

## A *Chlamydia pneumoniae* Component That Induces Macrophage Foam Cell Formation Is Chlamydial Lipopolysaccharide

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*Chlamydia pneumoniae* infection is associated with atherosclerotic heart and vessel disease, but a causal relationship between this pathogen and the disease process has not been established. Recently, it was reported that *C. pneumoniae* induces human macrophage foam cell formation, a key event in early atheroma development, suggesting a role for the organism in atherogenesis. This study further examines *C. pneumoniae*-induced foam cell formation in the murine macrophage cell line RAW-264.7. Infected RAW cells accumulated cholesteryl esters when cultured in the presence of low-density lipoprotein in a manner similar to that described for human macrophages. Exposure of *C. pneumoniae* elementary bodies to periodate, but not elevated temperatures, inhibited cholesteryl ester accumulation, suggesting a role for chlamydial lipopolysaccharide (cLPS) in macrophage foam cell formation. Purified cLPS was found to be sufficient to induce cholesteryl ester accumulation and foam cell formation. Furthermore, the LPS antagonist lipid X inhibited *C. pneumoniae* and cLPS-induced lipid uptake. These data indicate that cLPS is a *C. pneumoniae* component that induces macrophage foam cell formation and suggest that infected macrophages chronically exposed to cLPS may accumulate excess cholesterol to contribute to atheroma development.

Atherosclerosis is a chronic, inflammatory disease that results from arterial hemodynamic changes and complex interactions between a variety of lipids, cell types, and their soluble mediators (39, 44). The central cell mediating atheroma development may be the macrophage (32, 48). Atheroma macrophages accumulate excess cholesterol that is esterified and stored in the cytoplasm, and these cholesteryl ester-loaded macrophages, or foam cells, are the hallmark of early fatty streak lesions in atherogenesis (4).

Although multiple risk factors for atherosclerosis have been identified (21, 45, 46), 30 to 40% of disease complications result in the absence of these conditions and may involve previously unrecognized risk factors. Appreciation of atherosclerosis as an inflammatory disease (29, 38, 39) has renewed interest in the role that infectious agents may play in atheroma development. In particular, the obligate intracellular bacterium *Chlamydia pneumoniae* has been associated with atherosclerosis by seroepidemiology (41), identification of the organism within atheromas by immunohistochemistry (23) and within foam cells by electron microscopy (43), and identification of the *C. pneumoniae* genome within atheromas by PCR (24). In addition, the organism has been isolated from atheromas (19), and antibiotic treatment trials have shown significant reductions of complications in patients with atherosclerosis (15, 16).

Although ample clinical data exist to suggest a role for *C. pneumoniae* in atherosclerosis, how the organism may initiate or promote the disease remains unclear. Rabbit and apolipoprotein E-deficient murine animal models have been developed to study atherosclerosis following *C. pneumoniae* infection (30, 31) and may prove useful to examine mechanisms of

disease in vivo. In vitro, *C. pneumoniae* has been shown to infect and multiply within smooth muscle cells, endothelial cells, and macrophages (12), suggesting that the organism can survive and persist within atheromas. Furthermore, infected mononuclear cells release inflammatory cytokines (22) that may contribute indirectly to atheroma development.

Evidence linking *C. pneumoniae* infection to events thought to contribute directly to atherogenesis has emerged only recently. *C. pneumoniae* has been shown to induce human macrophage foam cell formation when cultured in the presence of low-density lipoprotein (LDL) (20), implicating this organism as a causative agent in atherosclerosis. However, detailed characterization of *C. pneumoniae*-induced foam cell formation by human monocyte-derived macrophages has been difficult due to the widely varying capacity to ingest lipids by monocytes/macrophages isolated from different subjects. In this study, we used the well-characterized murine macrophage cell line RAW-264.7 to further examine the role of *C. pneumoniae* in foam cell formation by macrophages.

### MATERIALS AND METHODS

**Reagents.** Cholesterol esterase was purchased from Boehringer Mannheim (Indianapolis, Ind.). Highly purified *C. trachomatis* chlamydial lipopolysaccharide (cLPS) extracted by the Galanos method (10), as modified by Qureshi et al. (18, 34), was kindly provided by Douglas Golenbock (Boston, Mass.). The purity of cLPS was determined by protein assay, thin-layer chromatography, and high-pressure liquid chromatography (18). *Escherichia coli* lipid X was synthesized by Peter Stutz (Vienna, Austria) and was a gift from Richard Proctor and Loren Denlinger (Madison, Wis.). All other reagents were purchased from Sigma Chemical Company (St. Louis, Mo.).

**Isolation of LDL.** LDL was isolated from normolipidemic donors by density gradient ultracentrifugation as previously described (37), extensively dialyzed against 0.15 M NaCl-0.05% EDTA, and concentrated by centrifugation in Centri/Por concentrators (Spectrum, Houston, Tex.). LDL protein and cholesterol were determined by use of a protein assay (Bio-Rad, Hercules, Calif.) and a total cholesterol test kit (Sigma), respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of LDL on a 4 to 20% gradient gel revealed a single protein (molecular weight, ~250,000) consistent with LDL apolipoprotein B-100. Agarose gel electrophoresis of LDL showed no increase in relative electrophoretic mobility within 2 weeks, indicating that isolated LDL did not become

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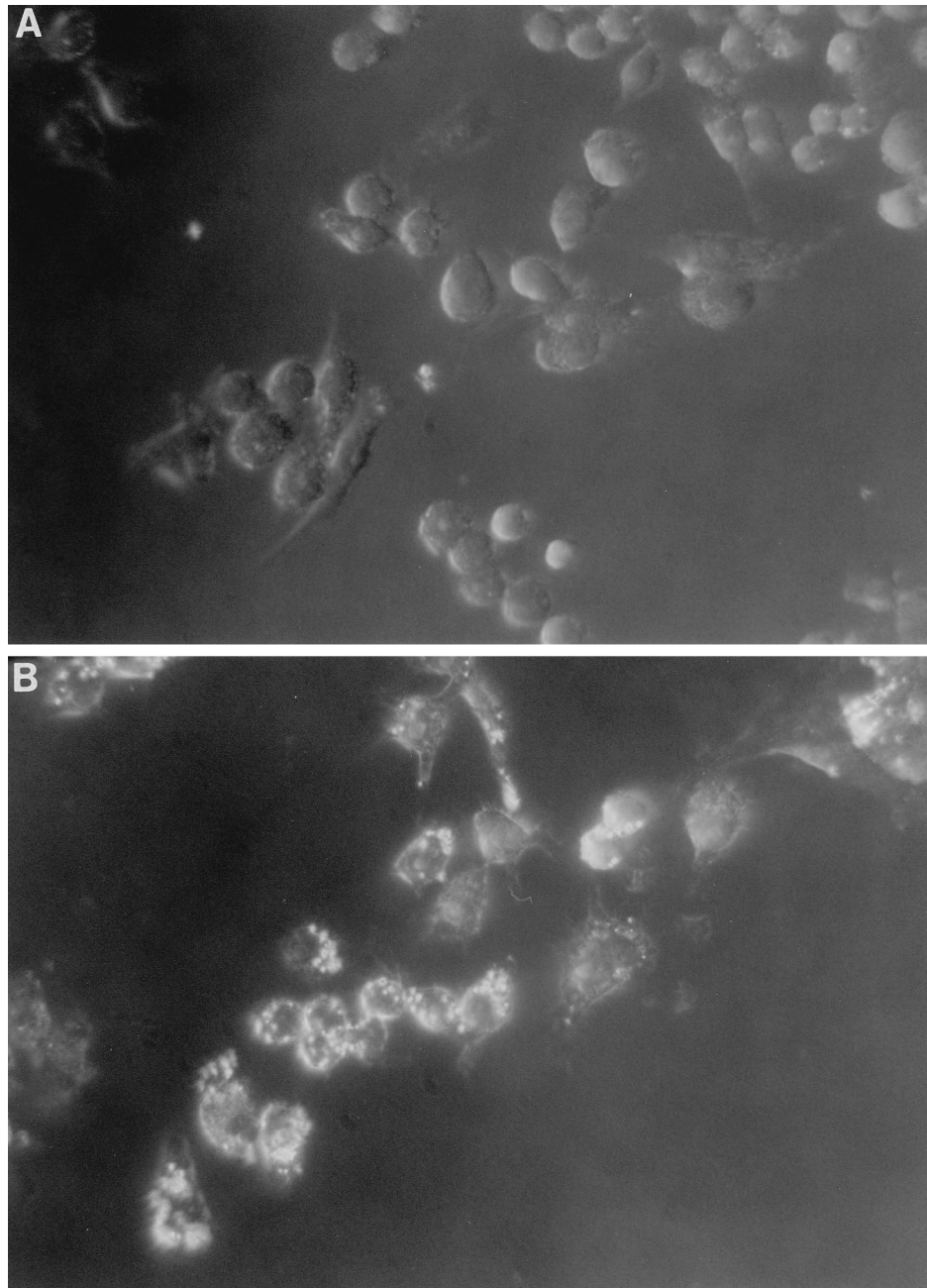


FIG. 1. Foam cell formation by uninfected (A) and infected (B) RAW macrophages. Cells were infected (MOI = 1) or not infected for 2 h, washed twice, and incubated with 100  $\mu\text{g}$  of LDL per ml for 24 h. Micrographs show oil red O-stained macrophages containing cytoplasmic neutral lipid droplets (magnification,  $\times 400$ ; dark-field microscopy).

oxidized (8). Isolated LDL was stored at 4°C and used within 2 weeks of isolation.

**Propagation of chlamydiae and growth of macrophages.** *C. pneumoniae* was purchased from the American Type Culture Collection (ATCC) (Manassas, Va.), propagated in HEp-2 cells (ATCC), and purified by Renografin gradient centrifugation as previously described (6). Propagated organisms were stored at  $-70^{\circ}\text{C}$  in sucrose buffer and titered for infectivity as described (20). Titers are reported here as multiplicity of infection (MOI), where one MOI equals one infectious *Chlamydia* organism/HEp-2 cell.

The murine macrophage cell line RAW-264.7 (ATCC) was grown in RPMI 1640 medium (BioWhittaker, Walkersville, Md.) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 50  $\mu\text{g}$  of vancomycin per ml, and 10  $\mu\text{g}$  of gentamicin per ml. Cells were maintained in a 37°C, 5%  $\text{CO}_2$  incubator and split every 2 days by gentle scraping.

**Infection and culture of macrophages.** Macrophages were plated at a density of either  $4 \times 10^4$  cells/well on 96-well trays (0.32-cm<sup>2</sup> growth area; Corning Costar Corporation, Cambridge, Mass.) or  $2 \times 10^5$  cells/well on 24-well trays (1.9-cm<sup>2</sup> growth area; Corning) and incubated overnight prior to infection. In some experiments, macrophages were plated overnight at  $2 \times 10^4$  cells/well on Lab-Tek chamber slides (0.32-cm<sup>2</sup> growth area; Nunc Inc., Naperville, Ill.) prior to infection. Macrophages were infected by incubating cells for 2 h with various doses of *C. pneumoniae* in 50  $\mu\text{l}$  (96-well trays) or 200  $\mu\text{l}$  (24-well trays) of RPMI 1640–10% FBS. Cells then were washed twice with phosphate-buffered saline (PBS; BioWhittaker) and incubated for 24 h in 200  $\mu\text{l}$  (96-well trays) or 500  $\mu\text{l}$  (24-well trays) of RPMI 1640–10% FBS in the presence or absence of heparin (100 U/ml) and/or various doses of LDL. Prior to infection in some experiments, chlamydiae were incubated in RPMI medium for 1 h either in a 100°C water bath or at 37°C with 0.25 M sodium periodate in 0.25 M sodium acetate buffer (pH

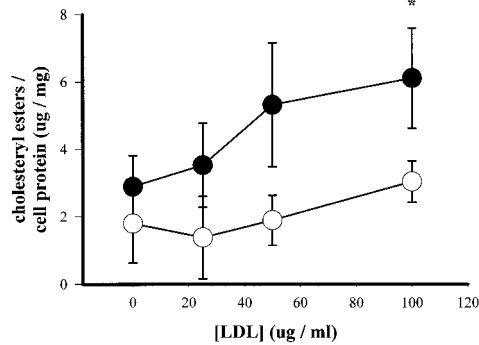


FIG. 2. Cholesteryl ester levels in uninfected (open circles) and *C. pneumoniae*-infected (solid circles) macrophages. Cells were infected (MOI = 2) or not infected for 2 h, washed twice, and incubated with various doses of LDL for 24 h. Cholesteryl ester levels were normalized to protein content. Data are representative of three experiments and are reported as means of triplicates with SD. \*, statistically significant difference compared with infected samples cultured without exogenous LDL,  $P < 0.05$  ( $t$  test).

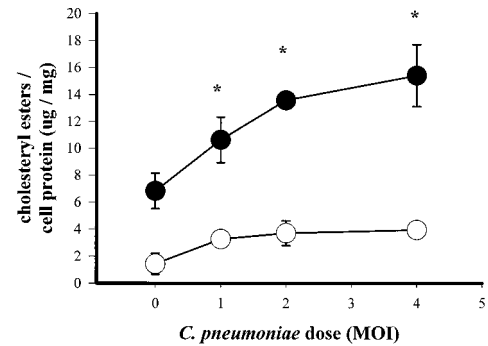


FIG. 3. Cholesteryl ester levels in heparin-treated (open circles) and untreated (solid circles) macrophages. Cells were infected with *C. pneumoniae* at various MOIs for 2 h, washed twice, and incubated with 100  $\mu$ g of LDL per ml for 24 h. Cholesteryl ester levels were normalized to protein content. Data are representative of three experiments and are reported as means of triplicates with SD. \*, statistically significant difference compared with uninfected, non-heparin-treated control,  $P < 0.05$  ( $t$  test).

5.5). In other experiments, macrophages were cultured with LDL in the presence or absence of cLPS and/or lipid X. All buffers were brought to 37°C before addition to macrophages. Trypan blue staining showed that none of the treatments affected macrophage viability at 24 h (>95% of macrophages were viable).

**Quantitation of foam cell formation.** Macrophages were washed twice with PBS, fixed for 15 min in 2% paraformaldehyde, and stained for 15 min in 1% oil red O (in 60% isopropanol). Cells then were washed three times in PBS and examined by light ( $\times 200$ ) or dark-field ( $\times 400$ ) microscopy (Diaphot 200 or Optiphot microscope; Nikon, Garden City, N.Y.). Cholesteryl ester-laden macrophages were scored by previously established criteria (17, 42).

**Quantitation of macrophage cholesteryl ester content.** Macrophage cholesteryl ester content was quantitated by a modification of the method of Gamble et al. (11). Briefly, macrophages were fixed in 0.5% paraformaldehyde, washed three times with PBS, and incubated in 200  $\mu$ l (24-well trays) of absolute ethanol for 30 min at 4°C to extract cellular lipids. Cholesterol content was determined by incubating 20  $\mu$ l of ethanol-extracted lipids with 180  $\mu$ l of assay solution (total cholesterol) (11) or 180  $\mu$ l of assay solution lacking cholesterol esterase (free cholesterol) for 1 h at 37°C and then measuring fluorescence (HTS-7000 microplate fluorometer; 340-nm excitation, 405-nm emission; Perkin-Elmer, Foster City, Calif.). Total and free cholesterol contents were calculated by using cholesteryl oleate and cholesterol as standards. Cholesteryl ester content was calculated by subtracting free cholesterol from total cholesterol for each sample and is reported as mean  $\pm$  standard deviation (SD). Lipid-extracted cells were dissolved in 0.1% sodium dodecyl sulfate–0.1 M NaOH for 30 min, and total cell protein was determined by the use of a Lowry protein assay kit (DC Protein Assay; Bio-Rad).

## RESULTS

***C. pneumoniae* induces foam cell formation and cholesteryl ester accumulation by RAW macrophages.** RAW macrophages exposed to *C. pneumoniae* accumulated cytoplasmic cholesteryl ester droplets in the presence of exogenous LDL (Fig. 1) in a manner similar to that previously observed for human macrophages (20). *C. pneumoniae*-infected macrophages also accumulated increasing levels of cholesteryl esters when cultured with increasing concentrations of LDL (Fig. 2). Furthermore, infection of macrophages with increasing doses of *C. pneumoniae* resulted in higher levels of cholesteryl ester formation (Fig. 3). As with human macrophages, RAW macrophages did not accumulate cholesteryl esters when cultured in the presence of heparin (Fig. 3), which binds LDL and prevents its association with a native LDL receptor (13). Heparin also inhibited baseline accumulation of cholesteryl esters by uninfected macrophages cultured with exogenous LDL (Fig. 3). In contrast, *C. pneumoniae*-induced foam cell formation was not inhibited when cells were cultured with fucoidan, which inhibits modified LDL uptake by class A scavenger re-

ceptors (14), or in the presence of the antioxidant butylated hydroxytoluene (not shown).

**Exposure of *C. pneumoniae* to periodate but not elevated temperatures inhibits macrophage foam cell formation.** To characterize the *C. pneumoniae* components involved in inducing macrophage foam cell formation, chlamydiae were exposed to elevated temperatures or periodate before being added to macrophages. Figure 4 shows that incubation of *C. pneumoniae* for 1 h at 100°C followed by cooling to 37°C did not inhibit the organism's capacity to induce foam cell formation, although exposure of macrophages to only medium treated in a similar manner did cause ~18% of macrophages to accumulate neutral lipids. However, *C. pneumoniae* that was treated for 1 h with 25 mM sodium periodate, which has been shown to oxidize antigenic carbohydrate residues on cLPS (3, 5), exhibited significantly reduced capacity for foam cell formation (Fig. 4). In a separate experiment designed to test if any *Chlamydia*-secreted products caused induce foam cell formation, chlamydiae were centrifuged and supernatants were added to macro-

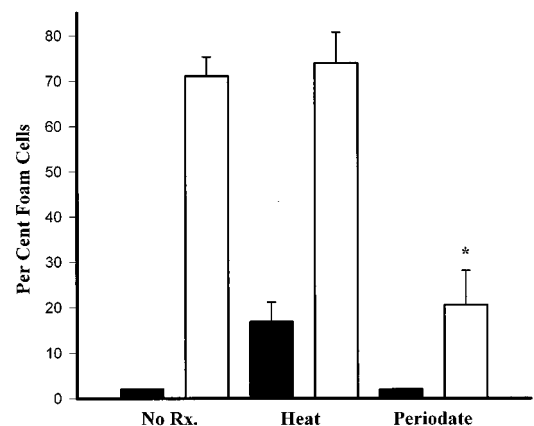


FIG. 4. Foam cell formation by uninfected (solid bars) and *C. pneumoniae*-infected (open bars) macrophages. Chlamydiae (MOI = 2) were incubated in RPMI medium for 1 h at 4°C (No Rx.), at 100°C (Heat), or with 0.25 M sodium periodate at 37°C (Periodate) before infection, cultured in the presence of 100  $\mu$ g of LDL per ml, and stained with oil red O. About 500 cells were counted from each well. Data are representative of three experiments and are reported as means of triplicates with SD. \*, statistically significant difference compared with untreated, infected and heat-treated, infected samples,  $P < 0.001$ .

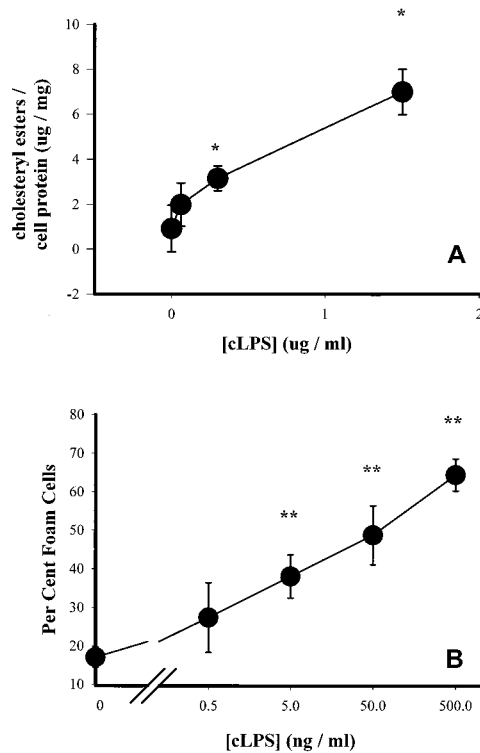


FIG. 5. Cholesteryl ester levels in (A) and foam cell formation by (B) cLPS-treated macrophages. Cells were exposed to various concentrations of cLPS for 1 h and cultured in the presence of 100  $\mu$ g of LDL per ml for 24 h. Data are representative of three experiments and are reported as means of triplicates with SD. \*, statistically significant difference compared with untreated samples,  $P < 0.05$ ; \*\*, statistically significant difference compared with untreated samples,  $P < 0.005$ .

phages in the presence of LDL; *Chlamydia* supernatants failed to induce macrophage foam cell formation (<10% oil red O-positive cells).

**Chlamydial LPS is sufficient to induce macrophage foam cell formation and cholesteryl ester accumulation.** To test whether purified cLPS could induce foam cell formation, macrophages were exposed to cLPS at various doses and incubated in the presence of LDL. The results indicate that cLPS was sufficient to induce cholesteryl ester accumulation (Fig. 5A) and foam cell formation (Fig. 5B).

**Lipid X inhibits *C. pneumoniae*- and cLPS-induced foam cell formation.** To determine whether a specific LPS antagonist could inhibit *C. pneumoniae*-induced macrophage lipid uptake, infected and uninfected macrophages were cultured in the presence of various concentrations of lipid X, a monosaccharide precursor of the lipid A component of LPS (35). Figure 6A shows that lipid X inhibited foam cell formation by *C. pneumoniae*-infected macrophages in a dose-dependent manner and also inhibited cLPS-induced foam cell formation (Fig. 6B).

## DISCUSSION

Recent appreciation of atherosclerosis as an inflammatory disease (1, 12, 28, 29, 38) has renewed interest in possible infectious etiologies of atherogenesis. Atherosclerosis and chlamydial diseases share some interesting similarities, including the fact that they are chronic, subclinical, and inflammatory in nature (39, 47), with macrophage activation playing a key

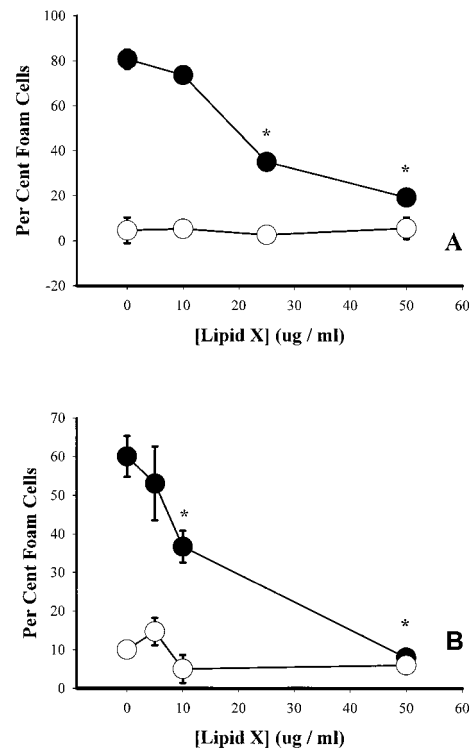


FIG. 6. Effect of lipid X on foam cell formation by *C. pneumoniae*-infected (A) and cLPS-exposed (B) macrophages. (A) Uninfected (open circles) or infected (MOI = 2; closed circles) cells were washed and incubated with 100  $\mu$ g of LDL per ml for 20 h in the presence or absence of various concentrations of lipid X. (B) Untreated (open circles) or cLPS-treated (100 ng/ml; closed circles) cells were incubated with 100  $\mu$ g of LDL per ml for 20 h in the presence or absence of various concentrations of lipid X. Macrophages were fixed and stained with oil red O, and about 500 cells were counted from each well. Data are representative of three experiments and are reported as means of triplicates with SD. \*, statistically significant difference compared with untreated samples,  $P < 0.005$ .

role in the inflammatory process (40). Formation of macrophage foam cells in the arterial intima is the hallmark of early lesions in atherosclerosis (39), and the pivotal step in foam cell formation is the uptake of excess cholesterol from LDL (4). Recently, *C. pneumoniae* was shown to induce macrophage foam cell formation in the presence of exogenous LDL (20), suggesting a causal role for the organism in atherogenesis. The present work further characterizes *C. pneumoniae*-induced foam cell formation by determining the *C. pneumoniae* component responsible for macrophage lipid accumulation. Chlamydiae incubated for 1 h at 100°C retained the capacity to induce foam cell formation, suggesting that the *C. pneumoniae* component that induced lipid accumulation was nonproteinaceous and heat stable (Fig. 4). In contrast, chlamydiae treated with periodate, which oxidizes antigenic carbohydrate residues on cLPS, had decreased capacity to induce macrophage foam cell formation (Fig. 4), suggesting that the *C. pneumoniae* heat-stable component causing macrophage lipid accumulation was cLPS. Indeed, cLPS extracted from chlamydiae was sufficient to induce macrophage foam cell formation and cholesteryl ester accumulation (Fig. 5), and *C. pneumoniae*- or cLPS-induced lipid uptake was inhibited by the LPS antagonist lipid X (Fig. 6). These data indicate that cLPS is a *C. pneumoniae* component that induces macrophage foam cell formation and suggest that inhibiting the activity of cLPS in vivo with monoclonal antibodies or LPS analogues may be sufficient to

reduce *C. pneumoniae*-induced foam cell formation and atheroma development.

Persistence of cLPS in the atheroma, either within intact *C. pneumoniae*-infected cells or in the exogenous subendothelial milieu following cell lysis, may promote atherosclerosis by continual macrophage activation and foam cell formation. Indeed, circulating cLPS-specific immune complexes, which may result from cLPS shedding from the atheroma, have been detected in patients with coronary heart disease (27). Compared to enterobacterial LPS, cLPS is a weak inducer of tumor necrosis factor alpha secretion (18), and the decreased capacity of cLPS to induce an acute inflammatory response may be necessary for chlamydial survival and establishment of chronic infection (2). In contrast, cLPS and enterobacterial LPS may be equally effective in inducing other macrophage activities that are not central to bacterial clearance, such as lipid uptake and foam cell formation. LPSs extracted from *E. coli* and *Salmonella typhimurium* have been shown to induce macrophage lipid accumulation when cultured in the presence of native LDL (9, 33), but the relative contribution to foam cell formation of endotoxins isolated from various bacteria is not known. Although endotoxin-mediated foam cell formation is not restricted to cLPS, it is improbable that other gram-negative organisms can invade and survive within the arterial intima to provide a continual source of antigen necessary to induce chronic inflammation and foam cell formation; unlike other gram-negative bacteria, *C. pneumoniae* has been detected within and isolated from atheromas (7, 25, 36), and it can survive and multiply within all cell types found in the atheroma (12). However, gram-negative septicemia may provide sporadic infiltration of enterobacterial LPS into the arterial intima (26), and therefore a possible role for enterobacterial LPS in foam cell formation in vivo cannot be discounted. Studies are under way to compare the effects of enterobacterial LPS versus cLPS and *C. pneumoniae* on foam cell formation in vivo in a murine model of atherosclerosis.

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