**Chlamydia pneumoniae Infection Does Not Induce or Modify Atherosclerosis in Mice**

Giuseppina Caligiuri, MD, PhD; Martin Rottenberg, PhD; Antonino Nicoletti, PhD; Hans Wigzell, MD, PhD; Göran K. Hansson, MD, PhD

**Background**—Seroepidemiological studies have linked *Chlamydia pneumoniae* (CP) to coronary heart disease, and recent experimental studies suggest that it may accelerate or even induce atherosclerosis. We therefore evaluated the effect of CP infection on atherosclerosis in atherosclerosis-prone apolipoprotein E–knockout (apoE-KO) and wild-type C57BL/6J mice.

**Methods and Results**—Six- to 8-week-old female mice were infected intranasally with live CP and then fed a standard chow diet for 22 weeks. A subgroup of mice was reinfected 18 weeks after primary infection. Polymerase chain reaction analysis of lung tissue confirmed successful infection with CP, and ELISA assays demonstrated development of a humoral immune response. Despite this, no statistically significant differences in aortic atherosclerotic lesions were found between CP-infected and control apoE-KO mice. Furthermore, CP infection did not induce atherosclerosis in C57BL/6J mice.

**Conclusions**—CP does not induce atherosclerosis in wild-type mice and does not accelerate atherosclerosis in chow-fed apoE-KO mice. Further studies will be necessary to clarify the explanation for the seroepidemiological association between CP and coronary heart disease in humans. (*Circulation. 2001;103:2834-2838.*)

**Key Words:** atherosclerosis ■ hypercholesterolemia ■ infection

Atherosclerosis is an inflammatory disease. Its lesions are filled with infiltrating macrophages and T cells, together with accumulating cholesterol, proliferating smooth muscle cells, and collagen deposition. It is strongly linked to hypercholesterolemia and increased levels of cholesterol-rich LDL. Deposition and oxidation of LDL in the arterial intima can elicit activation of endothelial cells, expression of leukocyte adhesion molecules, secretion of chemokines, and recruitment of mononuclear cells. Furthermore, components of oxidized LDL may be proinflammatory and antigenic. Immune/inflammatory responses to lipoproteins can therefore explain many of the cellular features of atherosclerosis. Cardiovascular disease resulting from atherosclerosis can be reduced substantially by lipid-lowering therapy. None of these protocols, however, have achieved a reduction of cases by >30% to 40%; hence, there is a need for new therapeutic and preventive approaches to this disease.

Recent seroepidemiological studies have detected a correlation between cardiovascular disease and antibody responses to the microorganism *Chlamydia pneumoniae* (CP). This microbe is a common pathogen in respiratory infections but has also been detected in atherosclerotic lesions. It can survive intracellularly in macrophages, which could be important for transport of CP in the human organism.

Experimental studies in rabbits and mice have suggested that CP is an important pathogenetic factor for atherosclerosis. CP causes vascular inflammation, but it does not induce atherosclerosis alone. Infection with CP, however, has been reported to accelerate cholesterol-induced atherosclerosis significantly in several models. It has been proposed that CP aggravates atherosclerosis by activating macrophages to secrete tumor necrosis factor and metalloproteinases and/or by eliciting production of antibodies that cause endothelial cytotoxicity. In addition, it has been shown that molecular mimicry between proteins of chlamydiae and structural proteins of the myocardium can cause autoimmune myocarditis; this may also result in increased heart disease in CP-infected individuals.

We used a gene-targeted mouse model to evaluate the effect of CP on atherosclerosis. The apolipoprotein E–knockout (apoE-KO) mouse has severe hypercholesterolemia due to targeted deletion of the apoE gene and develops spontaneous atherosclerosis. To study the effect of CP on atherosclerosis, we infected 6- to 8-week-old C57BL/6J and apoE-KO mice intranasally, reinfected a subgroup of apoE-KO mice after 18 weeks, and analyzed atherosclerotic lesions in all mice 22 weeks after primary infection. Our results show that the CP infection could not induce athero-
sclerosis in C57BL/6J mice or affect lesion development or composition in apoE-KO mice. Therefore, CP does not appear to be a major pathogenic factor for atherosclerosis in this model.

**Methods**

**Mice and CP Infection**

ApoE-KO female mice backcrossed 10 times on the C57BL/6J background were obtained from M&B Laboratories, Bomholtgaard, Denmark. Wild-type C57BL/6J female mice obtained from the same source were used as controls. When the animals were 6 to 8 weeks old, groups of mice were infected as described below. Groups of mice were maintained on standard mouse chow.

For infection, a Finnish CP isolate was used. Bacteria were propagated in HL cells. Infected cells were disrupted by sonication, centrifuged, and divided into aliquots. The inoculum preparations were resuspended in sucrose phosphate glutamate solution and stored at −70°C until used. Mice were mildly sedated with metofane and inoculated intranasally with 10⁶ inclusion-forming units diluted in 40 μL of PBS. Subgroups of apoE-KO mice were reinfected 18 weeks after primary infection to assess the role of CP reinfection on progression of atherosclerosis.

Mice were euthanized by exsanguination under carbon dioxide anesthesia 22 weeks after primary infection. The blood was collected and allowed to clot. Serum was separated by centrifugation and stored at −20°C. The vasculature was perfused with PBS, and the root of the aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning covering 0.8 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Five sections 10 μm thick were harvested per slide; thus, 16 slides per mouse were prepared. Sections 400, 500, 600, and 700 μm distant from the first cusp were stained with oil red O, counterstained with hematoxylin, and mounted under coverslips. Blinded lesion quantification was performed as previously described.

**Immunohistochemistry**

Frozen sections (250, 300, and 350 μm from the first appearance of the cusps) were fixed in acetone and stained for CD4⁺ T cells and I-A antigens with monoclonal antibodies. Rat anti-CD4 and biotinylated anti–I-Ab were obtained from PharMingen. After a blocking step for endogenous peroxidase with PBS/H₂O₂ 0.3%, antibody binding was visualized by biotin-labeled anti-rat IgG followed by avidin–horseradish peroxidase and diaminobenzidine. For detection of I-A, no second antibody was used, because the primary antibody was biotinylated. Primary antibodies were omitted in control sections. Peroxidase-positive cells were counted as a percentage of all hematoxylin-stained cells in 3 fields per section by use of a computer program written in Quips language.

**CP Serology**

The serum levels of antibodies to the major CP antigen OMP-2 were measured by ELISA. CP outer membrane protein OMP-2 was produced as a recombinant fusion protein and purified as described. The plates were coated overnight with 0.7 mg/mL of the Trx-ABP-OMP fusion protein. After blocking, sera from individual mice were added at 1:100 or 1:400 dilutions. The plates were subsequently developed with horseradish peroxidase–conjugated rabbit anti–mouse IgG (Sigma Chemical Co). The assay was standardized between plates by including the titration of a pooled serum rabbit anti–mouse IgG (Sigma Chemical Co). The assay was standardized between plates by including the titration of a pooled serum rabbit anti–mouse IgG (Sigma Chemical Co). The assay was standardized between plates by including the titration of a pooled serum rabbit anti–mouse IgG (Sigma Chemical Co). The assay was standardized between plates by including the titration of a pooled serum rabbit anti–mouse IgG (Sigma Chemical Co).

**Polymerase Chain Reaction Assay of CP DNA**

DNA was extracted from frozen lungs with the Qiagen DNA tissue extraction kit. It was amplified by thermal cycling for 54 cycles at a 64°C annealing temperature and with the following sequences as primers: sense OMP-2, 5’-AGCGGGGTATAGGCCGCTGTA-3’; antisense OMP-2, 5’-AGTCTGTGCTTTATGGGTGCA-3’.

The polymerase chain reaction (PCR) products were resolved in a 1.5% agarose gel and photographed.

**Statistical Analysis**

Results are expressed as mean±SEM. Data were analyzed by ANOVA and linear regression analysis. Differences between groups were considered significant at a value of *P* <0.05. Statview 4.1 software (Abacus Concept) was used for all statistical analysis.

**Results**

**Chronic CP Infection and Anti-CP Immune Responses Achieved in Infected Mice**

Both apoE-KO and wild-type (C57B6/J) mice were successfully infected with CP, because all infected mice developed antibodies to the major CP antigen, OMP-2 (Table 1). Twelve of 13 apoE-KO mice infected once and all of 14 mice infected twice had persistent CP DNA in their lungs 22 weeks after primary infection (Table 1). This indicates that chronic infection was achieved by both infection protocols. IgG antibodies to OMP-2 were significantly increased in infected mice compared with controls (Table 1). Antibody titers, however, did not correlate with lesion size (Figure 1).

Immunohistochemical staining for OMP-2 revealed the presence of CP material in occasional macrophages of arterial lesions (data not shown). Such macrophages were very rare,

**Figure 1.** Lesion size does not correlate with serum IgG titers to OMP-2 or CD4⁺ T-cell infiltration into lesions. a, Regression analysis between lesion size (%) and IgG OMP-2 titers (OD, dilution 1:400, means of triplicate analysis for each mouse). b, Linear regression analysis between lesion size (%) and infiltrated CD4⁺ cells (expressed as percentage of CD4⁺-positive cells among total number of cells in three ×400 microscopic fields).
Insignificant Effects of CP Infection on Lesion Size

As shown in Figure 2, apoE-KO mice developed large fibrofatty atherosclerotic lesions in the aorta during their lifetime of 28 to 30 weeks. No apparent differences in gross histology were observed between CP-infected and noninfected apoE-KO mice. Infection with CP was not sufficient to induce atherosclerosis, because C57Bl6/J mice did not develop atherosclerotic lesions on infection (Figure 2). Minute intimal cell masses could occasionally be detected in these mice, but no fibrofatty lesions or significant fatty streaks were observed. The effect of CP on atherosclerosis was estimated quantitatively by a standardized morphometric analysis of lesions in the aortic root (Figure 3). ApoE-KO mice infected twice had a 5.5% increase in lesion size ($P=0.2420$, NS), whereas those infected once had a 6.5% reduction in lesion size ($P=0.2729$, NS) compared with uninfected mice. Similar results were obtained when the cross-sectional area of lesions was analyzed rather than the ratio between lesion area and total vessel area (Table 2).

Effects on the Composition of Lesions

The composition of lesions was estimated semiquantitatively by scoring the size of the lipid core, cellularity, and fibrous cap in each lesion. No significant difference could be discerned between infected and uninfected mice (Table 3). The score for fibrotic tissue tended to be higher in mice infected twice with CP than in those infected once or those that remained uninfected (Table 3). Finally, the cellularity of lesions was not modified by CP infection (Table 3). Thus, no major effect of CP could be detected with regard to lesion composition.

Cellular Immune Response in Lesions After CP Infection

The extent of vascular inflammation was assessed by staining frozen sections for CD4$^+$ T cells and expression of the MHC class II antigen I-A$^b$. As shown in Figure 4, there was a tendency toward increased CD4$^+$ T-cell infiltration in lesions of infected apoE-KO mice. Similarly, I-A$^b$ expression was increased in atherosclerotic lesions of CP-infected apoE-KO mice (data not shown). CD4$^+$ infiltration, however, did not correlate with lesion size (Figure 1). These data do not support the notion that CP induces atherosclerosis by promoting vascular inflammation.

### Table 2. Morphometric Analysis of Lesions in CP-Infected and Control Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Infection</th>
<th>n</th>
<th>Lesion, $\mu m^2$</th>
<th>Lesion, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-E KO</td>
<td>No</td>
<td>16</td>
<td>428 489 ± 45 765</td>
<td>24.07 ± 1.92</td>
</tr>
<tr>
<td>Apo-E KO</td>
<td>Once</td>
<td>13</td>
<td>376 047 ± 35 806</td>
<td>22.49 ± 1.62</td>
</tr>
<tr>
<td>Apo-E KO</td>
<td>Twice</td>
<td>14</td>
<td>446 364 ± 30 086</td>
<td>25.39 ± 1.10</td>
</tr>
<tr>
<td>C57Bl6/J</td>
<td>Once</td>
<td>8</td>
<td>624 ± 301</td>
<td>0.05 ± 0.03</td>
</tr>
</tbody>
</table>

Lesion, $\mu m^2$ indicates average cross-sectional area of aortic lesions (see Methods for details in aortic root (4 sections per mouse), mean ± SEM; Lesion %, average cross-sectional area of lesion/cross-sectional area of vessel (see Methods), mean ± SEM. No significant effects of CP infections could be detected ($P=0.2531$).

### Table 3. Characteristics of Lesions in CP-Infected and Control ApoE-KO Mice

<table>
<thead>
<tr>
<th>Infection</th>
<th>Lesion Cellularity</th>
<th>Lipid Core</th>
<th>Lesion Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.6 ± 0.9</td>
<td>1.8 ± 1.3</td>
<td>2.6 ± 1.1</td>
</tr>
<tr>
<td>Once</td>
<td>2.5 ± 2.1</td>
<td>1.5 ± 0.7</td>
<td>2.0 ± 1.4</td>
</tr>
<tr>
<td>Twice</td>
<td>2.0 ± 1.1</td>
<td>2.2 ± 0.5</td>
<td>3.5 ± 0.6</td>
</tr>
</tbody>
</table>

Lesion cellularity, the size of the lipid core, and the amount of fibrosis were scored in apoE-KO mice on standard chow diet after CP infection (0, 1, or 2 times). Sections were stained with Masson’s trichrome and scored arbitrarily (1 to 4) for each characteristic by 2 blinded independent observers. No differences reached statistical significance.
In the present study, CP infection did not initiate atherosclerosis in C57BL/6J mice, nor did it accelerate atherogenesis in apoE-KO mice to any significant extent. Although the former finding is in line with the findings of others using the same model, those investigators observed an accelerating effect of CP on disease development in the hypercholesterolemic apoE-KO mouse. The reason for this discrepancy is unclear; it cannot be due to lack of infection, because our PCR analysis and serology confirm that the mice were successfully infected with the microbial isolate. It remains possible that CP strains differ in their capacity to infect arterial macrophages. Indeed, Molestina et al demonstrated that strains of CP isolated from coronary atheromas display divergences in their ability to infect arterial macrophages. Therefore, the difference in lesion size in infected apoE-KO mice may be relevant, because Moazed et al showed that the difference in lesion size in infected apoE-KO mice decreased with time. In that study, infected mice fed a chow diet exhibited greater lesions than controls at 16 or 20 weeks of diet. Lesions were evaluated, however, in unstained en face sections from the inner curvature of the aortic arch. This measurement reflects the percentage of vascular surface area covered by opaque lesions. It detects not only "mature" atherosclerotic plaques but also fatty streaks. Because not all of the latter may progress into plaques, measurement of fibrofatty lesions in cross sections may be more appropriate for estimating atherosclerotic disease. Furthermore, blinded, computer-assisted analysis of the cross-sectional area of atherosclerotic lesions might be better suited for quantifying disease burden and the effects of factors such as infections.

Whether CP affects atherosclerosis in humans remains unclear. An interesting speculation is that CP infection may cause bouts of arteritis and plaque inflammation. This could increase the proteolytic and cytotoxic activity in the plaque, perhaps leading to plaque rupture and myocardial ischemia. In addition, CP may hamper endothelial NO production; this might increase the risk for vasospastic events and precipitate myocardial infarction. Thus, CP could be important for ischemia in the atherosclerotic heart rather than accelerating the early phase of atherosclerosis. The present results certainly urge caution in interpretation of experimental studies reporting associations between CP and atherosclerosis.

Acknowledgments

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References


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