

Chlamydia pneumoniae in Abdominal Aortic Aneurysms Abundance of Membrane Components in the Absence of Heat Shock Protein 60 and DNA

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Abstract—In this article, we describe the results of a comparative study for the detection of *Chlamydia pneumoniae* in abdominal aortic aneurysm specimens of 19 patients through the use of immunocytochemistry (ICC), in situ hybridization (ISH), and polymerase chain reaction (PCR), along with the detection of cytomegalovirus (CMV) and herpes simplex virus (HSV) by ICC and PCR. *C pneumoniae*-specific membrane protein was detected in specimens of all 19 (100%; 95% confidence interval [CI] 82% to 100%) and of 15 (79%; 95% CI 54% to 94%) patients with monoclonal antibodies RR-402 and TT-401, respectively. Chlamydial lipopolysaccharide was detected in specimens of 15 (79%; 95% CI 54% to 94%) patients when the results of 4 different monoclonal antibodies were combined. Surprisingly, chlamydial heat shock protein 60 was not detected in any of the specimens by ICC. Furthermore, *C pneumoniae* DNA was not detected by ISH when a *C pneumoniae* major outer membrane protein gene fragment was used as probe, nor was it reproducibly detected by PCR on extracted DNA. These results may be explained either by different kinetics of degradation of the different components of *C pneumoniae* after infection of the vessel wall or by the involvement of other *Chlamydia*-like microorganisms. Coexistence of *C pneumoniae* antigens and HSV antigens but not CMV antigens was observed in specimens from 10 of 18 (56%; 95% CI 31% to 78%) patients by ICC. CMV and HSV DNAs were not detected by PCR. In conclusion, we have demonstrated the presence of antigens of *C pneumoniae* in the absence of specific DNA in abdominal aortic aneurysms, suggesting persistence of the antigens rather than a persistent infection. (*Arterioscler Thromb Vasc Biol.* 1999;19:2680-2686.)

Key Words: *Chlamydia pneumoniae* ■ herpes simplex virus ■ cytomegalovirus ■ abdominal aortic aneurysms
■ atherosclerosis

Chlamydia pneumoniae, an important respiratory tract pathogen first isolated in 1965, has been recognized as the third species of the genus *Chlamydia* since 1989.¹ In the late 1980s, *C pneumoniae* infection was associated serologically with chronic coronary heart disease and acute myocardial infarction.² Since then, *C pneumoniae* has been demonstrated in atherosclerotic lesions of the coronary,³⁻⁷ carotid,⁸⁻¹⁰ and popliteal/femoral¹¹ arteries; in lesions of the abdominal aorta¹²; and in abdominal aortic aneurysms (AAAs)¹³⁻¹⁵ through the use of several techniques. Ramirez et al⁵ showed that only when *C pneumoniae* was isolated from a coronary artery by culture on cell monolayers were the results of immunocytochemistry (ICC), polymerase chain reaction (PCR), in situ hybridization (ISH), and electron microscopy also positive. Recently, Maass et al⁷ also showed that when isolation of *C pneumoniae* from coronary arteries was successful, *C pneumoniae* could be detected by PCR. However, in culture-negative specimens, discrepant results

were obtained by ICC, PCR, ISH, or electron microscopy.⁵ In general, in studies in which >1 technique was applied, a large number of discrepant results were observed.^{3,4,6,8-11,14} Most authors explain these discrepancies by sample error, PCR inhibition, or different avidities of the antibodies used in ICC.^{4-6,8-11} To evaluate the differences in detection of *C pneumoniae* in vessel walls, we applied ICC with different monoclonal antibodies, PCR assays on different target genes, and ISH to a series of specimens obtained from AAAs. Here we report the detection of *C pneumoniae*. Detection of cytomegalovirus (CMV) and of herpes simplex virus (HSV) was included for reference purposes.

Methods

Patients and Specimens

In the period from June to October 1995, 19 patients (17 male and 2 female; mean age 69 years; age range 57 to 81) with asymptomatic AAAs undergoing elective surgical repair were enrolled in this

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study. These studies were approved by the medical ethics committee of the St. Radboud University Hospital. None of the patients showed overt signs or symptoms of infection. Routine preoperative laboratory investigations showed no evidence of infection in any of the patients. The patients underwent surgical repair of their AAAs, which had a mean transverse diameter of 6.7 cm (range 5 to 10 cm). Several vessel wall specimens were obtained during the operation and immediately frozen at -80°C . From each patient, the medical history was recorded and a serum specimen was obtained at the time of surgery. The AAA specimens were thawed on ice, and for each patient 3 or 4 specimens 0.5 to 1 cm^3 were fixed for a maximum of 24 hours in 10% buffered formalin and embedded in paraffin for analysis by ICC and ISH; 4 to 9 (117 in total) specimens of 50 to 300 mg were taken for PCR. One 4- μm section of each paraffin-embedded specimen was stained with hematoxylin-eosin for characterization of the microscopic pathology.

In Situ Detection of *C pneumoniae*, CMV, and HSV

Adjacent to the hematoxylin-eosin-stained sections, 4- μm sections were used for detection of antigens by ICC through the use of an indirect immunoperoxidase method. For detection of *C pneumoniae*, 2 *C pneumoniae*-specific anti-membrane protein monoclonal antibodies, RR-402¹⁶ and TT-401¹⁶ (Washington Research Foundation [WRF], Seattle); 4 *Chlamydia* genus-specific anti-lipopolysaccharide (LPS) monoclonal antibodies, 3 (16.3B6, 16.1D10, and 2.5F10) produced and characterized in our laboratory as previously described¹⁷ and CF-2¹⁸ (WRF); and 1 *Chlamydia* genus-specific anti-heat shock protein 60 (hsp60) monoclonal antibody (A57-B9¹⁹; Affinity Bioreagents Inc, SanverTECH, Breda, The Netherlands) were used. For identification of macrophages, the anti-CD68 monoclonal antibody PG-M1 (DAKO A/S, ITK Diagnostics BV, Uithoorn, The Netherlands) was used. Toluidine blue (Sigma-Aldrich Chemie) was used for staining of mast cells. Irrelevant primary monoclonal antibodies of the same isotype were used as negative controls. Peroxidase-labeled goat anti-mouse, anti-isotype antibodies (Southern Biotechnology Associates, Inc, ITK Diagnostics, Uithoorn, The Netherlands) were used as secondary antibodies. Before detection of CMV, the sections were treated with trypsin. Then they were successively incubated with a monoclonal antibody reactive with CMV early antigen (Dako A/S, code CCH2), peroxidase-labeled rabbit anti-mouse antibodies (Dako A/S), and peroxidase-labeled swine anti-rabbit antibodies (Dako A/S) according to the instructions of the manufacturer. For detection of HSV, a polyclonal rabbit antiserum raised against an HSV-1 extract and reactive with type-specific as well as type-common HSV antigens (Dako A/S, code B0114) followed by peroxidase-labeled swine anti-rabbit antibodies (Dako A/S) was used. For ICC with the 16.3B6 anti-LPS monoclonal antibody and for ICC detection of CMV or HSV, tyramide signal amplification (NEN Life Sciences Products) was used according to the instructions of the manufacturer. Peroxidase was visualized with a diaminobenzidine/nickel substrate (Sigma). The sections were counterstained with nuclear fast red (Sigma).

Detection of *C pneumoniae* DNA by ISH in 4- μm sections adjacent to those used for ICC was carried out with a digoxigenin (DIG)-labeled *C pneumoniae* major outer membrane protein (MOMP) gene fragment (426 bp, from nucleotide 354 to nucleotide 779 of the MOMP gene; GenBank accession No. M69230) as a probe. A DIG-labeled minute virus of mouse genome fragment (202 bp, from nucleotide 586 to nucleotide 787; GenBank accession No. J02275) was used as a negative control probe. Both probes were labeled by PCR with DIG-labeled dUTP (Boehringer Mannheim) according to the instructions of the manufacturer. After the sections were dewaxed, they were digested for 15 minutes at 37°C with 5 mg/L proteinase K (Sigma) in 0.1 mol/L Tris-HCl (pH 7.6) containing 2 mmol/L CaCl_2 ; washed twice with PBS for 5 minutes, once with 0.01% Triton X-100 in PBS for 10 minutes, and twice with PBS for 5 minutes; dehydrated; and dried. Hybridization mix (42% deionized formamide, 9.4% dextran sulfate, $5.8\times$ saline-sodium citrate buffer [SSC]; $1\times$ SSC is 15 mmol/L $\text{Na}_2\text{H}_2\text{C}_6\text{O}_6$ containing 150 mmol/L NaCl], $4.7\times$ Denhardt's solution [$50\times$ Denhardt's solution is 1% BSA, 1% Ficoll, and 1% polyvinylpyrrolidone in water; Sigma], 94 mg/L denatured herring sperm DNA, and 50 $\mu\text{g}/\text{L}$

probe) was heated for 10 minutes at 100°C , cooled on ice, and added to the sections. The slides were incubated for 5 minutes at 95°C to denature the DNA in the sections, immediately cooled on ice, and incubated overnight at 45°C . After being washed at 65°C , twice with $2\times$ SSC for 15 minutes, once with $1\times$ SSC for 5 minutes, and once with $0.5\times$ SSC for 5 minutes, the hybridized probes were visualized with alkaline phosphatase-labeled anti-DIG Fab fragments (Boehringer Mannheim) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate incubation overnight. The sections were counterstained with nuclear fast red (Sigma).

Sections of HEP2 cells (code CCL23; American Type Culture Collection [ATCC], Manassas, Va) infected with *C pneumoniae* strain TW-183 (WRF) at a multiplicity of infection of 0.1 inclusion-forming units (IFU) and of mock-infected HEP2 cells, fixed and embedded in paraffin similar to the clinical specimens, were used as positive and negative controls, respectively, for in situ detection of *C pneumoniae*. Paraffin sections of lung tissue infected with CMV or HSV (Dako A/S) were used as positive controls for in situ detection of CMV or HSV, respectively.

Clinical specimens were considered positive for *C pneumoniae* by ICC or ISH when a definite cell-associated cytoplasmic staining was observed and for CMV and HSV when definite staining within the nucleus and/or cytoplasm was observed. Specimens were scored positive when at least 1 clearly positive cell was observed.

PCR Assays for Detection of *C pneumoniae*, CMV, and HSV

DNA isolation and PCR assays were performed as described previously.²⁰ DNA was isolated from finely minced vessel wall tissue by using the Easy-DNA kit (Invitrogen BV) with additional silica purification of the DNA. Five microliters of the specimen was added to 20 μL of PCR mixture including the dUTP-uracil-*N*-glycosylase contamination prevention system and anti-*Taq* polymerase antibodies. All possible measures were taken to avoid false-positive results, in agreement with recently published guidelines.²¹ For detection of *C pneumoniae*, 1 primer set in the 16S rRNA gene and 1 in the MOMP gene; for detection of CMV, 1 primer set in the immediate-early 1 gene and 1 in the glycoprotein B gene; and for detection of HSV, 1 primer set in the immediate-early 2 gene and 1 in the glycoprotein B gene were used.²⁰ The specificity of the amplicons was confirmed by Southern blotting and hybridization with specific probes. Positive specimens were repeated in the PCR assay with the same DNA preparation. A positive control (200 mg of vessel wall tissue spiked with 100 IFUs of *C pneumoniae* TW-183 and 100 50% tissue-culture infectious dose units of CMV strain AD169 [ATCC VR-538], HSV-1 strain F [ATCC VR-733], and HSV-2 strain G [ATCC VR-734], respectively) and negative controls (empty tubes), one after the specimens of each patient, were included in each DNA isolation. Negative PCR controls consisting of 5 μL of Tris-EDTA buffer (10 mmol/L Tris-HCl [pH 7.5] containing 1 mmol/L Na_2EDTA) were included after every fifth specimen in the PCR assay. All DNA preparations were checked for inhibitors by adding DNA equivalent to 1 IFU of *C pneumoniae* TW-183 directly to each PCR. A PCR assay amplifying a 536-bp fragment of the human β -globin gene²² and gel electrophoresis of the isolated DNA were used to assess the integrity of the DNA in a random sample of 8 DNA specimens.

Detection of Antibodies Against *C pneumoniae*, CMV, and HSV

Serum IgG antibodies to *C pneumoniae* were determined with an enzyme immunoassay (EIA) developed in-house, the RIVM (National Institute of Public Health and the Environment) EIA. The RIVM EIA was carried out as previously described for *C trachomatis*,^{17,23} with minor modifications.²⁴ The RIVM EIA performed equally well compared with the microimmunofluorescence assay for detection of antibodies against *C pneumoniae*.²⁴ Untreated and sodium periodate-treated antigens of *C pneumoniae* TW-183 and control antigen of mock-infected HEP2 cells were used. Patient sera were tested at a dilution of 1:1000. A reference serum was included in each assay. Titers of the patient specimens were calculated relative to this reference serum.

TABLE 1. Detection of Microbial Antigens in AAA Specimens by ICC of 19 Patients

Antigen	Antibody	No. positive (%; 95% CI)*
<i>Chlamydia pneumoniae</i> membrane protein	MoAb RR-402	19 (100; 82–100)
	MoAb TT-401	15 (79; 54–94)
Chlamydial LPS	MoAb 16.3B6	9 (47; 24–71)
	MoAb 16.1D10	6 (32; 13–57)
	MoAb 2.5F10	14 (74; 49–91)
	MoAb CF-2	6 (32; 13–57)
Chlamydial hsp60	MoAb A57-B9	0 (0; 0–18)
CMV early antigen	MoAb CCH2	0 (0; 0–18)
HSV	PoAb rabbit anti-HSV-1	10 (56; 31–78)†

MoAb indicates monoclonal antibody; PoAb, polyclonal antibody.

*Results of 3 or 4 sections per patient from different locations within the specimen.

†One specimen not interpretable.

Serum IgG antibodies to CMV and HSV were determined with Enzygnost Anti CMV/IgG and Enzygnost Anti HSV/IgG test kits according to the instructions of the manufacturer (Behringwerke AG).

Statistical Analysis

The geometric mean titer was calculated by taking the antilogarithm of the mean of the log titers. The difference between geometric mean titers was tested by an unpaired Student's *t* test with the computer program Statistica for Windows, version 5.0 (StatSoft Inc). A *P* value <0.05 was considered statistically significant. Ninety-five percent CIs were calculated for percentages by using Geigy Scientific Tables²⁵ and for geometric mean titers by using Student's *t* distribution.

Results

All AAA specimens from all patients showed signs of inflammation and the histological characteristics of advanced atherosclerotic type VI lesions (plaques with thrombus, hematoma, and/or surface defect) as defined by the American Heart Association.²⁶

Control readings of ICC and ISH on sections of these specimens incubated with the control antibody and control probe, respectively, were negative. Control sections of HEp2 cells infected with *C pneumoniae* TW-183 were positive on ICC, with similar signal intensity for the different antibodies, and were also positive on ISH. Control sections of lung tissue infected with CMV or HSV were positive on ICC with the anti-CMV or anti-HSV antibodies, respectively.

The results of the detection of chlamydial, CMV, and HSV antigens in the AAA specimens are summarized in Table 1. Staining for chlamydial antigens was predominantly found in pathological regions with an inflammatory infiltrate and was observed in large macrophage-like cells as a granular cytoplasmic staining (Figure 1). Cells stained for *C pneumoniae*-specific membrane protein were found in the same area as cells stained for the macrophage marker CD68 (Figure 2) but not in the same area as cells stained with toluidine blue, a marker for mast cells, in adjacent sections. Although *C pneumoniae*-specific membrane protein was detected in all patient specimens with the monoclonal antibody RR-402, chlamydial LPS was less frequently positive (Figure 2), and chlamydial hsp60 was detected in none of the specimens. In general, the signal intensity and number of cells stained with the monoclonal antibody TT-401 were less than with the

monoclonal antibody RR-402. In addition, usually a higher number of cells was stained more intensely with the anti-*C pneumoniae* membrane protein antibodies than with the anti-chlamydial LPS antibodies (Figure 1). The anti-LPS antibodies showed a patient-dependent immunoreactivity, a summary of which is given in Figure 3. Specimens from 4 patients were completely negative, and specimens from only 3 patients were positive with all 4 anti-LPS antibodies.

Staining of HSV antigens was found in the cytoplasm and nucleus of lymphocytes in pathological regions with an inflammatory infiltrate in specimens from 10 patients (Figure 2), whereas the staining in 1 specimen could not be interpreted. Thus, 10 of 18 (56%; 95% CI 31% to 78%) patients showed evidence of the coexistence of *C pneumoniae* membrane protein and HSV antigens by ICC. However, although both antigens were observed in the same region of the lesion, they were found in different cell types.

The DNA specimens of 1 patient (No. 9) were lost during DNA isolation. In a random sample of 8 DNA specimens, the size of the isolated DNA was ≈30 to 40 kbp, and a 536-bp β -globin fragment could be amplified by PCR from all of these specimens. Positive controls of spiked tissue and negative controls of the DNA isolation and the PCR assays reacted appropriately. No PCR inhibitors were detected in any of the DNA specimens. The results of the detection of *C pneumoniae*, CMV, and HSV DNAs by PCR in these specimens are summarized in Table 2. In total, from 4 patients 1 specimen was positive, 3 in the *C pneumoniae* 16S rDNA PCR assay and 1 in the HSV immediate-early 2 gene PCR assay. However, on retesting, these specimens were negative. They were also negative in the *C pneumoniae* MOMP gene PCR assay and in the HSV glycoprotein B gene PCR assay, respectively, even after retesting. Furthermore, *C pneumoniae*-specific MOMP gene DNA was not detected in any of the paraffin-embedded AAA specimens of all patients by ISH.

Specific IgG antibodies to *C pneumoniae* were observed in sera from 15 of the 19 patients (79%; 95% CI 54% to 94%). The titers were between 1:800 and 1:12 800. Specimens from 2 of the 4 patients without IgG antibodies to *C pneumoniae* showed a high number and from the other 2 patients a moderate number of positive cells per section by ICC with monoclonal antibody RR-402. IgG antibodies to CMV were observed in sera from 15 of the 19 patients (79%; 95% CI 54% to 94%) and IgG antibodies to HSV in sera from 18 of the 19 patients (95%; 95% CI 74% to 100%). Specimens from the only patient without IgG antibodies to HSV showed a positive reaction on ICC. The geometric mean titer of IgG to HSV of the 10 ICC-positive and the 8 ICC-negative patients was not significantly different (reciprocal geometric mean titers of 3499 [95% CI 390 to 31 374] and of 11 527 [95% CI 5589 to 23 778], respectively; *P*=0.3).

Discussion

In this study, we showed that *C pneumoniae* membrane protein antigens were detected more frequently than LPS antigens and that chlamydial hsp60 was not detected in any AAAs. In addition, we showed that *C pneumoniae*-specific DNA could not be demonstrated by PCR nor by ISH. Furthermore, we demonstrated the coexistence of *C pneu-*

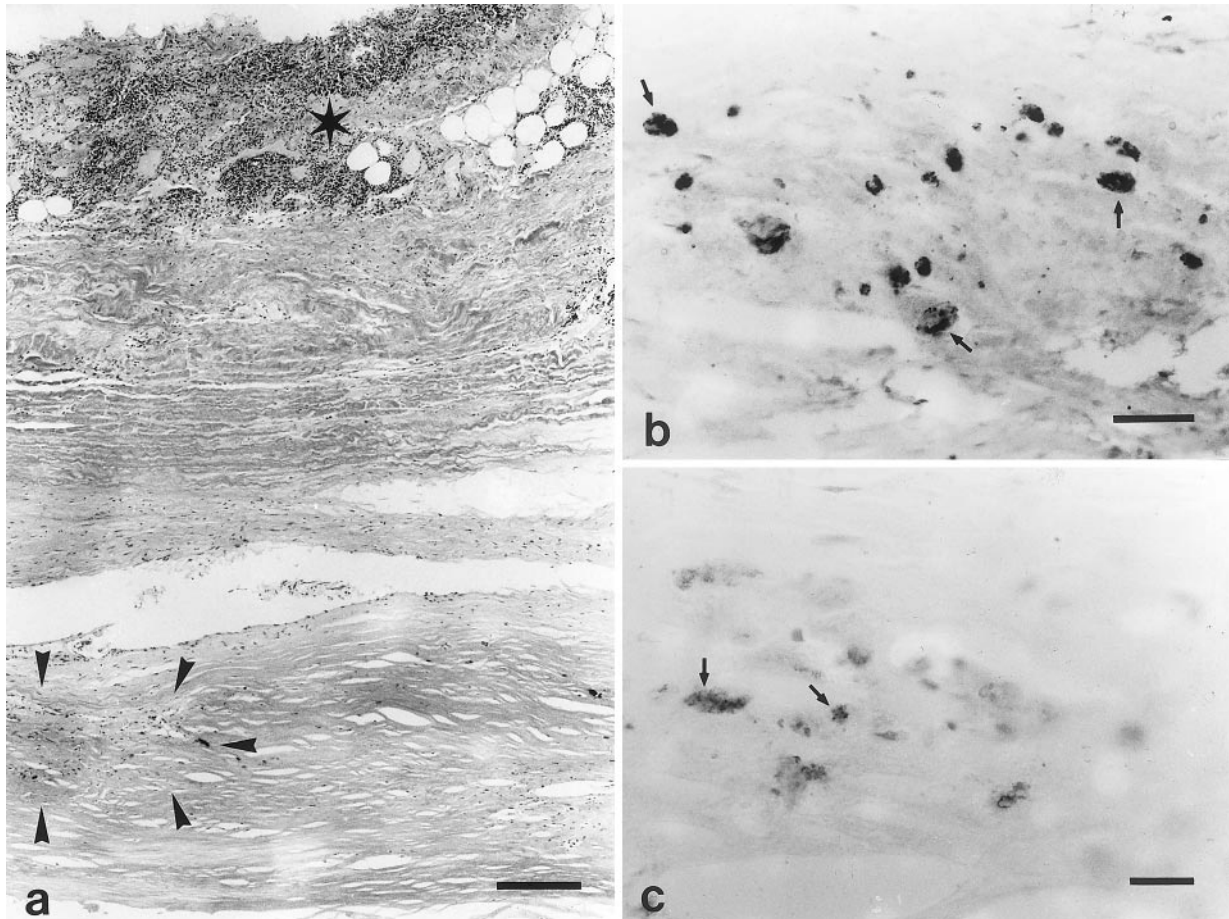


Figure 1. Example of demonstration of *C pneumoniae* membrane protein and LPS in an AAA specimen. a, Section stained with hematoxylin-eosin (bar=200 μ m). The adventitia is marked with an asterisk. The sclerotic area enclosed by the arrowheads is shown in panels b and c. b, Section stained with monoclonal antibody RR-402 for *C pneumoniae* membrane protein (bar=50 μ m). Several immunostained cells are shown, of which some are indicated with arrows. c, Section stained with monoclonal antibody 16.3B6 for chlamydial LPS (bar=20 μ m). The immunostaining for LPS (some positive cells indicated with arrows) is less intense than that for membrane protein in panel b. Note that the immunostaining pattern for *C pneumoniae* membrane protein (b) and for chlamydial LPS (c) is granularly dispersed in the cytoplasm. Sections shown in panels b and c were counterstained with nuclear fast red.

moniae antigens and HSV antigens in AAAs of some patients, whereas CMV antigens were not detected.

Specimens positive with the CF-2 antibody were confirmed by the RR-402 and TT-401 antibodies, in agreement with the results of Grayston and coworkers^{4,6,8} who used the same antibodies. In their strategy, the genus-specific antibody CF-2 either is used alone^{9,11} or for screening followed by confirmation with the species-specific antibody RR-402 or TT-401.^{4,6,8} However, only 32% (95% CI 13% to 57%; 6 of 19) of our specimens positive with the RR-402 antibody and only 31% (95% CI 11% to 59%; 5 of 16) of our specimens positive with the TT-401 antibody were positive with the CF-2 antibody. By using 3 additional anti-LPS antibodies, these percentages were raised to 79% (95% CI 54% to 94%; 15 of 19) and 75% (95% CI 48% to 93%; 12 of 16), respectively. All CF-2-positive specimens were confirmed by at least 1 of the additional anti-LPS antibodies. The percentage of positive patients by ICC (19 of 19; 95% CI 82% to 100%) is in agreement with that reported by Juvonen et al¹⁴ for AAAs (12 of 12) by ICC. However, in contrast to our results, they detected chlamydial LPS in all patients and *C pneumoniae* protein in 8 of the 12 patients. An explanation might be the use of different monoclonal antibodies.

In this study, *Chlamydia* genus-specific anti-hsp60 antibodies were used to detect chlamydial hsp60 in atherosclerotic tissue. The absence of chlamydial hsp60 is not consistent with the assumption of persistent infection with *Chlamydia*.²⁷ Although chlamydial hsp60 has been implicated in the pathogenesis of complications of chlamydial infections²⁷ and induction of atherosclerosis in rabbits by the mycobacterial homologue of hsp60 has been demonstrated,²⁸ our results do not support a major role for chlamydial hsp60 in advanced AAAs.

A possible explanation for the difference in detection of LPS and membrane protein antigen is a more rapid degradation of LPS than of membrane protein, resulting in the absence of all LPS epitopes in some patients. All anti-LPS monoclonal antibodies recognize oxidation-sensitive epitopes on the LPS molecule. The presence and colocalization with macrophages of myeloperoxidase in atherosclerotic lesions might explain the loss of oxidation-sensitive epitopes.²⁹ Although these observations were based on studies of atherosclerotic lesions, chronic inflammation, as present in all our specimens, is the main cause of these oxidative reactions. Chronic inflammation is a hallmark of both atherosclerosis and aneurysm.^{30,31} However, because AAA is not as "pure" a

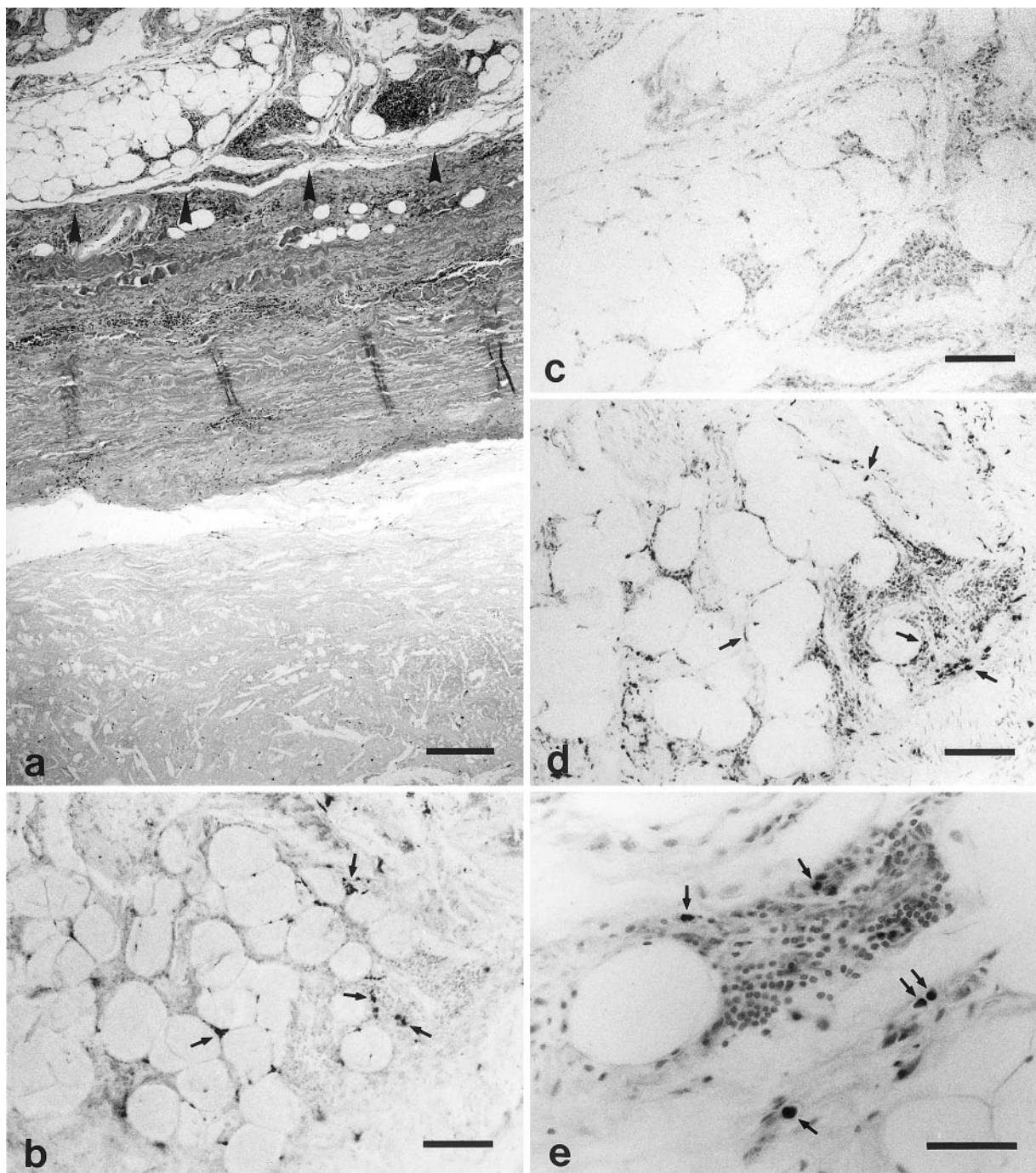


Figure 2. Example of demonstration of *C pneumoniae* membrane protein in the absence of LPS and of HSV in an AAA specimen. a, Section stained with hematoxylin-eosin (bar=200 μ m). Positive cells were typically found in the area of the adventitia above the arrowheads. Typical examples of ICC results are shown in panels b through e. b, Section stained with monoclonal antibody RR-402 for *C pneumoniae* membrane protein (bar=100 μ m). Several immunostained cells are shown, some of which are indicated with arrows. c, Section stained with monoclonal antibody 16.3B6 for chlamydial LPS (bar=100 μ m). No staining was observed. d, Section stained for the macrophage marker CD68 (bar=100 μ m). Numerous immunostained cells are shown, some of which are indicated with arrows. Note that the distribution pattern of cells positive for *C pneumoniae* membrane protein in panel b is similar to that of the CD68-positive cells. e, Section stained with anti-HSV antibodies (bar=50 μ m). Several stained cells are shown (arrows) that can be recognized as lymphocytes. The sections in panels b through e were counterstained with nuclear fast red.

model for the study of atherosclerosis^{31,32} as are plaques in other arteries, extrapolation should be done with caution. Sample error is a less likely explanation for the observed differences, because from all patients at least 3 specimens were taken from different locations in the aneurysmal vessel wall and the results were combined for each anti-LPS and anti-protein monoclonal antibody. Because all antibodies

showed similar signal intensities on paraffin sections of cultured cells infected with *C pneumoniae*, differences in antibody avidity also cannot explain our results. In addition, because we clearly demonstrated the presence of chlamydial LPS and membrane protein, the most likely explanation for the absence of hsp60 is its rapid degradation after an infection with *C pneumoniae*. Alternatively, the presence of *C pneu-*

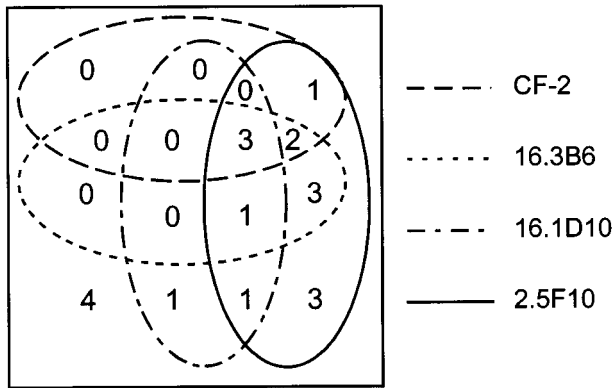


Figure 3. Venn diagram showing the relation in reactivity of AAA specimens of 19 patients with 4 different anti-chlamydial LPS monoclonal antibodies (CF-2, 16.3B6, 16.1D10, and 2.5F10). Numbers within an ellipse or within overlapping parts of 2 or more ellipses represent the numbers of patients positive with the indicated antibody or antibodies. The number outside the ellipses represents the number of patients not positive with any of the indicated antibodies.

moniae remnants in macrophages may be explained by the "traveling" of macrophages, which have ingested and degraded bacteria at other sites in the body infected by *C pneumoniae*, to the inflammation process in the aortic wall, as suggested by Capron.³³ Yet another possibility that may explain the difference in detection of *C pneumoniae* antigens is the involvement of other *Chlamydia*-like microorganisms. Recently, we detected new 16S rDNA sequences clustering together with the recently discovered *Chlamydia*-like organisms "Z"³⁴ and "*Parachlamydia acanthamoebae*"³⁵ by phylogenetic analysis.³⁶ As the antigenic makeup of these new chlamydial microorganism has not been determined yet, cross-reactions of the currently used monoclonal antibodies with components of these new organisms cannot be excluded.

In contrast to many studies in which PCR assays have been used^{4-9,11,13-15} but in agreement with the reports of Weiss et al³⁷ studying coronary atheromas, Lindholt et al³⁸ studying AAAs, and Paterson et al³⁹ studying carotid and coronary atheromas, the presence of *C pneumoniae* DNA could not be demonstrated. However, our PCR assay should be positive if we assume that the observed positivity for *C pneumoniae* antigens (frequently >10 cells per AAA section) is caused by intact *Chlamydia* particles. Previously, we demonstrated the reliability of our DNA isolation procedure and PCR assays to detect microbial DNA in vessel wall specimens.²⁰ To exclude sample error, 4 to 9 specimens of 50 to 300 mg per patient

were analyzed. Furthermore, our ISH results confirmed the absence of *C pneumoniae* DNA, in agreement with the findings of Ramirez et al.⁵ Therefore, we concluded that *C pneumoniae* DNA is not present or is heavily degraded after infection and that the positive immunoreactivity does not represent intact *Chlamydia* particles. In addition, in line with the molecular revision of Koch's postulates by Fredericks and Relman,⁴⁰ we suggest that confirmation of the presence of *C pneumoniae* DNA in vessel walls should be obtained by in situ techniques showing the localization of DNA in the cells in which other components of *C pneumoniae* are also detected.

In agreement with previous reports,^{4,10,14} there was no correlation between the presence of antigen-positive cells in paraffin sections of vessel wall specimens and the presence or titer of serum IgG antibodies. In addition, Maass et al⁴¹ recently showed that serology with the use of the microimmunofluorescence assay is not a reliable parameter to predict individual vascular *C pneumoniae* infection.

HSV antigens were detected in specimens of 56% (95% CI 31% to 78%) of the patients by ICC, but HSV DNA could not be detected by PCR. CMV antigen was not detected by ICC, nor was CMV DNA by PCR. These results are in agreement with those from Raza-Ahmad et al⁴² in coronary arteries by ICC (HSV 48%) and Satta et al⁴³ in AAAs by ICC and electron microscopy (CMV not detectable). However, our results were different from those of Chiu et al¹⁰ in carotid arteries by ICC (CMV 36% and HSV 11% positive) and Tanaka et al⁴⁴ in AAAs by PCR (CMV 68% and HSV 27% positive).

In conclusion, detecting *C pneumoniae* in vessel wall specimens using only 1 technique might cause misinterpretations about the presence of *C pneumoniae*. Results of several techniques combined provide a better view of the actual situation at a certain point in time of a long and continuing disease process. The most likely explanation for our results is a difference in kinetics of degradation of chlamydial components after an infection, rather than persistence of viable *Chlamydia*. Our findings suggest a rapid degradation of hsp60 and DNA, followed by degradation of LPS, and the persistence of membrane proteins. How this process is influenced by the underlying disease remains to be investigated. Also, because atherosclerosis and aneurysm could be distinct diseases, extrapolation should be done with caution.

TABLE 2. Detection of Microbial DNA in AAA Specimens by PCR

Patient No. (n=18)	Target Gene (No. Positive/No. of Specimens Tested Per Patient)					
	<i>Chlamydia pneumoniae</i>		CMV		HSV	
	16S rDNA	MOMP	IE1	gB	IE2	gB
1	1/9*	0/9	0/6	0/6	0/6	0/6
3	1/6*	0/6	0/6	0/6	0/6	0/6
4	0/6	0/6	0/6	0/6	1/6*	0/6
8	1/9*	0/9	0/6	0/6	0/6	0/6
Other (n=14)	0/4-9	0/4-9	0/4-6	0/4-6	0/4-6	0/4-6

IE indicates immediate early; gB, glycoprotein B.

*On retesting, the PCR assay was negative.

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