**Chlamydia pneumoniae** Seropositivity Is Associated With Increased Plasma Levels of Soluble Cellular Adhesion Molecules in Community-Dwelling Subjects

The Shimanami Health Promoting Program (J-SHIPP) Study

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Background and Purpose—In vitro studies have demonstrated that Chlamydia pneumoniae infection of the endothelium increases the expression of adhesion molecules and chemokines, indicating that C pneumoniae infection affects the adhesion and recruitment of leukocytes to the endothelium, which is believed to be involved in the initial steps of atherosclerosis. However, whether chronic C pneumoniae infection increases these molecules in vivo has not been elucidated.

**Methods**—The association between C pneumoniae seropositivity and plasma concentrations of soluble adhesion molecules and a chemokine was investigated in 200 community-dwelling residents free from cardiovascular diseases and medication. Plasma levels of IgA and IgG antibodies to C pneumoniae were measured by enzyme-linked immunosorbent assay. Indices of IgG and IgA antibodies were determined as the ratio to the standardized positive control. The subjects were divided into 3 groups according to the indices of antibodies: C pneumoniae seronegative (n=57, IgA<1.0 and IgG<1.0), C pneumoniae intermediate (n=81, 1.0≤IgA<1.1 or 1.0≤IgG<1.1), and C pneumoniae seropositive (n=62, IgA≥1.1 and IgG≥1.1). Plasma concentrations of soluble forms of intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1, and monocyte chemoattractant protein-1 were determined by enzyme-linked immunosorbent assay.

**Results**—Plasma concentrations of ICAM-1 (392±118, 398±94, 470±154 ng/mL, P=0.0004) and vascular cellular adhesion molecule-1 (402±146, 419±130, 472±181 ng/mL, P=0.03) were significantly different among the C pneumoniae seronegative, intermediate, and seropositive groups respectively. However, plasma monocyte chemoattractant protein-1 was not significantly different among the 3 groups. Stepwise regression analysis showed that plasma concentration of ICAM-1 was significantly associated with C pneumoniae seropositivity, independent of other known risk factors for atherosclerosis and carotid intima-media thickness.

**Conclusion**—These findings indicate that C pneumoniae seropositivity is associated with higher plasma concentrations of soluble forms of adhesion molecules in the general population. The increase in circulating adhesion molecules may underlie the mechanisms linking C pneumoniae infection and atherosclerosis in vivo. (Stroke. 2002;33:1474-1479.)

**Key Words:** atherosclerosis ■ Chlamydia pneumoniae ■ intercellular adhesion molecule-1 ■ monocyte chemoattractant protein-1 ■ vascular cellular adhesion molecule-1

There is a growing body of evidence showing that persistent Chlamydia pneumoniae infection could contribute to the pathogenesis of atherosclerosis. Several articles have reported that C pneumoniae has been detected in the plaque of atherosclerotic lesions. Seroepidemiological studies have shown a linkage between C pneumoniae seropositivity and cardiovascular disease. Early atherosclerotic changes evaluated by carotid ultrasonography as well as increased progression of carotid atherosclerosis have also been shown to be related to C pneumoniae seropositivity, although prospective studies failed to demonstrate a direct association between C pneumoniae seropositivity and atherosclerosis. Recently, a possible treatment effect of roxithromycin on restenosis after coronary arterial stenting has been reported in patients with a higher titer of IgG antibody for C pneumoniae. These findings provide observational evidence of the association between C pneumoniae infection and the development of atherosclerosis.

Many in vitro studies have been performed to elucidate the underlying mechanisms linking C pneumoniae infection and atherosclerosis. Activation of endothelial cells is one of the hallmarks of atherogenesis. Several lines of evidence pro-

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vided by in vitro studies have established endothelial cells to be the target of *C pneumoniae* infection.14–18 Infection of human endothelial cells with *C pneumoniae* results in stimulation of a wide variety of cytokines, adhesion molecules, chemokines, and proteins with procoagulant activity.14 Enhanced expression of endothelial adhesion molecules by *C pneumoniae* infection is associated with increased rolling, adhesion, and transmigration of leukocytes and monocytes.15 Recently, it has also been reported that *C pneumoniae* infection increases the expression of monocyte chemotactic protein-1 (MCP-1),14,17 which plays a pivotal role in initiating the recruitment of monocytes to atherosclerotic lesions. These findings indicate possible mechanisms by which *C pneumoniae* infection leads to endothelial damage and atherosclerosis. However, whether chronic *C pneumoniae* infection activates endothelial cells and increases the expression of adhesion molecules and chemokines in vivo remains to be addressed.17

Although the origin is not clear, there are circulating forms of adhesion molecules. Shedding from the surface of endothelium and macrophages into the blood is one possible source.19 Plasma levels of the circulating soluble forms of adhesion molecules have been shown to be markers of systemic atherosclerosis.20–23 The plasma level of MCP-1 has also been shown to be associated with atherosclerotic disorders.24–26

Based on this background, we hypothesized that chronic persistent *C pneumoniae* infection constantly stimulates the expression of adhesion molecules and chemokines, which could be reflected by increases in the plasma concentrations of these substances. To test this hypothesis, we evaluated the association between seropositivity for *C pneumoniae* infection and plasma concentrations of soluble intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) as well as MCP-1 in community-dwelling healthy residents free from medication and cardiovascular disease.

### Subjects and Methods

#### Subjects

The Shimanami Health Promoting Program (J-SHIPP) was started in 1999 in the Shimanami district, located in the southern part of Japan.27 J-SHIPP is a longitudinal study evaluating factors relating to cardiovascular disease, dementia, and death. The present study is a part of J-SHIPP performed in 1 community that participated in the study. The total population of the community is 948. All residents older than 50 years were invited to participate in the program, which consisted of an interview, anthropometric measurement, blood sampling, and carotid ultrasonography. About 50% of residents older than 50 years participated in the program. Information about medical history, present conditions, and medications was obtained by interview with each subject. Among all participants, those who agreed to the entire procedure and had no history or symptoms of cardiovascular disease (except for hypertension) and were free from medication (including anti-inflammatory drugs) were enrolled.

Two hundred residents completed the entire procedure. Written informed consent for the study was received from all subjects. The study procedures were approved by the ethical committee of Ehime University School of Medicine.

#### Carotid Ultrasonography

The right carotid artery was evaluated with an SSD-900 (Aloka Co, Ltd) using a 7.5-MHz probe.28 After having the subject rest for at least 10 minutes in the supine position with the neck in slight hyperextension, we evaluated an optimal visualization of the right common carotid artery, carotid bulb, and extracranial internal and external carotid arteries. From multiple approaches, we detected plaque as the presence of wall thickening at least 50% greater than the thickness of the surrounding wall. From anterior, lateral, and posterior approaches, intima-media thickness (IMT) of the far wall was measured in the right common carotid artery 1 cm proximal to the bulb and averaged to obtain mean IMT. Measurements were never taken at the level of a discrete plaque. Two dimensionally guided M-mode tracings of the right common carotid artery 1 cm proximal to the bulb were recorded. Peak-systolic internal dimension was obtained by continuous tracing of the intima-luminal interface of the wall of the common carotid artery in 3 cycles and averaged. The axial resolution of the M-mode system was 0.1 mm.

#### Evaluation of Risk Factors

Systolic and diastolic brachial blood pressure were measured twice at a 5-minute interval with the subject in the supine position with an automatic oscillometric blood pressure recorder (HEM-705CP, OMRON Co) during the carotid echo examination. The mean value of 2 measurements was obtained. The validity and reproducibility of the device have been reported.29 Total cholesterol, high-density lipoprotein cholesterol, and glucose were determined by conventional methods.

#### Measurement of Plasma Levels of Soluble Forms of Intercellular Adhesion Molecules and MCP-1

Blood was collected in a tube containing EDTA. Plasma was obtained by centrifugation for 2 minutes at 2500 rpm, and aliquots were stored at −80°C until measurement. Plasma levels of ICAM-1 and VCAM-1 were measured with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). The plasma level of MCP-1 was also measured with an ELISA kit (R&D System). Intra-assay variabilities for ICAM-1, VCAM-1, and MCP-1 were 4%, 5%, and 6%, respectively. Interassay variabilities for ICAM-1, VCAM-1, and MCP-1 were 6%, 8%, and 6%, respectively.

#### Serological Study for *C pneumoniae*

The levels of serum IgA and IgG antibodies to *C pneumoniae* were determined using a specific ELISA kit (HITAZYME *C pneumoniae*, Hitachi Chemical Co, Ltd).30–33 This ELISA method detects antibodies to the chlamydial outer membrane complex, which has been isolated from purified elementary bodies of *C pneumoniae* KY-41 strain.33 Antibody used in the ELISA has weak cross-reactivities with *C trachomatis* (1/32) and *psittaci* (1/4).31 The levels of IgA and IgG to *C pneumoniae* in each sample were expressed as the IgA or IgG index. The IgA and IgG indices were determined by calculating the corrected optical density (OD) (405 nm) of the IgA and IgG samples divided by the IgA and IgG cutoff values. The corrected OD (405 nm) of a sample = OD (405 nm) of sample X reference value of positive control/mean OD (405 nm) of the positive control. The cutoff value = mean OD (405 nm) of negative control X reference value of the positive control/mean OD (405 nm) of the positive control + 0.20. Index values of more than 1.10 were scored as positive for IgA or IgG antibodies, whereas those below 1.10 were scored as negative. Detection rates of IgA and IgG antibodies to *C pneumoniae* by this ELISA method were compared with the microimmunofluorescence method: sensitivity was 90.4% for IgG and 84.6% for IgA, and specificity was 89.9% for IgG and 87.4% for IgA.32,33 In addition, the rate of agreement between the ELISA method and Western blotting analysis was 80.0% for IgG and 87.5% for IgA.32,33 Subjects were divided into 3 categories according to the indices of IgA and IgG. *C pneumoniae* seropositivity was diagnosed when both IgG and IgA indices were >1.1 (*C pneumoniae* seropositive group; n = 62). *C pneumoniae* seronegativity was defined when both IgA and IgG indices were <1.0 (seronegative group; n = 57).
Subjects with 1.0≤IgA≥1.1 or 1.0≤IgG≥1.1 or both were categorized as the intermediate group (n=81). The advantage of this assay system is that quantification is more objective. Because it has been suggested that a higher titer of C pneumoniae antibody would more likely reflect chronic persistent and active infection, we analyzed plasma levels of soluble CAMs and MCP-1 in subjects in the C pneumoniae seropositive group with higher antibody index (n=30), ie, IgA index >2.5 or IgG index >2.5 (approximately equivalent to 128 in the microimmunofluorescence method\(^\text{15}\)).

**Statistical Analysis**

All values are expressed as mean ± SD unless otherwise specified. Statistical comparisons among groups were performed by ANOVA. Differences in prevalence among groups were analyzed by the chi-squared method. Forward stepwise multiple regression analysis for C pneumoniae seropositivity was performed with the following parameters: age, gender, systolic blood pressure, total cholesterol, blood glucose, smoking status, carotid IMT, and plasma concentrations of ICAM-1, VCAM-1, and MCP-1. All analyses were performed using software packages (JMP; SAS Institute). A probability value less than 0.05 was considered statistically significant.

**Results**

### Seropositivity for C pneumoniae and Clinical Characteristics

Clinical characteristics of the 3 groups of C pneumoniae seropositivity are summarized in Table 1. There were significant differences among the 3 groups in age, gender, total cholesterol, and prevalence of current smokers. Carotid arterial IMT and dimension in the 3 groups are also summarized in Table 1. Carotid IMT and internal dimension were significantly different among the 3 groups of C pneumoniae seropositivity.

### Seropositivity for C pneumoniae and Plasma Concentration of Adhesion Molecules and Chemokine

Plasma concentrations of ICAM-1, VCAM-1, and MCP-1 in the 3 groups of C pneumoniae seropositivity are depicted in Figure 1. The C pneumoniae seropositive group had significantly higher plasma concentrations of ICAM-1 and VCAM-1 than the C pneumoniae seronegative and intermediate groups. However, there was no significant difference in plasma concentration of MCP-1 among the 3 groups.

The relationship between plasma concentration of adhesion molecules and chemokine and seropositivity was separately analyzed between IgA and IgG index. IgG index, as a continuous variable, showed a significant positive association with plasma ICAM-1 (r=0.23, P=0.001), VCAM-1 (r=0.16, P=0.021), MCP-1 (r=0.183, P=0.0095), and carotid IMT (r=0.174, P=0.0014). Although index of IgA showed a significant positive association with ICAM-1 (r=0.17, P=0.019) and VCAM-1 (r=0.17, P=0.019), there were no associations with MCP-1 and carotid IMT.

Stepwise regression analysis was performed to evaluate the independent association between seropositivity for C pneumoniae infection and plasma levels of adhesion molecules and chemokines (Table 2). It revealed that plasma ICAM-1 was significantly and independently associated with C pneumoniae seropositivity in addition to gender and total cholesterol.

Thirty subjects in the C pneumoniae seropositive group had a higher Ig index for C pneumoniae (IgA≥2.5 or IgG≥2.5). In those subjects, plasma concentration of ICAM-1 (481±139 versus 407±121 ng/mL, P=0.003), VCAM-1 (488±201 versus 421±142 ng/mL, P=0.028) and
MCP-1 (217±138 versus 188±56 pg/mL, P=0.046) were significantly increased compared with the rest of the population.

**Discussion**

Epidemiological studies have implicated bacterial infection in the pathogenesis of atherosclerosis.1,7 Seroepidemiological observation reported the association between seropositivity for *C pneumoniae* and atherosclerotic disorders including myocardial infarction and stroke.1,7 Morphological and microbiological demonstration of *C pneumoniae* in the atheromatous plaque provide strong evidence for an association between *C pneumoniae* and atherosclerosis.1,7 Although the exact mechanism of how *C pneumoniae* affects the vasculature is not fully understood, endothelium and vascular smooth muscle have been shown to be targets of *C pneumoniae* infection.1,14–18 It has been demonstrated that pulmonary macrophages infected with *C pneumoniae* can disseminate the organisms throughout the body, including the cardiovascular system and atheroma.35 These in vitro results suggest that persistent *C pneumoniae* infection could involve systemic endothelial cells.

In in vivo study, it has also been reported that repeated *C pneumoniae* infection in apoE knockout mice resulted in significant attenuation of endothelial-dependent arterial dilation,36 although *C pneumoniae* infection failed to induce atherosclerosis itself.37 Recently, Sharma et al38 evaluated the association between *C pneumoniae* seropositivity and endothelial function in healthy individuals. Although there was no significant association between *C pneumoniae* seropositivity and flow-mediated vasodilatation, they showed that subjects with detectable IgA and C-reactive protein tended to have less marked endothelium-dependent vasodilatation. In the present study, we observed that *C pneumoniae* seropositivity was associated with higher plasma concentrations of soluble adhesion molecules, independent of carotid atherosclerosis. These findings provide clinical evidence to support the hypothesis that persistent *C pneumoniae* infection continues to activate the systemic endothelium, resulting in increased expression of adhesion molecules.

Although seroepidemiological observation had provided useful information about the possible association between *C pneumoniae* seropositivity and atherosclerosis, *C pneumoniae* seropositivity itself does not reflect chronic persistent infection.1,7 Although we used both IgA and IgG indices in the present study for the definition of *C pneumoniae* seropositivity, the dissociation between IgA and IgG has been also reported.39 Several studies reported the usefulness of IgA,40,41 whereas it has been also reported that the IgA titer for *C pneumoniae* did not reflect chronic *C pneumoniae* infection.39 In the present study, we also evaluated the associations between IgA and IgG indices and plasma levels.

**TABLE 2. Stepwise Regression Analysis for Seropositivity for Chlamydia pneumoniae**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1 (ng/mL)</td>
<td>0.225</td>
<td>11.23</td>
<td>0.0010</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>0.195</td>
<td>8.18</td>
<td>0.0047</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>0.147</td>
<td>4.63</td>
<td>0.0327</td>
</tr>
</tbody>
</table>

ICAM indicates intracellular adhesion molecule-1. The following parameters were not found to be significantly associated with seropositivity and, therefore, were not included in the equation: vascular intercellular adhesion molecule-1, monocyte chemoattractant protein-1, intima-media thickness, glucose, systolic blood pressure, smoking status, and age.
of CAMs and MCP-1. Although IgG index showed significant positive associations with carotid IMT and plasma concentrations of all 3 parameters, IgA was associated only with ICAM-1 and VCAM-1 and was not associated with IMT and MCP-1. These findings may indicate the difference between IgA and IgG indices in reflecting \textit{C pneumoniae} infectious state.

It has been suggested that a higher titer of \textit{C pneumoniae} antibody reflects chronic and persistent \textit{C pneumoniae} infection. We also analyzed subgroups with higher indices of antibody for \textit{C pneumoniae} infection. Those subjects had significantly higher plasma levels of ICAM-1 and VCAM-1 as well as chemokines. This finding, together with the findings of linear associations between antibody indices and plasma concentrations of adhesion molecules and chemokine, may indicate that persistent \textit{C pneumoniae} infection is associated with an increase in the plasma levels of adhesion molecules and chemokines.

Stepwise regression analysis showed that the plasma concentration of ICAM-1 but not VCAM-1 was independently associated with seropositivity for \textit{C pneumoniae}. Although we do not have a clear explanation for this dissociation, it has been reported that endothelial activation by \textit{C pneumoniae} was associated with a lesser response in VCAM-1 compared with ICAM-1 in in vitro study. The lesser response by chronic \textit{C pneumoniae} infection in vitro might underlie the differences between ICAM-1 and VCAM-1.

In vitro studies demonstrated that \textit{C pneumoniae} infection stimulates MCP-1 production, resulting in trans-endothelial migration of monocytes. It has also been demonstrated that \textit{C pneumoniae} infection facilitates monocyte adherence to endothelial cells and vascular smooth muscle cells. A higher plasma concentration of MCP-1 has been shown to be associated with myocardial infarction, unstable angina, venous thrombosis, and re-stenosis after percutaneous transluminal coronary angioplasty. In the present study, the IgG index for \textit{C pneumoniae} showed a significant positive association with plasma concentration of MCP-1. However, the IgA index did not correlate with plasma MCP-1. As a result, \textit{C pneumoniae} seropositivity was not associated with the plasma concentration of MCP-1. The dissociation between IgA and IgG may indicate that past \textit{C pneumoniae} infection, not repeated or persistent \textit{C pneumoniae} infection, affects the circulating MCP-1 level. The finding that a more strict definition of seropositivity for \textit{C pneumoniae} is associated with higher plasma MCP-1 concentration may support this explanation. Another possibility is that the origin of soluble adhesion molecules may differ from that of MCP-1. The antibodies detected by the ELISA used in the present study are against the outer membrane complex of \textit{Chlamydia}, which is released from infected monocytes and macrophages.

Accordingly, \textit{C pneumoniae} positivity in the present study indicates persistent \textit{C pneumoniae} infection of monocytes and macrophages in the blood. Since macrophages could be another source of soluble adhesion molecules, the increased circulating levels of adhesion molecules in the \textit{C pneumoniae} seropositive group may not have originated from the endothelium.

The present results should be interpreted with caution. How do circulating soluble adhesion molecules contribute to the progression of atherosclerosis? Do the increased plasma levels of adhesion molecules reflect stimulated production on the endothelial surface? Although the plasma level of ICAM-1 has been shown to be associated with future coronary events, the causative role of circulating adhesion molecules in cardiovascular disorders needs to be determined. Furthermore, a cross-sectional study also has limitations in interpreting the results. We also could not rule out the possibility that a systematic bias was introduced, because the study population did not include younger generations and because those taking medications and having cardiovascular complications were excluded from the study. The causality of \textit{C pneumoniae} infection on the higher plasma levels of adhesion molecules and chemokine is another issue to be determined. A recent report that 6 months of administration of azithromycin to patients positive for \textit{C pneumoniae} failed to show a change in plasma concentrations of VCAM-1 and ICAM-1 may contradict our hypothesis. Whether the change in \textit{C pneumoniae} seropositivity after treatment with antibiotics is associated with a change in the circulating levels of adhesion molecules and a decrease in cardiovascular events needs to be evaluated in a larger population.

There is a methodological limitation in a seroepidemiological approach to evaluate the causality of \textit{C pneumoniae} infection on atherosclerosis. A second prevention trial using antibiotics for the progression of atherosclerosis could evaluate the causative role of \textit{C pneumoniae} infection on atherosclerosis. Recently, Neumann et al. reported that roxithromycin administration for 28 days after coronary stenting was effective in reducing the rate of restenosis >70% in patients with higher IgG titers (1/512) for \textit{C pneumoniae}. However, the primary end point of the study, i.e., the rate of restenosis >50%, was not significantly affected by roxithromycin administration even in patients with higher IgG titers for \textit{C pneumoniae}. These findings, together with previous observations, indicate that second prevention trials have also been inconclusive.

The confounding factors could also be bias. A higher prevalence of smokers among subjects with \textit{C pneumoniae} seropositivity, which is a consistent finding observed in the seroepidemiological study, is also observed in the present study. Protective immunity to \textit{C pneumoniae} has been shown to be impaired in smokers. Chronic inflammation caused by smoking could also precipitate \textit{C pneumoniae} infection. Because smoking is an independent risk factor for atherosclerosis, these pathological changes associated with smoking could link \textit{C pneumoniae} infection and atherosclerosis. Increased prevalence of \textit{C pneumoniae} seropositivity with aging could also be a confounding factor. Because prevalence of \textit{C pneumoniae} seropositivity increases with age, advanced age could link \textit{C pneumoniae} seropositivity and atherosclerosis. These confounding factors could also interfere with the findings in the present study.

In summary, \textit{C pneumoniae} seropositivity was associated with higher plasma concentrations of soluble adhesion molecules in community-dwelling subjects. Whether the association is a causative mechanism in atherosclerosis needs to be further determined.
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References

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