A 6 week course of azithromycin treatment has no beneficial effect on atherosclerotic lesion development in apolipoprotein E-deficient mice chronically infected with *Chlamydia pneumoniae*

E. Blessing†, L. A. Campbell, M. E. Rosenfeld, B. Chesebro‡ and C.-C. Kuo*

Department of Pathobiology, University of Washington, Seattle, WA 98195, USA

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**Objectives:** To evaluate whether antimicrobial chemotherapy prevents acceleration of atherosclerotic lesion development induced by infection with *Chlamydia pneumoniae*.

**Methods:** ApoE-deficient mice which develop hyperlipidaemia and atherosclerosis spontaneously were inoculated intranasally with *C. pneumoniae*. Animals were treated with azithromycin for 6 weeks after the third inoculation and the atherosclerotic lesion areas in the aortic sinus were measured by computer-assisted morphometry.

**Results:** At 12 weeks post-infection, infected untreated animals developed significantly larger lesion areas compared with sham-inoculated controls (8.7 ± 2.3 × 10^4 μm^2 versus 5.6 ± 2.4 × 10^4 μm^2). However, there were no differences in lesion size of infected mice treated with azithromycin in comparison with untreated infected controls (11.0 ± 3.0 × 10^4 μm^2 versus 8.7 ± 2.3 × 10^4 μm^2).

**Conclusions:** Antibiotic treatment against *C. pneumoniae* has no beneficial effects on hyperlipidaemia-induced atherosclerosis accelerated by *C. pneumoniae* in a mouse model.

**Keywords:** *C. pneumoniae*, atherosclerosis, hyperlipidaemia, treatment, prevention

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**Introduction**

An association of *Chlamydia pneumoniae* with atherosclerosis has been well established by seroepidemiology, detection of the organism in atherosclerotic lesions and animal models. Experiments in mouse models have shown that *C. pneumoniae* promotes atherosclerosis in hyperlipidaemic but not in normolipidaemic mice. The mechanisms by which *C. pneumoniae* promotes atherosclerosis are yet to be defined. As *C. pneumoniae* infection contributes to atherosclerosis, the risk of atherosclerosis could potentially be reduced by treatment with macrolide antibiotics that are known to be effective against *C. pneumoniae*. The potential protective effects of this type of antibiotic have previously been evaluated in animals and humans. However, these studies have yielded variable results. Therefore, the purpose of this study was to evaluate a long-term treatment of chronic *C. pneumoniae* infection with azithromycin on atherosclerotic lesion development in hyperlipidaemic apolipoprotein E (apoE)-deficient mice, which develop atherosclerosis spontaneously on a normal chow diet. The study design took into consideration the epidemiological observations of age of *C. pneumoniae* infection and development of hyperlipidaemia and atherosclerosis in humans. Seroepidemiological studies have shown that *C. pneumoniae* infections are rare in children under 5 years of age. Age specific incidence of infection has shown that everyone gets infected between the age of 5 and 14. Thus, first infection with *C. pneumoniae* occurs at the age hyperlipidaemia and atherosclerosis have not fully developed. Therefore, in this study antibiotic treatment was started after chronic infection had been established in young mice in which early atherosclerotic lesions are forming.

**Materials and methods**

**Experimental animals**

Eight-week-old pathogen-free male apoE−/− mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed under modified specific pathogen free conditions in filter-top...
Inoculation and treatment
Mice were sedated by intraperitoneal injection of a mixture of ketamine (Fort Dodge Laboratories, Shenandoah, IA, USA) and xylazine (Lloyd Laboratories, Shenandoah, IA, USA). To induce chronic C. pneumoniae infection in the aorta, mice were inoculated intranasally with 3 × 10^7 inclusion forming units of purified C. pneumoniae (strain AR-39) organisms at 8, 9 and 10 weeks of age. Control mice were sham-inoculated with sterile PBS. The inoculated and sham-inoculated animals were further divided into treatment and no-treatment groups. The treatment regimen consisted of administration of azithromycin (Zithromax; Pfizer Pharmaceuticals, Inc., Groton, CT, USA) at 30 mg/kg body weight by intramuscular injection into the gluteal muscle at days 3, 4 and 5 after the 3rd inoculation and once a week for 5 weeks thereafter. Sham injections were performed with sterile saline. Susceptibility of AR-39 to azithromycin has been tested in vitro and the MIC was determined to be 0.5 mg/L. The study design is illustrated in Figure 1.

Tissue collection
Necropsies were performed at 16 and 20 weeks of age (8 and 12 weeks post first inoculation). Mice were sedated with a mixture of acepromazine maleate (15 mg/kg; Fermenta Animal Health Co., Kansas City, MO, USA) and ketamine HCl (150 mg/kg; Phoenix Pharmaceutical Inc., St Joseph, MO, USA). Blood was collected by exsanguination from the femoral arteries at necropsy. The heart and aorta were perfusion fixed with 10% buffered formalin administered through the left ventricle. The lungs, heart and thoracic aorta with its main branches attached were dissected intact. The aorta was separated from the heart, and the base of the heart was frozen in OCT medium (OCT™, Sakura Finetek, Torrance, CA, USA), and sectioned on a cryostat at the level of the aortic sinus. Once the atrioventricular valves were identified, 8 μm sections were taken and mounted on gelatin-coated slides. Sections of the aortic sinus were collected up to the point where the valves disappeared. Every other section throughout the sinus was used for atherosclerotic lesion analysis.

Quantification of lesion area
The cross-sectional area of an atherosclerotic lesion was determined in a cryostat at the level of the aortic sinus. Once the atrioventricular valves were identified, 8 μm sections were taken and mounted on gelatin-coated slides. Sections of the aortic sinus were collected up to the point where the valves disappeared. Every other section throughout the sinus was used for atherosclerotic lesion analysis.

Serology and blood cholesterol measurement
Plasma was separated from heparinized blood and frozen at −70°C for serology and lipid measurements. C. pneumoniae-specific IgG and IgM antibody titres were determined by the micro-immunofluorescence test. Total plasma cholesterol was measured using a commercial enzymic test kit (Sigma, St Louis, MO, USA).

Statistical analysis
Data are expressed as means ± SD. Group data were analysed by the unpaired Student’s t-test. A value of P < 0.05 was considered statistically significant.

Results
Clinical response and lipid profiles
Clinical signs of infection included increased respiratory rate and nasal and ocular discharge. The symptoms were most severe within the first few days after the first and second inoculation. These clinical symptoms were resolved within 2 weeks after the third inoculation. No adverse reactions were observed following antibiotic treatment. There were no significant differences in body weights or cholesterol levels between any of the groups at the time of necropsy (data not shown).

Serology
All infected mice developed IgG antibodies against C. pneumoniae. The titres ranged from 1:128 to 1:512. Antibiotic treatment did not significantly reduce the antibody titres. No IgM antibodies were detected. All of the control mice were antibody negative (<1:8).

Quantification of lesion area
There were no significant differences in lesion areas between the infected and uninfected mice at 8 weeks post-infection either in the untreated group (5.0 ± 10^4 ± 3.0 ± 10^4 μm^2, n = 15 versus 4.1 ± 10^4 ± 1.6 ± 10^4 μm^2, n = 10; P > 0.05) or treated group (5.5 ± 10^4 ± 3.4 ± 10^4 μm^2, n = 15 versus 4.8 ± 10^4 ± 1.8 ± 10^4 μm^2, n = 9; P > 0.05) (Figure 2). At 12 weeks post-infection, the infected untreated animals developed significantly larger lesion areas compared with controls (8.7 ± 10^4 ± 2.3 ± 10^4 μm^2, n = 14 versus 5.6 ± 10^4± 2.4 ± 10^4 μm^2, n = 8; P = 0.026) as did the infected treated animals versus controls (11.0 ± 10^4 ± 3.0 ± 10^4 μm^2, n = 15 versus 6.0 ± 10^4 ± 1.5 ± 10^4 μm^2, n = 8; P = 0.003) (Figure 2). However, there were no differences in lesion size of infected mice treated with azithromycin in comparison with untreated infected controls (11.0 ± 10^4 ± 3.0 ± 10^4 μm^2, n = 15 versus 8.7 ± 10^4 ± 2.3 ± 10^4 μm^2, n = 14; P > 0.5) (Figure 2).

Discussion
This study confirmed our previous studies that C. pneumoniae infection in hyperlipidaemic mice accelerates the progression of atherosclerosis. However, a 6 week course of treatment with azithromycin at 30 mg/kg by intramuscular injection starting 3 days after the third weekly inoculation with C. pneumoniae did not reduce the acceleration of lesion development by C. pneumoniae infection in hyperlipidaemic
apoE−/− mice. Whether a 6 week course of treatment is long enough to be effective is not known. However, considering that the life span of mice is 2.5 years, 6 weeks of treatment represents 4.6% of the life span of mice, which would be equivalent to 3.3 years of treatment in humans.

Our results are similar to those reported by Rothstein et al., using the same mouse model but with a shorter treatment regimen. In their study, mice were inoculated with C. pneumoniae twice at 2 week intervals and azithromycin was administered orally at 24 mg/kg once a week for 2 weeks starting 2 weeks after the second inoculation. No reduction in lesion development induced by C. pneumoniae was observed at either 10 or 14 weeks post-infection.

In contrast, in New Zealand white rabbits, weekly treatment by intramuscular injection of 30 mg/kg of azithromycin for 7 weeks prevented the acceleration of intimal thickening induced by C. pneumoniae in rabbits given a diet supplemented with a small amount (0.25%) of cholesterol. In this model, animals were inoculated three times at 3 week intervals and the antibiotic treatment was started immediately after the final inoculation.

Human therapeutic trials with macrolides have also yielded variable results. Of eight small clinical trials, three trials showed positive results, four negative results and one equivocal results. A large-scale randomized control trial of 7747 adults who had previous myocardial infarction with a 3 month course of azithromycin [Weekly Intervention with Zithromax for Atherosclerosis and Related Disorders (WIZARD)] was recently completed and reported. This study showed that this treatment regimen did not reduce the overall clinical sequelae of coronary heart disease after a median of 14 months follow-up. However, a beneficial effect was observed with a 30% reduction in the incidence of death or non-fatal re-infarction at 6 months after stopping treatment. The ACES (Azithromycin and Coronary Events Study) study has just been completed in 2004. In this study, patients were treated with 600 mg of azithromycin orally once a week for 1 year. No significant clinical benefit in the secondary prevention of coronary heart disease events has been observed. (Presented by J. T. Grayston at the Fifth European Society for Chlamydia Research, September 1–4, 2004, in Budapest).

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References


