

Detection of *Chlamydia pneumoniae* but Not *Helicobacter pylori* in Atherosclerotic Plaques of Aortic Aneurysms

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Received 17 April 1996/Returned for modification 29 May 1996/Accepted 15 August 1996

Recent reports suggest an association between *Chlamydia pneumoniae* and *Helicobacter pylori* bacteria and atherosclerosis. We studied 51 patients (mean age, 68.3 years) who underwent abdominal aortic aneurysm surgery. For each patient we performed a microimmunofluorescence test for immunoglobulin G (IgG), IgA, and IgM antibodies to *C. pneumoniae* specific antigen (TW-183). Anti-*H. pylori* antibodies were determined by means of an EIA-G test. Each aortic aneurysm surgical specimen was sampled into multiple sections of 0.3 cm² each and frozen at -20°C. Two samples of each aneurysm were used for a nested PCR with two sets of *C. pneumoniae* and two sets of *H. pylori* specific primers. Specimens were treated with a solution containing 20 mM Tris-HCl, Tween 20–Nonidet P-40 (0.5% [vol/vol] each), and 100 µg of proteinase K per ml and incubated at 60°C for 1 h and at 98°C for 10 min. DNA was extracted twice with phenol-chloroform-isoamyl alcohol and precipitated with sodium acetate-ethanol by standard methods. Forty-one patients were seropositive for *C. pneumoniae* with past-infection patterns in 32 patients (16 ≤ IgG < 512; 32 ≤ IgA < 256) and high antibody titers in 9 patients (IgG ≥ 512). 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*. In all cases PCR showed no evidence of *H. pylori* presence in plaque specimens. This study provides data on a possible *C. pneumoniae* involvement in the pathogenesis of aortic aneurysm and additional evidence for an association between this agent and atherosclerosis. Conversely, notwithstanding a high *H. pylori* seroprevalence observed, our results tend to rule out the possibility of a direct involvement of *H. pylori* in atherosclerosis.

Chlamydia pneumoniae is one of the leading causes of acute respiratory tract infections in humans worldwide (16, 25, 32). This obligate intracellular, gram-negative bacterium is involved in a wide spectrum of respiratory tract diseases ranging from a flu-like syndrome with a long-lasting dry cough to severe pneumonia (2, 7, 13, 17). A flood of recent data has documented the importance of this agent in the development of respiratory disease, showing a high specific antibody seroprevalence in adults, reaching more than 60% in elderly subjects (6). Recent evidence indicates the possibility of *C. pneumoniae* chronic infection in patients with chronic bronchitis, adult-onset asthma, and sarcoidosis (4, 8, 12). Moreover, seroepidemiological evidence, immunocytochemistry, and molecular biology studies have suggested an association between *C. pneumoniae* and coronary artery disease (14, 15, 27, 29). Saikku and coworkers (27) have demonstrated elevated levels of antibody to *C. pneumoniae* in patients with acute myocardial infarction and chronic coronary heart disease (CHD), and the same group reported the detection of immune complexes containing chlamydial lipopolysaccharides in more than 60% of patients with acute myocardial infarction (15). Furthermore, Shor et al. (29) and Kuo et al. (14) found *C. pneumoniae* in coronary artery atherosclerotic plaques using immunocytochemistry, nucleic acid detection, and electron microscopy. Other evidence of a possible involvement of this agent in the

pathogenesis of atherosclerosis was reported by Melnick et al. (19), who indicated a possible association between past infection by *C. pneumoniae* and asymptomatic carotid atherosclerosis. *C. pneumoniae* involvement in extracoronary atherosclerosis is reported only in a seroepidemiological study on asymptomatic carotid atherosclerosis (19), whereas, to our knowledge, no data on the role of *C. pneumoniae* in aneurysmal lesions have been reported in the literature. We therefore studied the possibility of identifying the presence of *C. pneumoniae* in aneurysmal plaques of abdominal aorta in a sample of subjects who underwent surgical excision of an aortic aneurysm. Moreover, recent studies have shown an association between *Helicobacter pylori* infection and CHD. Mendall et al. (20), in a case-control pilot study of 111 consecutive patients with documented CHD and 74 controls, found serological evidence of a link between *H. pylori* seropositivity and CHD. In this study *H. pylori* appeared as a possible independent risk factor for CHD. Patel et al. (23) reported a study of 72 patients showing that *H. pylori* infection is associated with an increase in fibrinogen level, which in turn is considered an important risk factor for CHD. Both studies relied on seroepidemiological evidence in suggesting an association between *H. pylori* and CHD. Epidemiological data indicate an *H. pylori* seroprevalence in Italy ranging from 20% (20 to 29 years) to more than 40% (≥60 years) (33). So far, no data documenting the direct involvement of *H. pylori* in the pathogenesis of atherosclerotic plaques is present in the literature.

We report the evidence of an association between *C. pneumoniae* and atherosclerotic plaques of abdominal aortic aneurysms as detected by the use of a nested-PCR technique.

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TABLE 1. Main clinical characteristics of the 51 patients enrolled

Characteristic(s)	n
Smoking	22
Past smoking	19
Hypertension ^a	28
Hypercholesterolemia ^b	30
Obesity	8
Diabetes mellitus	5
Risk factor clustering	
Smoking + hypertension	6
Smoking + hypercholesterolemia	7
Smoking + hypertension + hypercholesterolemia	4
Past smoking + hypertension	7
Past smoking + hypercholesterolemia	3
Past smoking + hypertension + hypercholesterolemia	4

^a Diastolic arterial pressure ≥ 95 mm Hg.^b ≥ 6 mmol/liter.

MATERIALS AND METHODS

Between November 1994 and May 1995 we studied 51 consecutive subjects (43 males and 8 females; m5qge, 68.3 years; age range, 54 to 81 years) who underwent abdominal aortic aneurysm surgery. Table 1 shows the main clinical characteristics of enrolled patients. For each patient, serology for *C. pneumoniae* was performed by a microimmunofluorescence test for immunoglobulin G (IgG), IgA, and IgM antibodies using a specific antigen (TW-183) purchased from the Washington Research Foundation, Seattle, Wash. (36). Microimmunofluorescence results were classified as past (chronic)-infection pattern ($16 \leq \text{IgG} < 512$; $32 \leq \text{IgA} < 256$) and high antibody titer ($\text{IgG} \geq 512$; $\text{IgA} \geq 256$). In all patients the anti-*H. pylori* antibody titer was determined by an EIA-G test (Pyloriset; Orion Diagnostic).

After surgical excision aortic aneurysm specimens were immediately sampled, with a sterile blade, into multiple sections of about 0.3 cm² each and frozen at -20°C . For each patient two contiguous samples were analyzed. Aortic aneurysm specimen sections were treated with a solution containing 20 mM Tris-HCl, Tween 20–Nonidet P-40 (0.5% [vol/vol] each), and 100 μg of proteinase K per ml and incubated at 60°C for 1 h and at 98°C for 10 min. DNA was extracted twice with phenol-chloroform-isoamyl alcohol and precipitated with sodium acetate-ethanol by standard methods. We applied a nested PCR using two sets of primers designed to detect a fragment of the 16S rRNA gene of *C. pneumoniae* reported by Black et al. (5): external primers 5' ATAATGACTTCGGTTGTTAT 3' (sense, 71 to 91) and 5' TATAAATAGGTTGAGTCAAC 3' (antisense, 1448 to 1468) and internal primers 5' AGTGTAATTAGGCATCTAATAT 3' (sense, 177 to 199) and 5' GCTGTATTTCTACAGTTG 3' (antisense, 1018 to 1035).

DNA was amplified in 50- μl volumes containing 200 μM each deoxynucleoside triphosphate, 2 μM each primer, 2 U of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany), 10 mM Tris-HCl, 2 mM MgCl₂, 50 mM KCl, and 5 μl of the sample DNA.

The first amplification was performed in an automated thermocycler (Hybaid) for 35 cycles at 94°C for 1 min (3 min for the first cycle), 45°C for 1 min, and 72°C for 1 min (10 min for the last cycle). The second amplification was performed in the same way starting with 2 μl of the first amplificate. This PCR technique allows the detection of a single *C. pneumoniae* elementary body.

For the detection of *H. pylori* DNA we applied a nested PCR using two sets of primers as described by Wang et al. (35). Briefly, two primer sets designed to identify the urease gene of *H. pylori* DNA were used: external primers 5' GCC AATGGTAAATTAGTTCC 3' (sense) and 5' CTCCTTAATTGTTTTACAT 3' (antisense) and internal primers 5' AGTTCCTGGTGAGTTGTTCT 3' (sense) and 5' AGCGCCATGAAAACACGCT 3' (antisense). DNA was amplified in 50- μl volumes containing 200 μM each deoxynucleoside triphosphate, 1 μM each primer, 2 U of *Taq* polymerase (Boehringer Mannheim), 10 mM Tris-HCl, 1.5 mM MgCl₂, and 50 mM KCl. The first amplification was performed in an automated thermocycler (Hybaid) 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 around 5 *H. pylori* cells (Fig. 1).

Amplification products, 858 and 361 bp for *C. pneumoniae* and *H. pylori*, respectively, were visualized by electrophoresis in a 3% agarose gel containing ethidium bromide at 0.2 $\mu\text{g}/\text{ml}$. To avoid the risk of contamination, tissue preparation, PCR amplification, and electrophoresis were performed in separate rooms. In each assay a negative control and a positive control were run. The negative control contained all of the PCR reagents and sterile distilled water. As positive control we used *C. pneumoniae* purified elementary bodies at a concentration of $10^3/\mu\text{l}$ and a 10-fold dilution of an *H. pylori* bacterial culture (around 5×10^5 bacterial cells).

Statistical analysis. Student's *t* test and the chi-square test were used for

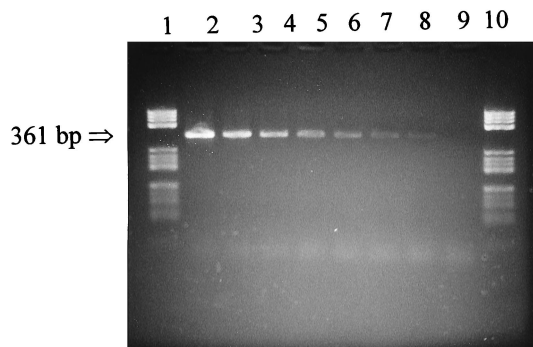


FIG. 1. Amplification products of *H. pylori* serial dilutions of culture, negative, and control samples in agarose gel. Lanes 1 and 10, DNA molecular weight marker V (Boehringer Mannheim) (660 to 8 bp); lanes 2 to 8, 5×10^6 , 5×10^5 , 5×10^4 , 5×10^3 , 5×10^2 , 5×10^1 , and 5×10^0 *H. pylori* cells, respectively; lane 9, negative control.

continuous and discrete variables, respectively. To evaluate the correlation between PCR positivity and other risk factors, a multivariate analysis was performed (SPSS package).

Informed consent was obtained from all subjects prior to admission to the study. The study was approved by the Ethics Committee of the University of Milan.

RESULTS

In all cases nested PCR showed no evidence of *H. pylori* presence in plaque specimens. Forty-seven of 51 patients were seropositive for *H. pylori*.

C. pneumoniae DNA was detected in aortic aneurysm plaques from 26 of 51 patients (51%). Figure 2 shows amplification products of positive, negative, and control samples in agarose gel. In 21 of 26 cases both samples gave a positive result, whereas only one of the two samples was positive in 5 cases. In 25 cases both samples were PCR negative.

In both PCR-positive and PCR-negative subjects no evidence of *C. pneumoniae* DNA was found in lesion-free arterial specimens (two samples for each patient).

C. pneumoniae-specific serologic patterns are shown in Table 2. Most patients (23 of 26) with PCR-positive plaques showed a *C. pneumoniae* antibody pattern indicating past or chronic infection, two had high antibody titers, and one was seronegative. Among patients with PCR-negative plaques, seven had high *C. pneumoniae* antibody titers, nine had anti-

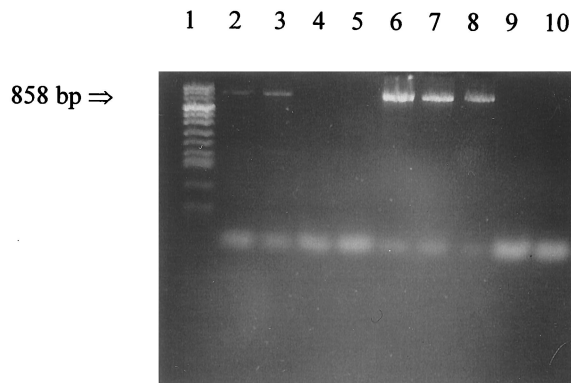


FIG. 2. Amplification products of *C. pneumoniae* positive, negative, and control samples in agarose gel. Lane 1, DNA molecular weight marker VIII (Boehringer Mannheim) (1,114 to 19 bp); lanes 2, 3, 7, and 8, positive samples; lanes 4, 5, and 9, negative samples; lane 6, positive control; lane 10, negative control.

TABLE 2. *C. pneumoniae* serologic patterns in the 51 studied patients

Serologic result ^a	No. of patients with ^b :	
	PCR-Positive plaques (26)	PCR-Negative plaques (25)
16 \geq IgG < 512 and/or 32 \leq IgA < 256	23 ^c	9
IgG \geq 512	2	7
Seronegative	1	9

^a None of the patients were positive for IgM.

^b The totals are in parentheses.

^c Significantly different from the number of patients with PCR-negative plaques ($P < 0.01$; Fisher's exact test [two-sided]).

body patterns indicating past or chronic infection, and nine were seronegative. Seropositivity for *C. pneumoniae* was significantly ($P < 0.01$) higher in subjects with PCR-positive plaques than in subjects with PCR-negative plaques. IgM antibodies were never detected.

No significant association was observed between known risk factors and *C. pneumoniae* DNA detection or seropositivity, taking into account the limited sample size.

DISCUSSION

Known risk factors for atherosclerosis (e.g., smoking, hypercholesterolemia, hypertension, etc.) do not completely explain the pathogenesis of the disease (18). Therefore, the study of atherosclerosis has taken new directions, one of the most intriguing of these being the infective theory of atherosclerosis that has been addressed in recent years. Epidemiological studies have suggested a role of infections in the pathogenesis of atherosclerosis (1, 3, 21, 22, 30), and bacterial DNA and viral structures have been identified in atherosclerotic lesions (34). Furthermore, Pesonen and Siitonen (24) observed a correlation between flu-like infection and acute myocardial infarction, and Spodick et al. (30) found a close association between respiratory infections and acute myocardial infarction.

C. pneumoniae infection has been recently indicated as a possible independent risk factor in acute myocardial infarction (26). This hypothesis was put forward following the appearance in the literature of seroepidemiological studies linking *C. pneumoniae* and coronary atherosclerosis (27, 31), in which patients with CHD showed higher *C. pneumoniae* seroprevalence than controls. Additional evidence of *C. pneumoniae* involvement in CHD came from the findings of Shor et al. (29) and Kuo et al. (14), who found the organism in atherosclerotic plaques of the coronary artery.

C. pneumoniae antibody titers were determined by a microimmunofluorescence test (36), and DNA was detected by means of a nested-PCR technique (5). The microimmunofluorescence serologic test used in this study, although quite time-consuming, is a specific and sensitive diagnostic method for *C. pneumoniae* infection. Stable elevated IgG and IgA antibody titers suggest chronic infection (27). PCR seems to be a first-choice method for *C. pneumoniae* detection in clinical specimens, particularly in chronic infection in which culture lacks necessary sensitivity (9, 11). In order to further enhance sensitivity and specificity of DNA detection (10, 37), we applied a nested-PCR technique that proved very useful in direct testing of clinical specimens for *C. pneumoniae* (5).

Our results show the presence of *C. pneumoniae* in a high percentage (51%) of aortic aneurysm plaques. Furthermore, all but three patients with PCR-positive plaques had serologic

patterns consistent with *C. pneumoniae* chronic infection, compared with only 9 of 25 PCR-negative plaque patients ($P < 0.01$). These data are consistent with previous studies indicating a chronic *C. pneumoniae* antibody pattern as a possible risk factor marker for atherosclerosis (28). Conversely, notwithstanding the high *H. pylori* seroprevalence observed, our results tend to rule out the possibility of a direct involvement of *H. pylori* infection in the pathogenesis of atherosclerosis.

Our findings suggest a link between *C. pneumoniae* chronic infection and the development of aneurysmal lesions. However, our data do not allow any definitive conclusion about a cause-effect relationship between infection and lesion. Nevertheless, this study provides data on a possible involvement of *C. pneumoniae* in the pathogenesis of aortic aneurysm and additional evidence for an association between this agent and atherosclerosis.

ACKNOWLEDGMENT

This study was supported by National Research Council (CNR) targeted project Prevention and Control Disease Factors PF 41 SP2.

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