Foam Cell Formation Inhibits Growth of Chlamydia pneumoniae but Does Not Attenuate Chlamydia pneumoniae–Induced Secretion of Proinflammatory Cytokines

Erwin Blessing, MD; Cho-chou Kuo, MD, PhD; Tsun-Mei Lin, MD; Lee Ann Campbell, PhD; Florian Bea, MD; Brian Chesebro; Michael E. Rosenfeld, PhD

Background—It has not yet been determined whether lipid-loaded macrophages (foam cells), a major cellular component of atherosclerotic lesions, have the capacity to support growth of Chlamydia pneumoniae and be activated to secrete proinflammatory cytokines in response to C pneumoniae infection.

Methods and Results—Lipid loading of RAW 264.7 cells and mouse peritoneal macrophages with either oxidized or acetylated LDL significantly inhibits the growth of C pneumoniae. Modified forms of LDL are not directly toxic to C pneumoniae and do not inhibit either the initial binding or internalization of C pneumoniae by macrophages. Lipid loading does not reduce infection of macrophages with Chlamydia trachomatis. Treatment of lipid-loaded macrophages with live, heat-killed, or UV-inactivated C pneumoniae stimulates secretion of cytokines. C pneumoniae also induces expression of the mRNA for tumor necrosis factor-α in foam cells despite inhibition of nuclear factor-κB binding to DNA by prior treatment with oxidized LDL.

Conclusions—Foam cell formation is not conducive to growth of C pneumoniae but does not inhibit the C pneumoniae–induced secretion of proinflammatory cytokines. (Circulation. 2002;105:1976-1982.)

Key Words: atherosclerosis ■ lipids ■ leukocytes ■ inflammation ■ infection

An association between Chlamydia pneumoniae infection and cardiovascular disease has been demonstrated by epidemiological studies and by detection of the organism in atherosclerotic lesions. It is currently unclear whether C pneumoniae infection contributes directly to the development of atherosclerotic lesions. Several recent studies have shown that C pneumoniae infection accelerates the development of fatty streaks in hyperlipidemic animal models, although this is still controversial. These studies suggest that C pneumoniae infection can contribute to the atherogenic process, but the underlying mechanisms remain unknown.

Because lipid-loaded macrophages (foam cells) are a major cellular component of atherosclerotic lesions, chronic infection of foam cells with C pneumoniae could exacerbate the inflammatory response that is associated with the initiation and progression of atherosclerotic lesions. This hypothesis is supported by recent studies demonstrating that C pneumoniae infection stimulates lipid accumulation and lipoprotein oxidation and induces expression of proinflammatory cytokines by macrophages in vitro. However, it is currently unknown whether established foam cells can be infected with C pneumoniae and whether foam cells respond to C pneumoniae infection comparably to non–lipid-loaded macrophages. This question has important implications because inflammatory activation and death of macrophages contribute to the progression of atherosclerotic lesions and formation of unstable plaques. Therefore, the objectives of this study were to determine whether established foam cells retain the capacity to support chronic C pneumoniae infection and whether foam cells can still be activated by C pneumoniae to express and secrete cytokines and chemokines.

Methods

Preparation of Reagents
C pneumoniae (strain AR-39) and Chlamydia trachomatis (strain E/UW-5/Cx) were grown in HL cells and HeLa 229 cells, respectively, and purified by density gradient centrifugation (Hypaque-76; Winthrop-Breon Laboratories, New York, NY). The purified organisms were resuspended in sucrose phosphate glutamic acid (SPG) and frozen at −70°C until use. For attachment studies, chlamydial...
organisms were metabolically labeled with $^{35}$S-methionine\textsuperscript{1,3} and purified as described above.\textsuperscript{12} LDL was isolated from plasma of normal human volunteers by preparative ultracentrifugation as described previously.\textsuperscript{14} Oxidized LDL was generated by incubation of LDL (300 \(\mu\)g/mL) in the presence of 5 \(\mu\)mol/L copper sulfate for 18 hours at 37°C. Acetylated LDL was made by adding acetic anhydride to LDL at a ratio of 1.5 mL per milligram of LDL protein over a period of 1 hour followed by dialysis in normal saline containing 1.0 \(\mu\)mol/L EDTA for 8 hours (Spectra/Por, Spectrum Laboratories).

RAW 264.7 mouse macrophages (American Type Culture Collection) were seeded at \(5 \times 10^5\) cells per well in 24-well culture plates containing glass coverslips and preincubated for 48 hours in DMEM (GIBCO-BRL) containing 10% FBS, 100 \(\mu\)g/mL vancomycin/streptomycin, and native, oxidized, or acetylated LDL (30 \(\mu\)g protein/mL). Cells were inoculated with \textit{C pneumoniae} at a multiplicity of infection (MOI) of 10. Inoculated cells were absorbed at 37°C for 2 hours on a rocker platform (Bellco Biotechnology) at 3 amplitudes per minute. For sham infection of cells, only SPG was used. Primary macrophages were obtained through peritoneal lavage with ice-cold PBS from C57BL/6J, LDL receptor--deficient (LDLR\textsuperscript{−/−}), or type A1 scavenger receptor--deficient (SR\textsuperscript{−/−}) mice on a C57BL/6J background and preincubated for 24 hours with native or modified forms of LDL before infection. In some experiments, cells were infected with \textit{C trachomatis}, with the same conditions and titer used as with \textit{C pneumoniae}.

**Infectivity, Toxicity, and Binding Assays**

The number of infected cells was determined after direct immunofluorescent staining of inclusions after 3 days of culture. Inclusions were stained with a fluorescein isothiocyanate conjugated, \textit{Chlamydia} genus--specific monoclonal antibody (CF-2) that recognizes chlamydial lipopolysaccharide\textsuperscript{(4)} (Figure 1). Chlamydial inclusions in macrophages are very small and difficult to count accurately. Thus, the percentage of cells containing inclusions per coverslip was used to evaluate the infectivity of the organism rather than a total count of the number of inclusions per cell. Three coverslips were counted for each treatment and were done in a blinded fashion. To further determine the viability and growth of \textit{C pneumoniae} in foam cells, cells were harvested 3 days after infection and sonicated, and the infectivity titers were assayed in HL cells. The toxicity of oxidized LDL to \textit{C pneumoniae} was tested by incubating purified \textit{C pneumoniae} with increasing concentrations of either native or oxidized LDL at 37°C for 1 hour. The subsequent viability of the oxidized LDL-treated \textit{C pneumoniae} was determined on the basis of the capacity to grow in HL cells.

To assess \textit{C pneumoniae} binding and internalization, RAW cells were seeded overnight on coverslips, incubated for 48 hours with native or oxidized LDL, and subsequently incubated with \textsuperscript{35}S-labeled \textit{C pneumoniae} at \(20\) organisms per cell. A higher MOI was used as compared with the infectivity assay (MOI 10) because of the low efficiency of metabolic labeling. Cells were incubated at either 37°C for 2 hours, kept at 4°C for 30 minutes and subsequently incubated at 37°C for 1 hour and assayed. Cell lysates were air-dried on glass fiber filter paper (2.4 cm, Ahlstrom), added to scintillation fluid (Formula-989, Biotechnology Systems), and counted (Packard Tri-carb scintillation spectrophotometer, Packard Instruments). The data are listed as counts per minute per microgram of cell protein and are the average of triplicate determinations.

**Measurement of Intracellular Cholesterol and Assays of Secreted Chemokines and Cytokines**

Lipids were extracted with 2:1 chloroform/methanol and solubilized with 1% Triton X-100/chloroform (vol/vol). The cholesterol concentrations were measured with a commercial colorimetric kit (Sigma Diagnostics), and values were normalized to cellular protein concentrations. Interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and monocyte chemotactic protein (MCP)-1 were measured in the macrophage-conditioned media with commercial ELISA kits (R&D Systems). The cytokine and chemokine contents in the media were normalized to cellular protein concentrations.

**Electrophoretic Mobility Shift Assay**

RAW cells were pretreated with oxidized LDL or native LDL for 48 hours (30 \(\mu\)g protein/mL) and harvested 3 hours after incubation with \textit{C pneumoniae} or SPG. Nuclear extracts were isolated by the method of Hoppe-Seyler et al.\textsuperscript{15} A double-stranded oligonucleotide (Promega, Madison, Wis) representing the consensus binding site for nuclear factor (NF)\textsuperscript{κB} was radiolabeled with \(\gamma\)-\(\text{P}\)-ATP by means of T-4 polynucleotide kinase for 20 minutes at room temperature. Five micrograms of nuclear proteins and labeled oligonucleotides were incubated together and loaded on a 4% nondenaturing acrylamide gel for separation from the unbound oligonucleotides according to the manufacturer’s specifications (Promega). Gels were analyzed by means of phosphorimaging (Cyclone, Packard Instruments).

**Reverse Transcriptase–Polymerase Chain Reaction for TNF-α mRNA in RAW Cells**

Total RNA was extracted from the RAW cells (RNasey, Qiagen), treated with RNase inhibitor (RNasin Ribonuclease Inhibitor, Promega), and reverse-transcribed at 42°C for 50 minutes with random hexamer primers (Promega). Each cDNA sample was then treated with Ribonuclease H (Promega) at 37°C for 20 minutes. Analysis by reverse transcriptase–polymerase chain reaction (RT-PCR) was ac-
completed through the use of a gene-specific relative RT-PCR kit (Ambion), using the mouse TNF-α primer set and an 18S ribosomal RNA (Primer:Competimer 1:9). An aliquot of each reaction was subjected to electrophoresis on 1.5% agarose gels. Bands were visualized by ethidium bromide staining. Quantification of the TNF-α and 18S bands was performed with the Quantity One gel quantification system (BioRad). Results were expressed as the ratio of intensity of the TNF-α to the 18S bands.

Statistical Analyses

Data were analyzed by 1-way ANOVA. Post hoc comparisons were made with least-squared difference tests. Differences between means were considered significant at a value of \( P < 0.05 \). Linear regression analysis was used to correlate the intracellular cholesterol content with the percentage of infected cells.

Results

Susceptibility of Lipid-Loaded Macrophages to Infection by \textit{C} \textit{pneumoniae}

The number of RAW cells infected with \textit{C} \textit{pneumoniae} was significantly reduced in cells pretreated with oxidized and acetylated LDL as compared with cells pretreated with native LDL or without lipid (Figure 2A). Oxidized LDL inhibited infection to a greater extent than did acetylated LDL. The degree of infection was inversely correlated with the intracellular cholesterol concentration of the lipid-loaded cells (Table 1 and Figure 2B; \( r = -0.86 \)).

To evaluate the viability and growth of \textit{C} \textit{pneumoniae} in the foam cells, infectivity was assayed in HL cells after sonication of the foam cells. As shown in Figure 3, there was a significant reduction in the number of HL cells that became infected. However, the capacity of the \textit{C} \textit{pneumoniae} that did grow in the foam cells to subsequently infect HL cells was not impaired, inasmuch as the burst size was equivalent (burst size = 104 and 106 in HL cells treated with \textit{C} \textit{pneumoniae} forms of LDL).

### Table 1. Intracellular Cholesterol Concentrations of Lipid-Loaded RAW Cells

<table>
<thead>
<tr>
<th>LDL Added, ( \mu g ) protein/mL</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular cholesterol, ng cholesterol/( \mu g ) protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>39.6±4.3</td>
<td>69.1±8.7</td>
<td>56.0±6.2</td>
<td>98.5±8.5</td>
</tr>
<tr>
<td>Acetylated LDL</td>
<td>23.1±2.7</td>
<td>27.9±1.3</td>
<td>37.0±6.5</td>
<td>42.6±0.4</td>
</tr>
<tr>
<td>Native LDL</td>
<td>9.6±1.3</td>
<td>8.9±0.4</td>
<td>9.5±1.2</td>
<td>8.9±1.6</td>
</tr>
</tbody>
</table>

Values are mean±SD of triplicate determinations. RAW cells were incubated for 48 hours with various concentrations of oxidized, acetylated, or native forms of LDL. Values were normalized to protein concentration.
isolated from an equal number of infected macrophages treated with oxidized and native LDL, respectively.

To determine whether the reduction in the number of infected foam cells was due to a direct toxic effect of the modified forms of LDL to Lipid loading of macrophages inhibits passage of C. pneumoniae infection to HL cells. HL cells were infected with C. pneumoniae re-isolated from an equivalent number of RAW cells pretreated for 48 hours with the indicated concentrations of oxidized LDL (Ox-LDL) or native LDL (N-LDL) and infected with C. pneumoniae for 3 days. Infection of HL cells was assessed by immunofluorescent staining of inclusions 3 days after infection. Data shown are the mean±SD of triplicate determinations (*P<0.01 compared with native LDL–treated RAW cells).

Figure 3. Lipid loading of macrophages inhibits passage of C. pneumoniae infection to HL cells. HL cells were infected with C. pneumoniae re-isolated from an equivalent number of RAW cells pretreated for 48 hours with the indicated concentrations of oxidized LDL (Ox-LDL) or native LDL (N-LDL) and infected with C. pneumoniae for 3 days. Infection of HL cells was assessed by immunofluorescent staining of inclusions 3 days after infection. Data shown are the mean±SD of triplicate determinations (*P<0.01 compared with native LDL–treated RAW cells).

To verify that the accumulation of lipids by type 1 scavenger receptors in part inhibits C. pneumoniae growth, the percentage of infected peritoneal macrophages obtained from type 1A scavenger receptor knockout mice and LDL receptor knockout mice were compared with macrophages obtained from wild-type C57BL/6 mice (Figure 4). The percentage of infected peritoneal macrophages isolated from the wild-type and LDLR/– mice was substantially reduced after lipid loading with oxidized LDL. In contrast, a significantly greater percentage of the cells isolated from the SR–/– mice contained C. pneumoniae inclusions after loading with oxidized LDL (P<0.05).

To determine whether the inhibition of C. pneumoniae infection of lipid-loaded macrophages was due to an inhibition of attachment or internalization of the organism, RAW cells were inoculated with 35S-labeled C. pneumoniae. No significant differences were observed between lipid-loaded and non-lipid-loaded cells in the rate of attachment (adherence at 4°C) and internalization (adherence at 4°C, followed by internalization of adherent organisms at 37°C) or in cells continuously incubated at 37°C (Figure 5).

To determine whether the growth of other chlamydial species in lipid-loaded macrophages is also suppressed, RAW cells loaded with oxidized LDL were infected with C. trachomatis. No significant differences in infectivity were observed. Prior lipid loading of primary peritoneal macrophages from C57BL/6J mice also did not reduce infectivity with C. trachomatis (data not shown).

**C. pneumoniae Induction of Cytokine Secretion by Lipid-Loaded Macrophages**

Despite the reduction in the percentage of lipid-loaded macrophages infected with C. pneumoniae, there was a significant increase in the secretion of the proinflammatory cytokines IL-1β, IL-6, TNF-α, and MCP-1 by the lipid-loaded RAW cells within the first 24 hours after infection (Table 2). Furthermore, the addition of heat-killed and UV-inactivated C. pneumoniae also stimulated the secretion of TNF-α (data not shown).

Inoculation of RAW cells with C. pneumoniae resulted in a >4-fold increase of NF-κB binding activity to DNA compared with sham-infected cells (Figure 6A). No reduction in NF-κB binding activity was observed after prior incubation with native LDL and inoculation with C. pneumoniae. However, there was an approximately 2.5-fold reduction in NF-κB binding activity in RAW cells exposed to oxidized LDL for 24 hours before inoculation (Figure 6A). Despite the fact that oxidized LDL inhibition of C. pneumoniae induced NF-κB binding to DNA, C. pneumoniae was still able to modestly stimulate the expression of TNF-α mRNA in the lipid-loaded macrophages (Figure 6B).

**Discussion**

At present, it is unknown how C. pneumoniae infection contributes to the atherogenic process and whether direct or indirect mechanisms are involved. One possible scenario involves the dissemination of C. pneumoniae from the lungs to the artery wall, followed by sustained, widespread, chronic infection of all of the cell types within atherosclerotic lesions.
A sustained infection probably would augment the lipid-induced inflammatory response, accelerate cell death, and contribute to the expansion of the necrotic core, thinning of the fibrous cap, and increase in plaque instability. Previous in vitro and in vivo studies have provided support for this hypothesis by demonstrating that \textit{C pneumoniae} can infect endothelial cells, smooth muscle cells, and macrophages.\textsuperscript{2–4,16,17} In addition, we have shown in passive transfer experiments in mice that macrophages can disseminate \textit{C pneumoniae} infection from the lungs to the aorta.\textsuperscript{18} However, the results of this study indicate that growth of \textit{C pneumoniae} in lipid-loaded macrophages is suppressed. These findings are consistent with the in vivo observations that very few cells are actually infected in atheromatous lesions and with reported difficulties in culturing \textit{C pneumoniae} from coronary artery specimens reflecting a low-grade rather than widespread infection of atheroma.\textsuperscript{3}

In contrast to our findings in macrophages, Knoebel et al\textsuperscript{19} have reported an increase in infectivity of oxidized LDL–treated smooth muscle cells with \textit{C pneumoniae}. One possible explanation for these contrasting observations is the smaller number of scavenger receptors expressed and the reduced capacity of smooth muscle cells to accumulate lipids.\textsuperscript{20} Furthermore, unlike the present investigations, cycloheximide, an inhibitor of eukaryotic cell protein synthesis, was used in the studies with smooth muscle cells and is known to enhance susceptibility of many cell lines to chlamydial infections.\textsuperscript{21}

The mechanism by which accumulation of modified forms of LDL reduces the number of \textit{C pneumoniae}–infected macrophages is not clear. Our data rule out a direct toxic

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Effects of oxidized LDL on binding and internalization of \textit{C pneumoniae} by macrophages. RAW cells were incubated for 48 hours with oxidized or native LDL (30 \(\mu\)g protein/mL) and subsequently infected with \textsuperscript{35}S–labeled \textit{C pneumoniae}. Absorptions were done at \(4^\circ\)C for 30 minutes \(\left(4^\circ\right.\), at \(4^\circ\)C followed by transfer to \(37^\circ\)C \(\left(4^\circ\right.\rightarrow\left.37^\circ\right.\), and at \(37^\circ\)C continuously for 2 hours.}
\end{figure}

\begin{table}
\centering
\caption{\textit{Chlamydia pneumoniae} Infection Stimulates Secretion of Cytokines and Chemokines by Foam Cells}
\begin{tabular}{lcccc}
\hline
\multicolumn{5}{c}{LDL Concentrations, \(\mu\)g/mL} \\
\hline
& Native & & Oxidized & \\
0 & 10 & 30 & 10 & 30 \\
\hline
Cytokine secretion, pg/\(\mu\)g protein & \\
TNF-\(\alpha\) & + & 4.47±0.54 & 4.76±0.02 & 4.98±4.79 & 4.53±0.06 & 4.19±0.31 \\
& − & 0.31±0.005 & 0.27±0.04 & 0.24±0.0004 & 0.26±0.02 & 0.47±0.01 \\
IL-1\(\beta\) & + & 0.12±0.02 & 0.08±0.17 & 0.07±0.002 & 0.16±0.005 & 0.06±0.006 \\
& − & <0.01 & <0.01 & <0.01 & <0.01 & <0.01 \\
IL-6 & + & 3.47±0.51 & 3.78±0.13 & 4.08±0.08 & 3.47±0.07 & 2.38±0.02 \\
& − & <0.01 & <0.01 & <0.01 & <0.01 & <0.01 \\
MCP-1 & + & 7.89±1.13 & 7.55±1.26 & 8.99±0.04 & 8.00±0.31 & 6.84±0.19 \\
& − & 2.12±0.16 & 1.99±0.05 & 1.84±0.15 & 2.66±0.08 & 4.59±0.05 \\
\hline
\end{tabular}
\end{table}

Values are mean±SD of duplicate measurements. + indicates with infection; −, without infection.

Conditioned media were obtained 1 day after inoculation. Values were normalized to protein concentrations in the corresponding cells.
effect of oxidized lipids on *C pneumoniae* as well as inhibition of the initial binding and internalization of the organisms. On the basis of the inverse correlation between the intracellular content of cholesterol and percentage of infected cells, we speculate that the intracellular milieu of foam cells is in some way less conducive for growth of *C pneumoniae*.

Resistance of lipid-loaded macrophages to *C pneumoniae* growth suggests that widespread chronic infection of atherosclerotic lesions is an unlikely explanation for how *C pneumoniae* contributes to this disease process. Previous in vitro studies have shown that treatment of a variety of different cell lines with *C pneumoniae* rapidly induces the release of various proinflammatory cytokines and chemokines. Therefore, another possibility is that *C pneumoniae* is released into the artery wall by macrophages infected in the lungs and that the initial binding of *C pneumoniae* to artery wall cells stimulates the release of proinflammatory factors without requiring a widespread sustained infection. This possibility is supported by the passive transfer experiments previously cited and by the fact that endothelial cells in atherosclerotic lesions express adhesion molecules that would target infected alveolar macrophages to the artery wall. It is also supported by our present data demonstrating that preincubation of macrophages with native or oxidized LDL does not prevent *C pneumoniae* from inducing a rapid cytokine release. The fact that cytokines were secreted within the first

**Figure 6.** Combined effects oxidized LDL and *C pneumoniae* infection on activation of NF-κB and expression of TNF-α by macrophages. A, NF-κB activation. Electrophoretic mobility shift assay with nuclear extracts obtained from RAW cells 2 hours after infection. Lane 1: HeLa cell positive control; lane 2: negative control; lanes 3, 4, 5: RAW cells infected with *C pneumoniae*; lanes 6, 7, 8: sham-infected cells. O indicates non-foam cells; n, native LDL-induced foam cells; and ox, oxidized LDL-induced foam cells. B, Expression of TNF-α. RAW cells were incubated for 48 hours with oxidized or native LDL (30 μg protein/mL) and subsequently infected with *C pneumoniae* (solid bar) or sham inoculated with *Chlamydia* transport medium (open bar). Results are expressed as the ratio of the intensity of the bands for TNF-α vs the ribosomal housekeeping gene 18S in duplicate samples.
24 hours after infection and that both UV-inactivated and heat-killed \textit{C. pneumoniae} also induce cytokine secretion indicates that \textit{C. pneumoniae}–induced activation of foam cells does not require completion of the \textit{C. pneumoniae} growth cycle and probably is mediated through chlamydial lipopolysaccharide or heat shock protein-60.\textsuperscript{23} Furthermore, despite the inhibition of NF-$\kappa$B binding to DNA by oxidized LDL,\textsuperscript{24} \textit{C. pneumoniae} is still capable of increasing the expression and release of cytokines in foam cells.

In summary, our present study demonstrates that lipid accumulation by macrophages is not conducive to the growth of \textit{C. pneumoniae}. Despite the resistance to infection, foam cells retain their capacity to secrete proinflammatory cytokines and chemokines in response to binding of \textit{C. pneumoniae}. This suggests that \textit{C. pneumoniae} can contribute to the atherogenic process without requiring a widespread, sustained infection.

**Acknowledgments**

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**References**

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