

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

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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).¹⁻⁸ Furthermore, *Cp* has been demonstrated in AAAs and in atherosclerotic lesions of coronary, carotid, and femoral arteries by a variety of tests, including immunohistochemical techniques, ELISA, the polymerase chain reaction (PCR), electron microscopy, and isolation in tissue culture.⁹⁻¹⁵ However, detection rates of *Cp* in atherosclerotic lesions by PCR vary between 0% and 100%,¹⁶ and the correlation between demonstration of *Cp* DNA or antigen in tissue from aneurysms or plaque material and the antibody titers in serum is not convincing.^{17,18} Furthermore, we have recently shown that there was no clear evidence of the presence of outer membrane protein (OMP) in the wall of AAAs but a likely cross-reaction between rabbit anti-OMP and hemoglobin.¹⁹ Finally, intervention trials with macrolides seem to have only a transient effect, if any.²⁰

Consequently, the relationship between *Cp*, AAAs, and atherosclerotic disease remains unknown, and the clinical

impact of detecting the organism is unresolved. However, we have demonstrated in prospective studies that antibodies against *Cp* are associated with the progression of AAAs and lower-limb atherosclerosis.^{1,8,21,22} An explanation could be that pathogenetically important antibodies cross-react with *Cp* OMP. The aim of the present study was to detect OMP in AAA wall tissue by use of purified *Cp* OMP antibodies from AAA patients and to search for potential cross-reacting proteins in the wall of AAAs.

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*.^{1,8} 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.²² Isolation and purification of specific antibody

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TABLE 1. Patient Characteristics

	AAA Size, cm	
	5–6	>6
No.	12	5
Age in years, median (range)	71.1 (46–85)	72.7 (69–85)
Current smoking	8	7
Diagnosis of hypertension	4	4

ies against *Cp* OMP were as follows: 100 μ L of serum sample was diluted 1:2 in PBS buffer (in mmol/L: 2.7 KCl, 1.8 KH_2PO_4 , 10.1 Na_2HPO_4 , 140 NaCl, pH 7.4) and added to the wells coated with *Cp* OMP. 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 μ L 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 μ mol/L pepstatin, and 1 μ mol/L leupeptin with an Ultra-Turax homogenizer. Cell debris was removed by centrifugation (14 000g 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.²³ For 1D gels, ≈ 25 μ g 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 polyacrylamide gels (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] $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$), and rinsed 2 times for 3 minutes in water. Staining of gels was performed for 20 minutes in 0.2% (wt/vol) AgNO_3 , 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] Na_2CO_3 , 0.0185% [vol/vol] formaldehyde, 0.0004% [wt/vol] $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) 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

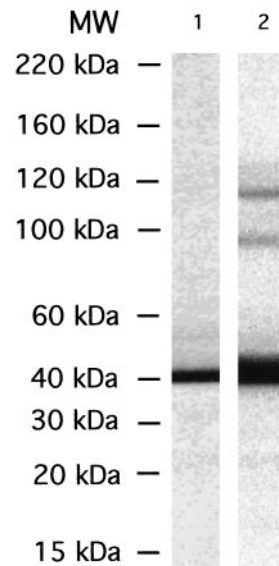


Figure 1. Immunoblot analysis of affinity-purified *Cp* OMP antibody. Lane 1, Pure recombinant OMP was electrophoresed and silver-stained. Protein is very pure, with only 1 band present at ≈ 40 kDa, as expected. Lane 2, Western blot of pure recombinant OMP reacted with affinity-purified anti-OMP antibody, showing that anti-OMP reacts with OMP antigen.

as described above. The blots were washed and reacted with horseradish peroxidase-conjugated secondary antibody (sheep anti-human antibody from the LOY-EIA ELISA kit) diluted 1:1000. The bands were visualized by use of an enhanced chemiluminescence kit and enhanced chemiluminescence hyperfilm (Amersham Pharmacia Biotech).²⁴

Protein Identification

Protein spots were excised from silver-stained 2D gels and digested by use of the protocol described by Shevchenko et al.²⁵ Briefly, the gel spots were washed with 50 mmol/L NH_4HCO_3 /acetonitrile (1:1), followed by dehydration with acetonitrile. The proteins were reduced in 10 mmol/L dithiothreitol/50 mmol/L NH_4HCO_3 for 1 hour at 56°C and alkylated in 55 mmol/L iodoacetamide/50 mmol/L NH_4HCO_3 for 2 hours at room temperature. The gel pieces were washed several times in 50 mmol/L NH_4HCO_3 , followed by dehydration with acetonitrile. The proteins were digested overnight with trypsin (Promega, modified trypsin) at 37°C, and the resulting peptide mixtures were analyzed by tandem mass spectrometry at Alphalyse A/S (Odense, Denmark). The peptide masses obtained were used to query the non-redundant sequence database (NRDB) for protein identification. The NRDB database contains more than 360 000 entries and is maintained and updated by the European Molecular Biology Laboratory. It was specifically tested whether the proteins could be fragments of *Cp* OMP.

Results

Anti-OMP antibodies present in patient sera were isolated by affinity purification by allowing the antibodies to react with immobilized *Cp*-specific OMP. After purification, we checked that the recovered antibodies still possessed the OMP reactivity by Western blot analysis of pure recombinant OMP. As demonstrated in Figure 1 (lane 1), recombinant major OMP runs in a silver-stained gel as a single band of ≈ 40 kDa. Immunoblotting was performed by use of the affinity-purified anti-OMP antibody (lane 2). A strong signal was obtained, corresponding to 40 kDa. Two very faint, higher-molecular-mass bands were also observed on the

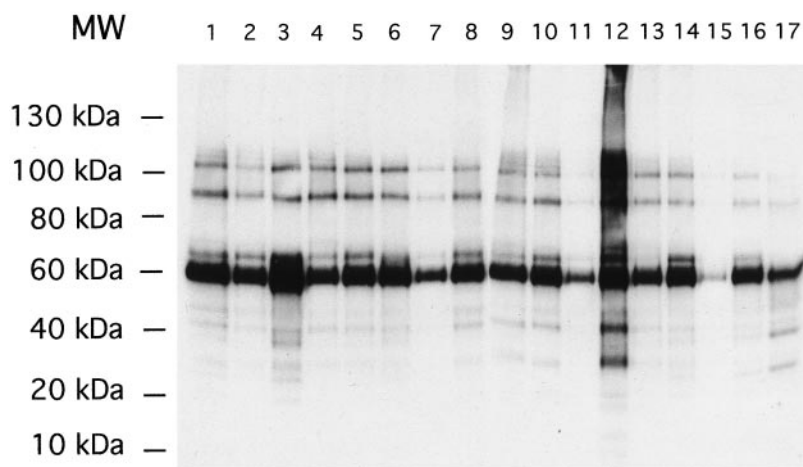


Figure 2. Western blot analysis of proteins from AAA wall proteins (lanes 1–17). Blot was reacted with affinity-purified *Cp* OMP serum antibodies. It was not possible to detect OMP as a 40-kDa protein. However, several cross-reacting bands were found, especially one at ≈ 50 kDa, together with some minor bands with slightly higher molecular mass.

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,²⁶ 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,²⁷ 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.¹ 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.

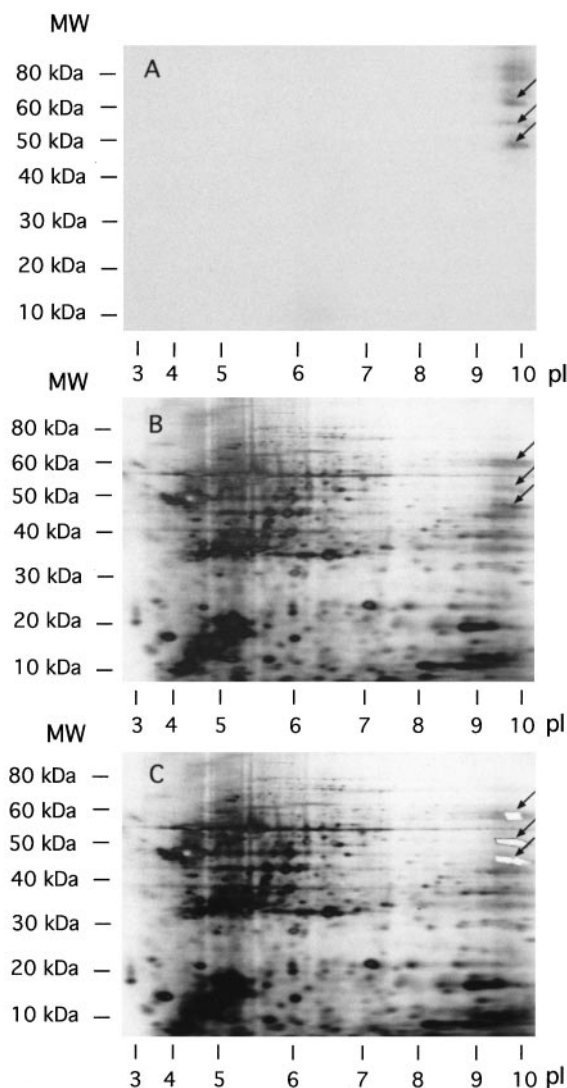


Figure 3. 2D gel analysis of AAA wall proteins. A, 2D Western blot of proteins from sample 1 (same as shown in Figure 2) reacted with anti-OMP antibody. A positive reaction was seen with protein spots at \approx pI 10 (arrows) and molecular masses $>$ 50 kDa. B, Silver-stained 2D gel of AAA wall proteins (sample 1) serving as reference for Western blot. C, Same gel as shown in B after 3 spots had been excised from gel. Excised protein spots were digested with trypsin, and peptides were identified by tandem mass spectrometry, giving sequences shown in Table 2. All peptides are present in heavy chain of human immunoglobulin.

The role of *Cp* remains controversial; we have earlier failed to demonstrate the presence of *Cp* in AAA walls by PCR methods and immunoblotting.^{19,28} The PCR test is a diagnostic method for *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, Apfalter¹⁶ showed that attempts to detect intravascular *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 *Cp* than PCR techniques, and the method also has a poor correlation

with PCR findings.²⁹ However, we have demonstrated by Western blotting that positive signals might be a result of cross-reacting proteins.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 *Cp* OMPs or *Cp* DNA in the mural thrombus.^{19,28} On the basis of the discrepancy of results regarding the presence of *Cp* in AAAs and the methodological problems in direct detection of the organism, it may be that *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 al³⁰ showed in an animal model that *Cp* seems to stimulate the influx of macrophages and dilatation of the abdominal aorta. We have shown in earlier studies^{1,8} that AAA expansion was positively correlated with the presence of antibodies against *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 *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 Hashimoto's thyroiditis.^{31–33} Furthermore, cytokines that modulate the immune response and activate proteolysis,³⁴ and substantial increases of IgG compared with aortic occlusive diseases and normal aortas,³² are found in AAA walls. An autoimmune reaction could be triggered by an initial *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.

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TABLE 2. Identification of Proteins Reacting With the Cp-OMP Antibody by Peptide Sequencing Using Tandem Mass Spectrometry

Protein Spot No.	Peptide Sequence Obtained by Tandem Mass Spectrometry	Identification of Protein	Sequence Coverage
1	GPSVFPLAPSSK	Immunoglobulin γ -1 heavy chain constant region (gi No. 184747)	24%
	STSGGTAALGCLVK		
	DTLMISR		
	FNWYVDGVEVHNAK		
	ALPAPIEK		
	EPQVYTLPPSR		
	EPQVYTLPPSRDELTK		
2	GPSVFPLAPSSK	Similar to immunoglobulin heavy constant γ -3 (gi No. 19263707)	13%
	STSGGTAALGCLVK		
	FNWYVDGVEVHNAK		
	ALPAPIEK		
	EPQVYTLPPSR		
	EPQVYTLPPSRDELTK		
	3		
GPSVFPLAPSSK			
STSGGTAALGCLVK			
DTLMISR			
FNWYVDGVEVHNAK			
ALPAPIEK			
EPQVYTLPPSRDELTK			

Peptides were obtained by trypsin digestion. Similarity was searched in the NRDB database.

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