Serum Antibodies Against *Chlamydia pneumoniae* Outer Membrane Protein Cross-React With the Heavy Chain of Immunoglobulin in the Wall of Abdominal Aortic Aneurysms

Jes S. Lindholt, MD, PhD; Jette Støvring, MD; Lars Østergaard, MD; Sigitas Urbanavicius, MD; Eskild W. Henneberg, MD; Bent Honoré, MD; Henrik Vorum, MD, PhD

**Background**—*Chlamydia pneumoniae* (*Cp*) has been demonstrated in arteries and abdominal aortic aneurysms (AAAs). However, the validity of the methods used is questioned, and antibiotic treatment trials have thus far shown disappointing results. Nevertheless, antibodies against the *Cp* outer membrane proteins (OMPs) have been associated with progression of atherosclerosis and AAAs. The aim of this study was to detect *Cp* OMPs in the wall of AAA patients by use of purified serum antibodies directed against *Cp* OMP and to assess potential cross-reacting proteins in AAA walls.

**Methods and Results**—Seventeen patients undergoing infrarenal AAA repair were studied. Full AAA thickness tissue was collected from the anterior wall of the aneurysm. Anti-OMP was extracted from seropositive AAA patients by use of an ELISA kit (Labsystems). Analysis was performed by use of 2D polyacrylamide gel electrophoresis, immunoblotting, and mass spectrometric protein identification. OMP antigens were not detected in 16 of 17 AAA walls. However, 3 major AAA proteins cross-reacted with anti-OMP. The proteins were all identified as heavy chains of human immunoglobulin.

**Conclusions**—We could not find evidence of *Cp* OMP in 16 of 17 AAA walls, but instead, all samples showed a strong cross-reaction between *Cp* OMP antibodies and human immunoglobulin. This might indicate that AAA is an autoimmune disease, perhaps triggered by an initial *Cp* infection. (*Circulation. 2004;109:2097-2102.)*

**Key Words:** aneurysm ■ antibodies ■ *Chlamydia pneumoniae* ■ pathogenesis ■ inflammation

Antibodies against *Chlamydia pneumoniae* (*Cp*) have been associated with atherosclerosis, acute myocardial infarction, and expansion of abdominal aortic aneurysms (AAAs).

However, the validity of the methods used is questioned, and antibiotic treatment trials have thus far shown disappointing results. Nevertheless, antibodies against the *Cp* outer membrane proteins (OMPs) have been associated with progression of atherosclerosis and AAAs. The aim of this study was to detect *Cp* OMPs in the wall of AAA patients by use of purified serum antibodies directed against *Cp* OMP and to assess potential cross-reacting proteins in AAA walls.

Methods

Isolation of Specific Antibodies Against the OMP of *Cp* From Patient Sera

Sera were taken from 5 patients with an AAA, an IgA titer >64, and an IgG titer >128. The patients had taken part in one of our previous studies on the correlation between the progression of lower-limb atherosclerosis, the natural history of AAAs, and antibodies against *Cp*. The sera were applied to a new commercially available ELISA test from Labsystems (LOY-EIA). The LOY-EIAs for *Cp* IgG and IgA are indirect solid-phase enzyme immunoassays with *Cp* OMP as immobilized antigen. Isolation and purification of specific antibod-
ies against CpfOMP were as follows: 100 μL of serum sample was diluted 1:2 in PBS buffer (in mmol/L: 2.7 KCl, 1.8 KH₂PO₄, 10.1 Na₂HPO₄, 140 NaCl, pH 7.4) and added to the wells coated with CpfOMP. The plates were incubated at 37°C for 1 hour under gentle shaking and washed 10 times with PBS. The purified antibodies were eluted by addition of 100 μL elution buffer (0.1 mol/L glycine, pH 3.0) to each well. The eluates were collected and quickly neutralized to pH 7.4 with 1 mol/L Trizma base, pH 9.0.

AAA Samples
AAA wall samples were studied in 17 male patients undergoing infrarenal AAA repair at the Department of Vascular Surgery, Viborg Hospital, Denmark. Data on age, smoking, and hypertension are shown in Table 1. Their mean age was 71.9 years (range, 46 to 85 years). The maximal cross-sectional diameter of the AAA was measured by ultrasound and/or CT scanning performed immediately before the operation.

The study was approved by the local scientific ethics committee and reported to the data protection authorities. All patients gave informed consent.

Full AAA thickness tissue was harvested in a standardized manner from the central part of the anterior wall of the aneurysm and stored immediately at −22°C. Approximately 1 g was rinsed 3 times with PBS for 10 minutes. The specimens were crushed in 1 mL cold sucrose buffer (300 mmol/L sucrose, 10 mmol/L Tris, pH 7.4) containing the protease inhibitors 1 mmol/L Na-EDTA, 1 mmol/L PMSF, 1 μmol/L pepstatin, and 1 μmol/L leupeptin with an Ultra-Turax homogenizer. Cell debris was removed by centrifugation (14 000 g for 20 minutes). Clear protein solutions were used for subsequent gel electrophoresis.

Gel Electrophoresis and Immunoblotting
The total AAA protein content of each sample was estimated by Bio-Rad Protein Assay according to the method of Bradford. For 1D gels, ∼25 μg of prepared sample was loaded in each well and run on 10% to 20% gradient polyacrylamide minigels (NOVEX). For 2D gels, the serum was dissolved in lysis buffer. Rehydration buffer was added, and the first dimension was run with immobilized pH-gradient (IPG) strips (pH 3 to 10, non-linear) from Amersham Biosciences. The second dimension was performed on homemade gradient (12% T, 3% C) that were fixed in 50% (vol/vol) methanol for at least 1 hour or overnight. They were then washed 3 times with 12% acetic acid (vol/vol) lysis buffer. Rehydration buffer was added, and the first dimension was run with immobilized pH-gradient (IPG) strips (pH 3 to 10, non-linear) from Amersham Biosciences. The second dimension was performed on homemade gradient (12% T, 3% C) that were fixed in 50% (vol/vol) methanol, 12% (vol/vol) acetic acid, and 0.0185% (vol/vol) formaldehyde for at least 1 hour or overnight. They were then washed 3 times for 20 minutes in 35% (vol/vol) ethanol, pretreated for 1 minute in pretreatment solution (0.02% [wt/vol] Na₂S₂O₅ · 5H₂O), and rinsed 2 times for 3 minutes in water. Staining of gels was performed for 20 minutes in 0.2% (wt/vol) AgNO₃, 0.028% (vol/vol) formaldehyde, after which they were rinsed 2 times for 20 seconds in water. Development was performed in development solution (6% [wt/vol] Na₂CO₃, 0.0185% [vol/vol] formaldehyde, 0.0004% [wt/vol] Na₂S₂O₅ · 5H₂O) for ~3 minutes, and was stopped in stop solution (50% [vol/vol] methanol, 12% [vol/vol] acetic acid). Finally, the gels were dried between cellophane sheets and sealed in plastic bags.

From 1D and 2D gels, proteins were transferred to nitrocellulose membranes that were blocked for 1 hour with 0.5% skim milk and 0.05% Tween-20 in PBS and incubated at room temperature for 1 hour with pure anti-OMP antibody (1:100) isolated from patient sera.
Western blot. These may be because of small amounts of aggregates of the recombinant protein or because of small amounts of impurities in the protein sample cross-reacting with the antibody. Immunolabeling controls performed by use of anti-OMP preabsorbed with the recombinant OMP exhibited no labeling (not shown).

We then investigated whether we could detect the presence of OMP in the AAA wall from 17 patients by use of the anti-OMP antibody. Western blots with AAA homogenate and anti-OMP antibody are shown in Figure 2. No specific reaction was seen at 40 kDa according to the molecular mass of OMP. Only patient 13 showed some reactivity at ≈40 kDa that we cannot completely exclude as being due to the presence of OMP. However, this is an exception, because generally a much stronger reaction (also from patient 13) was seen with cross-reacting proteins >50 kDa from all AAA patients. In addition, other less strongly reacting bands were noticed with still slightly higher molecular masses. The reaction was equally strong irrespective of age, current smoking, diagnosis of hypertension, and AAA size.

To identify the reacting proteins, we first performed a high-resolution purification of aortic wall proteins from 1 selected patient (patient 1) by 2D gel electrophoresis. One of the gels was Western blotted and reacted with the anti-OMP antibody (Figure 3A), and another gel was silver-stained and served as reference (Figure 3B). By aligning the blot to the silver-stained gel, 3 proteins with pI of ≈10 were cut out from the silver-stained gel (Figure 3C) to be identified by sequence determination by use of tandem mass spectrometry. The mass spectrometry analysis revealed the sequence of several peptides from each of the 3 proteins cut out. All peptides were found to be present in the heavy chain of human immunoglobulin (Table 2). It was specifically analyzed whether the proteins could originate from the Cp OMP protein. This was not the case.

**Discussion**

We isolated and affinity-purified anti-OMP antibody from sera of patients with AAAs. After verifying that the anti-OMP antibody had retained its activity for recognizing recombinant OMP, we used the antibody to analyze the aortic walls of 17 AAA patients by Western blotting. We found a positive reaction in the samples from all 17 patients. However, OMP could not be detected in the AAA wall, because no signal was seen at ≈40 kDa except in 1 sample (patient 13), in which several bands from 30 to 100 kDa were reacting, including a minor band at ≈40 kDa. However, this reaction is apparently very unspecific, because a much stronger band is found above 50 kDa. All the other samples also contained a number of cross-reacting proteins beyond 50 kDa.

Theoretically, these bands could arise from a 40-kDa protein that is modified, eg, glycosylated, or has reacted with a component of a matrix protein that adds 10 to 20 kDa to the protein. However, OMP synthesized in vivo forms large oligomeric structures stabilized by disulfide bonds, but it is expected to migrate in the monomeric form under electrophoresis, because the disulfide bonds will be disrupted by the addition of the reducing agent dithiothreitol to the samples before analysis. The monomer could still migrate with a molecular mass other than 40 kDa if it is modified in vivo by, eg, glycosylations. However, this does not seem to be the case, because native OMP, immunoprecipitated from infected HeLa cells, migrates as an ≈40-kDa band, indicating that the protein is not substantially modified in HeLa cells. Thus, it is expected that putative OMP also present in the AAA wall should migrate as a 40-kDa band.

In summary, to the best of our knowledge, there is no indication that such modifications take place. Furthermore, the 2D gel Western blots showed that the cross-reactivity at ≈50 to 60 kDa was not seen at the expected pI of OMP of ≈6 but instead at a pI of ≈10. In addition, the cross-reacting proteins were all identified by mass spectrometry sequencing as the heavy chain of immunoglobulin. Unfortunately, we did not have sera from the patients who had undergone surgery. Thus, the negative result with regard to the presence of OMP could be because we had examined the AAA walls only from seronegative patients. This is not very likely, however, because 62% to 83% of AAA patients are reported to be seropositive for Cp, depending on the definition. The fact that 16 walls examined showed the same pattern with no signs of OMP present, and in 1 sample perhaps only very faint amounts present, makes it very unlikely that we, by coincidence, examined walls only from seronegative patients.
The role of \textit{Cp} remains controversial; we have earlier failed to demonstrate the presence of \textit{Cp} in AAA walls by PCR methods and immunoblotting.\textsuperscript{19,28} The PCR test is a diagnostic method for \textit{Cp} infection, which claims to yield data of high sensitivity compared with other methods, including culturing. However, to validate the PCR technique between different laboratories, Apfalter\textsuperscript{16} showed that attempts to detect intravascular \textit{Cp} DNA directly varies between 0\% and 100\%. One reason for this discrepancy can be contamination of samples, because several laboratories have reported positive findings in the negative controls. Apparently, immunohistochemistry yields more positive results for \textit{Cp} than PCR techniques, and the method also has a poor correlation with PCR findings.\textsuperscript{29} However, we have demonstrated by Western blotting that positive signals might be a result of cross-reacting proteins.\textsuperscript{19}

In principle, the presence of cross-reacting proteins could be related to late colonization of the mural thrombus, but we have not been able to detect either \textit{Cp} OMPs or \textit{Cp} DNA in the mural thrombus.\textsuperscript{19,28} On the basis of the discrepancy of results regarding the presence of \textit{Cp} in AAAs and the methodological problems in direct detection of the organism, it may be that \textit{Cp} is not present in AAA walls at a late stage. This, however, does not exclude the possibility that it may have initiated the inflammatory reaction that subsequently leads to production of cross-reacting antibodies.

Conversely, Tambiah et al\textsuperscript{30} showed in an animal model that \textit{Cp} seems to stimulate the influx of macrophages and dilatation of the abdominal aorta. We have shown in earlier studies\textsuperscript{1,8} that AAA expansion was positively correlated with the presence of antibodies against \textit{Cp}. In the present study, we have shown that these antibodies react with the OMP protein and, in addition, shown that other proteins, possibly immunoglobulins, are cross-reacting with the anti-OMP antibody. The correlation between antibodies against \textit{Cp} and the progression of small AAAs strongly suggests a pathogenetic role of the protein that caused the antibodies to be produced. From this study, we cannot conclude that this protein was OMP or another protein with epitopes similar to OMP. The missing presence of OMP suggests that another protein may be involved. The demonstrated cross-reaction with immunoglobulins could indicate an autoimmune reaction, because antibodies reacting on antibodies are known only in autoimmune diseases.

This hypothesis is supported by histological examination of AAA walls, which shows the abundant presence of cells of chronic inflammation and Russell bodies, as in the autoimmune disease Hasimoto’s thyroiditis.\textsuperscript{31–33} Furthermore, cytokines that modulate the immune response and activate proteolysis,\textsuperscript{34} and substantial increases of IgG compared with aortic occlusive diseases and normal aortas,\textsuperscript{32} are found in AAA walls. An autoimmune reaction could be triggered by an initial \textit{Cp} infection, explaining the fact that the antibodies react with OMP and apparently cross-react with immunoglobulins. An autoimmune disease instead of an infective disease would have a dramatic impact on the choice of potential medical treatment, because antiinflammatory drugs rather than antibiotics might be effective.

In addition, the observations might indicate that this cross-reaction is unique for AAAs or a result of coexisting atherosclerosis, which also has been associated with chlamydial infection. Unfortunately, we did not harvest material from other tissues such as native vessels or occlusive atherosclerotic arteries. Future studies are directed toward such tissues and examining whether rheumatic factors are associated with AAAs and aneurysmal progression and whether rheumatic factors cross-react with OMP.

Acknowledgments
Financial support for this study was provided by the Danish Heart Foundation, the Foundation of Asta and Rosa Jensen, the Danish Medical Research Council, the University of Aarhus Research...
Foundation, the Novo Nordic Foundation, and the Health Department of Viborg County. The authors thank Kirsten Peterslund for expert technical assistance.

References


Serum Antibodies Against *Chlamydia pneumoniae* Outer Membrane Protein Cross-React With the Heavy Chain of Immunoglobulin in the Wall of Abdominal Aortic Aneurysms

Jes S. Lindholt, Jette Støvring, Lars Østergaard, Sigitas Urbonavicius, Eskild W. Henneberg, Bent Honoré and Henrik Vorum

*Circulation*. 2004;109:2097-2102; originally published online April 26, 2004; doi: 10.1161/01.CIR.0000127772.58427.7E

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2004 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circ.ahajournals.org/content/109/17/2097

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/