Antigens of persistent Chlamydia pneumoniae within coronary atheroma from patients undergoing heart transplantation

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Abstract: AimsIn order for Chlamydia pneumoniae to play a causative role in chronic human disease, it would need to persist within infected tissue for extended periods of time. Current theory suggests that C pneumoniae may persist at the site of infection via an alternative replicative form, known as an aberrant body.MethodsA panel of C pneumoniae-specific antibodies upregulated by the aberrant body was used to probe tissue specimens from the coronary atheroma from 13 explanted hearts to identify patterns of reactivity in these tissues, as well as to determine the presence and prevalence of C pneumoniae aberrant bodies.ResultsSix of 13 patients had an ischaemic cardiomyopathy secondary to coronary atherosclerosis, while another six patients had an idiopathic, dilated cardiomyopathy. One additional patient, a young (24 years) woman with cardiomyopathy, had no history of atherosclerotic disease. Eleven patients were positive by immunohistochemistry with at least one antibody. Coronary arteries of the two other patients were negative by immunohistochemistry with all antibodies. One of these patients was the 24-year-old woman with grade I disease and no risk factors for coronary artery disease.ConclusionsThe protein antigens of persistent C pneumoniae infection found in the atheromatous lesions from patients in this study could potentially be used as markers to detect such infections and some may be virulence factors or immunogens specific to C pneumoniae, thus serving as target molecules for diagnostic use or therapeutic intervention.

DOI: https://doi.org/10.1136/jclinpath-2011-200270

Journal of
CLINICAL PATHOLOGY

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Journal:	*Journal of Clinical Pathology*
Manuscript ID:	jclinpath-2011-200270.R1
Article Type:	Original Article
Date Submitted by the Author:	n/a
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Specialty:	Inter-disciplinary
Keywords:	ANTIBODIES, atherosclerosis, CARDIOVASCULAR, IMMUNOHISTOCHEMISTRY

Antigens of Persistent *Chlamydia pneumoniae* within Coronary Atheroma from Patients Undergoing Heart Transplantation

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Abstract

Aims: In order for *Chlamydia pneumoniae* to play a causative role in chronic human disease, it would need to persist within infected tissue for extended periods of time. Current theory suggests that *C. pneumoniae* may persist at the site of infection via an alternative replicative form, known as an aberrant body (AB).

Methods: A panel of *C. pneumoniae*-specific antibodies up-regulated by the AB was used to probe tissue specimens from the coronary atheroma from 13 explanted hearts to identify patterns of reactivity in these tissues, as well as to determine the presence and prevalence of *C. pneumoniae* AB.

Results: Six of 13 patients had an ischemic cardiomyopathy secondary to coronary atherosclerosis, while another six patients suffered from an idiopathic, dilated cardiomyopathy. One additional patient, a young (24 years) woman with cardiomyopathy, had no history of atherosclerotic disease. Eleven patients were positive by immunohistochemistry (IHC) with at least one antibody. Coronary arteries of the two other patients were negative by IHC with all antibodies. One of these patients was the 24 year-old woman with Grade I disease and no risk factors for coronary artery disease.

Conclusions: The protein antigens of persistent *C. pneumoniae* infection found in the atheromatous lesions from patients in this study could potentially be used as markers to detect such infections and some may be virulence factors or immunogens specific to *C. pneumoniae*, thus serving as target molecules for diagnostic use or therapeutic intervention.

Keywords: *Chlamydia pneumoniae,* coronary atheroma, heart transplantation, persistence

This paper describes a detailed, prospective investigation on the prevalence and expression of a panel of chlamydial proteins in coronary artery tissue specimens of 13 human heart transplantation patients. Immunohistochemistry examinations were complemented by a thourough investigation of coronary artery tissue specimens by histopathology, culture, PCR and IMG. The specificity and suitability of the antibodies for immunohistochemistry was shown in previous studies (Borel et al., 2006 & 2008). Patient sera were investigated by MIF and Western blot with the idea to find a correlation between direct antigen or DNA detection and antibody reaction. The aim of this study was to find markers to detect and diagnose persistent chlamydial infections in patients with atherosclerosis. A panel of C. pneumoniae-specific antibodies up-regulated by the aberrant body was suitable to detect such infections and could serve as diagnostic markers in the future.

1. Introduction

Chlamydia pneumoniae is an obligate intracellular pathogen which causes acute and chronic respiratory infections in humans (1-4). Over the last decade, a significant amount of data have suggested a role for *C. pneumoniae* in chronic human disease, in particular, atherosclerosis; however, a direct causal role remains to be established (5). In order for *C. pneumoniae* to play a causative role in chronic human disease, it would need to persist within infected tissue for extended periods of time. One hypothesis suggests that *C. pneumoniae* persists at the site of infection via an alternative replicative form, known as an aberrant body (AB) defined as viable but noncultivable form of chlamydiae (6). These ABs may represent the mechanism by which this organism persists and causes the chronic inflammatory-type infections characteristic of *C. pneumoniae*, and other chlamydiae. *In vitro*, alterations of the normal developmental cycle of

C. pneumoniae can be induced (7). Although these *in vitro* models of persistence have been characterized in detail, convincing evidence for the *in vivo* existence and prevalence of *C. pneumoniae* AB is lacking. Demonstration of a high prevalence of ABs in chronically-infected human tissue would establish this form of *C. pneumoniae* as a likely participant in the pathogenesis of disease.

Chlamydial ABs have been shown to exist, *in* vitro (7), and we have detected AB-like structures of *C. suis* in infected pigs (8). We have also identified evidence of persistent *C. pneumoniae* in the atheroma of patients with coronary artery disease (CAD) by TissueMicroarray and Immunogoldelectron (IMG) microscopy (9,10), however, these data were collected on archived specimens. Therefore, we conducted a prospective examination of human atheromatous tissue from explanted hearts of patients receiving heart transplants.

The objective of this study was to use a panel of *C. pneumoniae*-specific antibodies, the majority of which are reactive to antigens upregulated in the persistent state of the organism, to probe tissue specimens from the coronary arteries from explanted hearts to identify patterns of reactivity in these tissues, as well as to determine the presence and prevalence of *C. pneumoniae* ABs. Antibodies directed against chlamydial proteins upregulated during the persistent stage *in vitro* (LPS/MOMP, GroEL and GroES) and suitable to screen atheromatous samples *in* vivo (9,10) were applied in the present study. In addition, recently described antibodies (11,12) against other chlamydial proteins such as incA, cpaf and HtrA were used to investigate coronary artery specimens. Immunohistochemistry examinations were complemented by a thourough investigation of adjacent coronary artery tissue specimens by histopathology, culture, PCR and

immunogoldelectron microscopy. Patient sera were investigated by microimmunofluorescence and Western blot with the idea to find a correlation between direct antigen or DNA detection and antibody reaction.

2. Methods

2.1. Patients

Explanted hearts from 13 patients (IDL-01 to IDL-13) seeking heart transplantation were investigated. This study was approved by the University of Louisville Institutional Review Board and the required informed consent was obtained for each patient. Medical records from each patient were analyzed for: (i) demographics: sex, age at transplantation date; (ii) past medical history: neoplastic disease (active or within the last year), congestive heart failure, cerebrovascular disease, renal disease/chronic renal failure, liver disease/cirrhosis, neurologic diseases/mental illness, history of community-acquired pneumonia (CAP), chronic obstructive pulmonary disease (COPD); (iii) selected laboratory findings: homocysteine levels, C-reactive protein; (iv) established risk factors for atherosclerosis and cardiovascular events: family history of coronary artery disease (CAD), active CAD, essential arterial hypertension, hyperlipidemia, prior myocardial infarction, prior angioplasty/bypass surgery/peacemakers, atrial fibrillation, history of smoking, history of alcoholism, diabetes; (v) prior cardiovascular medications: aspirin, beta-blockers, ACE inhibitors, anticoagulants (heparin, warfarin), antiplatelets other than aspirin, statins, and (vi) antibiotic treatment received within the 30 days prior to transplantation. The age range of 12 patients at transplantation date was 47.1 and 70.2. A single patient was 24 years old. There were two female and eleven male patients. In total, eight patients (IDL-01, IDL-04, IDL-06, IDL-07, IDL-08, IDL-09, IDL-10, IDL-12) were on left

ventricular assistant device (LVAD). Of these, five patients (IDL-01, IDL-06, IDL-07, IDL-08, IDL-09) had recurrent drive line infections and were treated with antibiotics within 30 days prior to transplant. *Pseudomonas aeruginosa* was detected in IDL-08 and Methicillin-resistant *Staphylococcus aureus* (MRSA) in IDL-09. For the three other patients, neither the organism(s), nor the antibiotics used for treatment, was recorded. Aminoglycosides, fluorquinolones, rifamycins, tetrazyclines and linezolid was used for antibiotic treatment in these two patients.

Prior antibiotic treatment was recorded in five patients (IDL-01, IDL-06, IDL-07, IDL-08, IDL-09). Antibiotic treatment was needed due to line infections of left ventricular assistant devices in all five patients.

2.2. Histopathology

At least two pieces of the left and right coronary arteries were collected from the excised tissue samples and immediately fixed in 4% buffered formalin. Each was routinely processed and stained with hematoxylin and eosin. Atheroma grading was performed by a cardiac pathologist and was assigned a Grade I through V occlusion score.

2.3. Antibodies

Antibodies directed against *C. pneumoniae*-specific proteins are shown in Table 1. All formalin-fixed, paraffin-embedded coronary artery specimens were screened by immunohistochemistry with each antibody. Based on the immunohistochemistry results, and results obtained in previous studies (9,10), the *Chlamydiaceae*-family specific antibody directed

against the LPS and MOMP (LPS/MOMP antibody; Cygnus Technologies, Inc., Southport, NC) was selected for immunogold electron microscopy labeling.

2.4. Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections were stained with the panel of primary anti-chlamydial-antibodies as in Table 1 and detection was performed with the Detection Kit (Dako ChemMate™ Detection Kit, Glostrup, Denmark) according to the manufacturer's instructions. The optimal dilution for each antibody was previously determined on HEp-2 cell monolayers infected with *C. pneumoniae* for 72 hrs.

Antigen retrieval was performed by 20 minutes pressure cooking with HIER target retrieval solution (pH 6,0; Target Retrieval Solution (x 10), Dako ChemMate™, Glostrup, Denmark) for all antibodies. For inhibition of the endogenous peroxidase activity, the slides were immersed in peroxidase-blocking solution (Dako ChemMate™, Glostrup, Denmark) for 5 min at room temperature (RT). Two additional blocking solutions were added to the slides, which were incubated with the polyclonal antibodies GroEL and GroES: Dako Protein Block Serum-free for 5 min at room temperature (Dako ChemMate™, Glostrup, Denmark) and 20 min Avidin D solution followed by 20 min Biotin solution at room temperature (Vector Laboratories Inc., Burlingame, CA). The slides were then incubated with the primary antibody for 60 min (LPS/MOMP, incA, cpaf, HtrA) or over night (GroEL, GroES) at room temperature in a moist chamber. Following that, the sections were incubated for 20 min at RT with the link-antibody, developed in 3-amino, 9-ethyl-carbazole (AEC) substrate solution for 10 min at RT, and counterstained with hematoxylin. By using the antibody diluent instead of the primary antibody

a negative control of each section was performed. Positive controls consisted of HEp-2 cells infected with *C. pneumoniae* for 72 hrs. Patients with at least one positive localization per antibody were defined as positive.

2.5. Transmission electron microscopy (TEM)

Glutaraldehyde-fixed coronary artery specimens were post-fixed in osmium tetroxide and then embedded in epon. Appropriate areas for ultrastructural investigation were selected using semi-thin sections (1 µm) stained with toluidine blue (Fluka, Buchs SG, Switzerland). Ultra-thin sections (80 nm) for TEM were mounted on gold grids (Merck Eurolab AG, Dietlikon, Switzerland), contrasted with uranyl acetate dihydrate (Fluka) and lead citrate (lead nitrate and tri-natrium dihydrate; Merck Eurolab AG) and examined for the presence of chlamydial developmental stages in an electron microscope (Philips CM10).

2.6. Immunogold electron microscopy labeling (IMG)

For IMG labeling, sections were labeled with the primary antibody LPS/MOMP at a dilution of 1:500 at RT overnight. After washing with PBS/TBS buffer for 30 min, samples were incubated with a 18-nm colloidal gold-conjugated polyclonal IgG anti-rabbit secondary antibody (dilution 1:15) for 2 h. The sections were then washed for another 20 min, rinsed with deionized water, and contrasted as described (10). Uninfected and infected (72h) HEp-2 cell pellets were used as negative and positive controls, respectively.

2.7. Culture

Culture was performed at two laboratories in HEp-2 cell monolayers using standard methods. In brief, patient samples were mechanically disrupted and centrifuged at 1,000 x *g* onto sub-confluent HEp-2 cell monolayers for 1h in serum free MEM-Eagles medium (Invitrogen, Carlsbad, CA, USA) in 24 well plates. Subsequently, MEM-eagles, containing 10% FBS and 2 g/ml cycloheximide, was added. Following 72 h of incubation at 37°C, the entire contents of each well was harvested and sonicated for 5 min at 4°C. This was divided into two wells of a 24-well plate and inoculation was carried out as described above. Immunofluorescence microscopy was carried out on the contents of one well using the Pathfinder Chlamydia Culture Confirmation System (BioRad, Hercules, CA, USA). The remaining well was passaged into two new wells after 72h of additional incubation.

2.8. DNA extraction and Polymerase chain reaction (PCR)

PCR was as performed at two separate laboratories. Prior to PCR, tissue specimens from coronary atheroma in M4 transport medium were processed for DNA extraction using a commercial DNA extraction kit (DNeasy Tissue kit; Qiagen, Hilden, Germany).

At one site, a real-time PCR was conducted on an ABI 7500 instrument (Applied Biosystems, Foster City, CA) using a modified version of the procedure of Everett et al (13). This 23S-based *Chlamydiaceae* family-specific real-time PCR as described previously (14) includes primers Ch23S-F (5'-CTGAAACCAGTAGCTTATAAG CGGT-3'), Ch23S-R (5'-ACCTCGCCGTTTAACTTAACTCC-3'), and probe Ch23S-p (FAM-CTCATCA TGCAAAAGGCACGCCG-TAMRA) and yields a 111-bp product specific for members of the family *Chlamydiaceae*. In each reaction, 2.5 µl of extracted DNA was added to a mix of reagents containing 12.5 µl of 2X TaqMan® Fast Universal PCR Master Mix (Applied

Biosystems, Darmstadt, Germany), with final concentration of 5pmol/µl of each primer and the probe (Microsynth, Balgach, Switzerland) to yield a final volume of 25 µl. The cycling profile included initial denaturation (95°C, 10 min) followed by 45 cycles of denaturation at 94°C for 15 s, 60°C for 60 s. A cycle threshold (Ct value) of < 38.00 was considered as positive, and all samples were tested at least in duplicate.

At the second site, a real-time multiplex PCR with 3 probes that can detect *C. trachomatis, C. pneumoniae* and *C. psittaci* was used (15).

2.9. Microimmunofluorescence (MIF)

Serum samples were collected immediately prior to heart transplantation from all patients. Blood samples were centrifuged (3000 × *g*, 10 min) and sera were stored at -80 °C prior to testing. IgG and IgM antibody titers were determined by MIF using commercial kits (Focus Diagnostics, Cypress, CA) according to manufacturer's instructions.

2.10. Western Blot

In a previous study (16), the serological response to novel *C. pneumoniae* antigens associated with persistent *C. pneumoniae* infections was characterized. According to these results, the molecular chaperone Hsp70 (DnaK), the RNA polymerase α-chain (RpoA) and the hypothetical protein CpB0704 were found to be immunodominant and thus selected to test human sera from the 13 transplanted patients. Serum reactivity towards RpoA, DnaK and the hypothetical protein CpB0704 was analyzed as described previously (17). In brief, the recombinant proteins were separately applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by electroblotting onto Bio Trace NT nitrocellulose membranes (Pall, Port Washington, NY, USA).

The membranes were blocked using 5% nonfat dry milk, cut into strips with 4 mm width of which each was incubated with a serum sample of the 13 donors at a dilution of 1:1,000. After four subsequent washing steps, the strips were incubated with a peroxidase-conjugated rabbit anti-human IgG antibody (1:2,000 dilution, DakoCytomation) for 45 min followed by another four washing steps. Immunoreactive bands were visualized and quantified by enhanced chemiluminescence detection using a LAS-3000 imaging system (Fuji) and the AIDA software package (Raytest/Fuji). An intensity threshold of 1,000 counts above the background level was used as the cutoff.

3. Results

Detailed results of individual patients are shown in Table 2. Six of 13 patients had an ischemic cardiomyopathy secondary to coronary atherosclerosis (IDL-01, IDL-02, IDL-06, IDL-07, IDL-10, IDL-11) while another six patients suffered from an idiopathic, dilated cardiomyopathy (IDL-04, IDL-05, IDL-08, IDL-09, IDL-12, IDL-13). One patient, a young (24 y.o.) woman (IDL-03), had no history of atherosclerotic disease. All patients were free of neoplastic disease (active or within the last year prior to transplantation), cerebrovascular disease (i.e. previous stroke), renal diseases other than chronic renal failure, neurologic diseases/mental illness and liver disease/cirrhosis. In contrast, all patients (n=13) suffered from congestive heart failure and nine out of 13 patients had chronic renal failure. Three patients had a previous history of community-acquired pneumonia (IDL-06, IDL-07, IDL-08) and five patients had chronic obstructive pulmonary disease (IDL-5, IDL-06, IDL-08, IDL-12, IDL-13), respectively. Several patients had risk factors for cardiovascular events, including: family history of CAD (n=8), active CAD (n=11), hyperlipidemia (n=11), arterial hypertension (n=10), prior myocardial

infarction (n=10), history of smoking (n=10), diabetes (n=9), atrial fibrillation (n=7), or history of alcoholism (n=1). Prior cardiac surgery was performed in five patients (IDL-02, IDL-06, IDL-07, IDL-10, IDL-11).

Homocysteine levels were elevated in eleven patients (ranged from 4.4 to 23.5 uMol/l, mean value was 14.4 uMol/l) and not determined in two patients (IDL-01, IDL-12). C-reactive protein was high in a single patient (12.1 mg/dl, patient IDL-07), but not tested in all other patients. Cardiovascular medications received within 30 days prior to transplantation included: aspirin (n=8), beta-blockers (n=12), ACE inhibitors (n=7), anticoagulant (n=11), antiplatelet (n=6) and statins (n=8). Prior antibiotic treatment was recorded in five patients (IDL-01, IDL-06, IDL-07, IDL-08, IDL-09). Antibiotic treatment was needed due to line infections of LVADs in all five patients. Results regarding chlamydial infections were not different between patients with line infections and antibiotic treatment and patients with non-infected LVAD or without LVAD.

All 13 patients exhibited atherosclerotic lesions in their coronary arteries (Grade I to V). One patient (IDL-03) had minimal atherosclerotic lesions (Grade I). Four patients had between Grade II and III (40-70% occlusion), six patients showed Grade IV lesions (80-95% occlusion) and Grade V (100% occlusion) was seen in two patients.

Representative positive immunohistochemistry labeling with the antibodies LPS/MOMP, GroES, incA and HtrA on patient coronary specimens, in comparison with infected HEp-2 cell pellets, is shown in Figure 1. Coronary arteries of two patients were negative by IHC with all antibodies (IDL-03, IDL-09). One of these patients was the 24 year-old woman (IDL-03) with Grade I disease and no risk factors for CAD. All other eleven patients were positive by immunohistochemistry with at least one antibody. Likewise, all eleven patients showed positive reactions in at least one tissue specimen with the LPS/MOMP antibodies, ten patients were

positive by the GroEL antibody and nine patients revealed positive labeling with the GroES, incA and HtrA antibodies. Only three patients were positive with the cpaf antibody.

A rigorous examination of a minimum of four grids for each tissue specimen, for a total of 52 grids, by TEM and IMG using the LPS/MOMP antibody failed to detect the presence of any normal (elementary, intermediate and reticulate bodies) or altered replicative state of *C. pneumoniae*, including AB (data not shown).

Culture and PCR for *C. pneumoniae* was negative in all 13 patients in both laboratories (data not shown).

All 13 patient sera were negative for IgM antibodies by MIF (data not shown). IgG titers were lower (n=2) or equal (n=2) to 1:64, or higher: 1:128 (n=1), 1:256 (n=7) and highest in patient IDL-08 (1:512). Western blot analysis of sera showed positive results for RpoA (n=3), DnaK (n=2) or both (n=6). Only three sera showed reactivity to the hypothetical protein CpB0704.

4. Discussion

Atheromatous tissues from patients with Grade III to V pathology demonstrated reactivity to antibodies to *C. pneumoniae* proteins that are upregulated in models of *in vitro* persistence. One young patient, who could conceivably be viewed as a "negative control" in this study, showed no reactivity to any of the serum antibodies tested. This patient was transplanted due to cardiomyopathy at a very young age and also had no risk factors for CAD. Eleven of the 12 other patients showed a variable degree of reactivity with one or more of the antibodies tested, indicating the presence of these antigens within the atherosclerotic lesions of these patients. These chlamydial antigens could be responsible for triggering inflammatory responses via receptor-dependent manner in the absence of replicating chlamydial organisms. Furthermore,

ongoing inflammatory and necrotic processes in chronically-infected atherosclerotic lesions can co-stimulate pattern recognition receptors by danger-associated molecular patterns. Though, in the light of negative culture results in all 13 patients, it is possible that *C. pneumoniae* antigens persisted in chronically-infected coronary atheroma in the absence of normal replication. The persistence of *C. pneumoniae* antigens rather than viable bacteria has been reported in the literature *in vitro* and *in* vivo (18,19). As observed in our study, Meijer et al. (19) found a granular staining pattern for MOMP, hsp60 and LPS in infected mice by IHC instead of visible inclusions. The granular staining pattern of antigens in the absence of staining for DNA by in situ hybridization was caused by non-viable bacteria as stated by the authors and these findings are in accordance with negative PCR and culture results but positive labeling for different chlamydial proteins in the majority of the heart transplantation patients. Furthermore, rapid degradation of DNA in atherosclerotic lesions but persistence of LPS and MOMP in the vessel walls as reported by Meijer et al. (18) could support our results.

By immunohistochemistry, antibodies against chlamydial proteins whose increased expression has been demonstrated in *in vitro* models of persistence (7), were applied to detect persistent infections. Antibodies against LPS/MOMP, GroEL and GroES were suitable to detect aberrant bodies of *C. pneumoniae* by IHC and IMG in two previous studies on archived material (9,10) and similarly showed positive labeling in the majority of patients in the present study. By proteomic analyses, MOMP and heat shock proteins such as GroEL and GroES have been shown to be upregulated in various induced persistence models of *C. pneumoniae* (20,21) and these findings were confirmed by our study of coronary artery samples.

The chlamydial protease-like activity factor (cpaf) is a major factor of chlamydial pathogenicity, *Chlamydia*-associated cell damage and inflammation (22) and has different localizations in the cell depending on acute or persistent *C. pneumoniae* infection (23). Antibodies to cpaf have been detected in women with *C. trachomatis* cervicitis (24) and may promote *C. trachomatis* persistence (25). In addition, the highest increase in serum reactivity in *C. pneumoniae*-positive donors was found towards cpaf in a recent study (16) implicating cpaf as a persistence marker candidate. However, only three patient specimens were positive for cpaf by IHC and preliminary experiments by Western blot showed few positive serum samples (data not shown), thus cpaf seems not to be strongly expressed in *C. pneumoniae* persistence, *in vivo*. In contrast, the antibody against HtrA reacted positive in nine patients comparable to the labeling with the LPS/MOMP, GroEL and GroES antibodies. HtrA is a virulence and stress response periplasmic serine protease and molecular chaperone and is up-regulated during *C. trachomatis* persistence *in* vitro (11,26). Chlamydial chaperone proteins such as HtrA, GroEL and GroES may be used as a panel marker to detect persistent *C. pneumoniae* infections *in vivo*. The regulation of the incA protein, an effector protein secreted by the chlamydial type III secretion system (27), under chlamydial persistence has not been reported so far, but positive labeling in nine out of 13 patients indicate its possible expression during chlamydial persistence.

A rigorous and labor-intensive examination by TEM and IMG failed to reveal the presence of any normal or altered replicative forms of *C. pneumoniae*, including AB. One conclusion would be that there are indeed no AB to be found in any of these tissue specimens or patients, and that a larger group of patients would need to be examined. However, this would go against the theory of a relatively high prevalence of AB participating in the atherogenic response in

humans. The possibility exists that AB are present and prevalent in human coronary atheroma, but that a more complete TEM and IMG examination is needed. By IMG, intracellular, atypical, round to oval structures of variable diameter resembling reticulate bodies of *Chlamydia*, located within smooth muscle cells, macrophages or fibroblasts, were described in a previous study (10) on atherosclerotic tissue from five patients seeking heart transplantation. The absence of typical chlamydial inclusions and their developmental forms (elementary and reticulate bodies) and the presence of necrotic cellular debris mixed with lipids and calcified material made it demanding to screen TEM sections and limits the screening for chlamydial structures by TEM and IMG. Admittedly, one ultra-thin section only reveals a very small area of the entire atheroma, leaving many areas of the atheroma unscreened. A thorough investigation of the whole complete area of the coronary artery specimens was not possible in this study due to time and cost restraints.

Selected *C. pneumoniae* proteins with antigenic properties, such as RpoA, DnaK and the hypothetical protein CpB0704 were used to test patient sera by Western Blot. Of these, highest increase in serum reactivity of PCR-positive donors was found toward RpoA in a previous study (16). This protein was reported to be the most up-regulated protein during IFN-γ-mediated *in vitro* persistence (21). Consistent with the finding of increased expression of RpoA under persistence were the positive results towards this protein in nine out of 13 heart transplantation patients by Western Blot in our study. Corresponding immunohistochemistry results on patient tissue specimens were lacking due to unavailability of an antibody against the RpoA protein. The molecular chaperone Hsp70 (DnaK), another strongly upregulated protein under *in vitro* persistence, was one of the immunodominant proteins used for screening of patient sera resulting in eight positive samples. In contrast to RpoA, seroreactivity to DnaK was not

significanlty increased in sera of PCR-positive donors in the immunoproteomic identification study (16). However, results in our study were comparable for these two proteins. This could be explained by distinct antibody response patterns toward persistence-associated *C. pneumoniae* antigens *in vivo* as shown by variable presence or absence of dual IFN-γ - and IL-2-producing T-cell responses among *C. pneumoniae*-seropositive individuals (17). Only three patient sera showed reactivity to the hypothetical protein CpB0704 that was previously described to have unaltered seroreactivity among persistently infected patients (16). Generating antibodies against RpoA and DnaK for their use in IHC on atheromatous tissues is planned for comparative studies on tissues and serum samples.

5. Conclusion

In summary, the various antigens of persistent *C. pneumoniae* infection found in atheromatous lesions in this study could potentially be used as markers to detect such infections. Some of these may be virulence factors or immunogens specific to *C. pneumoniae*, thus serving as target molecules for diagnostic use or therapeutic intervention.

6. Collaborating investigators

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7. Source of funding

Supported by grants from the American Heart Association (J.T. Summersgill) and the Bodenseehochschulförderung (N. Borel, A. Pospischil, S. von Aulock, C. Hermann, C. Bunk).

9. Competing interests

None declared.

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11. Figure Legends

Figure 1.

Comparative immunohistochemistry results of HEp-2 cells (left panel) and of patient tissue (right panel). Coronary artery specimens from patient number 1 (IDL-1) and patient number 4 (IDL-4) are shown. Antibodies against the lipopolysaccharide and the major outer membrane protein of the family *Chlamydiaceae* (LPS/MOMP), the heat-shock protein GroES, the inclusion membrane protein incA, and the DO serine protease HtrA were used. Positive labeling with the respective antibodies is present intracytoplasmic in chlamydial inclusions in HEp-2 cells (left panel) and intracytoplasmic and granular in macrophages and smooth muscle cells of coronary arteries of heart transplant patients (right panel).

Table 1. Antibodies used for immunohistochemistry (IHC) and immunogold labeling (IMG).

Antibody	Antigen	Upregulated in persistence	Type	Dilution	Source	Reference
LPS/MOMP	*Chlamydiaceae* family	yes	rabbit polyclonal	1:800[1]/1:500[2]	Cygnus	commercial
GroEL	heat-shock protein	yes	rabbit polyclonal	1:200	2D Gel plug	9
GroES	heat-shock protein	yes	rabbit polyclonal	1:400	2D Gel plug	9
incA	Inclusion membrane	not known	mouse monoclonal	1:50	G. Zhong	12
cpaf	protease-like activity	yes	mouse monoclonal	1:100	G. Zhong	12
HtrA	DO serine protease	yes	rabbit polyclonal	1:400	P. Timms	11

[1]dilution for immunohistochemistry

[2]dilution for immunogold labling

Table 2. Details of selected results in heart transplantation patients IDL-01 to IDL-13.

IDL	Pathology	Age	Sex	Risk factors for atherosclerosis										Immunohistochemistry						Western Blot			MIF[a]
				1	2	3	4	5	6	7	8	9	10	LPS/ MOMP	GroEL	GroES	incA	cpaf	HtrA	RpoA	DnaK	CpB 0704	IgG
01	Grd III/60%	58	m	yes	no	yes	yes	no	no	yes	yes	no	yes	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	Neg	Neg	**Pos**	1:64
02	Grd V/100%	58	m	yes	yes	yes	yes	yes	yes	no	no	no	yes	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	Neg	**Pos**	Neg	Neg	1:256
03	Grd I/10%	24	f	no	no	no	no	no	no	yes	no	no	no	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	1:64
04	Grd IV/90%	51	m	no	yes	yes	yes	yes	no	no	yes	no	yes	**Pos**	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg	**Pos**	Neg	1:64
05	Grd III/40%	57	f	no	yes	no	yes	no	no	no	yes	no	yes	**Pos**	**Pos**	**Pos**	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg	1:256
06	Grd IV/80%	62	m	yes	yes	yes	yes	yes	yes	yes	yes	no	yes	**Pos**	**Pos**	**Pos**	**Pos**	Neg	**Pos**	Neg	**Pos**	Neg	1:256
07	Grd IV/95%	47	m	yes	yes	yes	yes	yes	yes	no	yes	no	yes	**Pos**	**Pos**	**Pos**	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	1:64
08	Grd III/70%	60	m	no	yes	no	no	yes	no	yes	yes	yes	no	**Pos**	**Pos**	Neg	Neg	Neg	**Pos**	**Pos**	Neg	Neg	1:256
09	Grd III/50%	59	m	yes	yes	yes	yes	yes	no	yes	yes	no	yes	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	1:512
10	Grd IV/90%	70	m	yes	yes	yes	yes	yes	yes	yes	yes	no	yes	**Pos**	Neg	Neg	**Pos**	Neg	**Pos**	**Pos**	**Pos**	**Pos**	1:128
11	Grd IV/90%	53	m	yes	yes	yes	yes	yes	yes	no	yes	no	no	**Pos**	**Pos**	**Pos**	Neg	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**	1:256
12	Grd IV/80%	55	m	yes	yes	yes	yes	yes	no	yes	no	no	no	**Pos**	**Pos**	**Pos**	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg	1:256
13	Grd V/100%	60	m	no	yes	yes	yes	yes	no	no	yes	no	yes	**Pos**	**Pos**	**Pos**	**Pos**	Neg	**Pos**	**Pos**	**Pos**	Neg	1:64

1 family history of coronary artery disease (CAD)

2 active CAD

3 arterial hypertension

4 hyperlipidemia

5 prior miyocardial infarction

6 previous surgery

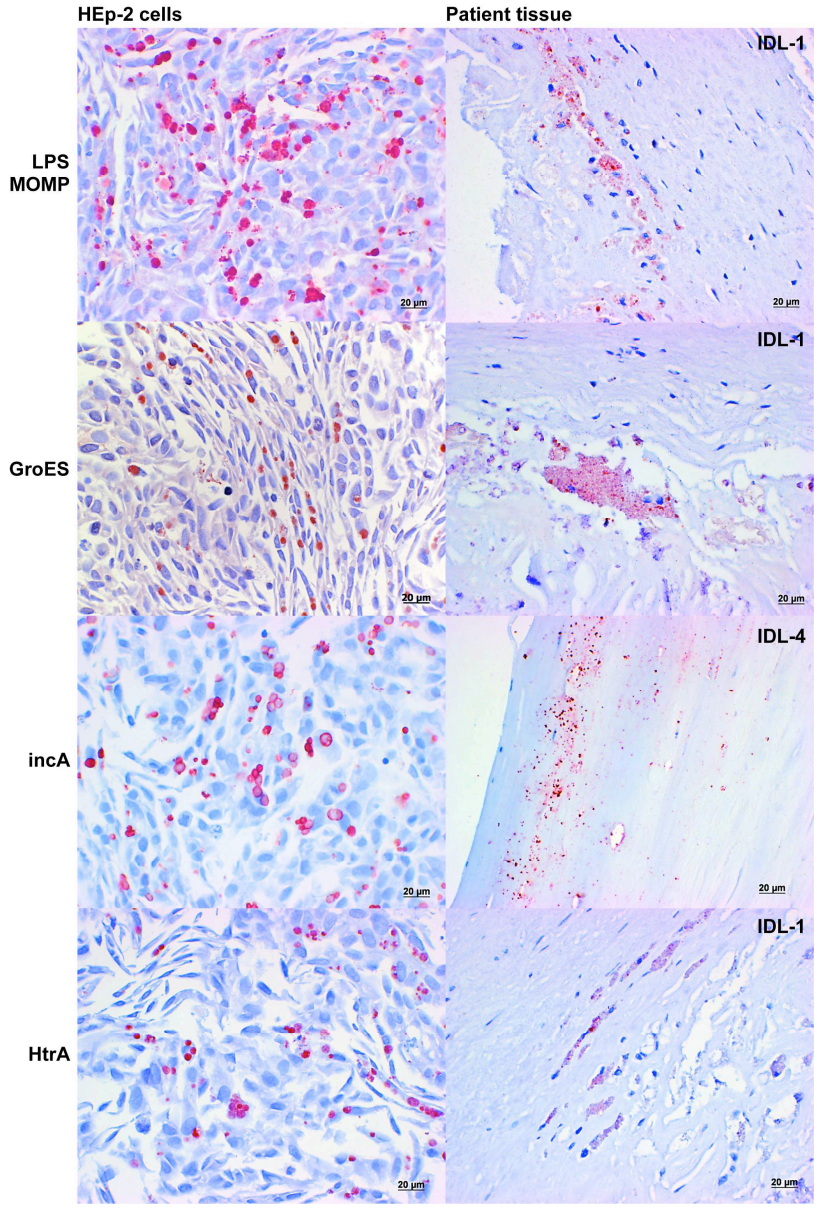
7 atrial fibrillation

8 history of smoking

9 history of alcoholism

10 Diabetes

[a]Microimmunofluorescence



Comparative immunohistochemistry results of HEp-2 cells (left panel) and of patient tissue (right panel). Coronary artery specimens from patient number 1 (IDL-1) and patient number 4 (IDL-4) are shown. Antibodies against the lipopolysaccharide and the major outer membrane protein of the family Chlamydiaceae (LPS/MOMP), the heat-shock protein GroES, the inclusion membrane protein incA, and the DO serine protease HtrA were used. Positive labeling with the respective antibodies is present intracytoplasmic in chlamydial inclusions in HEp-2 cells (left panel) and intracytoplasmic and granular in macrophages and smooth muscle cells of coronary arteries of heart transplant patients (right panel).
174x260mm (300 x 300 DPI)