Chlamydia Pneumoniae–Specific Circulating Immune Complexes in Patients With Chronic Coronary Heart Disease

Eila Linnamäki, MSc; Maija Leinonen, PhD; Kimmo Mattila, MD; Markku S. Nieminen, MD; Ville Valtonen, MD; and Pekka Saikku, MD

Background. An association of chronic Chlamydia pneumoniae infection to coronary heart disease has been suggested recently. In a recent study, we demonstrated circulating immune complexes containing chlamydial genus-specific lipopolysaccharide in patients with coronary heart disease. The objective of the present study was to investigate whether C. pneumoniae species-specific immune complexes are present in chronic coronary heart disease.

Methods and Results. The presence of Chlamydia-specific circulating immune complexes was studied in 46 patients with chronic coronary heart disease and in control subjects. Chlamydial lipopolysaccharide-containing immune complexes were detected with the antigen-specific capture method, and they were present in 41% of patients and 15% of control subjects (p < 0.01). The presence of C. pneumoniae antibodies in circulating immune complexes was studied by testing the specificity of antibodies derived from isolated and dissociated immune complexes by microimmunofluorescence testing and immunoblotting. The C. pneumoniae indexes based on the relative amount of immune complex–derived antibodies and free antibodies were significantly higher among patients compared with control subjects (median, 1/8 versus 1/16; p < 0.001). Immune complex bound antibodies showed specificity for 98-kd and 42-kd proteins of C. pneumoniae.

Conclusions. The results suggest that the majority of patients with chronic coronary heart disease have a chronic C. pneumoniae infection in which chlamydial components have an easy access to circulation to form immune complexes with preexisting antibodies. These findings give further evidence for the association of chronic C. pneumoniae infection with coronary heart disease. (Circulation 1993;87:1130–1134)

Key Words • Chlamydia pneumoniae • coronary heart disease • immune complexes

The known risk factors of coronary heart disease (CHD) such as age, smoking, hypertension, and dietary habits are not sufficient to explain all the epidemiological variations and fluctuations of the disease. During the last two decades, several studies have been published in which risk factors of infectious or immunologic origin have been proposed.1–5 Chlamydia pneumoniae is a newly recognized respiratory pathogen that causes a wide variety of mild upper respiratory infections and is involved in 5–10% of pneumonia cases in adults worldwide.6,7 Recent studies have also suggested that chronic C. pneumoniae infection could be involved in chronic bronchitis8 and adult asthma.8 Furthermore, we have shown previously a serological association of C. pneumoniae infection with acute myocardial infarction (AMI) and chronic coronary heart disease (CCHD).9 Compatible with a chronic chlamydial infection, more than half of these patients had elevated IgG and IgA serum antibody titers8 to C. pneumoniae and circulating immune complexes (CIC) containing chlamydial lipopolysaccharide (LPS) in their sera.10 We have also demonstrated in the prospective Helsinki Heart Study that markers of chronic C. pneumoniae infection—both elevated antibody titers and presence of CICs containing LPS—already are an independent risk factor for development of CHD 3–6 months before the coronary event.11 In addition, recent studies in Seattle have indicated a significant correlation between C. pneumoniae antibody titers and the extent of angiographically verified lesions in coronary arteries.12 In the present study, we investigated the presence of LPS-containing immune complexes (LPSICs) in CCHD patients. Since LPS is shared by all Chlamydia species, we also wanted to demonstrate antibodies specific to C. pneumoniae protein antigens in immune complexes (ICs) to confirm the involvement of C. pneumoniae in CCHD.

See p 1408

Methods

Patients and Random Control Subjects

Plasma samples of 46 patients with angiographically verified CCHD referred to Helsinki University Central
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<table>
<thead>
<tr>
<th>TABLE 1. Demographic Characteristics, Classic Coronary Risk Factors, and CPI in Patients With CCHD and in Their Control Subjects</th>
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</thead>
<tbody>
<tr>
<td>Controls (n=46)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
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<tr>
<td>Social class</td>
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<td>I</td>
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<tr>
<td>II</td>
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<tr>
<td>III</td>
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<tr>
<td>IV</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
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<tr>
<td>HDL cholesterol (mmol/L)</td>
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<td>Triglycerides (mmol/L)</td>
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<tr>
<td>Hypertensives</td>
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<tr>
<td>Smokers</td>
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<tr>
<td>Current</td>
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<td>Former</td>
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<tr>
<td>Never</td>
</tr>
<tr>
<td>Diabetics (types I/II)</td>
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<tr>
<td>Individuals with previous AMI</td>
</tr>
<tr>
<td>CPI titers:</td>
</tr>
<tr>
<td>≤1/32</td>
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<tr>
<td>1/16</td>
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<tr>
<td>1/8</td>
</tr>
<tr>
<td>1/4</td>
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<tr>
<td>1/2</td>
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</tbody>
</table>

CPI, *Chlamydia pneumoniae* index; CCHD, chronic coronary heart disease; BMI, body mass index; HDL, high density lipoprotein; AMI, acute myocardial infarction. Both previous and current users of antihypertensive medication were coded as hypertensives. Social class was defined on the basis of education as follows: I, salaried employees, higher level; II, salaried employees, lower level; III, specialized blue-collar worker; IV, unspecialized blue-collar worker.

Hospital during the period when the control group was enrolled were available (September 1985 and March 1986).

For each CCHD patient, a control subject representing the same sex and age range was selected from the control group collected for our previous study. These individuals were randomly chosen from the Census of Helsinki, and 86% of those invited agreed to participate. The most common reason for refusal was "the job hurries," and no one refused because of an acute illness. The demographic characteristics of the participants are presented in Table 1.

Convalescent serum samples from four patients with pneumonia verified by chest x-ray were used as controls in immunoblotting. Patients had serological diagnosis for *C. pneumoniae* based on a microimmunofluorescence test (a fourfold IgG titer rise or an IgM titer of ≥16).

**Measurement of Chlamydial Antibodies**

IgG, IgA, and IgM antibodies to *C. pneumoniae* were measured by microimmunofluorescence method using TWAR strain as antigen (University of Washington Research Foundation, Seattle), as described previously. IgG antibodies to *C. trachomatis* were determined with the same method using BED-, GFK- and CJH-antigen complexes and *C. psittaci* using avian (6BC) and mammalian (OA) strains.

**Detection of Chlamydial LPSICs**

Chlamydia LPSICs were detected by two antigen-specific enzyme immunoassays as described in detail previously. Briefly, for LPS capture assay, microtiter plates were coated with IgG2a mouse monoclonal antibody specific to chlamydial LPS; sera were diluted 1:100, and alkaline phosphatase–conjugated anti-human IgM was used as detecting antibody. For IgM capture assay, the microtiter plates were coated with rabbit anti-human IgM, and the sera were tested in dilution 1:100. The detection system included alkaline phosphatase–conjugated F(ab')2 fragment of monoclonal antibody to chlamydial LPS and an enzyme amplification system.

**Immune Complexes of Chlamydial Protein**

CICs were isolated by polyethylene glycol (PEG) precipitation, modified from the method of Schutzer et al. Briefly, 100 μL of the sample was added to an equal volume of 7% PEG in sodium borate buffer, pH 8.4, and the mixture was incubated overnight at 4°C followed by centrifugation at 7,500 rpm for 15 minutes. Pellets were then washed twice with 3.5% PEG-borate. Finally, the precipitates were dissolved to the original volume of 100 μL of sodium borate buffer, pH 12.0. The dissociated ICs were then analyzed in microimmunofluorescence testing for the presence of *C. pneumoniae* antibodies at twofold dilutions starting at 1/2. Serum antibody was measured in the original serum and compared with that of the dissociated IC. *C. pneumoniae* indexes (CPIs) were calculated as the ratio of antibody titers free in serum and in dissociated complexes.

Immunoblotting analysis was done for both serum samples and dissociated serum IC preparations. The sera tested included 1) two seropositive (based on the microimmunofluorescence test) CCHD patients with high-titer *C. pneumoniae* antibody (IgG ≥512); 2) one seropositive CCHD patient with low-titer antibody (IgG 32); and 3) four seropositive pneumonia patients (IgG ≥32). *C. pneumoniae* strain TW-183 (University of Washington Research Foundation, Seattle) was used as antigen and run on a homogenous 12.5% SDS-PAGE (Phast System, Pharmacia, Uppsala, Sweden). Separated proteins and molecular weight standards were transferred to nitrocellulose membranes (Phast Transfer Semi-dry Electrophoretic Transfer System, Pharmacia, Uppsala, Sweden), and 1/50 dilutions of serum samples and 1/10 dilutions of dissociated IC preparations were added as the first antibody. The second antibody was peroxidase-conjugated anti-human IgG (1/100) (Dako, Denmark).

**Statistical Analysis**

Rates and proportions were compared with the χ² test and quantitative variables with the t test or Mann-
Whitney test. Association of various risk factors to CHD was calculated with stepwise logistic regression analysis.

Results

Immune Complexes Containing Chlamydial LPS and Chlamydial Protein

The prevalence of positive IgG (≥32) titers to C. pneumoniae were high among patients and controls: IgG antibodies were found in 65% and 70% of plasma samples, respectively. One patient and five control subjects also had positive IgG antibody titer (≥32) to C. trachomatis antigens. Antibodies to C. psittaci were found in four control sera. Antibodies dissociated from the isolated CICs showed reaction with C. pneumoniae elementary bodies in the microimmunofluorescence test. The CIs based on the relative amount of IC-derived antibodies and free antibodies are presented in Table 1. CPI values were significantly higher in the patient group than in the control group (median, 1/8 versus 1/16; *p*<0.001).

Chlamydial LPSICs were detected with the LPS capture method in three (7%), with IgM capture method in 12 (26%) and with both methods in four (9%) of 46 plasma samples of CHD patients. In the control group, LPSICs were found with IgM capture in three (7%) and with LPS capture in four (9%) individuals. Altogether, LPS containing CICs were demonstrated in 19 of 46 CHD patients (41%) and in seven of 46 healthy control subjects (15%). The difference between patients and control subjects was statistically highly significant (*p*<0.01).

Figure 1 shows the presence of the ICs of chlamydial protein (CPI ≥1/4) was considered to indicate the definite presence of chlamydial antibodies in IC) and the LPSICs in the CHD patients group and in the control group. Altogether, 34 patients (74%) and 11 control subjects (24%) were positive for either LPSIC or protein IC (*p*<0.001).

Classic Risk Factors and CPI

Univariate statistics for CPI and the classic coronary risk factors are presented in Table 1. CPI levels were clearly higher in the CHD group than in their control group. In this material, the levels of total cholesterol were higher in the control group than among CHD patients, and in the multivariate analysis, the coefficient of total cholesterol was negative. The number of “ever-smokers” and the levels of serum triglycerides were higher in the CHD group. There was no association between CPI and smoking when only “current smokers” were coded as smokers. However, among ever-smokers, CPI levels were significantly higher compared with “never-smokers” (median, 1/8 versus 1/16; *p*<0.05). Multivariate analysis showed that the association of CPI to CHD was independent of other coronary risk factors (Table 2), and it was significant both among individuals with and without previous myocardial infarction (data not shown).

Immunoblotting Analysis of Dissociated ICs

After precipitation and dissociation of the circulating ICs of CHD patients, the liberated IgG antibodies in immunoblotting exhibited a strong reactivity against C. pneumoniae protein antigens with molecular weights of 98 kDa and 42 kDa (Figure 2). These proteins have been demonstrated earlier to be C. pneumoniae specific (see “Discussion”). The same specificity was seen in IC bound and free antibodies. The immunoblot pattern was similar in the CHD patients and in the C. pneumoniae patients used as controls.

Discussion

In a previous study, we demonstrated the presence of ICs containing chlamydial LPS in 57% of the AMI patients and in only 12% of their random control subjects. This result was in good agreement with earlier studies, which have indicated by nonantigen-specific methods that 50–70% of patients have circulating ICs after AMI. Furthermore, we have demonstrated in the prospective Helsinki Heart Study, in which a group of 4,000 dyslipidemic middle-aged men was followed for cardiac events, that chronic C. pneumoniae infection, as indicated by the presence of an elevated IgA titer and of LPSIC, is an independent risk factor for development of CHD. This finding excluded the possibility that the presence of LPSIC in AMI

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**Table 2. Stepwise Logistic Regression Analysis With CCHD as the Dependent Variable and CPI and Various Coronary Risk Factors as the Independent Variables**

<table>
<thead>
<tr>
<th>Factor</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPI</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum total cholesterol</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>NS</td>
</tr>
<tr>
<td>Age</td>
<td>NS</td>
</tr>
<tr>
<td>Social class</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes (types I/II)</td>
<td>NS</td>
</tr>
</tbody>
</table>

CCHD, chronic coronary heart disease; CPI, Chlamydia pneumoniae index; HDL, high density lipoprotein. Both previous and current users of antihypertensive medication were coded as hypertensives. Social class was defined on the basis of education.
patients could be due to the activation of a latent chlamydial infection by AMI.

In the present study, we were able to show that LPSICs are present also in about half of the patients with angiographically verified CCHD. LPSICs were detected by the IgM capture method in 63%, by the LPS capture method in 16%, and by both methods in 21% of positive cases. In our previous study, 70% of LPSICs in acute-phase AMI were demonstrable by the IgM capture method only, suggesting that the ICs were formed in LPS excess, whereas in the convalescent phase, 1 month after AMI, 65% of ICs were demonstrable only by LPS capture, now suggesting that ICs were formed in IgM antibody excess and that antibody response to LPS had happened after AMI. The predominance of IgM capture findings in CCHD patients points to the LPS excess in the sera, just as in acute-phase AMI.

In this study, we further characterized the circulating ICs from CCHD patients and their healthy control subjects by isolating CICs and testing them for antibody to C. pneumoniae outer membrane protein antigens (OMP). The ratio of antibody titers free in serum and in dissociated complexes was significantly higher among patients compared with control subjects. In antibody prevalence surveys, C. pneumoniae antibodies have been very common, seen in about 50% in middle-aged adults all over the world. In the present study, we found only 16 (35%) seronegative CCHD cases (IgG titer <32). These patients did not have OMP-specific ICs in their serum either, whereas the patients who had low titer (32–64) often had high titers of complexed antibody and positive CPI as well. There was no indication of an acute C. pneumoniae infection in the present patient material (IgM was negative in all patients), and C. pneumoniae components still were present in the circulation, suggesting continuous shedding of chlamydial components, both proteins, and LPS into circulation. In the current study, C. trachomatis antibodies were seen only rarely, which is in good agreement with earlier studies of non–sexually transmitted disease patient materials.18,19

Immunoblotting analysis of sera and dissociated ICs of CCHD patients revealed both free and complexed anti-C. pneumoniae antibodies to 98-kd and 42-kd antigens. Campbell et al20 have demonstrated that the antibodies reactive with a 98-kd protein are C. pneumoniae specific because this protein was never recognized by sera from patients with C. trachomatis or C. psittaci infection. Recently, Puolakkainen et al21 studied immunoreactivity of sera from 49 South African adults with coronary arterial fatty streaks or fibrolipid plaques in immunoblotting, and 42-kd protein was frequently found to be immunoreactive in this material. Also, they reported that reactivity with the 98-kd and 42-kd proteins were species specific.

The levels of total cholesterol were higher in the control group than among CCHD patients. A similar result was obtained in our previous study, in which the same control group was used.22 Besides chance, the possibility that individuals with symptomatic CCHD had changed their diet may explain this paradoxical result. There was no association between smoking and CPI when only current smokers were censored. However, an association was found between ever-smoking and CPI. The association between smoking and C. pneumoniae antibody has been reported also in the Helsinki Heart Study23 and in a recent study by a Seattle group.24 Hahn and Golubjatnikov24 recently reported a significant quantitative association between current smoking and C. pneumoniae serological titer. The reason for the association between smoking and C. pneumoniae antibody remains unclear. It could be speculated that because smoking has a harmful effect on the defense mechanisms on the respiratory tract, the lung tissue will become susceptible to chronic C. pneumoniae infection, which may lead to CHD.

There are several mechanisms by which chronic C. pneumoniae infection might contribute to the development of CHD. C. pneumoniae has been shown to multiply in alveolar macrophages25 and in endothelial cells;26 chlamydial components in chronic lung infections thus might have an easy access to circulation. These ICs could contribute to the development of atherosclerosis by deposition on the vessel wall, creating local inflammatory and procoagulant changes.1,27,28 Binding of ICs to endothelial cells, phagocytic cells, platelets, or erythrocytes could initiate a process leading to the release of a variety of mediators, including cell proliferative factors.1 LPS itself is also known for its deleterious effects on the blood coagulation system and on vascular endothelium.29–31

The CIC findings in the present study suggest that majority of CCHD patients have a chronic C. pneumoniae infection. The immunoblotting analysis as well as microimmunofluorescence tests confirmed that the antibody and IC findings were indeed specific for C. pneumoniae. The presence of C. pneumoniae protein-specific ICs in the sera of CCHD patients gives further

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**Figure 2. Immunoblot analysis of Chlamydia pneumoniae isolate TW-183 with human sera.** Chlamydia elementary bodies (EBs) were electrophoresed through 12% sodium dodecyl sulfate–polyacrylamide gel, electrophoretically transferred to nitrocellulose, and incubated with dissociated immune complex (IC) preparations and serum samples (S). Lane A represents chronic coronary heart disease (CCHD) patient with high-titer C. pneumoniae antibody (IgG titer 512); lane B represents CCHD patient with low-titer C. pneumoniae antibody (IgG titer 32); lane C represents patient with pneumonia caused by C. pneumoniae infection as control.
evidence for an association of a chronic C. pneumoniae infection with CHD.

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Chlamydia pneumoniae-specific circulating immune complexes in patients with chronic coronary heart disease.
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