Chlamydia pneumoniae DNA Detection in Peripheral Blood Mononuclear Cells
Is Predictive of Vascular Infection

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Abdominal aortic aneurysm tissue and peripheral blood mononuclear cells (PBMC) of 41 consecutive subjects undergoing abdominal aortic aneurysm surgery were analyzed by polymerase chain reaction (PCR) for the presence of Chlamydia pneumoniae, Mycoplasma pneumoniae, and Helicobacter pylori DNA. Twenty patients (49%) were positive for C. pneumoniae DNA—16 (39%) in both PBMC and aneurysm tissue, 3 (7.3%) in PBMC only, and 1 (2.4%) in the artery specimen only. Previous exposure to C. pneumoniae was confirmed in 19 (95%) of the 20 PCR positive subjects by C. pneumoniae—specific serology, using the microimmunofluorescence test. None was positive for H. pylori or M. pneumoniae DNA, either in the PBMC or in the artery specimens. In conclusion, carriage of C. pneumoniae DNA is common both in PBMC and in abdominal aortic tissue from patients undergoing abdominal aneurysm surgery. Blood PCR may be a useful tool for identifying subjects carrying C. pneumoniae in the vascular wall.

During the past decades, the understanding of lipid metabolism derangement (hypercholesterolemia and decreased high-density lipoprotein cholesterol concentration), together with a better control of hypertension, diabetes, and smoking habits, has resulted in a substantial decrease in coronary artery disease and stroke mortality [1]. Nonetheless, the above risk factors (together with age, sex, and family history) account for ~50%–70% of the pathogenesis of cardiovascular diseases [2]. Therefore, scientific attention has recently focused on investigating hypothetical additional risk factors and on achieving a deeper understanding of the development of atherosclerosis, the main pathological process involved in coronary artery disease.

Helicobacter pylori infection has recently been recognized as the prime cause of peptic ulcers. Features in common between the epidemiology of peptic ulceration and coronary artery disease have prompted seroepidemiological studies to test the association between infection with this agent and cardiovascular diseases. Case-control and cross-sectional studies showed that seropositivity to H. pylori is a risk factor for coronary artery disease [3]. It was also suggested that H. pylori infection may increase C-reactive protein and fibrinogen levels, two factors associated with cardiovascular diseases [4, 5]. However, a recent prospective cross-sectional analysis failed to associate H. pylori seropositivity with cardiovascular diseases in a population of 624 elderly subjects [6]. Likewise, in a study of healthy subjects, no link was found between H. pylori infection and increased concentrations of fibrinogen or other hemostatic factors, which does not support the possibility of this infection inducing a procoagulant state [7]. H. pylori genomic material was identified at autopsy in coronary arteries of persons who had died of myocardial infarctions in 1 series [8], but this finding was not confirmed in a study of aortic aneurysm atherosclerotic plaques [9].

Convincing evidence has now been put forward that Chlamydia pneumoniae is also associated with atherosclerosis and acute cardiovascular events. This evidence rests on seroepidemiological data, identification of genomic material by polymerase chain reaction (PCR), immunocytochemistry, in situ hybridization, electron microscopy, and culture [10]. C. pneumoniae has been identified in ~50% of atheromatous lesions but in only 5% of control samples of arterial tissue. Mycoplasma pneumoniae is another bacterium that is commonly involved in
human respiratory infections. Its possible involvement in atherosclerosis has not yet been investigated.

The purpose of the present study was to evaluate by PCR the presence of *C. pneumoniae*, *H. pylori*, and *M. pneumoniae* both in blood mononuclear cells and in artery samples obtained from patients with abdominal aortic aneurysms.

Materials and Methods

Between December 1998 and February 1999, 41 consecutive subjects (37 were men; mean age, 70.6 ± 5.9 years; age range, 57–81 years) who underwent abdominal aortic aneurysm surgery were enrolled. The main risk factor observed in our series was hypertension (31/41 patients). Sixteen patients were current smokers, and 21 were former smokers. Hypercholesterolemia (≥6 mM), obesity, and diabetes mellitus were observed in 11, 10, and 2 patients, respectively. For each patient, serology for *C. pneumoniae* was performed by using a microimmunofluorescence (MIF) test for IgG, IgA, and IgM antibodies (Labsystems, Helsinki, Finland). A titer of 1:64 was used as the cutoff for IgG, and 1:16 for IgA and IgM. In all patients the anti–*H. pylori* antibody titers were determined by an EIA-G test (Pyloriset; Orion Diagnostica, Espoo, Finland). Serology for *M. pneumoniae* was performed by an EIA test (Pantec, Turin, Italy). An 8-mL blood sample was collected at admission in Vacutainer CPT (Becton Dickinson, Franklin Lakes, New Jersey), for isolation of peripheral blood mononuclear cells (PBMC). The tube was immediately inverted 10 times to mix anticoagulant additive with blood and was kept at room temperature. The tube was then centrifuged (1500 g) for 20 min at room temperature in a horizontal rotor within 2 h. After centrifugation, red blood cells deposit below a gel band in the Vacutainer CPT, whereas PBMC form a pellet above the gel. The PBMC band above the gel was transferred by use of a disposable pipette to an Eppendorf tube and was stored at ~70°C. DNA was isolated by use of a High Pure PCR Template Preparation Kit (Boehringer, Mannheim, Germany).

PCR. PCR for *C. pneumoniae*, *H. pylori*, and *M. pneumoniae* was performed for each patient, both on PBMC and on artery specimens, by using a thermocycler (Robocycler; Stratagene, La Jolla, CA). To avoid the risk of contamination, sample preparation, PCR amplification, and product analysis were performed in separate rooms. In each assay, positive and negative controls were included. The negative controls contained all the PCR reagents and sterile distilled water. Serial dilutions of purified bacteria were used as positive controls for all runs to ensure successful nucleic acid amplification. All PCR-negative samples were analyzed by PCR for the presence of β-actin DNA to confirm the presence of DNA in the samples.

*C. pneumoniae*. Touchdown nested-PCR was performed essentially as described by Tong and Sillis [12], using primers designed to detect the major outer-membrane protein of *C. pneumoniae*. Extracted DNA solution (10 μL in a total volume of 50 μL) was used in the first PCR round; 5 μL of the PCR products amplified by the outer primers was then transferred to a new 50-μL PCR reaction mix for a second amplification using the inner primers. The first round consisted of 40 cycles and the second of 35 cycles. This PCR technique allowed the detection of ~1–5 *C. pneumoniae* elementary bodies.

*H. pylori.* For the detection of *H. pylori* DNA, we applied a nested-PCR by using 2 sets of primers, as described by Wang et al. [13]. Briefly, 2 primer sets designed to identify the urease gene of *H. pylori* DNA were used. DNA was amplified in 50-μL volumes containing 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer, 2 U of Taq polymerase (Sigma, St. Louis), 10 mM Tris-HCl, 1.5 mM MgCl₂, and 50 mM KCl. The first amplification was performed for 40 cycles at 96°C for 30 s, 56°C for 15 s, and 74°C for 30 s (10 min for the last cycle). The second amplification was performed in the same way, starting with 2 μL of the first amplification product. This PCR technique allowed the detection of ~5 *H. pylori* bacteria.

*M. pneumoniae.* PCR was performed essentially as described by Kai et al. [14] by using the sense primer designated MP8 and the antisense primer designated MP6, selected from a variable region of the *M. pneumoniae* 16S rRNA genome. The temperature program was as follows: 94°C for 5 min for initial denaturation, 94°C for 1 min for cycle (35 cycles) denaturation, 62°C for 1 min for annealing, and 74°C for 1 min for extension (10 min for the last cycle). This PCR technique allowed the detection of down to 10 *M. pneumoniae* bacteria.

Results

In 16 patients, *C. pneumoniae* DNA was detected in both PBMC and artery specimens; in 3 patients, in PBMC but not in artery specimens; and in 1 patient, only in the artery samples (table 1).

Microimmunofluorescence serology results including exposure to *C. pneumoniae* were found in 26 (63%) of 41 patients: *C. pneumoniae* IgG was found in 24 (58%) of the 41 patients, 5 of whom were positive for *C. pneumoniae* IgA, and 2 patients were positive for IgA without detectable IgG. IgM antibodies were not detected. Fourteen patients were negative both by *C. pneumoniae* serology and PCR on PBMC and artery specimens.

In the 19 patients with positive PCR on PBMC, MIF serology confirmed previous exposure to *C. pneumoniae*. In 16 of 17 patients with *C. pneumoniae* DNA detection in artery specimens, MIF was also positive. In 7 patients MIF positivity was not associated with *C. pneumoniae* DNA detection. No

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<th>Table 1. Chlamydia pneumoniae serology and polymerase chain reaction (PCR) results in the 41 patients studied.</th>
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<td>Serology results</td>
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NOTE. PBMC, peripheral blood mononuclear cells.
* IgG ≥ 1: 64 and/or IgA ≥ 1: 16.
difference between DNA positive and DNA negative patients was observed in terms of IgG and IgA antibody titers. The distribution of risk factors was similar in the 2 groups. By PCR there was no evidence of *H. pylori* or *M. pneumoniae* DNA, neither in the PBMC nor in the artery specimens. Positive IgG titers to *H. pylori* were found in 28/41 patients. *M. pneumoniae* IgG positivity was found in 4/41 patients.

**Discussion**

Given the increasing evidence of an association between *C. pneumoniae* and cardiovascular diseases, it becomes mandatory to define a method for the identification of long-term *C. pneumoniae* carriers. Traditionally, MIF has been considered to be the “gold standard,” although its inability to distinguish between chronic infection and previous infection indicates that it cannot be used for identifying subjects with an ongoing infection. Techniques that directly determine the presence of the microorganism are therefore preferable. So far, direct identification has relied mainly on PCR techniques. *C. pneumoniae* has been repeatedly identified in atheromatous plaques, but this method has little use as a large-scale clinical marker of chronic infection. Recently, Boman et al. [15] described a technique for the detection of *C. pneumoniae* DNA in PBMC that presents advantages and may be a potential marker of chronic infection. We evaluated the presence of *C. pneumoniae*, *H. pylori*, and *M. pneumoniae* both in artery samples and in blood mononuclear cells. The results of the study showed that MIF serology could confirm previous exposure to *C. pneumoniae* in all 19 patients who were positive for *C. pneumoniae* DNA on blood mononuclear cells, thus confirming the association between PCR and serology previously described by Boman et al. [15]. In no case was evidence of *H. pylori* DNA found, thus adding further negative evidence against the possibility of direct involvement of this microorganism in cardiovascular diseases. *M. pneumoniae* is a commonly occurring respiratory pathogen that has been shown to cause chronic infection. It therefore possesses similarities with *C. pneumoniae*. In all patients tested, no evidence of *M. pneumoniae* DNA was found in artery specimens or in PBMC. This indicates that not necessarily all commonly occurring respiratory pathogens capable of causing chronic infection can be found in the bloodstream or in the plaques. Consequently, this may be a specific characteristic of *C. pneumoniae*.

The rate of *C. pneumoniae* DNA detection in artery specimens and PBMC was similar (17/41 and 19/41 patients, respectively). Thus, PCR performed on peripheral blood may be a good marker for the identification of subjects carrying *C. pneumoniae* in the vascular wall. It would also be interesting to evaluate the utility of *C. pneumoniae* DNA detection on PBMC in other chronic diseases such as asthma and chronic bronchitis to determine whether this technique may be a general marker of chronic *C. pneumoniae* infection or of vascular infection only. In conclusion, PCR on PBMC may become the new “gold standard” for the diagnosis of chronic *C. pneumoniae* infection, at the least, a chronic vascular infection.

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**References**