The association between Chlamydia Pneumoniae and Atherosclerosis

John D Rutherford, MB ChB, FRACP

Internal Medicine Grand Rounds
University of Texas Southwestern Medical Center
Dallas, Texas

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Biographical information :

John D Rutherford, MB ChB, FRACP, FACC
Associate Dean, Clinical Affairs,
Professor of Internal Medicine,
Gail Griffiths Hill Chair in Cardiology,
President of Medical Staff , Parkland Health & Hospital Systems, 1998-9.

Interests:
Acute and Chronic Ischemic Heart Disease
Heart Disease in Pregnancy

THE ORGANISM

The Chlamydiae are obligate intracellular bacteria. Unlike viruses, the Chlamydiae have a cell wall similar to that of gram-negative bacteria, they contain both DNA and RNA, have prokaryotic ribosomes, and synthesize their own proteins, nucleic acids, and lipids. They divide by binary fission and are susceptible to antibiotics. They cannot replicate outside cells or synthesize high-energy adenosine triphosphate metabolites; they are "energy parasites". The Chlamydiae have a unique developmental cycle and are classified in a separate order; chlamydiales, with one family: chlamydiaceae, containing one genus Chlamydia, in which three species are recognized; Chlamydia trachomatis, Chlamydia psittaci, and Chlamydia pneumoniae (C. pneumoniae- formerly called the TWAR agent). All phylogenetic trees show a distinct line of descent of the family chlamydiaceae built of two main clusters : the C. Pneumoniae cluster and the Chlamydia psittaci cluster[1]. Chlamydia trachomatis is the more recently evolved species within the genus Chlamydia. C. Pneumoniae lacks appreciable DNA homology with either C.trachomatis or C.psittaci, it has different restriction endonuclease patterns, and it lacks extra chromosomal DNA, which both C.trachomatis and psittaci have.

Two morphologically distinct forms of Chlamydiae are recognized. There is a dense, spherical elementary body, which contains prokaryotic ribosomal RNA and has a rigid cell wall, which is the infectious form of the organism, and is capable of limited extra cellular survival. The other form is the osmotically fragile reticulate body, it is the intracellular metabolically active form and is incapable of existing outside cells. The closed circular DNA of both forms, compactly organized in a central nucleoid, has a molecular weight of 660 million, and codes for about 600 proteins.

The outer membrane of the chlamydial elementary body, like that of many gram-negative bacteria, has several components the most prominent being the major outer membrane protein (MOMP), a transmembrane protein with type-, subspecies-, and genus-reactive epitopes defined by monoclonal antibodies. Infection with Chlamydiae induces MOMP-specific antibodies, but their role in protective immunity and in diagnosis is unclear. The Chlamydia outer membrane also contains a lipopolysaccharide (LPS) antigen that is structurally similar to that of Salmonella Minnesota Re strains. The extractable Chlamydia LPS is the major antigen detected in genus-specific serologic tests for Chlamydiae, and monoclonal antibodies and monospecific polyvalent antisera to the LPS are used in enzyme immunoassays to detect Chlamydia antigen and clinical specimens.

The organism, Chlamydia pneumoniae, was initially considered to be a strain of Chlamydia psittaci and was named TWAR for the laboratory identifying letters of the first two isolates: TW-183, isolated from the eye of a control child in a trachoma vaccine study in Taiwan in 1965, and AR-39 recovered from the throat of a student with pharyngitis at the University of Washington in 1965. Data from DNA homology and electron microscopy studies demonstrated that these organisms were a separate species, Chlamydia pneumoniae. Strains of C.pneumoniae and C.psittaci have 10% or less DNA sequence homology, and the elementary body of C.pneumoniae is pear shaped and has a large periplasmic space, different from the typically round elementary bodies of C.psittaci and C.trachomatis.

ANIMAL MODELS

Models for the development of C.pneumoniae induced pneumonitis have been developed in mice and rabbits.

New Zealand white rabbits were inoculated intranasally and intratracheally with Chlamydia pneumoniae, strain AR-39 and after a single inoculation, lung pathology was characterized by a moderate self resolving interstitial pneumonia of 21 days duration[2]. Watanabe heritable hyperlipidemic rabbits were less susceptible to C. pneumoniae infection. After multiple inoculations C. Pneumoniae was detected by PCR and/or immunocytochemistry until day 21.

In another rabbit model,[3] (not fed a high cholesterol diet) New Zealand White rabbits were inoculated with C. Pneumoniae via the nasopharynx and lung inflammation similar to the prior model ensued. However , two rabbits demonstrated early and intermediate lesions of atherosclerosis (Day 7 - one animal showed accumulation of foamy macrophages [fatty streak or focal periaortitis] in the arch of the aorta and the other animal showed spindle cell proliferation of smooth muscle cells on Day 14) and C. Pneumoniae antibodies were demonstrated by immunocytological stain in the aorta (one of which corresponded to the intermediate lesion).

Laitinen et al [4], infected New Zealand white rabbits, on a normal diet, intranasally with Chlamydia pneumoniae. Reinfection was given 3 weeks later. Six of the nine reinfected animals showed inflammatory changes consisting of intimal thickening or fibroid plaques resembling atherosclerosis in 2 to 4 weeks after reinfection. One rabbit had calcified lesions. Immunohistochemistry for C. Pneumoniae was strongly positive in the three older affected animals. No lesions were seen in the controls. The results suggest that infection is capable of inducing inflammatory atherosclerosis-like changes in the aortas of infected rabbits.

Last Muhlestein et al [5], gave three separate innoculations to 30 New Zealand white rabbits of either C. pneumoniae (n = 20) or saline (n = 10) at 3-week intervals and fed chow enriched with a small amount (0.25%) of cholesterol. Immediately after the final inoculation, infected and control rabbits were randomized and begun on a 7-week course of Azithromycin or no therapy. Three months after the final inoculation, rabbits were sacrificed and sections of thoracic aortas were blindly evaluated microscopically for maximal intimal thickness, percentage of luminal circumference involved, and plaque area index of atherosclerosis. Vascular C. pneumoniae antigen was assessed by direct immunofluorescence. Maximal intimal thickness differed among treatment groups (P=.009), showing an increase in infected rabbits (0.55 mm; SE = 0.15 mm) compared with uninfected controls (0.16 mm; SE = 0.06 mm) and with infected rabbits receiving antibiotics (0.20 mm; SE = 0.03 mm) (both P<.025), whereas maximal intimal thickness in infected/treated versus control rabbits did not differ. Plaque area index differed significantly (P<.01) among groups with a similar pattern. Chlamydial antigen was detected in 2 untreated, 3 treated, and 0 control animals.

Apolipoprotein E-deficient transgenic mice, which spontaneously developed atherosclerosis, and C57BL/6J mice which only developed atherosclerosis on an atherogenic diet have been evaluated[6]. Following single and multiple intranasal inoculations of apo E-deficient transgenic mice, C. pneumoniae were detected in lung, aorta and spleen for 20 weeks after inoculation in 25-100% of mice. In the aorta, C. pneumoniae were detected within the atherosclerotic lesion. In the other mouse model C. pneumoniae were detected in the aorta only two weeks after a single intranasal inoculation in 8% of mice.

CELLS

The interaction of C. pneumoniae with various cell types has undergone limited study. Gaydos et al [7], studied in vitro growth of C. pneumoniae in two macrophage cell lines (human bronchoalveolar lavarge and peripheral blood monocyte derived), several endothelial cell lines and aortic smooth muscle cells. Five strains of C. pneumoniae were capable of three passages in human U 937 and in murine RAW 246.7 macrophages. Both types of macrophages were able to inhibit C. pneumoniae after 96 hours of growth. Eleven C. pneumoniae strains were capable of replicating in normal human aortic artery-derived human endothelial cells, umbilical vein derived endothelial cells and pulmonary artery endothelial cells. Infection in human aortic artery smooth muscle cells was established for 13 strains of Chlamydia pneumoniae.

Kalayoglu and Byrne [8], found that exposure of macrophages C. pneumoniae followed by low-density lipoprotein (LDL) caused a marked increase in the number of foam cells and accumulation of cholesteryl esters. Foam cell formation was not inhibited by the antioxidant butylated hydroxytoluene nor fucoidan, suggesting that lipid accumulation did not involve scavenger receptors. In contrast, addition of heparin, which blocks binding of LDL to the LDL receptor, inhibited C. pneumoniae -induced foam cell formation, suggesting that the pathogen induced lipid accumulation by dysregulating native LDL uptake or metabolism (or both). These data demonstrate that an infectious agent can induce macrophage foam cell formation and implicate C. pneumoniae as a causative factor in atherosclerosis.

Knoebel et al [9], evaluated the ability of C. pneumoniae to infect cells that make up atherosclerotic lesions, including endothelial cells, smooth muscle cells, and cholesterol-loaded smooth muscle cells. The organism readily infected rabbit, bovine, and human aortic smooth muscle cells. Cholesterol-loaded smooth muscle cells were even more susceptible to C. pneumoniae infection. Chlamydia trachomatis inefficiently infected smooth muscle cells, demonstrating that this is not a characteristic of all members of the genus Chlamydia. C. pneumoniae infected bovine endothelial cells poorly.

Last, Fryer et al [10], investigated whether Chlamydia pneumoniae, and Chlamydia trachomatis (types H and L2/434/BU) could infect cultured human venous endothelial cells. The ability of infected cells to express procoagulant (tissue factor) activity was also measured and adhesion of platelets to chlamydia-infected cells was a quantitated. They found that Chlamydia pneumoniae, Chlamydia trachomatis type H, and Chlamydia trachomatis L2/434/BU could infect cultured human umbilical vein endothelial cells and stimulate a 4-fold increase in expression of tissue factor, which reached a peak 18 hours postinfection. Tissue factor expression was enhanced even in the presence of tetracycline, suggesting that the chlamydial factor responsible for stimulating synthesis of endothelial cell tissue factor was preformed. Platelet adhesion was significantly enhanced when endothelial cells were infected by chlamydia species

EPIDEMIOLOGY AND CLINICAL MANIFESTATIONS

C. pneumoniae (TWAR) has a global distribution, with infection most common among children between the ages of 5 and 14 years [11]. In children, TWAR infection is usually mild or asymptomatic [12], but it may be more severe in adults. Immunoglobulin G (IgG) titers have been

detected in 40-60% of adults in seven different countries (40% in Nova Scotia, Canada, to over 60% in Taiwan and Panama). In Denmark and Seattle, Washington, antibody prevalence rates were low in children, increased sharply in teen-agers, continued to increase until middle age, and remained high until old age. In the seven countries evaluated, seropositivity rates were 10-25% higher for males. Data from retrospective and prospective serologic studies indicate that disease caused by C. pneumoniae is endemic in the United States and epidemic in Scandinavia and Finland and that infection does not occur with any consistent seasonal periodicity. The mechanism and place of transmission, the incubation period, and the infectiousness of the organism are unknown although there is evidence that C. pneumoniae is a primary human pathogen transmitted from human to human without an avian or animal reservoir. Chlamydia trachomatis causes sexually transmitted disease and eye disease and Chlamydia psittaci infects birds and causes a human pneumonitis.

Pneumonia and bronchitis are the most common clinical manifestations of C. pneumoniae infection, and TWAR is responsible for approximately 10% of cases of community-acquired and nosocomial pneumonias and 5% of cases of bronchitis in the United States. The infection is usually mild but may be severe especially in elderly patients with chronic disease. The illness frequently begins with upper respiratory tract infections particularly pharyngitis with hoarseness, followed by persistent cough and other symptoms of lower respiratory disease. Infections frequently are mild or asymptomatic and go unrecognized.

The microimmunofluorescence serologic assay is specific for TWAR and can distinguish between recent and past infections. The organism can be isolated in cell culture; however, PCR techniques have recently facilitated its detection in tissues and clinical specimens.
Pneumoniae.

INFLAMMATION, IMMUNITY ETC

Inflammation may have a role in both the initiation and progression of atherosclerosis [13, 14] and there is great interest in the potential role of inflammation in the pathogenesis of the acute coronary syndromes [15].
Plasma C-reactive protein - a marker for systemic inflammation - was measured in 545 apparently healthy men participating in the Physicians' Health Study in whom myocardial infarction, stroke or venous thrombosis subsequently developed and in 543 study participants who did not report these events in greater than 8 years of follow-up [16] . Those physicians in the highest quartile of baseline C-reactive values had 3 times the risk of myocardial infarction and 2 times the risk of ischemic stroke during follow-up. In addition, the use of aspirin was associated with a 56% reduction in the risk of myocardial infarction in physicians with the highest C-reactive protein values but this did not confer a benefit to those in the lowest quartile. Furthermore, in this same population, the C-reactive protein value added to the predictive value of total cholesterol and HDL in determining the risk of first myocardial infarction [17].

The European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group used new ultrasensitive immunoassays to measure C-reactive protein and serum amyloid A protein (SAA) concentrations in plasma from 2121 outpatients with angina (1030 unstable, 743 stable, the rest atypical) [18]. All patients underwent coronary and were followed up for 2 years. 75 individuals (41 with unstable, 29 with stable, and 5 with atypical angina) had a coronary event during follow-

up. Concentrations of C-reactive at study entry were associated with coronary events in patients with stable or unstable angina: there was about a 2 -fold increase in the risk of a coronary event in patients whose C-reactive concentration was in the fifth quintile (> 3.6 mg/L), compared with the first four quintiles. A third of the events occurred among patients who had a C-reactive concentration of more than 3.6 mg/L. C-reactive concentrations were positively correlated with age, smoking, body-mass index, triglycerides, extent of coronary stenosis, history of myocardial infarction, and lower ejection fraction.

To attempt to link an inflammatory marker (C-reactive protein) to infection blood samples were prospectively collected from 363 patients undergoing coronary angiography and both C-reactive protein and IgG titers to Chlamydia pneumoniae, Helicobacter pylori and Cytomegalovirus were measured [19]. C-reactive protein levels were higher in patients with coronary artery disease (> two-fold) and myocardial infarction (> four- fold) , infectious serology was highly prevalent in patients and controls and sero-positivity to both C. pneumoniae and Helicobacter pylori (but neither agent alone) may be associated with higher C-reactive protein levels and may predict risk of coronary artery disease and myocardial infarction. However, the link between specific chronic infections and coronary artery disease are tenuous [20] although the scattered evidence is intriguing [13, 21].

SEROLOGIC TESTS

Few of the studies examining serology and epidemiology have been performed prospectively or with a case-control design, the studies have been for the mostly descriptive and often confounding variables have not been accounted for [13].

Chlamydia pneumoniae, strain TWAR, has been associated with atherosclerotic cardiovascular disease in a series of seroepidemiological studies from Finland and the United States which have shown a statistically significant association between several types of TWAR antibody, including immune complexes, and atherosclerotic disease of the coronary and carotid arteries [22]. The laboratory diagnosis of infection with C. pneumoniae is based predominantly on serologic tests. The microimmunofluorescence tests with the TWAR antigen, capable of distinguishing IgM and IgG, is specific for C. pneumoniae . IgM appears about three weeks after the onset of the primary illness, usually declines over the next two to six months to a level that cannot be detected and may not reappear with infection. IgG detected six to eight weeks after the onset of the illness primarily persists for life and may arise one to two weeks following re-infection. A four-fold rise in IgG to between acute and convalescent phase serum samples, a single IgG titer of 1:512 or greater, or a IgM of 1:16 or greater is consistent with acute infection. C. pneumoniae can be isolated in cell culture.

In patients with carotid atherosclerosis [23] and myocardial infarction [24-28], compared to a variety of control populations, there appears to be a high prevalence of IgG (or IgA [24, 25, 28]) antibodies to C. pneumoniae indicating a past or chronic infection. In some studies high titers of antibodies appear to be related to increased risk of an ischemic event (onset of angina or myocardial infarction) [27], or acute myocardial infarction may be associated with antibody conversion [24]. Other studies have demonstrated no relationship between high antibody titers and acute myocardial infarction [25].

In both controls and patients with recent ischemic cerebral infarctions, or transient ischemic attacks, a similar, a high prevalence of specific serum IgG antibodies to C. pneumoniae have been

demonstrated. However elevated IgA titers and specific IgG antibodies present in circulating immune complexes were more likely to be present in patients with cerebrovascular events than in controls - odds ratios of 1.71 and 2.0 respectively [29].

Antibodies to C. pneumoniae were measured in 176 patients with stroke or transient cerebral ischemia and in 1518 control subjects. It was found that 14% of stroke/transient ischemic attack (TIA) patients and 6 % of control subjects had antibody titers suggesting C. pneumoniae (re)infection, while 32% of stroke/TIA patients and 13% of control subjects had titers suggesting previous infection (P<.05). Stroke/TIA patients differed from control subjects in their levels of acute and previous infection, with adjusted odds ratios of 4.2 (95% CI, 2.5 to 7.1) and 4.4 (95% CI, 3.0 to 6.5), respectively. These data support the association of cerebral vascular disease with prior C. pneumoniae infection and acute events with recrudescence of infection [30].

In 34 male patients, who had undergone previous coronary artery bypass graft surgery, four months of doxycycline therapy - administered in a randomized, placebo-controlled trial - did not influence serological markers of C. pneumoniae infection [31].

Cell-mediated and humoral immune responses to Chlamydia in 93 patients with angiographically confirmed coronary heart disease and in 115 controls without angiographically demonstrable lesions were analyzed by measuring lymphocyte proliferative reactivity to whole elementary body antigens of C. pneumoniae [32-34]. Control antigens included C. trachomatis and purified protein derivative of tuberculin. Chlamydia-specific antibodies were measured using microimmunofluorescence assay. Marked C. pneumoniae-specific immune reactivity, demonstrated by the high incidence of elevated IgG and IgA antibodies and strong lymphocyte proliferative response, was associated with coronary heart disease in male but not in female patients or controls. In male patients, the cell-mediated responses were strong to C. pneumoniae. This suggests that the immune mechanisms triggered by Chlamydia are a possible contributing factor in the disease pathogenesis of coronary atherosclerosis in males

PATHOLOGIC EVIDENCE

Morphological-molecular studies have shown the C. pneumoniae organism in atheroma of the coronary arteries, aorta, carotid and peripheral arteries but not in normal arteries. The presence of the organism in atheroma has been demonstrated by electron-microscopy, immunocytochemical staining with TWAR-specific monoclonal antibody and by the polymerase chain reaction for TWAR-specific DNA [22]. Jackson et al, showed that C. pneumoniae is more frequently found in atherosclerotic than normal tissue. Cardiovascular and non-cardiovascular tissue samples from 38 autopsy cases were tested by polymerase chain reaction and immunocytochemistry. 33 granuloma biopsy specimens were also tested, as the organism has been detected in macrophages. C. pneumoniae was detected in coronary artery tissue in 34%, lung in 13%, liver in 10%, and spleen in 5% of the 38 autopsy cases ($P < 0.05$ for comparison of proportion of positive coronary arteries with that of each of the other types of tissue). Of the 21 cases with at least one positive tissue sample, 11 had only a positive cardiovascular tissue (coronary artery, venous bypass graft, or myocardium), 7 had both cardiovascular and non-cardiovascular positive tissues, and 3 had only a non-cardiovascular positive tissue [35].

Coronary Arteries

In 36 autopsy cases from Johannesburg, South Africa, C. pneumoniae was detected by immunocytochemistry (15/36) and by polymerase chain reaction (13/30). Sequence analysis of the C. pneumoniae rRNA genes amplified by polymerase chain reaction (PCR) confirmed that the amplified gene products were Chlamydia pneumoniae. Electron microscopy revealed typical pear-shaped C. pneumoniae elementary bodies in 6 of 21 atheromatous plaques [36]. In a multicenter study, called Pathobiological Determinants of Atherosclerosis in Youth (PDAY), a left anterior descending coronary artery sample was examined in 49 subjects ages 15-34 who had sudden or unexpected death, usually traumatic, and were not known to have atherosclerosis. Seven of the artery samples were found to have atheromatous plaque, 11 had intimal thickening and 31 had no lesions. Eight of the samples were positive for C. pneumoniae by immunocytochemistry (N=7) and/or PCR (N=3). Six of the 7 with atheroma, two of the 11 with intimal thickening and none of the 31 normal appearing coronary samples were positive [37].

Coronary specimens from atherectomy, endarterectomy and re-stenotic coronary artery bypass samples have also been tested.

90 patients with symptomatic coronary disease undergoing coronary atherectomy were tested for the presence of Chlamydia species using direct immunofluorescence [38]. Control specimens from 24 subjects were also examined. Coronary atherectomy specimens were definitely positive in 73%, equivocally positive in 6%, and only 1 (4%) of 24 non-atherosclerotic specimens showed any evidence of C. pneumoniae ($P = < 0.001$). Transmission electron microscopy was used to confirm the presence of appropriate organisms in 3 of 5 positive specimens. The Chlamydia strain TWAR was identified by PCR in two specimens.

In 70 patients, undergoing myocardial revascularization, atherosclerotic lesions from 53 coronary endarterectomy and 17 restenotic bypass samples were cultured and subjected to nested polymerase chain reaction (PCR) for Chlamydia pneumoniae. Antichlamydial immunoglobulin G (IgG), IgA and IgM was examined by microimmunofluorescence. Viable C. pneumoniae were recovered from 16% of 70 atheromata, and chlamydial deoxyribonucleic acid (DNA) was detected in 30% of 70 atheromata; 17 nonatherosclerotic control samples were PCR-negative ($p < 0.01$). Fifteen (28%) of 53 endarterectomy and 35% of 17 bypass samples were PCR-positive. DNA sequencing of six different PCR products did not reveal differences between coronary isolates and respiratory reference strains, suggesting that common respiratory strains gain access to the systemic circulation. Serologic results did not correlate with direct detection results and did not identify individual endovascular infection [39].

Last, C. pneumoniae has been isolated from the coronary artery of the native heart a patient undergoing cardiac transplantation[40]. In 12 consecutive patients undergoing heart transplantation culture for C. pneumoniae was attempted in HEp-2 cell monolayers. Other methods of detection included PCR, immunocytochemistry, transmission EM, and in situ hybridization. C. pneumoniae was cultured from atherosclerotic plaques in one patient and was found in the atheromas of this patient by PCR, immunocytochemistry transmission EM, and in situ hybridization. In addition at least one testing method showed C. pneumoniae in coronary artery tissue in 6 of 9 additional patients with coronary atherosclerosis.

Aorta

C. pneumoniae antigens were demonstrated using the immunocytochemical technique in atheroma of the aorta in autopsy patients from retrospective studies at the University of Washington [41]. The patients were 34 - 58 years old. Immunoperoxidase staining using Chlamydia-specific monoclonal antibodies showed 1 of 4 fatty streaks and 6 of 17 fibrous plaques which were positive for C. pneumoniae antigens. Four control aortic tissues were negative. Two of the positive plaques were from the same patient. Double-label immunocytochemical staining using Chlamydia-and tissue type-specific monoclonal antibodies demonstrated antigens in the cytoplasm of macrophages in smooth muscle cells in the atheromatous lesion.

In a study from Mexico 16 aortic specimens were obtained at autopsy in subjects who died with coronary disease [42]. The presence of C. pneumoniae was determined by means of an immunofluorescent technique using a specific monoclonal murine antibody. A positive reaction was found in advanced non-ulcerated fibrolipid lesions in just 2 patients (13%).

Carotid Arteries

Carotid endarterectomy specimans have been examined in a number of studies. Atherosclerotic carotid arteries were examined using a C. pneumoniae-specific nested polymerase chain reaction. Chlamydial DNA was detected in 9 of 61 (15%) arterial samples obtained from significant carotid stenoses. Chlamydial presence appeared limited to advanced atherosclerotic lesions ($P < 0.02$): since tissues from the same arteries with early subendothelial lesions did not harbor the pathogen [43].

Carotid endarterectomy specimens from 76 patients with carotid artery stenoses were stained for C. pneumoniae, cytomegalovirus (CMV), and (Herpes Simplex Virus-1) HSV-1 particles with specific IgG monoclonal antibodies by the avidin-biotin-peroxidase method. IgG antibodies to CMV and C. pneumoniae were also measured in the serum. These were correlated with plaque morphology and the presence of the microorganisms in the atherosclerotic plaques. C. pneumoniae was detected in 71%, CMV was detected in 35% and HSV-1 was detected 10% versus none of 20 (0%) control normal carotid artery and aortic tissue (autopsy) specimens ($P<.001$ for CMV and C. pneumoniae). At least one microorganism was detected in 78% of the specimans ,with a single microorganism present 46%, two microorganisms present in 24%, and all three present 8%. Atherosclerotic plaques with thrombosis were more likely to have C. pneumoniae (80%) or CMV (58%) than were plaques without thrombosis (57% and 17%, respectively; $P=.04$ and .007). There was no correlation between the presence of CMV and C. pneumoniae in the atherosclerotic vessels and serum antibody titers [44].

Carotid endarterectomy specimans stained immunohistochemically with monoclonal antibodies for a C pneumoniae-specific antigen, macrophages, and smooth muscle cells demonstrated immunoreactivity for the C pneumoniae-specific antigen in 11 of 20 specimens (55%), and intense immunoreactivity was observed in 7 of 20 (35%). C pneumoniae infection was observed in endothelial cells, macrophages and in smooth muscle cells that had migrated into the atheromatous plaque, as well as in smooth muscle cells and small arteries in the media underlying the atheromatous plaques. C pneumoniae infection was most prominently observed in smooth muscle cells [45].

Aortic aneurysm specimens

Some studies have demonstrated the presence of C. pneumoniae in symptomatic aortic aneurysm specimans and others have not. In 1996 Blasi et al [46], studied 51 patients who underwent abdominal aortic aneurysm surgery and performed a microimmunofluorescence test for IgG, IgA, and IgM antibodies to C. pneumoniae specific antigen (TW-183).Forty-one patients were seropositive for C. pneumoniae with past-infection patterns in 32 and high antibody titers in 9 patients. In 26 of 51 patients, C. pneumoniae DNA was detected in aortic aneurysm plaque specimens. Of these patients, 23 had a serologic past-infection pattern, 2 had an acute reinfection pattern, and 1 was seronegative. (Forty-seven of 51 patients were seropositive for H. pylori but PCR showed no evidence of H. pylori presence in plaque specimens in any of these patients). Juvonen, et al [47], examined specimens from abdominal aortic aneurysms for the presence of C. pneumoniae by immunohistochemical analysis, the polymerase chain reaction amplifying omp 1 gene, transmission electron microscopy, and culture methods with histologically atherosclerosis-negative human aortic tissues used as a control group. Chlamydial lipopolysaccharide and C. pneumoniae specific antigens were found by immunohistochemistry in 12 of 12 and 8 of 12 aneurysm specimens, respectively, and C. pneumoniae DNA could be demonstrated in 6 of 6 aneurysm specimens studied. Furthermore electron microscopy revealed the presence of Chlamydia-like elementary bodies in 3/4 aneurysm specimens tested. None of the control samples gave positive reaction in the polymerase chain reaction, and C. pneumoniae antigens were not detected in any of them. Last, Peterson et al [48], studied 40 patients operated transperitoneally for an infrarenal abdominal aortic aneurysm. Specimens from the aneurysm wall were taken peroperatively under sterile conditions. The control group consisted of 40 deceased persons without aortic aneurysms in whom specimens from the non-aneurysmal infrarenal aortas were collected within 48 h after death. The specimens from both groups were frozen at -70 degrees C immediately after collection. A nested polymerase chain reaction (PCR) method, using two sets of primers designed to detect a fragment of the major outer membrane protein gene of Chlamydia pneumoniae, was used. The detection of C. pneumoniae -specific DNA was significantly higher in the study group (14/40 = 35%) than in the control group (2/40 = 5%); ($p = 0.001$).

In contrast, Lindholt et al [49], performed an inhibitor-controlled nested polymerase chain reaction (PCR) amplifying fragments of the gene encoding the C. pneumoniae specific major outer membrane protein after optimization of DNA extraction procedures on 124 wall-specimens from 20 patients with symptomatic abdominal aortic aneurysms. None of the specimens contained C. pneumoniae -specific DNA. Minor inhibition of the PCR was noticed especially in media specimens. Using a sensitive and specific nested PCR, the investigators were not able to detect C. pneumoniae in patients with symptomatic abdominal aortic aneurysms.

Femoral and Popliteal Arteries

Arterial biopsy specimens obtained from femoral and popliteal arteries during bypass operations for claudication were examined by immunocytochemical analysis and PCR reaction for the presence of organisms. C. pneumoniae was detected in atherosclerotic plaques by either method in either artery of 11/23 patients. 8 of 21 popliteal and 3 of 18 femoral arteries had positive results [50].

Aortic valves

Nystrom-Rosander et al [51], performed a prospective study of the incidence of C. pneumoniae in the sclerotic valves of patients undergoing aortic valve replacement for aortic stenosis and of the aortic valves of patients dying of non-cardiac causes. The results were correlated to serological markers of past (IgG) or persistent (IgA) C. pneumoniae infection. Chlamydia pneumoniae, as determined PCR, was detected in the aortic valve in 19/39 (49%) patients and in 1/11 (9%) of autopsy controls ($p = 0.018$) and confirmed by electron microscopy in one patient. There was no significant difference in the incidence rate of IgG or IgA antibody positivity between PCR-positive and PCR-negative cardiac patients.

Juvonen et al [52], examined aortic valve specimens with varying degrees of macroscopic disease in 35 subjects--17 consecutive patients undergoing aortic valve replacement for treatment of nonrheumatic aortic stenosis and 18 age-matched subjects at autopsy. The possible presence of C. pneumoniae in aortic valves was studied by immunohistochemical analysis, polymerase chain reaction or transmission electron microscopy, or a combination of these. Positive immunohistochemical staining with C. pneumoniae specific antibody was found in 53% of 17 patients with advanced aortic valve disease requiring surgical treatment (group A), 80% of 10 cadavers with clearly macroscopic aortic valve pathology (group B) and 1 (12%) of 8 grossly normal cadaver control subjects (group C). Statistical significance with regard to the presence of C. pneumoniae was found when combined diseased subjects were compared normal control subjects ($p = 0.018$). However, when patients with advanced disease were compared with control subjects, there was only marginal statistical significance ($p = 0.088$). There was strong statistical significance ($p = 0.015$) when cadavers with macroscopic pathology were compared with normal cadaver control subjects. C. pneumoniae DNA was also found in 3 stenotic valves, and in two of the three tested valve specimens chlamydia-like particles were seen by electron microscopy

RISK FACTORS

To evaluate whether an association exists between chronic C. pneumoniae infection and serum lipid values lipid profiles and C. pneumoniae antibodies were measured from paired serum samples of 415 Finnish males taken 3 years apart [53]. Chronic infection, defined as persistent IgG and IgA antibodies, was found in 20%, and the antibodies were negative in 15% of the cases studied. The serum triglyceride and total cholesterol concentrations were higher in the subjects with a chronic C. pneumoniae infection than in the subjects with no antibodies (108 mg/dl versus 91 mg/dL and 248 mg/dL versus 244 mg/dL, respectively). The HDL cholesterol concentrations were reduced and the ratios total cholesterol to HDL cholesterol were significantly increased in the subjects with chronic infection.

4,000 dyslipidemic middle aged men in the Helsinki Heart Study were followed for cardiac events. C. pneumoniae infection as indicated by the presence of an elevated IgA titer and of LPS containing immune complexes in sera was an independent risk factor for the development of coronary heart disease. Patients with elevated IgA titers against C. pneumoniae or presence of immune complexes containing C. pneumoniae antigen were twice as likely to suffer a cardiac event within the next six months (odds ratio = 2.3). This increased risk was independent of age, hypertension and smoking [54].

PATIENT TREATMENT STUDIES

The in vitro susceptibilities of C. pneumoniae isolates to macrolide, tetracycline, and quinolone antibiotics have been determined. Tetracycline, clarithromycin, and erythromycin have the lowest MICs in the first cell culture passage and Azithromycin requires the lowest concentration for complete inhibition of inclusion formation on the second pass into antibiotic-free medium, probably reflecting its high intracellular concentrations [55].

A few, relatively small, trials of secondary prevention have been completed or are ongoing. It has been argued that since 80-90% of persons with coronary artery disease have C. pneumoniae antibody that testing for antibody as a prerequiste to randomization to therapy is a waste of time [56] and it is debated how long treatment should continue. The elementary body (EB) form of the organism is the infectious nonreplicating form and it is not susceptible to the action of antibiotics - indeed it may exist in the body for weeks or months and initiate new cellular infection[56].

200 myocardial infarct survivors were screened for elevated C. pneumoniae titers (IgG > 1/64) using the microimmunofluorescence test. 60 eligible patients were randomized into a double-blind prospective study and received a three day course of Azithromycin (Az) or placebo. A further course of Az was given after three months to 45 of the original 60 patients. Blood samples were taken at 1, 3 and 6 months. At 6 months the subset of patients randomized to double Az therapy (n= 11) showed a significant fall in total monocyte tissue factor and CD 11B expression compared to patients receiving placebo only. Repeat C-reactive protein serology at 6 months showed that 5/11 Az patients (compared to placebo/placebo patients) had a significant titer fall. These 5 patients also had a fall in total leukocyte count and serum neopterin - a marker of activated macrophages (compared to the 16 patients with unchanged titers). It was concluded that Azithromycin therapy reduces monocyte activation and C. pneumoniae antibody titers in post MI patients [57].

The ACADEMIC study [58] is a randomized, placebo-controlled trial of Azithromycin in patients with evidence of coronary artery disease and positive titers to C. pneumoniae. A total of 447 patients with coronary artery disease were screened. 302 had positive C. pneumoniae titers (≥ 1:16) were randomized to placebo (n=152) or Azithromycin (n=150). Treatment was continued for 3 months. The primary end-point of the study was the change in a composite of 4 inflammatory markers (C-reactive protein, IL-1, IL-6, and tumor necrosis factor) at 3 months. Additional follow-up was conducted at 6 months.
At 3 months : No significant changes in composite or individual inflammatory markers.
At 6 months : The Azithromycin group had a significant decrease in C- reactive protein and IL-6.
No significant differences in clinical outcomes.

Acute myocardial infarction survivors were screened for anti- C. pneumoniae antibodies [59]. Of these 213 were stratified into three groups:-

1. C. pneumoniae - ve = Cp -ve (N = 59) - no antibodies detectable.
2. C. pneumoniae - I = Cp I (N = 74) = intermediate titers of 1/8 to 1/32.
3. C. pneumoniae +ve = Cp +ve (N = 80). Seropositive > 1/64 dilution.

Cp +ve patients were randomized to either oral Azithromiacin = Cp +ve **A** (500 mg for 3 days, N = 28), 500 mg for 6 days (N = 12), or placebo = Cp +ve **P** (N = 20).
Follow-up was for 18 months for adverse cardiovascular events:- Cp -ve = 7%, Cp I = 15%, Cp +ve not randomized = 30% , Cp +ve Placebo = 25% (N = 5). In contrast the odds ratio for events in patients receiving azithromycin (Cp +ve **A**) and positive antibodies was the same as the Cp -ve group. Therefore, C. pneumoniae antibody positive patients not randomized or on placebo had odds ratio of 4.2, P = 0.03, for adverse cardiovascular events compared with C. pneumoniae antibody negative patients. The odds ratios for cardiac vascular events in patients receiving Azithromiacin was the same as for the C. pneumoniae antibody negative group (0.9, P = NS). Patients receiving Azithromycin were more likely to experience a decrease in IgG anti C. pneumoniae titers than were those in the placebo group (P = 0.02) .

The effect of Roxithromycin was assessed in 202 patients with unstable angina or a non Q wave myocardial infarction [60]. Patients were randomly assigned to either Roxithromycin 150 mg orally twice a day (N = 102) or placebo orally twice a day (N = 100). The treatment was continued for 30 days. Patients were followed up for six months. The primary clinical end points were (cardiac ischemic death, myocardial infarction, severe recurrent ischemia) assessed at day 31, in 202 patients on an intention to treat basis. A statistically significant reduction in the primary end point rates was observed in the Roxithromycin group, P = 0.032. The rate of severe recurrent ischemia, myocardial infarction, and ischemic death was 5.4%, 2.2%, and 2.2% in the placebo group and 1.1%, 0%, 0% in the treated group respectively.

Evidence supporting involvement of C. pneumoniae in atherosclerosis :-

Serology & epidemiology	+
Pathogen present in atheroma	+
Can produce atheroma in animals	±
Proof of causality	—

To distinguish a pathogenic from an adventitious microbe the following postulates of Robert Koch need to be fulfilled.
1. The microorganism is present invariably in the diseased individual.
2. The microorganism can be isolated (in pure culture on artificial media) from the diseased individual.
3. Inoculation of the etiologic agent into a healthy experimental animal should produce a disease similar to that observed initially.
4. The presumed pathogen should be isolated from the animal with the experimentally induced disease.

These conditions may not be met if the pathogen cannot be cultured in vitro or when it is not pathogenic for available laboratory animals. Some organisms (viruses) cannot be grown on artificial media and some organisms are pathogenic only for man.

Some comments on the evidence. Few of the studies examining serology and epidemiology have been performed prospectively or with a case-control design, the studies have been mostly descriptive and often confounding variables have not been accounted for [13]. While several studies have provided evidence for the presence of C. pneumoniae in human atherosclerotic specimens , as

outlined in the protocol, using a variety of techniques including immunochemistry, polymerase chain reaction detection of nucleic acid, and electron microscopy recovery of these organisms by culture has been less successful [61]. C. pneumoniae has been isolated from the coronary artery of the native heart a patient undergoing cardiac transplantation [40]. The organism was cultured from plaque and was found in the atheromata of this patient by PCR, immunocytochemistry, transmission EM, and in situ hybridization. However, this is anecdotal evidence. Even if infectious particles are demonstrated to be present in atheroma by the presence of antigens and / or characteristic nucleic acids a pathogenic role is not established. There is minimal evidence supporting a role for C. pneumoniae infection in animal models of atherosclerosis [2, 3, 5, 6]. The are data suggesting that C. pneumoniae can infect endothelial cells, smooth muscle cells [7, 9], and epithelial cells and induce the formation of foam cells [8]. Since under normal circumstances the life cycle of chlamydia involves two forms (the elementary body and, the replicative form, the reticulate body) it is possible that infection with Chlamydia may result in a chronic, persistent, non-lytic infection of cells rather than a lethal infection. It is unknown whether macrophages or vascular cell walls are susceptible to persistent infection [13]. It is unclear whether C. pneumoniae localizes more in atherosclerotic lesions than uninvolved arteries merely because macrophages are present or whether infection of macrophages might potentiate atherogenesis by enhancing cytokine and growth factor release from phagocytic cells within the involved atheromatous lesion. It is unclear whether infection with C. pneumoniae is a cause, a cofactor or an unimportant commensal in the context of atheroma [13, 21, 62-64].

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