question, we used a combination of delipidated growth medium and a mutant CHO cell line, CR-2. In the absence of exogenous cytidine, CR-2 cells are unable to synthesize CTP due to a mutation in their CTP synthetase (27). CTP is necessary for the synthesis of all glycerophospholipids in eukaryotic cells (18); therefore, glycerophospholipid synthesis in CR-2 is depressed under these growth conditions. The use of delipidated serum ensured that an exogenous source of lipids was not available for scavenging by the host cell. We found that *C. trachomatis* grew equally well in CR-2, indepen-

TABLE 1. Lipid composition of *Salmonella* sp., purified *C. trachomatis* RBs and EBs, and HeLa cells

Lipid		Phospholipid content ^a in:					
		<i>Salmo-</i> <i>nella</i> ^b	<i>C. tracho-</i> <i>matis</i> L2 RBs	<i>C. tracho-</i> <i>matis</i> L2 EBs	MI HeLa cells	<i>C. trachomatis-</i> infected HeLa cells	
						24 h p.i.	48 h p.i.
CL	17	2.6 ± 1.9	5.8 ± 3.0	3.4 ± 0.3	3.1 ± 0.9	3.8 ± 1.5	
PE	72	49.5 ± 0.5	44.3 ± 0.9	34.4 ± 2.5	37.5 ± 1.5	36.3 ± 1.2	
PG	11	4.7 ± 2.5	5.5 ± 3.9	0.8 ± 0.4	2.4 ± 0.9	3.9 ± 0.8	
PC		38.8 ± 2.0	38.8 ± 9.0	51.1 ± 4.8	45.1 ± 1.0	44.7 ± 0.7	
PS		1.3 ± 0.3	1.2 ± 0.4	5.5 ± 1.2	6.3 ± 1.2	5.3 ± 2.5	
PI		3.5 ± 1.3	5.0 ± 3.0	5.1 ± 1.0	5.5 ± 2.6	6.0 ± 2.9	
SM		2.4 ± 1.1	2.6 ± 1.3	2.7 ± 0.9	ND ^c	ND	
Cholesterol		34.0 ± 6.5	25.7 ± 0.9	42.5 ± 4.5	ND	ND	

^a Phospholipids were quantitated by measuring the phosphorus associated with a given glycerophospholipid and are expressed as a percentage of the total glycerophospholipid phosphorus. Cholesterol was measured by an enzymatic assay (micromoles of cholesterol per gram of cell protein) and expressed as a percentage of the total micromoles of phospholipid P and cholesterol per gram of cell protein. Results are expressed as the mean ± standard deviation of three separate experiments.

^b *Salmonella* was grown in Luria-Bertani broth and is representative of the typical phospholipid composition of a prokaryotic cell.

^c ND, not determined.

dent of the presence or absence of exogenous cytidine or delipidated serum (growth was assessed by the incorporation of radiolabelled adenine into chlamydial DNA and infectivity titration as previously described [reference 36 and data not shown]). This result indicates that neither de novo host phospholipid synthesis nor exogenous phospholipids are necessary for *C. trachomatis* replication.

Trafficking and modification of host-derived lipids and de novo lipid biosynthesis by *C. trachomatis*. The presence of eukaryotic lipids in purified *C. trachomatis* RBs and EBs (Table 1) suggested that trafficking of host-derived lipids to chlamydiae was occurring. To verify the presence of these lipids in chlamydial cells, we specifically labelled the lipids present in the *C. trachomatis* membrane. Fatty acid analysis of *C. trachomatis*-infected cells demonstrated the presence of branched-chain fatty acids. Fatty acids of this type are not synthesized in eukaryotic cells, indicating the presence of a branched-chain fatty acid synthetase in *C. trachomatis*. In contrast to priming of straight-chain fatty acids with acetyl coenzyme A (acetyl-CoA), synthesis of branched-chain fatty acids commences with a branched-chain acyl-CoA ester (isobutyryl-CoA, isovaleryl-CoA, or 2-methylbutyryl-CoA) and is followed by successive addition of two carbon units from malonyl-CoA. Isobutyryl-CoA, isovaleryl-CoA, and 2-methylbutyryl-CoA are provided to the system by α -ketoisovalerate dehydrogenase from α -ketoisovalerate, α -ketoisocaproate, and α -keto- β -methylvalerate, which in turn are derived from the amino acids valine, leucine, and isoleucine, respectively. By incubating chlamydia-infected cells with radiolabelled valine, leucine, or isoleucine, lipids synthesized or modified by *C. trachomatis* can be identified. Preliminary experiments were done with each of these amino acids. Although each amino acid showed incorporation into the glycerophospholipids isolated from *C. trachomatis*-infected cells, isoleucine showed by far the most extensive incorporation; therefore, only results for this amino acid are presented.

HeLa cells were MI or infected with *C. trachomatis* and incubated with [¹⁴C]isoleucine at 20 h p.i., and total lipids from the MI or *C. trachomatis*-infected cells were harvested 6 h later. MI cells incubated with label showed no incorporation of isoleucine into phospholipids, verifying that any observed isoleucine incorporation into lipids reflects *C. trachomatis* specific

activity. Figure 2 demonstrates that every glycerophospholipid isolated from the infected cells showed incorporation of radiolabel following incubation with [¹⁴C]isoleucine. In contrast to glycerophospholipids, there was no isoleucine labelling of SM. Labelling of eukaryotic lipids (e.g., PC and PI) suggests trafficking of host glycerophospholipids to the chlamydial cell. The radiolabelling studies described above were conducted with chlamydia-infected cells at 20 to 26 h p.i., a time of extensive RB multiplication. To determine if any changes in the pattern of isoleucine incorporation into *C. trachomatis* phospholipids occurred over time, we repeated our isoleucine-labelling studies with infected HeLa cells harvested at 12, 24, and 48 h p.i. (the cells were labelled 6 h prior to harvest). Total incorporation of isoleucine into the phospholipids of *C. trachomatis*-infected HeLa cells reflected increasing chlamydia cell number over time (12 h, 6,099 dpm/mg of protein; 24 h, 31,452 dpm/mg of protein, 48 h, 77,136 dpm/mg of protein). On a percentage basis, the pattern of isoleucine incorporation into the phospholipids of cells harvested at 24 and 48 h were similar (Fig. 3B and C). Cells harvested at 12 h showed a lesser incorporation of labelled isoleucine into PE and a greater incorporation into PC (Fig. 3A).

In addition to demonstrating trafficking of eukaryotic glycerophospholipids to chlamydiae, differences in the extent of incorporation of labelled isoleucine into the different species of phospholipids suggest de novo biosynthesis of some lipids by *C. trachomatis*. Although PE and PC were found in approximately equal proportions in the chlamydial cell (Table 1), isoleucine incorporation into PE was approximately fivefold that into PC. Similarly, although PG contributes only 5% to the total chlamydial phospholipids (Table 1), extensive incorporation of labelled isoleucine into PG suggests de novo synthesis of this lipid by *C. trachomatis*.

Further evidence to support the above conclusions was drawn from radiolabelling studies conducted with a mutant CHO cell line (strain 58) (6) as the host for chlamydial growth. Strain 58 carries a thermolabile CDP-choline synthetase. Following a temperature shift from 33 to 40°C, the PC content of this cell line decreases immediately, and it continues to decline in cells held at the nonpermissive temperature (6). We rea-

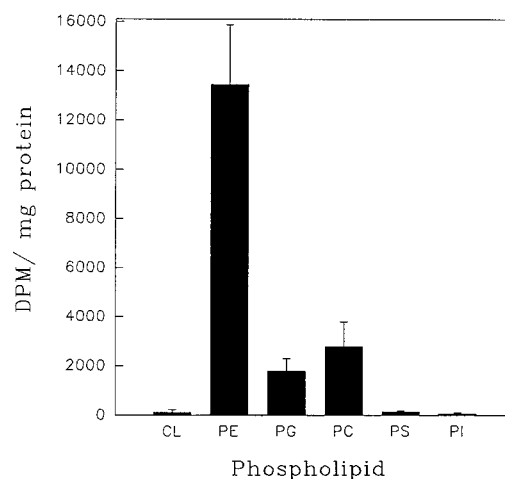


FIG. 2. Incorporation of [¹⁴C]isoleucine into glycerophospholipids of *C. trachomatis*-infected HeLa cells. The cells were labelled at 20 h p.i. and harvested at 26 h p.i. No incorporation of [¹⁴C]isoleucine into MI cells was observed; therefore, only data for infected cells is shown. In addition, SM was not labelled. The actual values for CL, PS, and PI are 135 ± 103, 161 ± 20, and 69 ± 32 dpm/mg of protein, respectively.

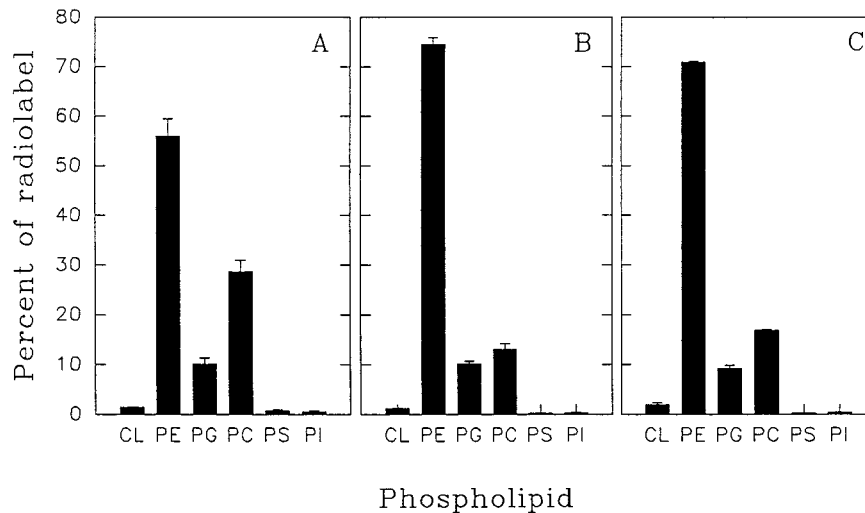


FIG. 3. Incorporation of [U-¹⁴C]isoleucine into glycerophospholipids of *C. trachomatis*-infected HeLa cells at 12 h (A), 24 h (B), or 48 h (C) p.i. The cells were labelled 6 h prior to harvest. The results for each phospholipid are expressed as a percentage of the total radioactivity incorporated into phospholipids.

soned that this decrease in the PC content would be reflected by a decreased incorporation of isoleucine into PC trafficked from the host cell to *C. trachomatis* and potentially by an increase in the label incorporated into phospholipids synthesized de novo by chlamydiae.

Strain 58 cells, after being held at 33 or 40°C for 72 h, were infected with *C. trachomatis*. Infected cells were held at their respective temperatures, labelled with [¹⁴C]isoleucine at 20 h p.i., and harvested 6 h later. The results showed decreased and increased isoleucine incorporation into PC and PG, respectively, at the nonpermissive temperature (Fig. 4). This change in isoleucine incorporation is consistent with chlamydial de novo synthesis of PG and decreased trafficking of host-derived PC to *C. trachomatis*.

Precursors utilized by *C. trachomatis* for de novo phospholipid synthesis. Given the apparent synthesis of PE, PG, and PS by *C. trachomatis*, we wished to determine the substrates utilized by this bacterium for biosynthesis of these phospholipids. Several of the labelling experiments described above provide data relevant to this question. Incorporation of labelled isoleucine, valine, and leucine into the lipids of infected cells suggests chlamydial uptake of branched-chain amino acids from the host for incorporation into branched-chain fatty acids. Additionally, the absence of any increase in the incorporation of acetate, glycerol, ethanolamine, or choline in *C. trachomatis*-infected cells suggested that none of these substrates were taken up by *C. trachomatis* for utilization in phospholipid biosynthesis.

Uniformly labelled glucose was tested as a potential carbon source for phospholipid biosynthesis by chlamydiae. There was an increase in the incorporation of label in *C. trachomatis*-infected cells in comparison to MI cells (Fig. 5). The most clearly apparent changes in incorporation occurred with PE and PG (10-fold), suggesting chlamydial transport and utilization of glucose from the host cell as a carbon source for the synthesis of these phospholipids. Smaller increases (twofold) in the labelling of PC, CL, PS, and PI were also observed. We attribute these increases to the reacylation of a host-derived lipid with a *C. trachomatis*-synthesized branched-chain fatty acid following incorporation of the host lipid into the chlamydial membrane. There was essentially no change in labelled-glucose incorporation into SM.

Since prokaryotic cells efficiently decarboxylate PS to PE, we examined the incorporation of [¹⁴C]serine into PE as a measure of parasite-specific decarboxylation activity. The results are shown in Fig. 6. MI cells incorporated serine into PS, PE, and SM. Serine is a direct precursor of PS and SM, while the radiolabel identified in PE results from PS decarboxylation activity which occurs in eukaryotic cell mitochondria (5, 23, 46). Following infection with chlamydiae, incorporation of the label into PS and SM was essentially unchanged while incorporation into PE increased 30-fold. The large increase in the

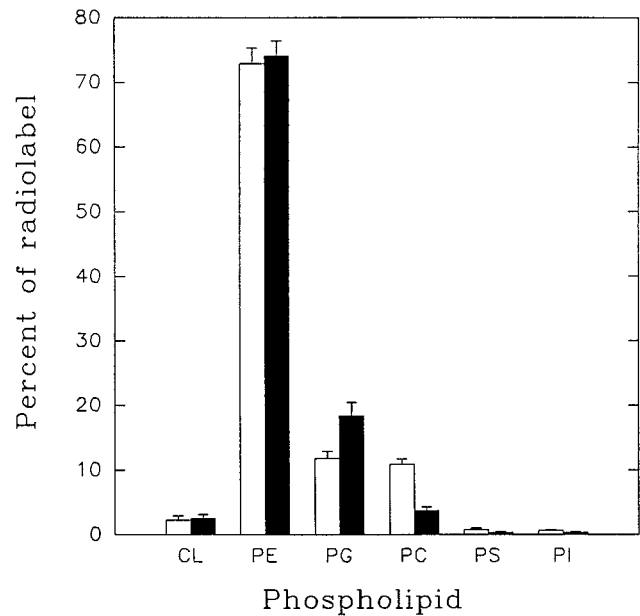


FIG. 4. Incorporation of [U-¹⁴C]isoleucine into glycerophospholipids of *C. trachomatis*-infected strain 58 cells (thermolabile CDP-choline synthetase) at 33°C (open bars) and 40°C (solid bars). The host cells were held at their respective temperatures for 72 h prior to infection. The temperature was maintained following infection. Infected cells were labelled at 20 h p.i. and harvested at 26 h p.i. The results for each phospholipid are expressed as a percentage of the total radioactivity incorporated into phospholipids.

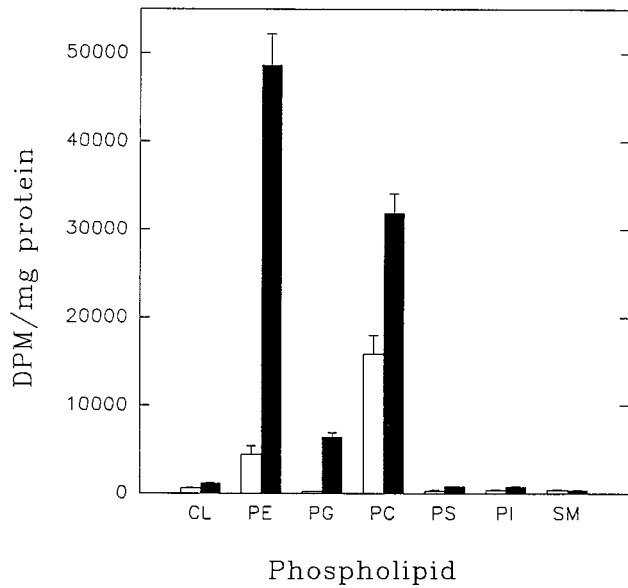


FIG. 5. Incorporation of [U - ^{14}C]glucose into phospholipids of MI (open bars) and *C. trachomatis*-infected (solid bars) HeLa cells. The cells were labelled at 20 h p.i. and harvested at 26 h p.i.

incorporation of labelled serine into PE following infection suggests that the observed changes in incorporation are due to a chlamydia-specific phosphatidylserine decarboxylase.

Infected cells labelled with serine showed no incorporation of radiolabel into PC (data not shown). Some prokaryotes synthesize PC by the addition of three methyl groups to the ethanolamine head group of PE, although this mechanism is rare (25, 55). If the PC present in purified chlamydiae was produced via this mechanism, [^{14}C]serine would label PC in addition to PE and PS.

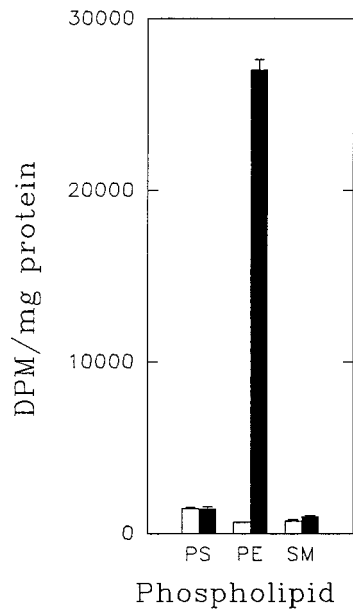


FIG. 6. Incorporation of [U - ^{14}C]serine into phospholipids of MI (open bars) and *C. trachomatis*-infected (solid bars) HeLa cells. The cells were labelled at 20 h p.i. and harvested at 26 h p.i.

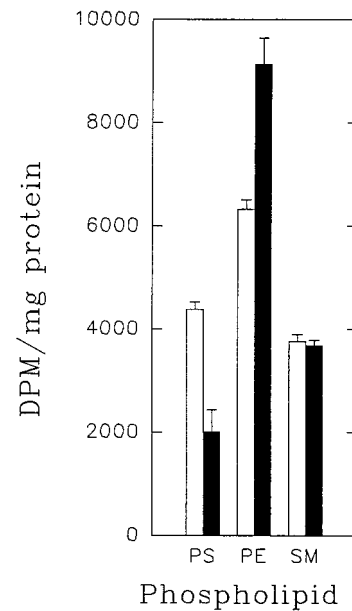


FIG. 7. Transfer and decarboxylation of host-derived PS by *C. trachomatis*-infected HeLa cells. HeLa cells were prelabelled with [U - ^{14}C]serine for 18 h. The cell monolayers were washed and then left as MI controls (open bars) or infected with *C. trachomatis* (solid bars), fresh medium was added, and the cultures were incubated for a further 44 h.

The above result indicates that *C. trachomatis* decarboxylates PS to PE. We took advantage of this fact to design an experiment that would provide further evidence supporting the trafficking of host phospholipids to chlamydiae. HeLa cells were prelabelled with [^{14}C]serine for 18 h, which results in radiolabelling of host PS, PE, and SM, (data not shown), the three phospholipids which have serine as their precursor (54, 55); then the [^{14}C]serine-containing medium was removed and the cell monolayers were extensively washed and infected with *C. trachomatis* or left as uninfected controls. Following the addition of fresh medium, the cultures were returned to the incubator for a further 44 h to allow chlamydiae to complete one growth cycle, and then the lipids were extracted and analyzed. We reasoned that if *C. trachomatis* could import and decarboxylate host-derived PS, increased radioactivity in PE and decreased activity in PS in infected cultures compared to those in uninfected HeLa cell controls would be found. The results show that this was indeed the case (Fig. 7). Infected cultures showed a 44% increase in radioactivity in PE and a 54% decrease in radioactivity PS compared to uninfected control cultures. This indicates that preexisting host cell PS is trafficked to chlamydiae and then decarboxylated to PE. In contrast to PE and PS, there was no change in the amount of radioactivity associated with SM between uninfected and *C. trachomatis*-infected cultures.

Route of phospholipid trafficking from the host cell to *C. trachomatis*. Given the clear indication of glycerophospholipid trafficking from the host cell to chlamydiae, we investigated the potential routes by which this lipid transfer may occur. Several potential routes of phospholipid trafficking in eukaryotic cells have been described previously (54). These include transfer of lipid monomers via lipid transfer proteins, vesicle-mediated transfer, and lateral transfer of lipids by transient direct membrane contact and fusion. Using a series of inhibitors and/or mutant host cell lines, we investigated which of these mecha-

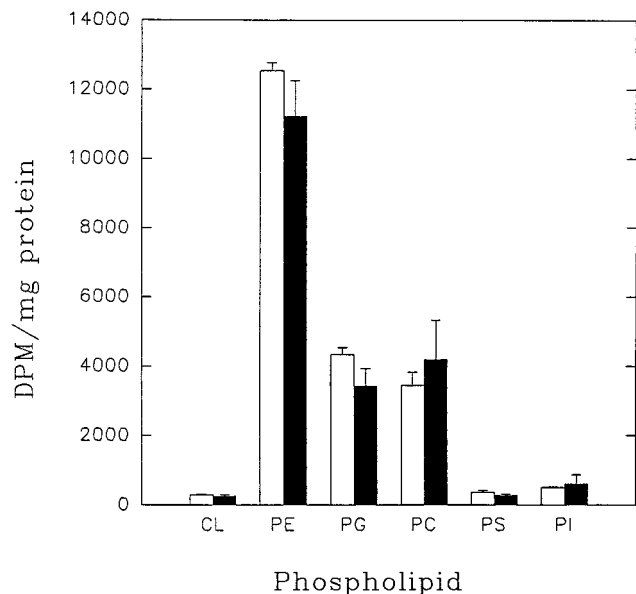


FIG. 8. Incorporation of [U-¹⁴C]isoleucine into glycerophospholipids of *C. trachomatis*-infected HeLa cells in the absence (open bars) or presence (solid bars) of 20 μ M brefeldin A. Brefeldin A was added at 19 h p.i., and radiolabelled isoleucine was added at 20 h p.i. The cells were harvested 6 h later.

nisms may play a role in glycerophospholipid trafficking between host cells and chlamydiae.

The antibiotics brefeldin A and monensin block the vesicle-mediated exocytic pathway originating from the Golgi apparatus (30, 37, 38). *C. trachomatis*-infected cells were treated at 19 h p.i. with brefeldin A (20 μ M) or monensin (1 μ M). These treatments had no significant effect on *C. trachomatis* growth as assessed by incorporation of radiolabelled adenine or infectivity titration (data not shown). Control infected cells were left untreated. The infected cells were labelled with [¹⁴C]isoleucine at 20 h p.i. and harvested 6 h later. Interruption of host phospholipid flow to chlamydiae following treatment would be expected to result in a decrease in isoleucine labelling of those phospholipids affected. No significant change was found in the extent of isoleucine incorporation into any of the lipids extracted from treated or untreated *C. trachomatis*-infected cells, suggesting that glycerophospholipid trafficking from host cells to chlamydiae is not dependent on the Golgi exocytic pathway (data for brefeldin A shown in Fig. 8; data for monensin not shown).

The potential influence of lipid transfer proteins was investigated with a mutant CHO cell line, ZR78 (53), which lacks an nsLTP. In vitro, nsLTP catalyzes the transfer of phospholipids, cholesterol, and glycolipids between membranes and is the only LTP known to be able to transfer PS (53, 56). *C. trachomatis* was grown in the mutant cell line ZR78 and its wild-type parent (CHO K1), labelled at 20 h p.i. with [¹⁴C]isoleucine, and harvested at 26 h p.i. No apparent change in the incorporation of label into any phospholipid was observed, suggesting that nsLTP is not responsible for the major flow of phospholipids to chlamydiae (data not shown). We repeated this experiment with [¹⁴C]serine to specifically investigate PS transfer from the host to bacteria. Again, no difference in the incorporation of serine into phospholipids in *C. trachomatis*-infected ZR78 or K1 cells was observed (data not shown). This leaves the possibility of direct membrane contact as the primary vehicle of phospholipid transfer from the host cell to chlamydiae.

C. trachomatis does not produce cholesteryl glucosides.

Analyses of the lipid composition of purified *C. trachomatis* resulted in the identification of cholesterol in both EBs and RBs. This identification was based on the enzymatic detection of cholesterol and by comparison of the R_f values of lipids separated by TLC with those of known cholesterol standards. Several human bacterial pathogens are known to modify cholesterol obtained from their host, producing unique cholesteryl glucosides (15). Using TLC, we compared the lipids of MI cells with those extracted from purified EBs and RBs. We detected no unique spots in purified *C. trachomatis* EBs or RBs which could be attributed to cholesteryl glucosides (data not shown), indicating that *C. trachomatis* does not modify the cholesterol it receives from the host cell.

Effect of *C. trachomatis* infection on the hydrolysis of oleic acid-radiolabelled phospholipids. The much lower levels of oleic acid ($C_{18:1}$, a fatty acid common in the sn-2 position of eukaryotic phospholipids) in purified *C. trachomatis* than in HeLa cells (Fig. 1), coupled with the incorporation of radiolabelled isoleucine into host-derived glycerophospholipids (Fig. 2), suggested that phospholipase A₂ activity may be required to deacylate host phospholipids prior to chlamydial reacylation with a parasite-derived branched-chain fatty acid. To determine if host cell phospholipid hydrolysis was affected by chlamydial infection, HeLa cells were prelabelled with oleic acid (incorporated primarily into the sn-2 position) for 18 h to obtain equilibrium labelling of all host glycerophospholipids. Prelabelled cells were infected with *C. trachomatis* or left as MI controls. After incubation for 40 h, the medium was collected and the lipids were extracted and analyzed for radioactive oleic acid by TLC. Approximately 2.5 times as much radioactive oleic acid was recovered from the medium of *C. trachomatis*-infected cultures (12,937 dpm/mg of cell protein) than from that of the MI controls (5,306 dpm/mg of cell protein). This result indicates that lipid hydrolysis was increased in chlamydia-infected cells.

Localization of branched-chain fatty acid on host-derived PC.

In total, the results of the above experiments suggest that during the trafficking of host cell phospholipids to *C. trachomatis*, the sn-2 position straight-chain fatty acid is removed and replaced with a parasite-derived branched-chain fatty acid. To directly test if the branched-chain fatty acid was located in the sn-2 position of host-derived phospholipids, we radiolabelled *C. trachomatis*-infected cultures with isoleucine for 40 h to allow for maximal incorporation of radioactivity into phospholipids and then extracted the lipids and separated the individual species by two-dimensional TLC. We chose PC for further analysis since our previous results indicated that despite not being synthesized by *C. trachomatis*, it is efficiently labelled by isoleucine, suggesting that it is readily trafficked to chlamydiae. The radiolabelled PC spot was removed from the TLC plate, and the PC was extracted from the silica gel. Half of the sample was left untreated, and the other half was subjected to treatment with commercial (*N. mossambica*) PLA₂. Following PLA₂ treatment, the lipids were extracted from the incubation mixture and both the treated and untreated control samples were analyzed by TLC under conditions suitable for separation of PC and lyso-PC (19). Consistent with the branched-chain fatty acid being in the sn-2 position, there was little radioactivity (514 dpm) associated with PC in the PLA₂-treated sample compared to the untreated control (22,340 dpm). Furthermore, in both the PLA₂-treated and untreated samples, there was little radioactivity associated with lyso-PC (1,500 to 2,200 dpm). There was a large amount of radioactivity in the fatty acid fraction from the PLA₂-treated sample (25,768 dpm). In contrast, in the untreated control sample, there was only a

small amount of radioactivity in the fatty acid fraction (367 dpm). Together, these findings indicate that most of the *C. trachomatis*-synthesized branched-chain fatty acid is located in the sn-2 position of host-derived PC.

DISCUSSION

The presence of a branched-chain fatty acid in *C. trachomatis* provides a unique way of identifying phospholipids incorporated into and modified in the chlamydial membrane and allows a clear differentiation of lipids present in the chlamydial membrane from contamination by host membranes. By using isoleucine to trace the incorporation of chlamydia-specific branched-chain fatty acids into phospholipids, we were able to demonstrate the transfer of host-derived glycerophospholipids to *C. trachomatis*. In addition, two other results, one obtained with a CHO cell line with a thermolabile CDP-choline synthetase infected with *C. trachomatis*, which shows decreased isoleucine labelling of PC at the nonpermissive temperature, and the second obtained with infected HeLa cells prelabelled with [¹⁴C]serine, which shows increased labelling of PE, provide additional evidence of trafficking of host-derived phospholipids to chlamydiae. A time course analysis of isoleucine incorporation into the phospholipids of *C. trachomatis*-infected cells indicates that the acquisition of host-derived lipids occurs throughout the infection. To our knowledge, this is the first biochemical evidence demonstrating intracellular pathogen modification of a host-derived phospholipid.

Our observations agree with the recent demonstration of SM trafficking to the chlamydial membrane; however, a key difference between our results and those of Hackstadt et al. (12, 13) relates to which host lipids are targeted to the chlamydial cell. Using a series of fluorescent phospholipid analogs, Hackstadt et al. (12) was able to demonstrate the presence of a fluorescent SM (NBD-SM) probe in the cell wall of *C. trachomatis* but not fluorescent analogs of PC (NBD-PC), PE (NBD-PE), or PS (NBD-PS). Conversely, our data indicate that chlamydial branched-chain fatty acids are incorporated into each of these glycerophospholipids but not into SM, a sphingolipid (Fig. 2). The inability to detect NBD-PC, NBD-PE, or NBD-PS may be related to the location of the fluorescent portion of the phospholipid analogs used by Hackstadt et al. (12). In each case, the fluorescent probe replaces the acyl group at the PLA₂-sensitive C-2 carbon, the position where the majority of the parasite-derived branched-chain fatty acid is located. Our results, i.e., increased hydrolysis of oleic acid-containing phospholipids in *C. trachomatis*-infected cells, isoleucine labelling of all glycerophospholipids but not SM, and loss of radiolabel from PC (isolated from isoleucine-labelled infected cells) after treatment with PLA₂ in vitro, establish the sn-2 position as the location of the branched-chain fatty acid and implicate PLA₂ as being responsible for the deacylation of the host-derived lipid prior to reacylation with a chlamydia-derived branched-chain fatty acid. As a result, much of the fluorescent label would be lost from the glycerophospholipid analogs but not from SM, which is insensitive to PLA₂ activity, prior to incorporation into the chlamydial membrane. Since Hackstadt et al. (12) detected NBD-PC, NBD-PE, and NBD-PS in the inclusion membrane but not in chlamydial cell walls, it is likely that the action of PLA₂ occurs at the vacuolar membrane. In support of this, Majeed et al. (32, 33) have shown that *C. trachomatis* infection causes a mobilization of intracellular Ca²⁺ stores and have localized accumulation of Ca²⁺ to chlamydia-containing endosomes. Calcium is a well-established regulator of cytoplasmic PLA₂ activity, being required for its translocation to membranes (3, 10, 28). Finally,

another possibility that cannot be ruled out is that *C. trachomatis* encodes a secretory PL that is responsible for host phospholipid deacylation.

Hackstadt et al. (12, 13) presented evidence that chlamydiae intercept host SM by interrupting an exocytic pathway from the Golgi apparatus to the plasma membrane. Treatment of cells with brefeldin A, a drug which causes disassembly of the Golgi stacks, prevented the trafficking of NBD-SM to chlamydiae. We could detect no significant change in isoleucine incorporation into glycerophospholipids of *C. trachomatis*-infected cells in the presence of brefeldin A or monensin. In addition, it is apparent from our results, as well as those of Hackstadt et al. (12) and Beatty and Stephens (1), that brefeldin A does not inhibit chlamydial replication or differentiation. For the glycerophospholipid species we examined, the results suggest that trafficking to the chlamydial cell is not dependent on a functional exocytic pathway. Furthermore, results with the ZR78 mutant CHO cell line indicate that phospholipid transfer proteins do not make a major contribution to the trafficking of phospholipids from the host to chlamydiae. This leaves the possibility of transient direct membrane contact as a mechanism of phospholipid trafficking to chlamydiae.

Vance and colleagues (44, 52) have provided evidence that direct membrane contact between the endoplasmic reticulum and mitochondria is at least partially responsible for the trafficking of phospholipids between the two organelles. Although definitive evidence for direct membrane contact between host cell membranes and chlamydiae is lacking, previous electron and confocal microscopic images have demonstrated a close association between the chlamydial inclusion and a variety of host subcellular structures (mitochondria, Golgi apparatus, endoplasmic reticulum, nucleus [13, 34, 42, 45, 47, 50]). In addition, metabolically active RBs maintain close contact with the inclusion membrane (13, 39). Taken together, these observations provide a plausible route for phospholipid trafficking between chlamydiae and the host cell.

In addition to acquisition of host cell phospholipids, there is evidence supporting de novo synthesis of PE and PG by *C. trachomatis*. This conclusion is based on the disproportionate labelling of PE and PG relative to their mole percentages and on the level of incorporation of isoleucine, glucose, and serine into PE and isoleucine and glucose into PG compared to other phospholipids (PI, PC, and CL) in *C. trachomatis*-infected HeLa cells. Direct evidence for de novo phospholipid biosynthesis by chlamydiae comes from our recent cloning of the PS decarboxylase gene from *C. trachomatis* L2 (GenBank accession no. U72715). In contrast to PE and PG, it appears that *C. trachomatis* does not synthesize CL, suggesting that CL in the chlamydial membrane is host derived. Host-derived CL must originate from the mitochondria, since CL is restricted to this organelle in eukaryotic cells (16). Evidence suggesting that there is no de novo CL biosynthesis in *C. trachomatis* is based on the observation that PG is the direct precursor of CL, yet despite similar percentages of the two phospholipids in the chlamydial membrane and extensive labelling of PG by isoleucine and glucose, little label is incorporated into CL. Loss of CL biosynthetic activity in *C. trachomatis* would not be unexpected, given the role of CL in oxidative phosphorylation (16) and the apparent lack of this metabolic process in chlamydiae (20, 35, 39). Further analyses are under way to verify the full complement of phospholipid biosynthetic enzymes present in chlamydiae.

Although *C. trachomatis* is capable of de novo phospholipid synthesis, it is unlikely that all of the PE, PS, and PG in the chlamydial membrane originate exclusively from chlamydia biosynthesis. To do this, chlamydiae would require a specific

mechanism for selecting or excluding particular phospholipids that it receives from the host. It is more likely that the composition of eukaryote-derived phospholipids in chlamydiae is a direct reflection of the composition of the host donor membrane. In this regard, it is interesting that the phospholipid composition of the extracellular form of chlamydiae (EB) and a purified mitochondrion (24) are similar.

Cholesterol was identified in purified EBs and RBs. To date, the presence of cholesterol in the cell membrane of bacteria is relatively rare, being found in *Acholeplasma* spp., *Mycoplasma gallinarum*, *Spiroplasma citri*, *Borrelia hermsii*, *Helicobacter* spp., and *Staphylococcus aureus* (14, 22). With the exception of *S. aureus*, these bacteria modify cholesterol obtained from the host cell by the addition of glucose to form cholesteryl glucosides. We found no evidence of cholesteryl glucosides in *C. trachomatis*. Since no modification of the cholesterol occurs, it may not serve a specific function in *C. trachomatis* but instead may be nonspecifically incorporated into the chlamydial cell membrane as a result of growth within a eukaryotic cell. In the presence of serum, *Escherichia coli* is also known to incorporate cholesterol into its membrane (15).

In conclusion, our data indicate that host cell phospholipids are trafficked to chlamydiae. Continued definition of the lipid flow between *C. trachomatis* and its host will be a major step toward understanding several aspects of intracellular bacterial parasitism. Identifying the eukaryotic phospholipid-trafficking pathways which chlamydiae intercept not only will advance the understanding of chlamydial biology but also may provide insights into bacterial evasion of host defenses. The essentially eukaryotic lipid composition of *C. trachomatis*, and presumably its vacuole, may allow this bacterium to appear to the host cell as a cytoplasmic organelle, which therefore would not be targeted for fusion with lysosomes. Additionally, chlamydiae may prove useful in the emerging field of cellular microbiology, a discipline based on the use of pathogens to address basic questions in cell biology (4). Since the transfer of host-derived lipids to pathogens is not universal among intracellular bacteria (21), *C. trachomatis* could prove useful in delineating eukaryotic phospholipid-trafficking pathways which normally occur within a eukaryotic cell.

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