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Infection With *Chlamydia pneumoniae* Accelerates the Development of Atherosclerosis and Treatment With Azithromycin Prevents It in a Rabbit Model

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Background—*Chlamydia pneumoniae* infection has been associated with atherosclerosis by serological studies and detection of bacterial antigen within plaque. We sought to evaluate a possible causal role in an animal model.

Methods and Results—Thirty New Zealand White rabbits were given three separate intranasal inoculations of either *C pneumoniae* (n=20) or saline (n=10) at 3-week intervals and fed chow enriched with a small amount (0.25%) of cholesterol. Immediately after the final inoculation, infected and control rabbits were randomized and begun on a 7-week course of azithromycin or no therapy. Three months after the final inoculation, rabbits were euthanized and sections of thoracic aortas were blindly evaluated microscopically for maximal intimal thickness (MIT), percentage of luminal circumference involved (PLCI), and plaque area index (PAI) of atherosclerosis. Vascular chlamydial antigen was assessed by direct immunofluorescence. MIT differed among treatment groups ($P=.009$), showing an increase in infected rabbits (0.55 mm; SE=0.15 mm) compared with uninfected controls (0.16 mm; SE=0.06 mm) and with infected rabbits receiving antibiotics (0.20 mm; SE=0.03 mm) (both $P<.025$), whereas MIT in infected/treated versus control rabbits did not differ. PLCI also tended to differ ($P<.1$) and PAI differed significantly ($P<.01$) among groups with a similar pattern. Chlamydial antigen was detected in 2 untreated, 3 treated, and 0 control animals.

Conclusions—Intranasal *C pneumoniae* infection accelerates intimal thickening in rabbits given a modestly cholesterol-enhanced diet. In addition, weekly treatment with azithromycin after infectious exposure prevents accelerated intimal thickening. These findings strengthen the etiologic link between *C pneumoniae* and atherosclerosis and should stimulate additional animal and human studies, including clinical antibiotic trials. (*Circulation*. 1998;97:633-636.)

Key Words: atherosclerosis ■ azithromycin ■ *Chlamydia pneumoniae*

Atherosclerotic cardiovascular disease is a major health problem, causing nearly half of all deaths in the United States. Several important risk factors for atherosclerosis have been discovered, but much of the risk remains unexplained. Recently, infectious agents have been proposed as a possible additional coronary risk factor. *Chlamydia pneumoniae* is a newly discovered third species of chlamydia shown to cause pneumonia, bronchitis, pharyngitis, and sinusitis.¹ *C pneumoniae* also has been associated with coronary heart disease and myocardial infarction in serological studies.²⁻⁴ More specifically, *C pneumoniae* antigen and elementary bodies have been found in atheromas from coronary arteries,⁵⁻⁷ carotid arteries,⁸ and aorta.⁹ However, these findings do not establish a causal role. Animal models would be useful in determining causality and assessing the role of antibiotic therapy. *C pneumoniae* causes pneumonitis in the rabbit,¹⁰ and the cholesterol-fed rabbit is an established model for accelerated atherosclerosis. Thus, the rabbit may be a suitable model to study a pathogenetic role of *C pneumoniae* in atherosclerosis. Fong et al¹¹ recently reported on a small study of rabbits nasally infected with *C pneumoniae*.

Two of 11 animals demonstrated early and intermediate histological lesions of atherosclerosis. We hypothesized that repeat infections and the addition of a small supplement of dietary cholesterol would yield more consistent and accelerated development of atherosclerotic lesions and that antibiotic therapy might prevent this process.

Methods

The objectives of the present study were (1) to determine whether repeated intranasal *C pneumoniae* infection of rabbits fed with chow supplemented with a small amount (0.25%) of cholesterol would result in significant acceleration of atherosclerosis compared with saline inoculation and (2) to assess the efficacy of azithromycin, an antibiotic known to be effective against *C pneumoniae*, in preventing accelerated development of atherosclerosis.

C pneumoniae Strain and Inoculum

The TWAR American Type Culture Collection strain VR 1310¹² was used. We harvested viable organisms from infected cultures of HeLa 229 cells by disrupting infected cells with glass beads and sonification. Organisms were partially purified by centrifugation, quantitated, and resuspended in sucrose phosphate glutamic acid with 10% dimethyl

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sulfoxide to provide a final inoculum of 1 to 5×10^6 inclusion-forming units per milliliter (method adapted from Mayer et al¹⁵).

Experimental Animals and Study Design

Thirty female New Zealand White rabbits (2 to 4 months old, pathogen free) were used. Rabbits were fed standard rabbit chow fortified with 0.25% cholesterol without antibiotics. Animal care and processing were performed under strict adherence to the Institutional Animal Care and Use Committee guidelines. Twenty rabbits were included in the infection arm of the study. Each was inoculated with 1 mL (1 to 5×10^6 inclusion-forming units) of *C pneumoniae* suspension via the nasal turbinates with a plastic catheter under light anesthesia using titrated intramuscular doses of ketamine. Three separate inoculations were performed at ≈ 3 -week intervals (average, 20 ± 1 [SE] days). Three days after final inoculation, 10 rabbits were randomized to a 7-week course of azithromycin. For the first week, a daily intramuscular injection of 30 mg/kg aqueous azithromycin (Pfizer Corp) was given. For the remaining 6 weeks, twice-weekly intramuscular injections of 30 mg/kg azithromycin were given.

Similarly, 10 control rabbits were intranasally inoculated three times at 2- to 3-week intervals with 1 mL of normal saline. Thereafter, 5 of the controls were randomized to the same 7-week course of azithromycin.

Three months after final intranasal inoculation (at 132 [SE, 6] days of study), all rabbits were euthanized. The aortas were removed, refrigerated, and sent for pathological evaluation.

Pathological Investigations

Aortic specimens were grossly inspected. Representative cross sections of thoracic and abdominal aortas were removed from each sample. Adjacent sections were submitted for either routine histological evaluation or frozen section staining for direct immunofluorescence. Histological specimens were fixed in 10% buffered formalin, paraffin imbedded, and stained with hematoxylin and eosin. The histological sections were evaluated by an experienced pathologist blinded to infection/treatment group. Quantitative evaluation was performed with the use of an Olympus BH-2 microscope equipped with an eyepiece micrometer. The degree of intimal atheromatous involvement was determined by use of three prespecified measures: (1) maximal intimal thickness (MIT), defined as the maximal measurement from the luminal surface to the internal elastic lamina of the vessel wall; (2) percentage of luminal circumference involved (PLCI) with atheroma (this measurement was determined by visual pathological estimate); and (3) plaque area index (PAI). This was defined as the product of MIT and PLCI.

Immunofluorescence

Specimens for immunofluorescence were frozen in optimal cutting temperature medium, cut at $4 \mu\text{m}$ onto glass slides, and air dried. After 30 minutes, direct immunofluorescence was performed with a prediluted monoclonal antibody (Baxter Scientific). This mouse monoclonal antibody is directed at a 3000-D lipoprotein common to all *Chlamydia* species (ie, *trachomatis*, *psittaci*, and *pneumoniae*). The antibody has been directly conjugated with fluorescein isothiocyanate. The genus-specific antibody was chosen over the species specific (*C pneumoniae*) because of its greater sensitivity.¹⁴ Slides were washed in PBS, incubated with the antibody for 30 minutes in a moist chamber at room temperature, and washed three times in PBS before cover slipping in aquamount. Slides were examined by use of an Olympus microscope equipped with epifluorescence and filters configured to detect fluorescein isothiocyanate. Positive and negative controls were run with each batch of slides. These consisted of the antigen controls received with the antibody, which were monkey kidney cells infected and uninfected with chlamydiae. Elementary bodies of chlamydiae fluoresce apple green and measure 0.35 to $0.45 \mu\text{m}$. Specimens were considered positive if any appropriately sized fluorescent elementary bodies were detected. The pathologist was blinded to treatment groups.

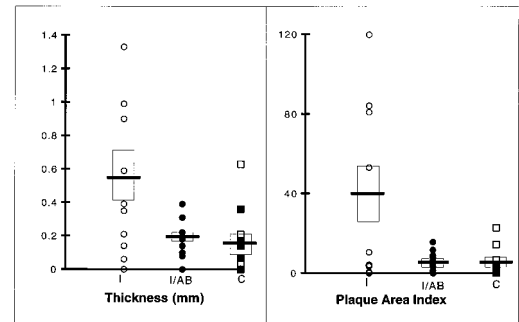


Figure 1. Indexes of aortic atheromatous involvement by infection and treatment group. Left, maximal intimal thickness in millimeters. Right, plaque area index, ie, maximal intimal thickness times plaque luminal circumference involved. I indicates infected; AB, antibiotic treated; and C, control. Individual data points (one per animal) are presented with group means and SEs. Circles represent infected animals, squares represent controls. Filled symbols represent animals treated with antibiotic, open symbols those that were not.

Statistics

MIT, PLCI, and PAI are expressed as mean \pm SE for the different treatment groups. MIT was prospectively selected as the primary end point, with PLCI and PAI as secondary end points. Differences among the three treatment groups were evaluated by ANOVA, followed by pairwise Student-Newman-Keuls testing (SPSS version 6.1). (Similar results were obtained using nonparametric Kruskal-Wallis testing, eg, $P = .032$ for MIT.) Immunofluorescence results are presented as simple proportions.

Results

Twenty-nine rabbits survived and could be evaluated, and 1 (in the infection/no treatment group) died under anesthesia. Fig 1 shows the results of intimal thickening measurements by infection/treatment group. Qualitatively, the lesions were uniformly characteristic of atheromas, with varying proportions of foamy cells, spindle (smooth muscle) cells, and extracellular matrix. The degree of MIT varied among the three treatment groups ($P = .009$ by ANOVA): MIT was increased in infected rabbits (0.55 mm [SE, 0.15 mm]) compared with uninfected controls (0.16 mm [SE, 0.06 mm]) and with infected rabbits receiving antibiotics (0.20 mm [SE, 0.03 mm]) (both $P < .025$ by Student-Newman-Keuls test), whereas MIT in infected/treated versus control rabbits did not differ. Response to infection varied among individual animals, with 4 of 10 showing prominent atherogenesis, indicating variability in success of infection or host response. PLCI tended to differ ($P = .1$) and PAI significantly differed ($P = .01$) among the three treatment groups: PLCI averaged 50% (SE, 12%) in infected/untreated animals versus 22% (SE, 8%) in controls and 32% (SE, 5%) in infected/treated animals. PAI averaged 40% (SE, 15%) in infected/untreated animals versus 5.0% (SE, 2.4%) in controls and 6.4% (SE, 1.5%) in infected/treated animals (both $P < .05$ versus infected/untreated animals). There was no difference between controls given antibiotics versus those given no antibiotics in these indexes ($P = .43$) (Fig 1). Photomicrographs of representative specimens from infected, infected/treated, and control animals are shown in Fig 2.

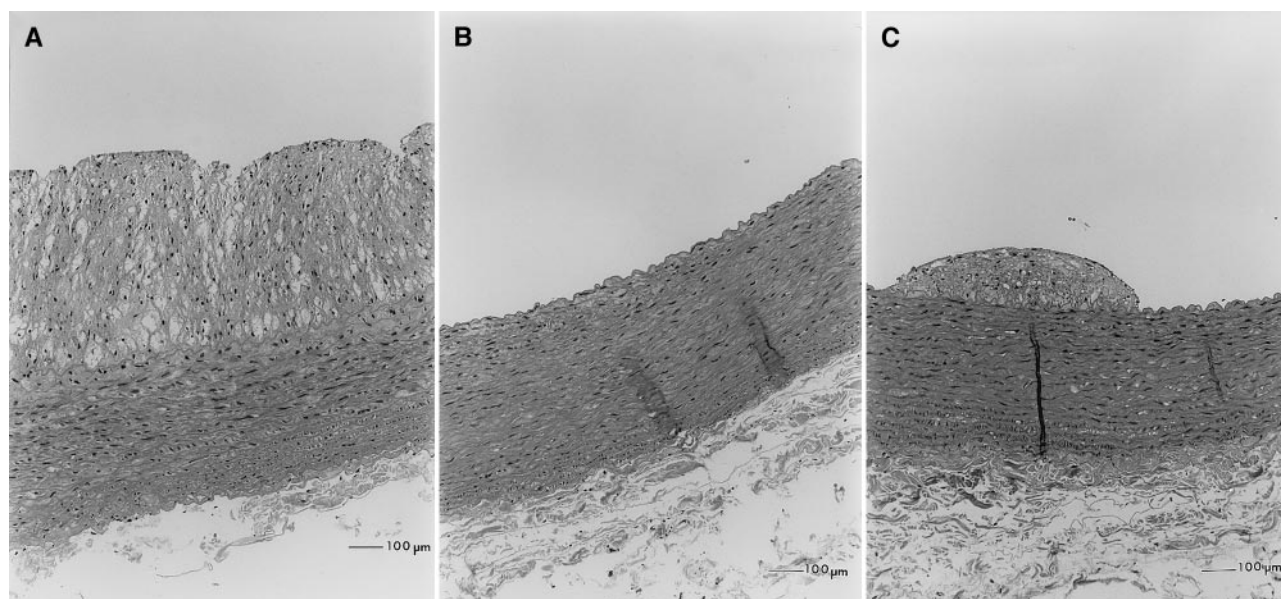


Figure 2. Photomicrographs of representative aortic sections from animals in the infected/untreated group (A); control (uninfected/untreated) (B); and infected/treated group (C). Hematoxylin and eosin–stained section, original magnification $\times 100$.

Chlamydial antigen was detected in 2 of 9 infected/untreated, 3 of 10 infected/treated, and 0 of 10 uninfected animals (differences not significant). Although not statistically significant, rabbits positive for chlamydial antigen tended to show an increased MIT, regardless of treatment group (MIT=0.54 mm, antigen positive; MIT=0.30 mm, antigen negative; $P=.2$). No association between age or weight of the rabbits and MIT was found.

Discussion

Study Summary

This study confirms and quantifies the capability of intranasal infection with *C pneumoniae* to accelerate atherosclerosis in a rabbit model. Additionally, it demonstrates the ability of azithromycin to prevent this accelerated process. These findings are best explained by assigning a causative role to *C pneumoniae* in the atherosclerotic process and a preventive or therapeutic role to azithromycin. The degree of atherosclerosis acceleration varied among individual animals, suggesting a variability in the success of establishing persistent infection or in host response to infection.

Vascular chlamydial antigen was not a useful quantitative marker of atheromatous effect in our model; it occurred in a small proportion of both treated and untreated animals and was not found in uninfected controls. Thus, infection-related atherosclerosis may occur in the absence of detectable local vascular antigen, and antibiotic therapy may not immediately eliminate chlamydial antigen.

Study Strengths and Limitations

This study was larger and more successful than a previous investigation¹¹ in demonstrating the potential of chlamydial infection to accelerate atherosclerosis. We attribute this to the multiple inoculations and to dietary cholesterol supplementation. Also, the design used randomization and blind-

ing to ensure objectivity. Importantly, the study showed that azithromycin can limit the atherogenic effects of chlamydial infection.

The number of animals was adequate for hypothesis testing, but additional observations will be welcome. The cellular and molecular mechanisms by which *C pneumoniae* accelerates atherosclerosis were not defined. Also, our data are too limited to exclude a small antiatheromatous effect of azithromycin apart from its antichlamydial actions. Whether *C pneumoniae* can cause atherosclerosis in the absence of cholesterol feeding was not addressed. The optimal dose and duration of antibiotic therapy are unknown, and further dose-ranging studies would be useful. However, the azithromycin regimen was selected to achieve dose concentrations clinically effective against chlamydiae and for a duration believed to suppress or eliminate chronic, persistent, and acute infection. Finally, it must be remembered that the rabbit model of atherosclerosis is not identical to that of human disease, and any extrapolations must be made with caution and confirmed by clinical studies.

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