Effect of Azithromycin on Murine Arteriosclerosis Exacerbated by *Chlamydia pneumoniae*

Neil M. Rothstein,1 Thomas C. Quinn,2,3 Guillermo Madico,2 Charlotte A. Gaydos,2 and Charles J. Lowenstein1

*Chlamydia pneumoniae* infection can exacerbate atherosclerosis in animals. To test the hypothesis that antibiotic therapy inhibits the atherogenic effects of *C. pneumoniae* infection, 10-week-old apolipoprotein E (ApoE) null mice were infected with *C. pneumoniae* or placebo, were treated for 2 weeks after infection with azithromycin or placebo, and were killed at 20 weeks of age. Infection did not affect the size of the aortic lesion, and antibiotic treatment had no effect. Another group of mice, 12-week-old ApoE mice, were infected with *C. pneumoniae* or placebo, were treated for 2 weeks after infection with azithromycin or placebo, and were killed at 26 weeks of age. *C. pneumoniae* infection increased the size of the lesion in infected mice, but azithromycin did not reduce the size of the aortic lesion in infected mice. Therefore, immediate therapy of acute infection may be necessary to prevent the proatherogenic effects of *C. pneumoniae* infection.

Atherosclerosis is a chronic inflammatory disease [1, 2], but the precise cause of inflammation that exacerbates atherosclerosis is unknown. Infection may play an inflammatory role in atherogenesis [3–11]. Many studies have assessed the possible connection to atherogenesis of several organisms, including *Chlamydia pneumoniae* [12–20]. Some seroepidemiologic studies found a correlation between titers of antibody to *C. pneumoniae* and the presence of atherosclerotic cardiovascular disease and between titers of antibody to *C. pneumoniae* and the prognosis of patients with recent myocardial infarctions [21–31]. However, other studies failed to detect any correlation between anti-*C. pneumoniae* titers and atherosclerosis or myocardial infarction, including 1 prospective study of 15,000 men [32–36]. Some studies showed the presence of *C. pneumoniae* within plaques by using immunohistochemistry, polymerase chain reaction (PCR), or electron microscopy [37–48]; others found a low prevalence of *C. pneumoniae* within atherosclerotic plaques [33, 49]. Neither the seroepidemiologic correlations nor the detection of *C. pneumoniae* in plaques can define a causative role for *C. pneumoniae* in atherogenesis.

In an attempt to define more clearly a causal role for *C. pneumoniae* in atherogenesis, several prospective studies explored the use of antibiotics and clinical outcomes among patients with coronary artery disease. These studies have had mixed results: In one, antibiotic usage was correlated with a reduction in adverse events following myocardial infarction [50], another found no association between antibiotic usage and clinical improvement [51], and a third showed a nonsignificant trend toward benefit in patients with unstable angina treated with antibiotics [52]. Several large prospective trials are now underway to measure the effect of antibiotics on the clinical outcomes of patients at high risk for coronary events [51, 53, 54]. These trials are based on the assumption that treatment with antibiotics chosen for their efficacy in treating acute *C. pneumoniae* infections will stabilize plaques and therefore decrease cardiovascular events.

Rabbit and mouse models of atherogenesis have been used to determine whether *C. pneumoniae* can induce de novo plaque formation [12, 18, 20, 55–59]. Muhlestein et al. [18] and Fong et al. [57] showed that infection with *C. pneumoniae* increases intimal thickness and causes atherosclerosis in 30%–40% of infected New Zealand White rabbits. This effect was diminished by treatment of acute infection with high-dose azithromycin administered once weekly, starting 5 days after inoculation and continuing for 10 weeks until death. In mice, *C. pneumoniae* has been implicated in increasing fatty streak lesion formation in apolipoprotein E (ApoE) null mice and low-density lipoprotein (LDL) receptor null mice [58, 59]. Serologic studies show that most people are infected with *C. pneumoniae* in the first 3–4 decades of life [60]. Since the majority of adolescents and young adults have visible fatty streaks
in their aorta and coronary arteries, C. pneumoniae infection may affect the development of fatty streaks. The lesions found in the early decades of life may be precursors of the fibrous plaques that cause cardiovascular events in later life; decreasing fatty streaks may prevent future unstable coronary syndromes.

This study was designed to test 2 hypotheses: (1) that C. pneumoniae infection accelerates fatty streak and fibrous plaque formation in mice and (2) that treatment with antibiotics after an acute C. pneumoniae infection prevents an increase in fatty streak and fibrous plaque formation. We administered azithromycin or placebo to ApoE null mice that either had been inoculated or had not been inoculated with C. pneumoniae and then observed the development of fatty streaks and fibrous plaques.

Materials and Methods

Animal studies. Eight- to 10-week old ApoE-deficient female mice (back-crossed >6 generations with C57BL/6J mice) were obtained from Jackson Laboratories and were fed standard mouse chow (Purina) throughout the experiment. The mice were housed in microisolator cages and were handled only in sterile hoods.

In each set of experiments, 10 mice were mock infected with sterile medium and were treated with placebo, 10 mice were mock infected with sterile medium and were treated with azithromycin, 10 mice were infected with C. pneumoniae and were treated with placebo, and 10 mice were infected with C. pneumoniae and were treated with azithromycin. This set of experiments was repeated for 2 different schedules (figure 1). For the first schedule, mice were inoculated with C. pneumoniae or sterile medium at 10 and 12 weeks of age, were treated with azithromycin or placebo at 14 and 15 weeks of age, and were killed at 20 weeks of age. For the second schedule, mice were inoculated with C. pneumoniae or sterile medium at 12 and 14 weeks of age, were treated with azithromycin or placebo at 16 and 17 weeks of age, and were killed at 26 weeks of age. The entire experiment then was repeated once, so that a total of 80 mice were treated according to the first schedule and 80 mice were treated according to the second schedule.

The 2 different schedules were developed according to the following rationale. We infected 1 group of ApoE null mice at 10–12 weeks of age when fatty streaks (collections of foam cells) first develop and the other group at 12–14 weeks of age when intermediate lesions (containing foam cells and spindle-shaped smooth muscle cells) begin to appear. The mice were inoculated at different ages, to determine whether fatty streaks or intermediate lesions have different susceptibilities to infection. Both groups then were treated with antibiotics 2 and 3 weeks after the last infection. The mice in the first group were killed at 20 weeks of age, the time at which fibrous plaques (necrotic core covered by a fibrous smooth muscle cell cap) appear, to determine whether infection or treatment affects fibrous plaque development. The mice in the second group were killed at 26 weeks of age, to determine whether infection or treatment affected advanced lesions (partial destruction of medial cells and calcification). The mice were studied at different ages to determine whether infection affects the development of different lesions, fibrous plaques, and advanced lesions.

All mice were killed after a 16-h fast. The mice were anesthetized with pentofane and then were exsanguinated via the inferior vena cava. Blood drawn from the inferior vena cava at the level of the renal veins was collected at the time of death in heparinized syringes, treated with EDTA, and centrifuged. Plasma was flash frozen and stored at −70°C. At death, the ventricles, aortic arch, right lung, and spleen were harvested and frozen at −70°C.

C. pneumoniae strain and inoculum. C. pneumoniae A-03, a human atheroma isolate [42], was grown in HEp-2 cells, as described elsewhere [61]. Purified elementary bodies were frozen in Chlamydia medium with glucose and antibiotics (CMGA) at a concentration of 108 inclusion-forming units (ifu)/mL and were stored at −70°C. All murine infections were done by using a single preparation of C. pneumoniae. Mice were anesthetized with pentofane and were intranasally administered 50 μL of either sterile CMGA or CMGA containing 5 × 10⁵ ifu of C. pneumoniae. Mice were infected twice 2 weeks apart.
**Table 1.** Detection of *Chlamydia pneumoniae* in mice by polymerase chain reaction (PCR) and by serology.

<table>
<thead>
<tr>
<th>Inoculation, treatment</th>
<th>PCR detection of <em>C. pneumoniae</em></th>
<th>Serology titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Any tissue</td>
<td>Aorta</td>
</tr>
<tr>
<td>Sham</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Placebo</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td><em>C. pneumoniae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>7/10</td>
<td>1/10</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>5/10</td>
<td>1/10</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. positive/no. tested. Mice at 12 and 14 weeks of age were sham inoculated or inoculated with *C. pneumoniae*, were treated with placebo or azithromycin at 16 and 17 weeks of age, and were killed at 26 weeks. Antibodies to *C. pneumoniae* were present in all mice inoculated with *C. pneumoniae* but were absent from sham-inoculated mice (n = 5 mice each of 4 groups). *C. pneumoniae* DNA was present in 20 mice inoculated with *C. pneumoniae*, whether they were treated with azithromycin or with placebo; *C. pneumoniae* DNA was absent from 20 sham-inoculated mice.

**Treatment with azithromycin.** Mice were given 24 mg/kg azithromycin in an aqueous suspension (gift of Pfizer Laboratories) by oral intubation at 2 weeks and at 3 weeks after the second infection. Untreated mice were given sterile water by oral intubation in the same manner as the treated mice.

**Serology.** Antibodies to *C. pneumoniae* were measured by the microimmunofluorescence test, as described elsewhere [62]. IgG antibodies were measured by using mouse serum sample dilutions of 1:16 to 1:1024. A goat anti-mouse IgG was used as the secondary antibody conjugated to fluorescein. Antibodies were measured in 20 of the 80 mice in each group, randomly selected from 10 mice in each of the following groups: the noninfected untreated, infected but not treated, noninfected but treated, and infected and treated groups. All tests were done by laboratory personnel who were unaware of the study treatment arms.

**Lipid profile.** Plasma samples from 3 mice in each of the 4 groups inoculated at 12 and 14 weeks were submitted to the Johns Hopkins Hospital Clinical Laboratory for the determination of total cholesterol, high-density lipoprotein (HDL), and triglycerides. The reported LDL was calculated as follows: total cholesterol = HDL + triglycerides/5 + LDL.

**PCR of *C. pneumoniae.*** DNA was extracted from tissue of 40 mice in each schedule by standard techniques and was analyzed by touchdown enzyme time release PCR [63]. The PCR reaction mix included DNA from murine tissues, primers CPN90 and CPN91 (which flank a 195-bp portion of the 16S rRNA genes), and Taq polymerase (HotStarTaq; Qiagen). The initial denaturing incubation at 95°C for 75 s was followed by 60 cycles of denaturation at 95°C for 45 s, annealing from 65°C to 55°C for 45 s, and extension at 72°C for 60 s. During the first 40 cycles, the annealing temperature of 65°C was lowered 1°C every 4 cycles, and then the annealing temperature was held constant at 55°C for the remaining 20 cycles. The PCR products were fractionated on 3% agarose Trisborate EDTA gels and were stained with ethidium bromide.

**Quantification of atherosclerotic lesions in the aortic root.** Aortas were harvested and quantified as described by Paigen et al. [64]. Mice were exsanguinated, and their hearts were perfused first with normal saline and then with optimum cutting temperature (OCT) compound (VWR Scientific Products). The hearts and ascending aortas were dissected, and the hearts were cut in half, perpendicular to the plane of the atria just below the atria, were mounted in OCT blocks, and were frozen. The blocks were cut in 10-μm sections, and every fifth section, starting from the first section distal to the aortic valve leaflets, was stained with oil red O (ORO) and counterstained with hematoxylin. Five sections were stained per aorta. The sections were photographed with a digital camera (Polaroid), using identical settings for exposure, lighting, and magnification. The number of pixels of staining was calculated with a macro created in Adobe Photoshop to eliminate observer bias. The values obtained for the 5 sections were averaged; this value represents the relative mean area index of ORO staining for a section of the aortic root in any given mouse and is termed the atherosclerotic lesion index. The mean and SD were calculated within each group of mice. The statistical significance of the differences among experimental groups was compared by Wilcoxon rank sum test.

**Results**

**Verification of infection by PCR and serology.** Serologic testing detected antibody to *C. pneumoniae* at an IgG titer of 1:128 in all mice infected at week 12 and killed at week 26 (table 1). No antibodies were detected in mock-infected mice.

We found that *C. pneumoniae* DNA persists in the lungs of infected mice, even after azithromycin treatment. The PCR detected *C. pneumoniae* DNA in 70% of tissues from untreated mice and in 50% of treated mice 8 weeks after infection (table 1). As expected, the PCR did not detect *C. pneumoniae* DNA in any sham-infected animals (table 1).

**Lipid profile.** There was no significant difference in total cholesterol, LDL, or HDL in the various experimental groups of mice that were killed at 26 weeks (table 2). However, triglyceride levels were significantly higher in infected versus sham-infected mice in both azithromycin treated and untreated groups (P = .05).

**Area of ORO stain of the aorta.** Lesions containing lipid appeared in aortas of 20- and 26-week-old mice (figure 2). The aortic lesion index was similar in 20-week-old noninfected or infected mice inoculated at 10 or 12 weeks of age (figure 3).

Treatment with azithromycin did not affect aortic lesion size.

**Table 2.** Lipid profile of apolipoprotein E null mice.

<table>
<thead>
<tr>
<th>Inoculation, treatment</th>
<th>Cholesterol</th>
<th>HDL</th>
<th>Triglycerides</th>
<th>LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>428 ± 66</td>
<td>108 ± 9</td>
<td>56 ± 6</td>
<td>372 ± 57</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>408 ± 61</td>
<td>117 ± 14</td>
<td>43 ± 4</td>
<td>282 ± 46</td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>448 ± 16</td>
<td>97 ± 1</td>
<td>69a ± 6</td>
<td>337 ± 16</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>399 ± 16</td>
<td>99 ± 4</td>
<td>66a ± 23</td>
<td>284 ± 18</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± SD. Mice at 12 and 14 weeks of age were sham inoculated or inoculated with *C. pneumoniae*, were treated with placebo or with azithromycin at 16 and 17 weeks of age, and were killed at 26 weeks of age. Serum was analyzed for total cholesterol, HDL, and triglycerides (mg/dL). No significant differences were found between groups, except for a slight increase in triglyceride levels in the *C. pneumoniae*-inoculated group (n = 3 ± SD). HDL, high-density lipoprotein; LDL, low-density lipoprotein.

* P = .05.
in infected mice. We also administered azithromycin to non-infected mice, since azithromycin and macrolide antibiotics have anti-inflammatory effects [65–67]; however, azithromycin had no effect on lesion size in noninfected animals (figure 3). In contrast, *C. pneumoniae* infection increased aortic lesion size in 26-week-old mice infected at 12 and 14 weeks of age, compared with that in sham-infected mice (*P* < .01; figure 3). However, treatment with azithromycin did not affect lesion size in infected mice, compared with that of placebo treatment.

**Discussion**

Our major findings were that *C. pneumoniae* infection increases aortic lesion size but that azithromycin treatment does not reduce this effect of infection. In other studies, *C. pneumoniae* increased aortic lesions in a murine model of atherosclerosis [58]. Our results are consistent with those of Hu et al. [59], who showed that *C. pneumoniae* infection increases aortic lesion area in LDL receptor knockout mice that were fed a high cholesterol diet, compared with those of uninfected mice. Similar to our results, the difference between infected and uninfected mice became apparent at 6–9 months of age but not at earlier time points. However, in our experiment, mice were infected twice 2 weeks apart, whereas Hu et al. infected their mice once every month for the duration of their experiment.

Another difference between our study and that of Hu et al. [59] was that *C. pneumoniae* infection had no effect on arteriosclerosis in LDL receptor null mice, unless the mice were fed an atherogenic diet. In contrast, *C. pneumoniae* infection increased arteriosclerosis in the ApoE null mice in our study when fed a normal mouse chow diet. One possible explanation for this difference is that the consequences of a lack of ApoE are more severe than of a lack of LDL receptor. The lesions found in LDL receptor knockout mice are primarily fatty streaks, an early lesion in arteriosclerosis. In contrast, most of the lesions in the ApoE mouse at 26 weeks have fibrous caps, a sign of a more mature arteriosclerotic lesion. If *C. pneumoniae* is more tropic to cells that contribute to the pathogenesis of fibrous plaques or if the microenvironment of the fibrous plaque favors the replication of *C. pneumoniae* more than that of a fatty streak, it is possible that *C. pneumoniae* could initially replicate to higher levels and cause more inflammation in ApoE than in LDL receptor null vessels.

Our findings extend the observations of Moazed et al. [58]. They showed that *C. pneumoniae* infection of ApoE null mice at 8, 9, and 10 weeks of age causes a 2.4-fold increase in the area of fatty streaks in the aortic arch at 16 weeks of age. However, this difference diminished to 1.5-fold at 20 weeks of age. In our study, infection caused an increase in fatty streak area at 26 weeks of age. In contrast to Moazed et al. [58], we could not detect a significant difference in aortic lesions of mice infected at 10 or 12 weeks of age and killed at 20 weeks of age. We did not investigate earlier time points after acute infection. These differences in results may be due to differences in strain, inoculum, or methodology. In both studies, the majority of mice 8 weeks after infection had *C. pneumoniae* detected by
infection may have no effect on atherogenesis. However, early
treatment of the acute infection may reduce the proatherogenic
effect of C. pneumoniae infection.

Azithromycin was chosen for this experiment because of its
long half-life and its high tissue concentrations. Previous studies
showed that a single 10-mg/kg dose is adequate to cure C.
Pneumoniae pneumonia in the mouse [68]. Although cultures
are negative after antibiotic treatment, C. pneumoniae DNA is
readily detected by PCR, and pulmonary infection can be re-
activated with steroid therapy [69–71]. The 24-mg/kg dose ad-
ministered to mice in our study is comparable with the 21-mg/
kg dose (1.5 g/70 kg) given to adults for chlamydial respiratory
infections.

Another finding of our study is that azithromycin treatment
has no effect upon aortic lesion size in noninfected mice. Ma-
crolide antibiotics have anti-inflammatory effects [72]. For ex-
ample, macrolide antibiotics can inhibit superoxide production
by activated neutrophils in vitro [67]. Macrolide antibiotics
reduced levels of tumor necrosis factor-α, interleukin-1β, pros-
taglandin E2, and nitrite in several in vivo models of inflam-
mation [65, 66]. Since atherosclerosis is an inflammatory
disease, we hypothesized that azithromycin would decrease the
size of aortic lesions in noninfected mice, compared with that
of noninfected and nontreated mice. However, azithromycin
had no effect on aortic lesion size in noninfected mice.

Our data suggest that an initial C. pneumoniae infection that
subsequently resolves can have long-term effects on atherogen-
esis. Treatment of this infection after 4–5 weeks with antibiotics
had no protective effect on the development of atherogenesis.
These 2 observations taken together support the hypothesis
that the initial injury of C. pneumoniae leads to an exacerbation
of atherogenesis, perhaps by stimulating an immune response or
by directly damaging the vessel wall and stimulating a repair
response within the vessel wall. Another possibility is that per-
sistent C. pneumoniae infection that is not eradicated by azith-
romycin contributes to atherogenesis by continually causing
inflammation of the vessel wall.

Several large clinical trials are underway to examine the role
of antibiotics in secondary prevention of heart disease by treat-
ing patients long after initial infection. The clinical implications
of our data suggest that the treatment of C. pneumoniae several
weeks after the acute infection might have no effect on the
proatherogenic effect of infection in a primary prevention trial.
However, our data did not determine whether antibiotics are
beneficial in secondary prevention trials, in which plaque in-
stability may be exacerbated by subsequent inflammation due
to reinfec tion or reactivation of C. pneumoniae infection.

Acknowledgments

We thank Kim Crotchfeldt (Division of Infectious Diseases, Dept.
of Medicine, Johns Hopkins University School of Medicine, Baltimore,
MD) for assistance in preparation of innocula and the polymerase chain
reaction and Mary Shepard (Division of Infectious Diseases, Dept. of
References


