Chlamydia pneumoniae in Atherosclerotic Middle Cerebral Artery

Dezso Virok, MD; Zoltan Kis, MPH; Laszlo Karai, MD; Laszlo Intzedy, MD; Katalin Burian, MD; Arpad Szabo, MD; Bela Ivanyi, MD, PhD; Eva Gonczol, MD, PhD

Background and Purpose—Atherosclerotic middle cerebral arteries are frequent sites of thrombosis, leading to stroke. Previous studies have suggested a role for *Chlamydia pneumoniae* in the pathogenesis of atherosclerosis. However, the presence of this pathogen in atherosclerotic middle cerebral arteries has heretofore not been documented. In the present study, we analyzed atheromatous plaques from middle cerebral arteries for the presence of *C pneumoniae*.

Methods—Atherosclerotic middle cerebral arteries from 15 cadavers who died of natural causes and corresponding nonatherosclerotic arteries from 4 otherwise healthy trauma victims were examined. Assays for *C pneumoniae* DNA were carried out by nested polymerase chain reaction (nPCR) specific for the *C pneumoniae* ompA gene. The presence of the bacterium was assessed by transmission electron microscopy.

Results—Five of the 15 atherosclerotic arterial samples and none of the control tissues were positive for *C pneumoniae* by nPCR. Particles similar in morphology and size to *C pneumoniae* elementary bodies were detected by transmission electron microscopy in 4 of the 5 nPCR-positive atherosclerotic samples.

Conclusions—The demonstration of *C pneumoniae* in atherosclerotic middle cerebral arteries is consistent with the hypothesis that this bacterium is involved in acute and chronic cerebrovascular diseases. **(*Stroke*. 2001;32:1973-1978.)**

Key Words: atherosclerosis ■ *C pneumoniae* ■ cerebrovascular disorders

Atherosclerosis is a multifactorial disease. The various explanations of the pathogenic process include chronic infection with certain pathogens. The microorganism most strongly implicated in the initiation/progression of atherosclerosis is the obligate intracellular bacterium *Chlamydia pneumoniae*, which commonly causes respiratory infections.

Evidence for a possible link between *C pneumoniae* infection and atherosclerosis at different vascular sites has come from seroepidemiology, analysis by polymerase chain reaction (PCR), electron microscopy, in situ hybridization, immunohistochemistry, culturing, and animal models.[1–4] However, the association of chronic *C pneumoniae* infection and cerebrovascular diseases has not been well investigated. Case-control studies revealed that specific anti–*C pneumoniae* antibody levels were significantly higher in patients with cerebrovascular disease than in control patients,[5–7] and a follow-up study indicated that high antibody titers to *C pneumoniae* were associated with an increased risk of future stroke.[8] Immunoreactivity to *C pneumoniae*–specific antigen was recently demonstrated in a low percentage of anterior and posterior cerebral arteries but not in middle cerebral arteries.[9]

The middle cerebral artery and internal carotid artery are frequent sites of thrombosis leading to stroke. One of the most important factors in the development of local thrombosis is the underlying atherosclerosis of the vessel wall. In the present study, we used nested PCR (nPCR) and transmission electron microscopy (TEM) to examine samples of middle cerebral arteries with atheromatous plaques and also samples of nondiseased vessels for the presence of *C pneumoniae*.

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Subjects and Methods

Atherosclerotic samples of middle cerebral arteries were obtained from 15 consecutively autopsied subjects. Samples were collected within 24 hours after death with the use of sterile instruments. Half of each sample was frozen at −70°C for nPCR analysis; the other half was fixed in 3% glutaraldehyde for TEM and histology. For control tissues, samples of 4 nonatherosclerotic middle cerebral arteries were collected from trauma victims who died (at ages 31 to 40) during the study period. Histological assessment of the vessel samples from the 15 patients indicated moderate or severe atherosclerotic stenosis. The control samples from the trauma victims were assessed as histologically normal. The study was approved by an institutional review committee.

DNA was extracted from frozen samples with the High Pure PCR Template Preparation Kit (Boehringer-Roche) according to the manufacturer's instructions. Samples from cases or controls were tested in a blinded fashion for *C pneumoniae* DNA with a GeneApm 2400 PCR system (Perkin-Elmer) with the use of nPCR primer pairs

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From the Department of Medical Microbiology (D.V., Z.K., K.B., E.G.), the Department of Pathology (L.K., L.I., B.I.), and the Department of Forensic Medicine (A.S.), University of Szeged, Szeged, Hungary, and The Wistar Institute (E.G.), Philadelphia, Pa.

Correspondence to Eva Gonczol, MD, PhD, Department of Medical Microbiology, University of Szeged, Dom ter 10, H-6720 Szeged, Hungary. E-mail gonczol@comser.szote.u-szeged.hu

***Stroke* is available at http://www.strokeaha.org**

Patient Characteristics Reviewed From Autopsy Records

Patient	Sex	Age, y	*C pneumoniae* PCR	Organ Manifestations of AT	Diseases Other Than AT	Cause of Death
1	M	95	+	CI, IHD, severe in aorta	Bilateral chronic pyelonephritis	Pneumonia
2	M	85	+	CI, severe in aorta, renovascular hypertension	Duodenal ulcer	Pneumonia
3	M	83	+	CI, IHD, severe in aorta	Cirrhosis of liver	Cardiac failure
4	F	76	+	IHD, severe in aorta and cerebral arteries	Essential hypertension	Sepsis
5	M	78	+	Mild in aorta and cerebral vessels	Chronic lymphoid leukemia	Purulent bronchiolitis
6	M	73	−	IHD, severe in aorta, gangrene of legs	Bilateral chronic pyelonephritis	Pneumonia
7	M	84	−	IHD, mild in aorta and cerebral arteries	Essential hypertension	Pneumonia
8	M	73	−	CI, IHD, moderate in aorta	Essential hypertension	Pneumonia
9	F	92	−	CI, IHD, atherosclerotic aneurysm in aorta	Bilateral chronic pyelonephritis	AMI
10	F	85	−	IHD, severe in aorta and moderate in cerebral vessels, mesenteric superior artery thrombosis	Essential hypertension	Small bowel infarction
11	F	90	−	IHD, severe in aorta and cerebral vessels	Perforated gastric ulcer	Peritonitis
12	M	70	−	IHD, severe in aorta and cerebral vessels	Diabetes, essential hypertension	Pneumonia
13	M	66	−	Moderate in aorta and cerebral vessels	Diabetes	Bilateral acute pyelonephritis
14	M	71	−	IHD, severe in aorta and cerebral vessels	Disseminated lung cancer	Pneumonia
15	M	59	−	IHD, severe in aorta and cerebral vessels, gangrene of leg	Hypopharynx cancer	Pneumonia

AT indicates atherosclerosis; M, male; F, female; CI, cerebral infarct; IHD, ischemic heart disease (critical stenoses in coronaries with or without microscopic foci of myocardial fibrosis and/or with or without myocardial scar); and AMI, acute myocardial infarction.

specific for the *C pneumoniae* ompA gene,[10] resulting in a 206-bp nPCR fragment. The presence of intact DNA was tested for each sample with the use of primers specific for the human β-actin gene. DNA extracted from the lysate of *C pneumoniae* (strain TWAR)–infected McCoy cells (both from American Type Culture Collection) was used as a positive control. The negative control was sterile distilled water subjected to the same extraction procedure as used for the tissue samples. For every set of 5 tested samples, nPCR including the negative control template was carried out. Strict precautions were taken to avoid contamination during DNA extraction and the preparation of the reaction mixture. Problems and limitations of PCR were considered as suggested.[11,12]

DNA samples amplified by the *C pneumoniae* primers were sequenced with the ABI Prism DNA Sequencing Ready Detection Kit (Perkin-Elmer). Fixed samples of the 5 *C pneumoniae* nPCR-positive arteries and also the 5 *C pneumoniae* nPCR-negative samples were postfixed in OsO_4 and embedded in Epon. Thin sections stained with uranyl acetate and lead citrate were examined by TEM. McCoy cells infected with *C pneumoniae* were treated similarly for morphological comparison.

Results

The patient characteristics reviewed from the autopsy records and the nPCR results are listed in the Table. *C pneumoniae* DNA was amplified in 5 of the 15 atherosclerotic samples, as demonstrated by a 206-bp DNA fragment visualized by agarose gel electrophoresis, whereas none of the 4 arterial samples from healthy trauma victims were nPCR positive (Figure 1). Sequencing of nPCR fragments from 2 of the 5 atherosclerotic samples revealed identity to the ompA sequences obtained from the National Center for Biotechnology Information (NCBI) database (which can be accessed online at http://www.ncbi.nlm.nih.gov). Three of the 5 nPCR-positive cases had symptomatic cerebrovascular disease (cerebral infarct), whereas only 2 of the 10 nPCR-negative cases had symptomatic cerebrovascular disease. The direct cause of death was related to infectious respiratory diseases in 9 of the 15 cases; there was uniform distribution among nPCR-positive and -negative individuals (Table).

TEM of intimal plaques showed structures resembling *C pneumoniae* elementary bodies in 4 of the 5 nPCR-positive atherosclerotic arterial samples. These structures had a pear-shaped appearance with a dense core (Figures 2A and 2B) and were $\approx$0.3 μm in diameter, ie, similar to the size of the elementary bodies detected in control infected tissue culture cells (Figure 2C). None of the 5 nPCR-negative atherosclerotic samples examined by TEM exhibited *C pneumoniae*–like structures.

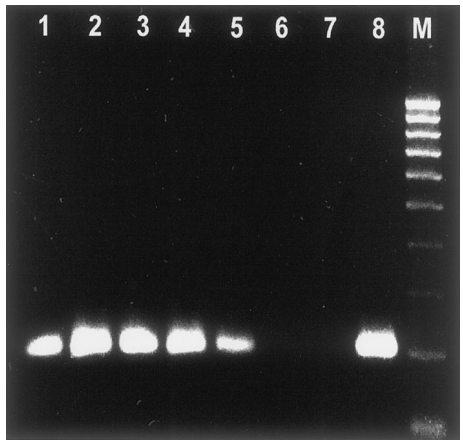

Figure 1. nPCR amplification of *C pneumoniae* ompA gene. Lanes are as follows: 1 to 5, nPCR-positive atherosclerotic samples (206-bp fragments); 6, nPCR-negative sample; 7, negative control; and 8, positive control. M indicates molecular size marker (100-bp DNA ladder, Sigma).

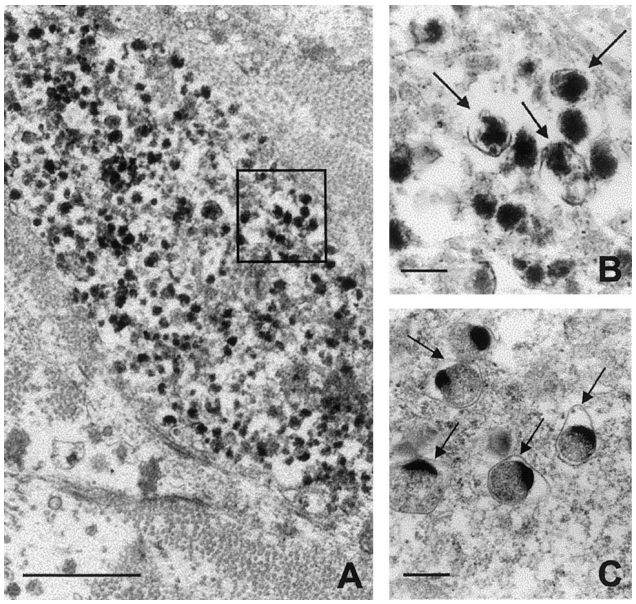

Figure 2. A, TEM of an aggregate of dense bodies in an intimal atheromatous plaque. The very close accumulation of the particles suggests an intracellular localization. The boxed area is shown in panel B. Bar=3.0 μm. B, Pear-shaped structures ≈0.3 μm in diameter and similar to *C pneumoniae* elementary bodies (arrows). Bar=0.3 μm. C, *C pneumoniae* elementary bodies (arrows) in a McCoy cell at 48 hours after infection. Bar=0.3 μm.

Discussion

C pneumoniae has several features that may lead to the chronic infection of vessel walls and ultimately to atherosclerosis with local thrombosis: the bacterium replicates in vitro in endothelial and smooth muscle cells and macrophages, induces the expression of adhesion molecules, elevates the levels of platelet adhesion and procoagulant activity in endothelial cells, and induces the production of cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6 in monocytes.[13–16]

C pneumoniae has been detected in atherosclerotic tissues at different sites of the vascular system, including the carotid and coronary arteries, the aortic valves, the aorta, and arteries in the lower extremities. A summary of the results from 23 studies indicated that *C pneumoniae* was detected in 52% (257 of 497) of diseased arteries and in 5% of control vessels by immunohistochemistry or PCR.[3] A recent study on the prevalence of *C pneumoniae* antigens at multiple locations in the arterial system within the same individual demonstrated a high prevalence of immunoreactivity in the abdominal aorta, iliac arteries, and coronary arteries and a low prevalence in cerebral anterior and posterior arteries but no immunoreactivity in samples of middle cerebral arteries.[9] We detected the presence of *C pneumoniae* DNA in 33% of the diseased middle cerebral arterial samples by nPCR. A review of the autopsy records revealed cerebral infarct in 3 of the 5 nPCR-positive cases, in contrast with only 2 of the 10 nPCR-negative cases. However, the small number of cases did not permit a statistical comparison. A further limitation of the present study is that the detectability of *C pneumoniae* DNA in the cerebrovascular vessels might be related to age. Because of the younger age of the control individuals and the small numbers of nPCR-positive and -negative cases, statistical comparison is not helpful, and this possibility cannot be excluded. The present study has demonstrated the presence of *C pneumoniae* DNA in some atherosclerotic samples from middle cerebral arteries, but further studies appear desirable to test for the presence of this organism in these vessels in relation to age and the occurrence of symptomatic cerebrovascular diseases. Because there was no difference in the incidence of respiratory diseases as the direct cause of death among the nPCR-positive and -negative cases, it is improbable that *C pneumoniae* nPCR positivity was secondary to the infectious diseases, which are rather considered to be terminal-stage diseases that developed a few days before death.

A commercial kit for the molecular detection of *C pneumoniae* is not available, but the considerations applied in our work suggest that our PCR assay is appropriate.[10–12]

The detection of pear-shaped structures in *C pneumoniae* nPCR-positive atherosclerotic samples by TEM suggests that not only was bacterial DNA present but that the complete pathogen was also present in the intimal plaque. A striking aggregation of dense particles indicates an intracellular accumulation of these structures. The additional presence of this microorganism extracellularly suggests either spontaneous autolysis of the cells followed by bacterial flow into the extracellular matrix or the accumulation of these pathogens outside the cell during a certain stage of their life cycle. *C pneumoniae* organisms were earlier found by electron microscopy in cells and interstitially in atherosclerotic lesions of the aorta or carotid or coronary arteries but not in the adjacent nonatherosclerotic tissue.[17]

The middle cerebral arteries are important sites of cerebral thrombosis. The presence of *C pneumoniae* in the atheromatous plaques of the middle cerebral artery does not necessarily mean that the organism is a causative agent of the disease; it rather raises the possibility of a role for this bacterium in the pathogenic process. Such a role would point to the value of antibiotic treatment as a means of attenuating cerebrovascular diseases relating to *C pneumoniae*. Tests on a larger number of cases and age- and sex-matched controls appear warranted, extending to the *C pneumoniae* serostatus and the presence of *C pneumoniae* DNA and antigens in vessels other than middle cerebral arteries in the same subjects.

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Editorial Comment

Chlamydia pneumoniae: In Your Heart and on Your Mind

Infections have long been recognized as causes of cerebrovascular disease. Infectious endocarditis, meningovascular syphilis, and varicella zoster virus–associated vasculitis are rare but well-known infectious syndromes of stroke. In the past decade, interest in the role of more common pathogens in the pathophysiology of atherosclerosis and stroke has grown. The best-studied of these infections is *Chlamydia pneumoniae*, but there is a growing body of literature implicating other organisms, including cytomegalovirus, *Helicobacter pylori*, and the multitude of bacteria that participate in periodontitis.

It is now widely believed that atherosclerosis is predominantly an inflammatory condition produced by a "response to injury."[1] A number of toxic stimuli can lead to endothelial injury, resulting in a cascade of events, culminating in smooth muscle cell proliferation and fibrous plaque formation. Oxidized LDL is a well-recognized cause of this endothelial injury, but homocysteine, toxic constituents of cigarette smoke, and high shear force have also been implicated. Infectious agents are hypothesized to be one more potential source of injury.

Since the original observations in 1988 that the prevalence of antibodies directed against *C pneumoniae* is higher among those with coronary artery disease than among controls,[2] a number of retrospective and prospective epidemiological studies have found an association of serological evidence of *C pneumoniae* infection and coronary disease risk. A smaller number of studies have found similar associations for risk of ischemic stroke,[3–6] though not all studies have been positive.[7] The conclusions drawn from these studies, however, have been limited by methodological considerations, including the heterogeneity of stroke subtypes enrolled and the absence of well-standardized tests for *C pneumoniae*. Although some data suggest that specific antibody isotypes, such as immunoglobulin A antibodies, may be more specific for the chronic infection thought to be associated with atherosclerotic disease,[3,6] this conjecture has not yet been validated. More recent efforts using PCR of peripheral blood mononuclear cells in larger populations may help resolve these epidemiological issues, although methodological concerns plague these studies as well.

Ultimately, however, because analytic observational studies cannot provide proof that infection with *C pneumoniae* causes atherosclerosis, investigators have relied on parallel areas of clinical and basic investigation, including pathology, in vitro and animal models, and clinical trials. The article by Virok et al addresses the pathology and thus the biological plausibility of the role of infection in cerebrovascular disease. The authors used nested PCR to assess the presence of *C pneumoniae* infection in middle cerebral arteries (MCAs) taken at autopsy from 15 individuals with atherosclerosis of the MCA and 4 healthy young individuals who died of traumatic causes. Histologically, the case subjects all had moderate or severe MCA atherosclerosis, while the MCA was normal in all the control subjects. Five of the 15 case specimens showed evidence of *C pneumoniae* DNA, whereas none of the control samples did. Moreover, 3 of the 5 polymerase chain reaction (PCR)-positive cases (60%) had symptomatic disease, whereas only 2 of the 10 PCR-negative cases (20%) were symptomatic. Electron microscopy, furthermore, demonstrated presence of *C pneumoniae* in 4 of 5 of the PCR-positive specimens.

While this study is limited by small numbers, it nonetheless offers evidence that *C pneumoniae* can be found in cerebral blood vessels. Because the controls selected were relatively young, however, it is possible that the presence of the

organism simply reflects age; it would have been ideal to have had age-matched controls without atherosclerosis. Still, the organism appears to have been found more often in the atherosclerotic cerebral vessels.

Discovery of *C pneumoniae* DNA by PCR in tissue from the MCA is not surprising. Several studies have found evidence of *C pneumoniae* in arterial tissue taken from sites throughout the body, including the coronary arteries,[8] aorta[9] and femoral[10] arteries. Closer to the brain, *C pneumoniae* has been identified using PCR and immunohistochemical techniques in carotid atherosclerotic tissue taken from endarterectomy specimens. The organism, notoriously difficult to culture, has also been cultured from the carotid artery.[11] A 1997 review[12] of studies of *C pneumoniae* in atherosclerotic tissue found that 257 of 495 samples of atheromatous tissue (52%) were positive for *C pneumonia*, while only 6 of 118 nonatheromatous specimens (5%) were positive. A more recent autopsy study[13] sampled 33 arterial sites throughout the body of each of 24 elderly individuals and found *C pneumoniae* immunostaining in at least one artery in all subjects. Almost all arteries were affected in at least some individuals, although the prevalence was greatest in the abdominal aorta, iliac arteries, and coronary arteries. Importantly, the prevalence was highest at sites of greatest luminal stenosis, and in sites typically affected clinically. The organism was found in only 2% of all large cerebral vessels in that study, however, compared with 33% of coronary vessels, and not in any of the MCA specimens. The present study is thus the first to find *C pneumoniae* in MCA tissue. It is of interest that the organism was found so much more commonly in the MCA tissue in the study by Virok et al, but this may simply reflect the different patient population, which in their sample included 15 patients with moderate or severe MCA stenosis.

Of course, the presence of *C pneumoniae* in arterial tissue cannot itself establish this, or any other organism, as a causative agent in atherosclerosis. Lipid-rich atherosclerotic tissue may simply be an attractive resting ground for the organisms, or they may be brought in by circulating macrophages entering the developing plaque. *C pneumoniae*, according to this critique, is simply an "innocent bystander," and not involved directly in the pathogenic process. A small but growing body of experimental and other animal evidence is available to support the pathogenic role of *C pneumoniae* in atherosclerosis, however. The presence of chlamydial lipopolysaccharide, for example, facilitates conversion of macrophages to foam cells and increases oxidative metabolism of LDL, potentially damaging endothelium.[14] *C pneumoniae* has also been shown in vitro to induce human peripheral blood monocytes to secrete proinflammatory cytokines known to participate in the atherosclerotic process.[1,15] In experimental models, rabbits inoculated with *C pneumoniae* develop atherosclerotic lesions while controls do not,[16] and azithromycin, a macrolide antibiotic active against chlamydiae, retards this process.[17]

There is also evidence to suggest that some of the adverse effects of infections on atherosclerosis could be caused indirectly by immunological mechanisms, without the persistence of organism itself in the vessel wall—what has been called a "hit and run" effect.[18] In rats undergoing arterial balloon injury and infected with rat cytomegalovirus, for example, arterial wall thickening progresses even after infection is completed and cytomegalovirus DNA is no longer detectable in the vessel.[19]

Currently, several large-scale clinical trials are ongoing among coronary disease patients to assess whether antibiotic therapy can prevent recurrent events. Atherosclerosis, however, has several logically distinct, if continuous, phases, including atherogenesis, progression, and plaque rupture, the most common precipitant of an acute event. Different mechanisms may be operative in each of these phases. A trial directed at reducing clinical events, therefore, even if negative, cannot prove that infectious agents are not involved in the earlier processes of atherogenesis or progression.

No clinical trials in stroke have yet been initiated. It is important to remember, however, that while the term "brain attack" has heuristic value, a stroke is not simply a "heart attack" of the brain. Again, even if negative, trials in heart disease cannot definitively answer the question of the association of *C pneumoniae* and stroke. The etiologies of stroke are more heterogeneous than those of coronary artery disease, and it is possible that there are differential effects on the two. Recent data from the National Health and Nutrition Examination Surveys, for instance, suggest an association of periodontal disease with stroke but not heart disease.[20,21] The study by Virok et al, then, is important in being the first to focus on the presence of this organism in the brain's vessels and thus in its recognition that research in stroke should begin with an investigation of the brain's arteries, and not simply those elsewhere in the body. Further studies of the prevalence of this organism in brain arteries and its clinical consequences are needed.

Mitchell S. Elkind, MD, *Guest Editor*
Department of Neurology
Columbia University
New York, New York

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