Oxidized LDL further enhances expression of adhesion molecules in *Chlamydophila pneumoniae*-infected endothelial cells

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Abstract ***Chlamydophila pneumoniae* is a common respiratory pathogen that has been shown to be associated with coronary artery disease. Recent studies have shown that one of the possible mechanisms of the atherogenicity of *C. pneumoniae* is overexpression of cell adhesion molecules (CAMs) in infected endothelial cells. We investigated whether exposure of *C. pneumoniae*-infected endothelial cells to oxidized LDL (oxLDL) leads to further upregulation of CAMs. Flow cytometry and immunoblot analysis of human aortic endothelial cells (HAECs) was performed for intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. ICAM-1 was expressed in 78.7% of *C. pneumoniae*-infected HAECs. The addition of oxLDL (100 μg/ml) to infected HAECs increased the proportion of ICAM-1-positive cells to 92%. VCAM-1 was only observed in 9.3% of infected HAECs, and the addition of oxLDL had no further effect on the surface expression of VCAM-1. *C. pneumoniae* also upregulated the surface expression of E-selectin on 52.2% of the cells, and incubation with oxLDL further increased the proportion of positive cells to 63.64%. In conclusion, *C. pneumoniae* upregulated the expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin on HAECs. The addition of oxLDL to the infected cells further enhanced the surface expression of ICAM-1 and E-selectin.**—Vielma, S. A., M. Mironova, J-R. Ku, and M. F. Lopes-Virella. **Oxidized LDL further enhances expression of adhesion molecules in *Chlamydophila pneumoniae*-infected endothelial cells. *J. Lipid Res.* 2004.** 45: **873–880.**

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During the past decade, several investigators have linked inflammation to atherogenesis and plaque disruption, and the concept that atherosclerosis is a chronic inflammatory process became well established (1, 2). Atherosclerotic plaques are known to contain not only lipids but also a heavy infiltrate of inflammatory cells (macrophages, T-lymphocytes) as well as smooth muscle cells and extracellular matrix (3, 4). The earliest step, however, in the sequence of events that leads to the development of the inflammatory reaction associated with atherosclerosis is endothelial dysfunction that starts with the upregulation of adhesion molecules (5, 6). That, in turn, promotes the attraction and adherence of monocytes and T lymphocytes to the endothelium, which is an essential first step in the formation of a perivascular inflammatory infiltrate (4, 6).

Of the multiple factors that contribute to the overexpression of adhesion molecules in the endothelium, modified lipoproteins, including oxidized LDL (oxLDL), have been the object of considerable attention. The incubation of macrophages with oxLDL results in the release of proinflammatory cytokines such as interleukin-1 and tumor necrosis factor (TNF), which are known to upregulate the expression of cell adhesion molecules (CAMs) (7–11). Another factor known to cause increased expression of cell adhesion molecules in the endothelium is *Chlamydophila pneumoniae* infection (12).

C. pneumoniae is one of the infectious agents most frequently associated with the presence of atherosclerosis and acute cardiovascular events. A role for *C. pneumoniae* in the pathogenesis of atherosclerosis was first suggested in 1988 by Saikku et al. (13), who demonstrated that patients with coronary artery disease were more likely to have serologic evidence of a previous *C. pneumoniae* infection than were matched controls. Several groups have demonstrated the presence of the organism in atherosclerotic plaques using various techniques, such as ELISA, PCR, electron microscopy, immunohistochemistry, and isolation in tissue culture (14–17). The presence of circulating *C. pneumoniae*-specific immune complexes in a high

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proportion of coronary heart disease patients suggests the persistence of a chronic infection (18, 19). However, none of these lines of evidence can be considered as proof of a causal relationship between *C. pneumoniae* and atherosclerosis.

The definition of mechanisms that could explain how *C. pneumoniae* might trigger or perpetuate perivascular inflammatory reactions is critical for establishing the pathogenic role of *C. pneumoniae*. The upregulation of CAMs in *C. pneumoniae*-infected endothelial cells has been proposed as one of the possible mechanisms (12) because it promotes the rolling and attachment of circulating leukocytes to endothelial cells, leading to the transmigration of monocytes into the subendothelial space (20–22). *C. pneumoniae* infection may also contribute to inflammation by enhancing oxidative stress and, as a consequence, oxidative modification of proteins, including LDL (23). Here, we investigate the possible synergism of *C. pneumoniae* infection and oxidized LDL on the expression of CAMs by endothelial cells.

MATERIALS AND METHODS

Cell cultures

Hep-2 cells [American Type Culture Collection (ATCC) CCL23] and human aortic endothelial cells (HAECs; Cascade Biologicals, Inc., Portland, OR) were cultured and maintained as previously described (24, 25).

C. pneumoniae propagation

C. pneumoniae AR-39 (ATCC 53592) was propagated in Hep-2 cell monolayers as previously described (25). After 72 h of incubation, *C. pneumoniae* was harvested by mechanical disruption followed by a low-speed (250 *g*) centrifugation. Elementary bodies present in the supernatant were pelleted at 30,000 *g* (JA-20 rotor; Beckman Instruments, Inc., Fullerton, NJ) for 30 min. The pellets were suspended in 0.01 M sodium phosphate (pH 7.2) containing 0.25 mol/l sucrose and 5 mmol/l L-glutamic acid (24, 26). Titration was performed in cycloheximide-treated Hep-2 cell monolayers grown on round coverslips (25). Infection of endothelial monolayers (2×10^5 cells) was attained by exposure of the cells to *C. pneumoniae* suspensions diluted in antibiotic-free Medium 200 (Cascade Biologicals) using infective concentrations ranging from 8×10^5 to 10×10^5 inclusion-forming units (IFUs). These infective concentrations did not cause significant cell death (as assessed by cell morphology, cell attachment, and trypan blue exclusion) and infected 30–40% of cells after 24 h, 60–70% of cells after 48 h, and 100% of cells at 72 h. At least one inclusion body was observed in every cell after 72 h of exposure to *C. pneumoniae*.

Because the elementary bodies of *C. pneumoniae* are isolated from Hep-2 cells, preparations of these cells were used as mock-infected cells, which consist of HAECs treated with crude preparations of noninfected Hep-2 cells processed in the same manner as infected Hep-2 cells. Experiments designed to differentiate the effects of active infection from those of surface binding were performed using killed *C. pneumoniae* elementary bodies obtained either by heating the organism at 95°C for 30 min or by ultraviolet (UV) irradiation for 30 min at 15 cm from the UV source [$1{,}200 \times 100$ mJ/cm^2 (UVC500; Hoefer, San Francisco, CA)].

Isolation of native LDL and preparation of oxLDL

Blood was collected from healthy volunteers to isolate LDL. Written informed consent as approved by the Review Board for Human Research of the Medical University of South Carolina was signed by all participants. Native LDL ($1.019 < d < 1.063$ g/ml) was isolated from the plasma pools by sequential ultracentrifugation at 60,000 rpm for 24 h at 10°C on a Beckman L-80 ultracentrifuge using a type 60-Ti rotor (Beckman, Palo Alto, CA). Afterward, native LDL was passed through a Sephadex G-25 column (Pharmacia Biotech, Piscataway, NJ) to remove EDTA and then incubated at 37°C in the presence of 40 mM $CuCl_2$. Oxidation of LDL was continuously monitored by measuring the formation of fluorescent compounds using a luminescence spectrometer at 360/430 excitation/emission (Bowman® series 2; Aminco, Rochester, NY) for 12–14 h as described (27). The freshly oxidized LDL was dialyzed against a solution containing 0.16 M NaCl and 300 mM EDTA and sterilized by passage through a 0.45 mm filter. The protein content of native LDL and oxLDL preparations was measured by the Lowry assay (28).

Incubation of HAECs with native and copper-oxidized LDL

To evaluate the effect of different types of modified LDL on intracellular adhesion molecule-1 (ICAM-1) expression by endothelial cells infected with *C. pneumoniae*, confluent HAECs (2×10^5 cells) were infected with 8×10^5 to 10×10^5 IFUs of *C. pneumoniae* elementary bodies at 37°C for 2 h on a rocker platform. After infection, native LDL or oxLDL was added to infected HAECs and noninfected controls. The cells were incubated in the presence of modified LDL for the times and concentrations indicated in the figures. In experiments in which HAECs were incubated with the lipoproteins for more than 48 h, the medium was removed at day 3 and 5 and substituted by fresh medium containing the same concentration of lipoproteins. In some experiments, HAECs were incubated with oxLDL for 72 hr before being infected with *C. pneumoniae*, and the cells were further incubated for 24 h in the presence of oxLDL.

Immunoblot analysis of ICAM-1

Total cell extracts were prepared using a Triton X buffer [10 mM HEPES, 200 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 1.5% (v/v) Triton X] containing 100 μg/ml PMSF (Sigma-Aldrich Co., St. Louis, MO) and 10 μg/ml leupeptin (Sigma-Aldrich Co.). Cell lysates were centrifuged and the protein concentration in the supernatant determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Western blot analysis was performed as described (29) using an anti-human ICAM-1 monoclonal antibody diluted at 1:500 (Calbiochem-Novabiochem, La Jolla, CA). A horseradish peroxidase-conjugated anti-mouse antibody (Calbiochem-Novabiochem) was used as a secondary antibody. ICAM-1 was visualized after incubation of the membranes in Chemiluminescence Reagent Plus (Perkin Elmer Life Science, Inc., Boston, MA) followed by exposure to X-ray film. The films were scanned (Foto/Analyst® Investigator; Fotodyne, Hartland, WI) (30), and densitometric analysis (optical density measurements) was performed on a Macintosh computer using the public domain NIH Image program developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/. The results are expressed as percentages of the optical density relative to baseline levels found in control HAECs.

Flow cytometry for detection of surface expression of CAMs

Flow cytometry analysis of adhesion molecules was performed as previously described (25). Confluent HAECs (1.5×10^6) were

infected for 4 h (for E-selectin determination) or 24 h [for ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) determination] with *C. pneumoniae* (15×10^6 IFUs). After incubation, $\sim 2 \times 10^5$ cells per experiment were incubated with a 1:100 dilution of ICAM-1 or VCAM-1 antibodies (phyco-erythrin-labeled; BD PharMingen, San Diego, CA) or E-selectin antibody solution (FITC-labeled; R&D System, Minneapolis, MA) for 25 min on ice. After one wash with fatty acyl-CoA (FACS) solution, a second wash was performed adding 10% (w/v) propidium iodide. Cells were resuspended in 300 μl of FACS solution and immediately processed using a FACS Calibur (Becton Dickinson, San Jose, CA), and the fluorescence emission profile was analyzed by the CellQuest program (Becton Dickinson).

Northern blot analysis

Human ICAM-1 cDNA and VCAM-1 cDNA were provided by Dr. Greve Jeffrey (Bayer Pharmaceutical Division, Berkeley, CA). E-selectin cDNA was provided by Dr. Vivian de Waard (Department of Biochemistry, Academic Medical Center, Amsterdam, The Netherlands). The cDNA probes were obtained after PCR (30 cycles at 95°C, 60°C, and 72°C) using a Qiagen Taq DNA Polymerase Kit (Qiagen, Valencia, CA). Primers for human ICAM-1 (5′-GAG ATC ACC ATG GAG CCA AT-3′ and 5′-GGG CCT CAC ACT TCA CTG TC-3′), VCAM-1 (5′-CAT CCA CAA AGC TGC AAG AA-3′ and 5′-CCT GGAT TCC CTT TTC CAG T-3′), and E-selectin (5′-TGA ACT GTA CAG CCC TGG AA-3′ and 5′-AAC TGG GAT TTG CTG TGT CC-3′) were used (Sigma-Genosys, The Woodlands, TX). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was purchased from the ATCC and radiolabeled as described below. Total cellular RNA was isolated from HAECs using an RNeasy Mini Kit (Qiagen) according to the instructions from the manufacturer. Purification and quantification of RNA were assessed by absorption at 260 and 280 nm, and an aliquot of RNA (10 μg) from samples with an absorbance ratio >1.6 was fractionated using a 1.2% agarose formaldehyde gel. Northern blotting of ICAM-1, VCAM-1, E-selectin, and GAPDH mRNA was performed using the Prime-it® Rmt Random Primer Labeling Kit according to the instructions provided by the manufacturer (Stratagene, La Jolla, CA) and as previously described (30).

Data analysis

All data are expressed as means of duplicate samples from three to five independently performed experiments. Data are presented as means ± SD of the percentage of optical density obtained from noninfected HAECs. Comparison between treatments was performed by ANOVA, and the degree of statistical significance was determined by either Mann-Whitney or Tukey-Kramer test. A value of $P < 0.05$ was considered significant.

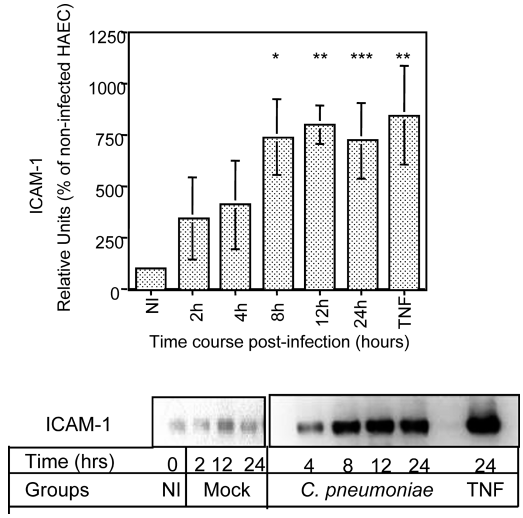

Fig. 1. Western blot analysis of the expression of intracellular adhesion molecule-1 (ICAM-1) in human aortic endothelial cells (HAECs) infected with *C. pneumoniae*. Confluent HAECs (2×10^5 cells) were infected with 8×10^5 inclusion-forming units (IFUs) of *C. pneumoniae* for 2 h. Infected cells were washed with HBSS and then incubated for 2, 4, 8, 12, and 24 h. Noninfected (NI), mock-treated, and tumor necrosis factor (TNF)-treated HAECs were used as controls. After incubation, cells were lysed, and 25 μg of protein was electrophoresed by 10% SDS-PAGE. Immunoblotting was performed using an antibody against human ICAM-1 as described in Materials and Methods. Immunoreactive bands were visualized by incubating the membranes with chemiluminescence reagents. The data represent the summary of five different experiments. ICAM-1 levels in noninfected cells were considered as 100%. Levels in *C. pneumoniae*-infected HAECs are expressed as percentages of the levels in noninfected cells (means ± SD). * $P = 0.017$. ** $P = 0.0012$. *** $P = 0.0104$.

RESULTS

Expression of ICAM-1 in *C. pneumoniae*-infected HAECs

Infection of HAECs by *C. pneumoniae* led to a time-dependent increase in the expression of ICAM-1. Relative to the ICAM-1 levels detected in noninfected HAECs (considered as 100%), increased expression of ICAM-1 was observed as early as 2 h after infection (339 ± 199%) and peaked at 12 h after infection (795 ± 93%, $P = 0.0012$ relative to baseline levels). At 24 h of infection, ICAM-1 levels remained markedly increased (720 ± 184%, $P = 0.0104$ relative to baseline levels) (**Fig. 1**).

The addition of rifampicin, an inhibitor of bacterial DNA-dependent RNA polymerase activity, to HAECs ei-

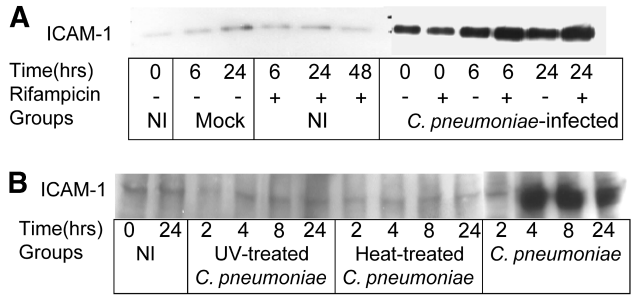

Fig. 2. Effect of rifampicin on the upregulation of ICAM-1 expression by HAECs. A: At left, control HAECs were incubated with rifampicin (10 μg/ml) for 6, 24, and 48 h. Noninfected (NI) and mock-treated cells were used as controls. At right, confluent HAECs (2×10^5 cells) were infected with viable *C. pneumoniae* (8×10^5 IFUs), and rifampicin (10 μg/ml) was added to the medium at 0, 6, and 24 h after infection. B: HAECs were infected with live *C. pneumoniae* or with either heat-treated or ultraviolet (UV)-treated elementary bodies for the periods indicated. Noninfected and mock-treated HAECs were used as controls. Twenty-five micrograms of protein was electrophoresed on 10% SDS-polyacrylamide gels, and the transferred blots were incubated with anti-human ICAM-1 monoclonal antibody. Western blots are representative of two experiments performed in duplicate.

ther at the time of infection or at two different times after infection (6 and 24 h) did not abrogate the *C. pneumoniae* induction of ICAM-1 expression in HAECs (**Fig. 2A**). In contrast, heat and UV treatment of *C. pneumoniae* elementary bodies completely abolished the upregulation of ICAM-1 (Fig. 2B).

Effect of native LDL and oxLDL on the expression of ICAM-1 by *C. pneumoniae*-infected HAECs

The results of the immunoblot experiments investigating the effect of native LDL and oxLDL on the expression of ICAM-1 by *C. pneumoniae*-infected HAECs are summarized in **Fig. 3**. Low levels of expression of ICAM-1 were observed in resting, noninfected HAECs. The expression of ICAM-1 by *C. pneumoniae*-infected HAECs was increased to 900% above the baseline levels detected in noninfected HAECs. The levels of ICAM-1 in infected HAECs exposed to oxLDL were 1,163% above baseline ($P = 0.001$ compared with the levels in infected cells not exposed to oxLDL). The addition of native LDL to *C. pneumoniae*-infected HAECs did not result in a significant increase of ICAM-1 levels.

Because oxLDL further increases the expression of ICAM-1 in *C. pneumoniae*-infected cells, we decided to determine whether or not this increase was time dependent.

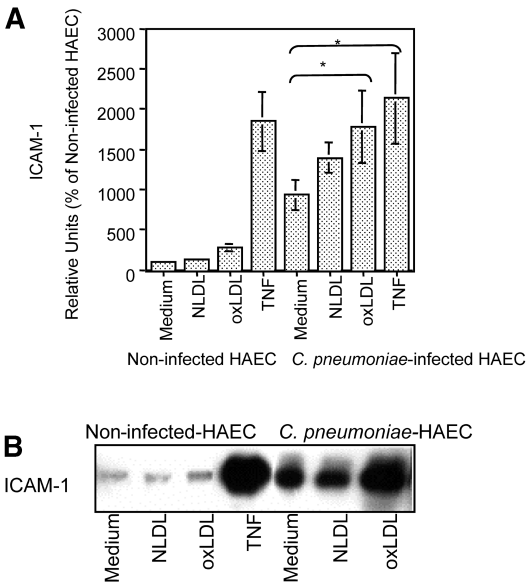

Fig. 3. Effect of modified LDL on the expression of ICAM-1 in *C. pneumoniae*-infected HAECs. Noninfected HAECs or HAECs infected with *C. pneumoniae* (8×10^5 IFU/ml) at 37°C and 5% CO_2 for 2 h were incubated for 20 h with medium alone, with 100 μg/ml of either native LDL (NLDL) or oxidized LDL (oxLDL), or with 25 U/ml TNF. After incubation, the cells were washed with PBS and lysed. Thirty micrograms of the cell lysates were electrophoresed on a 10% SDS-PAGE gel. Immunoblotting was performed with an antibody against ICAM-1 as described in Materials and Methods. Immunoreactive bands were visualized by incubating the membranes with chemiluminescence reagents. * $P = 0.0217$. B: Western blot representative of three separate experiments performed in duplicate (TNF in *C. pneumoniae*-infected cells not shown). Error bars, means ± SEM.

Figure 4A shows immunoblot analysis of ICAM-1 expression in resting, mock-treated, and oxLDL-treated noninfected HAECs. ICAM-1 expression was barely detectable in resting cells. In mock-treated cells, a slight upregulation of ICAM-1 was observed at both 6 and 24 h. Incubation of noninfected HAECs with 100 μg/ml oxLDL from 2 to 24 h induced a small increase in ICAM-1 expression compared with that in resting cells. However, as shown in Fig. 4B, a significantly higher level of ICAM-1 expression was noted when HAECs were infected for 4 h or more with *C. pneumoniae* (Fig. 4B, left). The highest level of expression was seen at 12 h after infection. Incubation of *C. pneumoniae*-infected cells with oxLDL led to even higher levels of expression, mainly at 12 and 24 h (Fig. 4B, right).

To investigate whether or not upregulation of ICAM-1 expression in *C. pneumoniae*-infected HAECs would be maintained for more than 24 h, longer time course experiments were performed. As shown in Fig. 4C, -infected HAECs show an increased expression of ICAM-1 up to 7 days after infection, although maximal levels were observed at 48 h. Similarly, enhancement of ICAM-1 expression was caused by exposure of the infected cells to oxLDL at all time points (Fig. 4C).

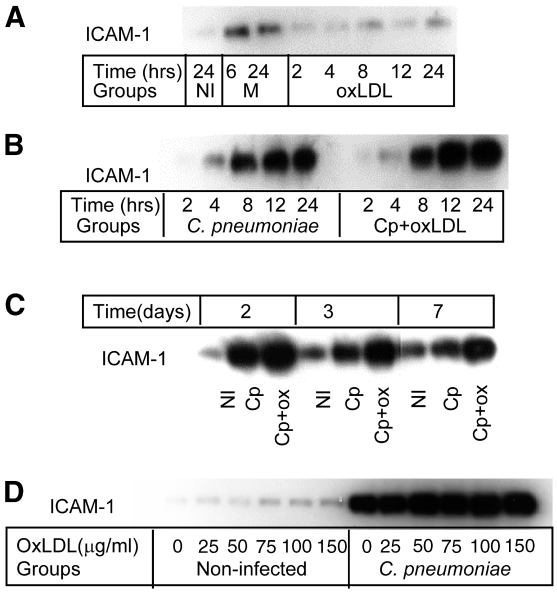

Fig. 4. Effect of oxLDL on the expression of ICAM-1 in *C. pneumoniae*-infected HAEC. A: Time course of ICAM-1 expression in control HAECs (NI), mock-treated cells (M), and noninfected cells stimulated with oxLDL. B: Time course of ICAM-1 expression on HAECs (2×10^5 cells) in the first 24 h of *C. pneumoniae* (Cp) infection (10×10^5 IFUs) incubated with oxLDL (left, 100 μg/ml). C: Long-term expression of ICAM-1 on control HAECs and *C. pneumoniae*-infected HAECs stimulated with 100 μg/ml oxLDL (Cp+ox). D: Dose-dependent expression of ICAM-1 on *C. pneumoniae*-infected HAECs stimulated with oxLDL. Noninfected-HAECs and *C. pneumoniae*-infected HAECs were incubated with 25, 50, 75, 100, or 150 μg/ml oxLDL for 20 h. Thirty micrograms of proteins of each cell extract was electrophoresed on a 10% SDS-polyacrylamide gel. Immunoblotting was performed with an antibody recognizing human ICAM-1 as described in Materials and Methods. The Western blot shown is representative of two separate experiments performed in duplicate.

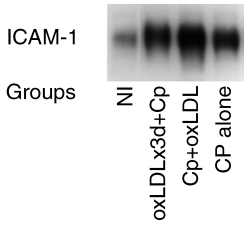

Fig. 5. Effect of pretreatment of HAECs with oxLDL before *C. pneumoniae* infection. Confluent HAEC monolayers (2×10^5 cells) were pretreated for 72 h with 100 μg/ml oxLDL followed by infection with *C. pneumoniae* (8×10^5 IFUs) for 24 h (oxLDLx3d+Cp) or infected with *C. pneumoniae* for 2 h and then incubated with oxLDL for 24 h (Cp+oxLDL). Noninfected (NI) and *C. pneumoniae*-infected HAECs were used as controls. Cell lysates were prepared, electrophoresed, and immunoblotted as described in Materials and Methods using an anti-human ICAM-1 antibody. Experiments were run in duplicate.

To determine whether the effect of oxLDL in the induction of ICAM-1 in *C. pneumoniae*-infected HAECs was concentration dependent, we treated infected and noninfected HAECs with increasing concentrations of oxLDL (25, 50, 75, 100, and 150 μg/ml). As observed in Fig. 4D, treatment of HAECs with oxLDL caused a dose-dependent upregulation of ICAM-1 expression in both infected and noninfected cells. The effect of oxLDL on ICAM-1 expression in *C. pneumoniae*-infected HAECs was greater than that observed in noninfected HAECs. In infected HAECs, treatment with 25 μg/ml oxLDL had a minimal effect on ICAM-1 expression, which was already enhanced as a consequence of infection. In contrast, concentrations of oxLDL equal to or greater than 50 μg/ml had a marked synergistic effect on the expression of ICAM-1 by *C. pneumoniae*-infected HAECs. Incubation of HAECs with 100 μg/ml oxLDL for 72 h before *C. pneumoniae* infection led to an upregulation of ICAM-1 expression (**Fig. 5**). The effect of oxLDL in upregulating ICAM-1 expression seems to be more marked in cells in which oxLDL was added after infection with *C. pneumoniae*.

Flow cytometry analysis of ICAM-1, VCAM-1, and E-selectin expression in *C. pneumoniae*-infected HAECs

Flow cytometry studies were performed to quantify the surface expression of ICAM-1 in *C. pneumoniae*-infected cells incubated in the presence or absence of oxLDL (**Fig. 6A**). Surface expression of ICAM-1 was observed in 51.2 ± 4.4% (n = 4) of noninfected endothelial cells, with an intensity of ICAM-1 per cell of 29.1 fluorescence units (geometric mean). A significant increase in the percentage of ICAM-1-positive cells was observed in *C. pneumoniae*-infected HAECs (78.7 ± 9.6%, $P < 0.01$ vs. noninfected cells, n = 4), with an increased density of ICAM-1 in the membrane (fluorescence intensity geometric mean of 184.7). Stimulation of infected HAECs with oxLDL further enhanced the proportion of ICAM-1-positive cells up to 92.06 ± 1.03% ($P < 0.0286$ vs. infected HAECs not exposed to oxLDL, n = 4) as well as the density of membrane ICAM-1 [in Fig. 6A (*y* axis), the geometric mean of fluorescence intensity (FL2-H) is represented as 226.5].

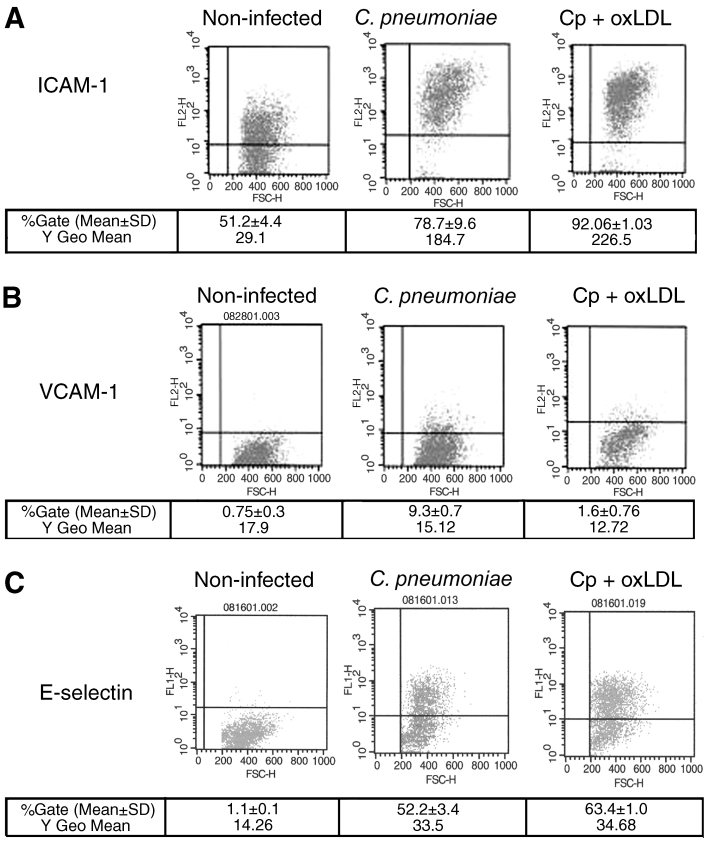

Fig. 6. Comparative flow cytometry analysis of the effect of oxLDL on ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin expression in *C. pneumoniae*-infected HAECs. Confluent HAECs (1.5×10^6 cells) were infected with *C. pneumoniae* (Cp; 10×10^6 IFUs) and incubated with medium alone or with oxLDL (100 μg/ml) for 18 h. Noninfected HAECs were used as controls. After incubation, cells were washed and detached. Cell suspensions were collected, and ~2×10^5 cells were incubated with Phyco-erythrin (PE)-labeled ICAM-1 (A), PE-labeled VCAM-1 (B), or FITC-labeled E-selectin (C) (1:100) antibodies or isotype control solutions. Cells were processed using a fatty acyl-CoA Calibur (Becton Dickinson). Results are representative of two separate flow cytometry analyses performed in duplicate for ICAM-1 or three separate flow cytometry analyses of VCAM-1 and E-selectin. Geo, geometric; % gate, percent of cells included in the defined gate; Y geometric, geometric mean of fluorescence intensity (FL2-H) in gated cells.

We also used flow cytometry analysis to measure VCAM-1 expression in *C. pneumoniae*-infected HAECs incubated with or without oxLDL (Fig. 6B). Our results showed that in contrast to ICAM-1, VCAM-1 was not constitutively expressed by HAECs. After HAEC infection by *C. pneumoniae*, only 9.3 ± 0.7% were positive for VCAM-1 expression after 24 h of infection (Fig. 6B), and VCAM-1 expression was no longer noted at 48 h after infection (data not shown). In contrast to ICAM-1, the addition of oxLDL led to a decrease in VCAM-1 surface expression (1.6 ± 0.7%) in *C. pneumoniae*-infected HAECs.

Finally, as shown in Fig. 6, in noninfected HAECs the baseline expression of E-selectin was very low (1.1 ± 0.15%). Stimulation of noninfected cells with oxLDL resulted in a significant increase in E-selectin expression relative to noninfected controls (7.72 ± 0.2%, $P < 0.05$, n = 3) (Fig. 6A). *C. pneumoniae* infection induced significant surface expression of E-selectin on 52.2 ± 3.4% of the cells ($P < 0.001$ relative to noninfected cells, n = 3) at 5 h of infection (**Fig. 7B**). In *C. pneumoniae*-infected HAECs, oxLDL (100 μg/ml) resulted in E-selectin expression in 63.64 ± 1.09% of the cells ($P < 0.001$ vs. *C. pneumoniae*-infected cells) (Fig. 6B).

Effect of oxLDL in the transcriptional activation of adhesion molecule mRNA in *C. pneumoniae*-infected HAECs

To further investigate whether the mechanism by which oxLDL-induced upregulation of adhesion molecules in infected cells occurred at the transcriptional level, ICAM-1, VCAM-1, and E-selectin mRNA levels in *C. pneumoniae*-infected cells incubated in the presence or absence of oxLDL (100 μg/ml) were assessed by Northern blot analysis. Confluent HAECs were infected with *C. pneumoniae* for 2 h and incubated with or without 100 μg/ml oxLDL at different time points after infection. As controls, we used noninfected cells, resting cells, and TNF-treated (25 U/ml) noninfected cells.

As observed in Fig. 7A, ICAM-1 mRNA levels were very low in noninfected control HAECs. Infection with *C. pneumoniae* alone induced a rapid increase in the level of ICAM-1 mRNA. The maximum increase (eight times the baseline level) was achieved within 2–4 h after the addition of *C. pneumoniae* to the endothelial cell monolayers, and the mRNA levels decreased to baseline levels after 8 h. The addition of 100 μg/ml oxLDL to infected cells had no discernible effect on ICAM-1 mRNA levels.

For VCAM-1 and E-selectin, Northern blot analysis showed a short-lived induction of mRNA transcripts by *C. pneumoniae* for both adhesion molecules. Maximum expression of VCAM-1 and E-selectin mRNA was observed at 2 h after infection, but it was markedly decreased at 4 h and not detectable at later time points. The addition of oxLDL to infected cells had no discernible effect on the level of VCAM-1 and E-selectin transcripts (Fig. 7A, B).

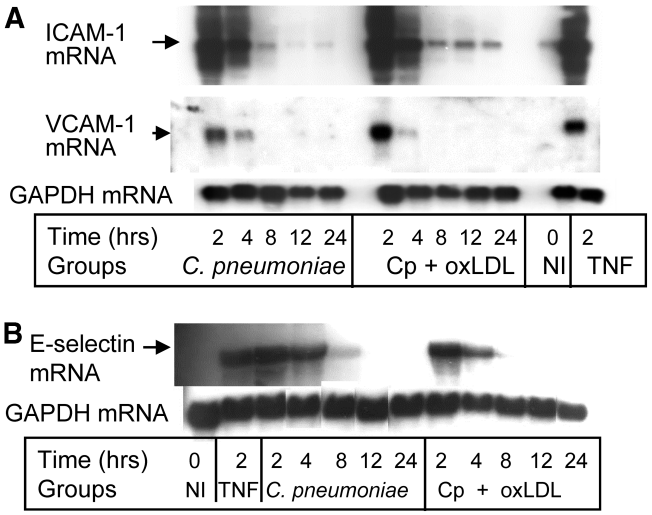

Fig. 7. Effect of oxLDL on the upregulation of ICAM-1, VCAM-1, and E-selectin mRNA in *C. pneumoniae*-infected HAECs. Confluent HAECs (1.5×10^6 cells) were infected with *C. pneumoniae* (10×10^6 IFUs) alone or *C. pneumoniae* (Cp; 3×10^6 IFUs/ml) and oxLDL (100 μg/ml) for 2, 4, 8, 12, and 24 h. Noninfected (NI) cells and HAECs treated with TNF (25 U/ml) were used as controls. Ten micrograms of RNA isolated from infected and noninfected cells was subjected to electrophoresis on a 1.2% agarose formaldehyde gel and transferred to a nylon membrane. The ICAM-1, VCAM-1, E-selectin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs immobilized on the membrane were hybridized with ^{32}P-labeled cDNAs as described in Materials and Methods. Data are representative of three separate experiments (n = 3).

DISCUSSION

Our results show that the expression of adhesion molecules in HAECs infected with *C. pneumoniae* AR39 is enhanced when the cells are incubated in medium containing oxLDL. The effect of oxLDL on ICAM-1 expression by *C. pneumoniae*-infected cells was concentration dependent, rapid and sustained, and more prominent when oxLDL was added after infection. In fact, the effect of oxLDL was seen as early as 12 h after infection and persisted for 7 days. The enhancement of ICAM-1 upregulation, however, was not observed in the presence of native LDL. Advanced glycation end product-LDL was also tested and did not enhance the upregulation of ICAM-1 (data not shown). These results strongly suggest that oxLDL plays a role in regulating the adhesion of monocytes to *C. pneumoniae*-infected endothelial cells by further enhancing the expression of ICAM-1 promoted by *C. pneumoniae*.

Interestingly, both *C. pneumoniae* and oxLDL have been shown to activate Nuclear factor κB (NF-κB). Given the well-established role of NF-κB as a transcription factor for CAM genes, including the genes encoding E-selectin, ICAM-1, and VCAM-1 (31), it seems likely that the synergistic effect on ICAM-1 expression observed in *C. pneumoniae*-infected HAECs is a consequence of the activation of NF-κB. Several reports indicate that oxLDL activates signaling pathway(s) upon ligation to the scavenger receptor and that NF-κB activation is mediated via protein kinase C (PKC)- and/or calcium-dependent pathways (32). In previously reported studies (33), we have demonstrated that HAECs, within 30 min of exposure to *C. pneumoniae*, show

translocation of PKC from the cytosol to the membrane followed by NF-κB activation, which resulted in increased ICAM-1 gene expression. The receptors to which *C. pneumoniae* binds to induce the activation of signaling pathways leading to ICAM-1 upregulation are not known.

The lack of effect on CAM expression observed in HAECs incubated with heat-treated and UV-inactivated *C. pneumoniae*, as shown in Fig. 2B and as previously described (33), strongly suggests that CAM expression requires the exposure of HAECs to viable *C. pneumoniae* elementary bodies or nonoxidized *C. pneumoniae* proteins. Heat inactivation does not affect chlamydial lipopolysaccharide (LPS); therefore, the lack of ICAM-1 upregulation in cells incubated with heat-inactivated *C. pneumoniae* excludes LPS as the mediator. UV irradiation, in contrast to heat inactivation, will not lead to the denaturing of proteins but may lead to their oxidative modification and therefore modify their behavior and binding activity. The present study also demonstrates that the effect of *C. pneumoniae* in ICAM-I upregulation is not secondary to the de novo synthesis of a chlamydial protein, because inhibition of bacterial protein synthesis by rifampicin at different stages of the chlamydial cell cycle did not affect the ability of *C. pneumoniae* to induce ICAM-1 expression.

The effects of *C. pneumoniae* by itself or in synergism with oxLDL on the expression of CAMs of the immunoglobulin superfamily are more pronounced for ICAM-1 than for any of the other adhesion molecules studied. This is not surprising. It has been previously reported (12, 21) that preferential upregulation of ICAM-1 takes place in *C. pneumoniae*-infected human umbilical venous endothelial cells (HUVECs) and HAECs. Also, exposure of arterial endothelial cells to oxLDL (11, 34, 35) or native LDL (10) has been shown to lead to a higher level of expression for ICAM-1 and to lower or no expression for VCAM-1 and E-selectin. Few studies examining the effect of modified lipoproteins in E-selectin expression have been reported, and the few studies published are not always concordant. Stimulation of HUVECs with endothelin-1 with and without the addition of oxLDL did not induce cell surface expression of E-selectin in endothelial cells (36). However, incubation of endothelial cells with native LDL (500 μg/ml) led to a small increase of E-selectin expression in human coronary artery endothelial cells (37, 38). In our experimental conditions, E-selectin expression was not significantly induced by high levels of native LDL (data not shown), but oxLDL was able to further increase the proportion of E-selectin-positive cells at 4 h after infection, further reinforcing the conclusion that oxLDL and *C. pneumoniae* act synergistically in the induction of CAM expression by HAECs.

Taken together, our findings indicate that *C. pneumoniae*-infected endothelial cells tend to express ICAM-1 and E-selectin and that exposure of infected cells to oxLDL further enhances the expression of these two CAMs. Adhesion molecules play a critical role in the progression of atherogenesis, particularly ICAM-1, which appears to be the adhesion molecule responsible for the earliest changes in vascular adhesiveness (38, 39). The exact molecular mechanisms involved in the upregulation of ICAM-1 by *C. pneumoniae* and oxLDL are not clear. The involvement of pathways leading to the PKC activation of NF-κB seems very likely, but the exact nature of the receptors involved and of the early signals delivered by *C. pneumoniae* and by oxLDL requires further investigation.

In conclusion, our data prove that, at least in vitro, two independent insults believed to play significant roles in the initiation and/or perpetuation of atherosclerotic vascular disease have a synergistic effect at the level of the endothelium. Of note is the fact that a synergistic interaction between hyperlipidemia and *C. pneumoniae* had been demonstrated in animal studies. In mice with low density lipoprotein receptor deficiency, *C. pneumoniae* failed to induce atherosclerosis by itself, but when the same mice were infected with *C. pneumoniae* AR39 and fed a high-cholesterol diet, infection significantly exacerbated the hypercholesterolemia-induced atherosclerosis (40). Recently, it was also shown that *C. pneumoniae* infection can enhance intimal thickening and atherosclerotic changes in New Zealand White rabbits fed 0.25% cholesterol-enriched chow (41). The direct correlation between hyperlipidemia and LDL oxidation (23, 42, 43) links the two separate observations, suggesting that an increased generation of oxLDL in hyperlipidemic animals may be one of the reasons behind the synergistic effects of *C. pneumoniae* and hyperlipidemia.

Further characterization of the complex regulatory mechanisms involved in the upregulation of ICAM-1 by *C. pneumoniae* in HAECs and other cells involved in the atherosclerotic process will further our understanding of the *C. pneumoniae*-induced inflammatory component in atherosclerosis. That may contribute to the development of new strategies to modulate the course of the chronic inflammatory process in atherosclerosis and likely to the development of therapeutic tools to halt the progression of arteriosclerosis.

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REFERENCES

1. Libby, P., G. Sukhova, R. T. Lee, and J. K. Liao. 1997. Molecular biology of atherosclerosis. *Int. J. Cardiol.* **62 (Suppl.):** 23–29.

2. Ross, R. 1999. Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340:** 115–126.
3. Mehta, J. L., T. G. P. Saldeen, and K. Rand. 1998. Interactive role of infection, inflammation and traditional risk factors in atherosclerosis and coronary artery disease. *J. Am. Coll. Cardiol.* **31:** 1217–1225.
4. Becker, A. E., O. J. de Boer, and A. C. van Der Wal. 2001. The role of inflammation and infection in coronary artery disease. *Annu. Rev. Med.* **52:** 289–297.
5. Chia, M. C. 1998. The role of adhesion molecules in atherosclerosis. *Crit. Rev. Clin. Lab. Sci.* **35:** 573–602.
6. Price, D. T., and J. Loscalzo. 1999. Cellular adhesion molecules and atherogenesis. *Am. J. Med.* **107:** 85–97.
7. Busse, R., and I. Fleming. 1996. Endothelial dysfunction in atherosclerosis. *J. Vasc. Res.* **33:** 181–194.
8. Cockerill, G. W., K. A. Rye, J. R. Gamble, M. A. Vadas, and P. J. Barter. 1995. High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1987–1994.
9. Hackman, A., Y. Abe, and W. Insull. 1996. Levels of soluble cell adhesion molecules in patients with dyslipidemia. *Circulation.* **93:** 1334–1338.
10. Smalley, D. M., J. H. C. Lin, M. L. Curtis, Y. Kobari, M. B. Stemerman, and K. A. J. Pritchard. 1996. Native LDL increases endothelial cell adhesiveness by inducing intercellular adhesion molecule-1. *Arterioscler. Thromb. Vasc. Biol.* **16:** 585–590.
11. Takei, A., Y. Huang, and M. F. Lopes-Virella. 2001. Expression of adhesion molecules by human endothelial cells exposed to oxidized low-density lipoprotein. Influences of degree of oxidation and location of oxidized LDL. *Atherosclerosis.* **154:** 79–86.
12. Kaukoranta-Tolvanen, S. S. E., T. Ronni, M. Leinonen, P. Saikku, and K. Laitinen. 1996. Expression of adhesion molecules on endothelial cells stimulated by *Chlamydia pneumoniae. Microb. Pathog.* **21:** 407–411.
13. Saikku, O., K. Mattila, M. S. Nieminen, P. H. Makela, J. K. Huttunen, and V. Valtonen. 1988. Serological evidence of an association of a novel Chlamydia TWAR with chronic coronary heart disease and acute myocardial infarction. *Lancet.* **2:** 983–986.
14. Gupta, S., and A. J. Camm. 1998. Is there an infective aetiology to atherosclerosis? *Drug Aging.* **13:** 1–7.
15. Kuo, C. C., A. Shor, and L. A. Campbell. 1993. Demonstration of *Chlamydia pneumoniae* in arteriosclerotic lesions of coronary arteries. *J. Infect. Dis.* **167:** 841–849.
16. Shor, A., C. C. Kuo, and D. L. Patton. 1992. Detection of *Chlamydia pneumoniae* in coronary arterial fatty streaks and atheromatous plaques. *S. Afr. Med. J.* **82:** 158–161.
17. Taylor-Robinson, D. 1998. *Chlamydia pneumoniae* in vascular tissue. *Atherosclerosis.* **140 (Suppl.):** 21–24.
18. Leinonen, M., E. Linnanmaki, K. Mattila, M. S. Nieminen, V. Valtonen, M. Leirisalo-Repo, and P. Saikku. 1990. Circulating immune complexes containing chlamydial lipopolysaccharide in acute myocardial infarction. *Microb. Pathog.* **9:** 67–73.
19. Linnanmaki, E., M. Leinonen, K. Mattila, M. S. Nieminen, V. Valtonen, and P. Saikku. 1993. *Chlamydia pneumoniae*-specific circulating immune complexes in patients with chronic coronary heart disease. *Circulation.* **87:** 1130–1134.
20. Kalayoglu, M. V., B. N. Perkins, and G. I. Byrne. 2001. *Chlamydia pneumoniae*-infected monocytes exhibit increased adherence to human aortic endothelial cells. *Microbes Infect.* **3:** 963–969.
21. Krüll, M., A. C. Klucken, F. N. Wuppermann, O. Fuhrmann, C. Magerl, J. Seybold, S. Hippenstiel, J. H. Hegemann, C. A. Jantos, and N. Suttorp. 1999. Signal transduction pathways activated in endothelial cells following infection with *Chlamydia pneumoniae. J. Immunol.* **162:** 4834–4841.
22. Molestina, R. E., R. D. Miller, J. A. Ramirez, and J. T. Summersgill. 1999. Infection of human endothelial cells with *Chlamydia pneumoniae* stimulates transendothelial migration of neutrophils and monocytes. *Infect. Immun.* **67:** 1323–1330.
23. Kalayoglu, M. V., B. Hoerneman, D. LaVerda, S. G. Morrison, R. P. Morrison, and G. I. Byrne. 1999. Cellular oxidation of low-density lipoprotein by *Chlamydia pneumoniae. J. Infect. Dis.* **180:** 780–790.
24. Wong, K. H., S. Skelton, and Y. K. Chan. 1992. Efficient culture of *Chlamydia pneumoniae* with cell lines derived from the human respiratory tract. *J. Clin. Microbiol.* **30:** 1625–1630.
25. Vielma, S., A. Gorod, G. Virella, and M. F. Lopes-Virella. 2002. *Chlamydophila pneumoniae* infection of human aortic endothelial cells induces the expression of Fc-gamma receptor II (FcgamaRII). *Clin. Immunol.* **104:** 265–273.
26. Kuo, C. C., and J. T. Grayston. 1990. A sensitive cell line, HL cells, for isolation and propagation of *Chlamydia pneumoniae* strain TWAR. *J. Infect. Dis.* **162:** 755–758.
27. Lopes-Virella, M. F., S. Koskinen, M. Mironova, D. Horne, R. Klein, C. Chassereau, C. Enockson, and G. Virella. 2000. The preparation of copper-oxidized LDL for the measurement of oxidized LDL antibodies by EIA. *Atherosclerosis.* **152:** 107–115.
28. Lowry, O. H., N. J. Rosebrough, A. I. Farr, and A. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
29. Rahman, A., K. N. Anwar, and A. B. Malik. 2000. Protein kinase C-zeta mediates TNF-alpha-induced ICAM-1 gene transcription in endothelial cells. *Am. J. Physiol. Cell Physiol.* **279:** C906–C914.
30. Huang, Y., M. Mironova, and M. F. Lopes-Virella. 1999. Oxidized LDL stimulates matrix metalloproteinase-1 expression in human vascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2640–2647.
31. Manning, A. M., F. P. Bell, C. L. Rosenbloom, J. G. Chosay, C. A. Simmons, J. L. Northrup, R. J. Shebuski, C. J. Dunn, and D. C. Anderson. 1995. NF-κB is activated during acute inflammation in vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. *J. Inflamm.* **45:** 283–296.
32. Han, C-Y., S-Y. Park, and Y. K. Pak. 2000. Role of endocytosis in the transactivation of nuclear factor-kappa B by oxidized low-density lipoprotein. *Biochem. J.* **350:** 829–837.
33. Vielma, S. A., G. Krings, and M. F. Lopes-Virella. 2003. *Chlamydophila pneumoniae* induces ICAM-1 expression in human aortic endothelial cells via protein kinase C-dependent activation of nuclear factor-κB. *Circ. Res.* **92:** 1130–1137.
34. Erl, W., P. C. Weber, and C. Weber. 1998. Monocytic cell adhesion to endothelial cells stimulated by oxidized low-density lipoprotein is mediated by distinct endothelial ligands. *Atherosclerosis.* **136:** 297–303.
35. Jeng, J. R., C. H. Chang, S. M. Shieh, and H. C. Chiu. 1993. Oxidized low-density lipoprotein enhances monocyte-endothelial cell binding against shear-stress-induced detachment. *Biochim. Biophys. Acta.* **1178:** 221–227.
36. Langenfeld, M. R., S. Nakhla, A. K. Death, W. Jessup, and D. S. Celermajer. 2001. Endothelin-1 plus oxidized low-density lipoprotein, but neither alone, increase human monocyte adhesion to endothelial cells. *Clin. Sci.* **101:** 731–738.
37. Allen, S., S. Khan, F. Al-Mohanna, P. Batten, and M. Yacoub. 1998. Native low density lipoprotein-induced calcium transients trigger VCAM-1 and E-selectin expression in cultured human endothelial cells. *J. Clin. Invest.* **101:** 1064–1075.
38. Haller, H., D. Schaper, W. Ziegler, S. Philipp, M. Kuhlmann, A. Distler, and F. Luft. 1995. Low-density lipoprotein induces vascular adhesion molecule expression on human endothelial cells. *Hypertension.* **25:** 511–516.
39. Davies, M. J., J. L. Gordon, A. J. H. Gearing, R. Pigott, N. Woolf, D. Katz, and A. Kyriakopoulos. 1993. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J. Pathol.* **171:** 223–229.
40. Hu, H., G. N. Pierce, and G. Zhong. 1999. The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to *Chlamydia pneumoniae. J. Clin. Invest.* **103:** 747–753.
41. Muhlestein, J. B., J. L. Anderson, E. H. Hammond, L. Zhao, S. Trehan, E. P. Schwobe, and J. F. Carlquist. 1998. Infection with *Chlamydia pneumoniae* accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. *Circulation.* **97:** 633–636.
42. Kalayoglu, M. V., and G. I. Byrne. 1998. Induction of macrophage foam cell formation by *Chlamydia pneumoniae. J. Infect. Dis.* **177:** 725–729.
43. Kalayoglu, M. V., G. S. Miranpuri, D. T. Golenbock, and G. I. Byrne. 1999. Characterization of low-density lipoprotein uptake by murine macrophages exposed to *Chlamydia pneumoniae. Microbes Infect.* **1:** 409–418.