MINIREVIEW

Involvement of *Chlamydia pneumoniae* in Atherosclerosis: More Evidence for Lack of Evidence

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During the past 15 years, many studies have been devoted to the relationship of *Chlamydia pneumoniae* and atherosclerosis: the serologic link has been investigated, and chlamydial organisms have been detected in lesions by electron microscopy, immunohistochemistry, in vitro cultivation, PCR, or in situ hybridization; efforts have been made to produce atherosclerosis experimentally in animals by inoculation of *C. pneumoniae* and therapeutic trials in humans have been undertaken. Some studies conclude that *C. pneumoniae* is present in cases of atherosclerosis, while others deny this, splitting the medical community into believers and disbelievers. The question, being a scientific one, should be resolved on a rational basis. After Boman and Hammerschlag reviewed the problem in 2002 (Clin. Microbiol. Rev. 15:1-20, 2002), a number of new studies were published on the subject. In this review, we critically evaluate the available data for the evidence or lack of evidence of a causal relationship for *C. pneumoniae* in atherosclerosis based on diagnostic and therapeutic studies performed with humans between 1992 and 2003, thereby searching mainly for concordance of evidence arising from different approaches used by different groups, at different times, and under different circumstances.

**SEROEPIDEMIOLOGIC STUDIES**

In 1988, Saikku et al. (61) reported that patients with coronary artery disease carry significantly more anti-*Chlamydia pneumoniae* immunoglobulin G (IgG) and IgA antibodies in their bloodstream than healthy controls. Since this initial study, a huge number of cross-sectional and case-control studies addressing the involvement of *C. pneumoniae* in atherosclerosis have been published. Several, but not all, of these studies found a similar positive association. Prospective studies, in which results were generally adjusted for the presence of traditional risk factors, seem to minimize the relationship between baseline *C. pneumoniae* IgG titers in the healthy population and the risk for a subsequent coronary event. Furthermore, the presence of elevated anti-*C. pneumoniae* antibodies in patients with preexisting vascular disease means no increased risk for future or recurrent cardiovascular events.

This serologic link between *C. pneumoniae* and vascular diseases has been studied by the microimmunofluorescence (MIF) test and enzyme-linked immunosorbent assays (ELISAs). There is, however, accumulating evidence that *Chlamydia* serology is less specific than was first assumed. Cross-reactivity between *C. pneumoniae* and other *Chlamydia* species has been demonstrated with the MIF test. In addition, neither the serologic procedures nor the criteria for defining an infection with *C. pneumoniae* are standardized. A standardization workshop held in 2001 (18) recommended that the MIF test should be considered the only acceptable serologic test for *Chlamydia* and that an IgG titer of ≥1/16 indicates past exposure but that neither elevated IgA titers nor any other serologic marker may be used as a validated indicator of persistent or chronic infections. As *C. pneumoniae* antibody seroprevalences in the general population are high, it remains questionable, however, whether seropositivity for *C. pneumoniae* results either from a chronic, active infection or from a past infection.

The MIF test has been criticized mainly because of improper interpretation of its results. The reproducibility of MIF among 14 different laboratories was examined by testing 22 identical sera, resulting in a 60 to 80% agreement with the results of the reference laboratory (53). Besides the problem of interlaboratory variation, discordant results between MIF tests were also obtained when they were analyzed and read by the same experienced technician. So, in addition to the subjective component, other factors (the type, purity, and concentration of the antigen used and the assay procedure) might contribute to the disagreement between the tests. In contrast to MIF tests, enzyme immunoassays are easier to perform, less time-consuming, and more objective because of the photometric reading involved. Nevertheless, three recent studies have demonstrated that the link between *C. pneumoniae* and coronary artery disease depends on the serologic method chosen to measure the *C. pneumoniae* antibodies (29, 45, 62).

In summary, although initial reports were positive, the later ones, often prospectively designed and adjusted for known cardiovascular risk factors, showed a negative or weak positive association overall between seropositivity for *C. pneumoniae* and atherosclerosis. Importantly, methodology has a strong impact on the link between *C. pneumoniae* and atherosclerosis. Inter- and intralaboratory variations and poor agreements between the different *C. pneumoniae* tests have been demonstrated.

**DETECTION OF *C. PNEUMONIAE* IN ATHEROSCLEROTIC LESIONS BY EM**

*C. pneumoniae* organisms were first detected by electron microscopy (EM) in atherosclerotic lesions by Kuo et al. and Shor et al. in 12 of 43 autopsy cases (36, 63, 64). Preparations

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often revealed organisms of various sizes and forms and also degenerative organisms. The organisms were situated in smooth muscle cells, foam cells, and extracellular debris and in areas of fibrosis and in ceroid bodies. Between 1993 and 2003, C. pneumoniae organisms were observed by EM in 63 of 155 (40.6%) atherosclerotic specimens in 11 studies and in none of 66 specimens examined in 4 studies. There are wide and significant variations between the studies: from 0 of 22 to 32 of 51 (62%) specimens were positive for C. pneumoniae (6, 73). Tissues with minimal lesions were positive as often as those with severe lesions (64). This finding may point to a low specificity of the procedure. Rose (60) mentioned abundant calcium hydroxylapatite crystals presenting as microvacular or microvesicular structures with a morphology which resembles that of chlamydial organisms. EM sensitivity can be estimated by comparing EM results with the PCR results in those studies in which specimens are independently studied by EM and PCR and when the results of both procedures are presented for each individual specimen (25, 36, 64, 72). It appears that the sensitivity of EM, in comparison to that of PCR, is very high, i.e., 64.5%, whereas in general EM is much less sensitive than PCR for the detection of microorganisms.

The interpretation of EM pictures is not convincing. It is thus impossible to conclude on the basis of EM studies that C. pneumoniae is present in atherosclerotic lesions.

CULTURE OF C. PNEUMONIAE FROM ATHEROSCLEROTIC LESIONS

Culture of C. pneumoniae remains an insensitive procedure. Gaydos et al. (22) compared the detection of C. pneumoniae by culture with detection by PCR using respiratory specimens and found a sensitivity for culture of 87.5% versus PCR. The specificity of culture is dependent on the ability of the laboratory worker to distinguish true C. pneumoniae inclusions from artifacts after fluorescent staining has been performed. Boman and Hammerschlag (12) insisted that true-positive C. pneumoniae cultures produce inclusions and not just elementary bodies.

A standardization workshop on C. pneumoniae assays (18) recommended two blind passages for respiratory specimens and four to six passages for tissue specimens; a finding of one or more inclusions per well or tube should be considered a presumptive positive result, and only if the strain can be propagated by means of subsequent passages or confirmed by an additional test such as PCR should the result be reported as confirmed positive.

The number of C. pneumoniae strains isolated from atherosclerotic specimens remains small: 46 isolates were obtained from 625 specimens (7.4%) by five research groups. In one study, an isolate was obtained in three different laboratories from fragments from the same coronary artery (58). The culture efforts made by six research groups with a total of 302 specimens remained negative (8, 23, 24, 32, 35, 36, 52, 70, 73). Strains were detected after two to five (39, 73) and five to eight passages (3, 30). Permanent propagation in tissue culture was successful for only 6 of 11 isolates (39). Afpalter et al. (3) mentioned that the difficulty in detecting C. pneumoniae elementary bodies and/or cell inclusions is due to the massive unpecific reactions caused by the inoculated material during the first five passages.

The small number of isolates of C. pneumoniae obtained after much effort is in sharp contrast with the high frequency of detection of C. pneumoniae by other approaches, such as immunohistochemistry (IHC) and PCR.

DETECTION OF C. PNEUMONIAE IN ATHEROSCLEROTIC ARTERIES BY PCR

Since the results of cultivation of C. pneumoniae from atherosclerotic lesions have been disappointing, most researchers have used the more-sensitive PCR method. Based on several studies (1, 14, 21) it appears that 0.1 inclusion-forming unit can be detected by this procedure.

Between 1992 and 2004, we identified 63 reports on the detection of C. pneumoniae. A variety of arteries as well as some veins were examined. Fresh, frozen, and fixed tissues were tested. Twenty studies included 665 control specimens. Single-format PCR, nested PCR (nPCR), and in two studies a reverse transcription (RT)-PCR were applied. Overall, validated primers were used (18). Amplicons were detected by agarose gel electrophoresis, which was not always confirmed by hybridization. It should be realized, however, that detection of specific amplicons solely by the inspection of agarose gels without hybridization using a specific probe may correspond to an unrecognized false-positive signal. In those studies, unpecific signals may be incorrectly interpreted as C. pneumoniae positive. This is particularly the case when regular Taq DNA polymerase rather than AmpliTaq Gold DNA polymerase is used (43). In 11 out of 33 studies reviewed by Maraha et al. (43), the detection rate was 31.6% when gel electrophoresis only was used to visualize the amplicons. In the 22 studies in which results were confirmed by hybridization, the detection rate was 24.5% (P = 0.0009). Furthermore, contamination by environmental chlamydia-like organisms has been detected (43). In a great majority of studies, C. pneumoniae or its DNA was included as a positive control in each amplification run or was added to PCR-negative specimens in a repeat reaction for the detection of PCR inhibitors. To avoid this potential source of contamination, an internal control was constructed in two studies (28, 31).

A total of 3,551 specimens were tested: 2,688 were negative and 863 (24.3%) specimens were positive for C. pneumoniae DNA. C. pneumoniae prevalence in atherosclerotic vessels ranged from 0 to 100%. However, a considerable proportion (up to 6.5%) of control arteries were also PCR positive. This high positivity in control arteries has led to skepticism concerning either the technique or the selection of the negative controls.

Results lack reproducibility within laboratories and between laboratories. Specimens tested in duplicate or triplicate were not consistently positive in the studies by Wong et al. (76), Petersen et al. (54), Thomas et al. (68), Jackson et al. (30), Weiss et al. (73), Ong et al. (50), and Blasi et al. (10). In contrast, all PCRs in the studies by Maass et al. (39, 40, 42) produced the same results. Ong et al. reported in 1996 (50) that 19 of 43 (44%) atherosclerotic vessels were positive for C. pneumoniae by PCR but failed to detect C. pneumoniae in 40 carotid artery specimens by applying the same technique in
2001 (51). Likewise, Maraha et al. (44) found 10 of 27 (37%) specimens positive for *C. pneumoniae* but could not detect any DNA in a second study using a more specific test (43). Subsequently, Maraha et al. concluded that the first positive results were probably due to contamination and methodological problems.

A few research groups applied to their samples a second PCR targeted at a different sequence in the genome. In four studies, the results were confirmed by the second PCR: positive results obtained by Kuo et al. (36) and negative results obtained by Bishara et al. (8), Wessely and Mall (74) and Hoymans et al. (28). Hoymans et al. applied two different single PCRs, a nested PCR, and a RT-PCR in two different laboratories with 197 specimens with negative results. Contradictory results were recorded by Jantos et al. for two patients (31).

Studies of contiguous arterial fragments also produced divergent results (10, 16, 40, 57, 68). The low reproducibility of many PCR results has been attributed to the presence of small numbers of *C. pneumoniae* organisms or to their random distribution in the tissues. If this is the case, the numbers of *C. pneumoniae* organisms present in the lesions should be very low in a considerable proportion of patients. The proportion of cases with low numbers of *C. pneumoniae* varies widely among the studies: from 5 of 26 (19%) in a study by Blasi et al. (10) to 5 of 21 (23.8%) in a study by Maass et al. (40), 7 of 21 (25%) in a study by Jackson et al. (30), and 42 of 52 (80%) in that by Thomas et al. (68). Tissue inhibitors were frequently evoked to be responsible for low sensitivity; however, the overwhelming majority of specimens do not contain PCR inhibitors, as was evidenced by the use of proper internal controls.

A real-time PCR technique not only produces amplification results more rapidly than traditional PCR but also is less prone to amplicon carryover. Ciervo et al. (15) tested three independent aliquots of 15 carotid plaques, both by an RT and a nPCR method. All samples were negative by the nPCR, and three samples were positive by the RT-PCR, with 50, 37, and 24 copy numbers. Ciervo et al. suggest that *C. pneumoniae* organisms are present infrequently and in low DNA copy numbers in atheromatous plaques. Hoymans et al. (28), using an RT-PCR with an analytical sensitivity of 0.02 inclusion-forming unit per reaction with 197 atherosclerotic and 8 control specimens, obtained negative results.

Two multicenter studies have been published. In a study by Ramirez et al. (58), samples taken from 10 different coronary arteries were tested by PCR in four laboratories. Positivity ranged from 4 of 10 specimens in one laboratory to 2 of 10, 1 of 10, and 0 of 10 specimens in the three other laboratories. Specimens from one patient were positive in three of four laboratories, but four positive results were solitary findings in two laboratories. This study was criticized because the laboratories did not investigate identical materials. In a study by Apfalter et al. (2), the detection rate in the individual laboratories did not correlate with the sensitivity of their assays as was determined with a panel of spiked control specimens: two of three of the most sensitive methods found all 15 atherosclerotic specimens negative, while one of the two laboratories that detected the most positive clinical specimens did not detect the lowest positive control. Contamination of negative controls occurred in two laboratories. The overall detection rate (9%) and the maximum concordant positive rate for the atheroma samples (25% for one carotid artery) were lower than expected. Statistical calculation concerning the observed distribution of positive results in the atheroma panel did not support the hypothesis that the discordant positivity rates were generated by a distribution problem. It was concluded that if *C. pneumoniae* was present at all in atheromatous specimens, it would be at a very low target level.

Although there are no quantitative data available for PCR results, some correlations with the intensity of the reaction and the degree of atherosclerosis have been made. In the studies by Bartels et al. (5), Berger et al. (7), Davidson et al. (17), Taylor-Robinson et al. (67), Wong et al. (76), and Thomas et al. (68), *C. pneumoniae* organisms were not more common in atherosclerotic vessels than in nonatherosclerotic internal mammary arteries.

In at least 15 studies, a lack of correlation between PCR detection of *C. pneumoniae* and serology has been noted. *C. pneumoniae* organisms were frequently detected in patients with no or low antibody titers and were often absent in the presence of high antibody titers (4, 7, 9, 13, 16, 31, 33, 36, 37, 41, 42, 55, 56, 77). For Kuo and Campbell (34), “this remains an unsolved paradox with no good explanation.”

It can be concluded that PCR offers no straightforward evidence for the presence of *C. pneumoniae* in atherosclerotic lesions.

### DETECTION OF *C. PNEUMONIAE* IN ATHEROSCLEROTIC TISSUE BY IN SITU HYBRIDIZATION

With in situ hybridization, results were negative in two studies (31, 47), results with an isolated specimen were positive in a third study (22), and some specimens were found to be positive in two additional studies (48, 58).

In situ hybridization does not offer straightforward results.

### *C. PNEUMONIAE* IN PERIPHERAL BLOOD MONOCYTES

Smieja et al. (65) recently reviewed 18 studies (to which the study of Hoymans et al. [28] should be added) of the presence of *C. pneumoniae* in the bloodstream: 9 studies were concerned with a simultaneous investigation of cardiovascular patients and control persons (healthy blood donors, medical students, and persons with a normal angiogram), and 10 studies were concerned with either vascular patients, healthy blood donors, or patients without vascular problems. Plasma was uniformly negative for *C. pneumoniae* (11, 65).

Haranaga et al. (26) and Bodetti and Timms (11) performed immunofluorescence assays on cytospin preparations of peripheral blood mononuclear cells (PBMCs) with genus- and species-specific monoclonal antibodies, respectively. Although few in numbers, small chlamydial inclusion bodies were seen in the PCR-positive samples, but none were found in the PCR-negative samples.

PCR-positive blood samples were present in all but three groups, of which one was a control group. The prevalences ranged between 0 and 59.4% among cardiovascular patients and between 2.5 and 47% among control persons. Most authors found a positive relationship between PBMC PCR and
the presence of *C. pneumoniae* seropositivity, but Rassu et al. (59) and Bodetti and Timms (11) did not. The relationship between *C. pneumoniae* DNA detection in PBMCs and in atheromas is unclear: Blasi et al. (9) found a correlation, but Berger et al. did not (7). The results of repeated testing of samples by Rassu et al. (59) and Fridank et al. (20) are analogous with those with arteries. Tondella et al. (69) used two RT-PCRs and two nPCRs and detected *C. pneumoniae* by one RT-PCR and one nPCR in two single but different PBMC specimens.

Investigating the presence of *C. pneumoniae* in peripheral blood monocytes has led to great skepticism, particularly when the high positivity rates in healthy blood donors are reported as “tending to stretch the credibility to the limit” (66).

DETECTION OF *C. PNEUMONIAE* IN Atherosclerotic Tissue by IHC Reactions

IHC methods with genus- and species-specific monoclonal antibodies as well as anti-heat shock proteins have been applied to large numbers of arterial specimens by many investigators. Subjectivity in reading immunofluorescence tests is a cause of concern and is hindered by nonspecific background staining and heterogeneity of *C. pneumoniae* elementary bodies (50, 68). In some studies, the results obtained with genus-specific antibodies could not be confirmed by the use of species-specific antibodies. It should be realized that IHC is essentially an antigen-antibody reaction with its inherent problems of sensitivity and specificity, and the question is whether positive reactions result from improved sensitivity or worse specificity.

The sensitivity of the IHC technique is unknown, but it can be compared to that of a Gram stain of smears, with a sensitivity of 10⁴ organisms per ml in a suspension. A tissue section with an estimated volume of 1 µl could thus correspond to a sample containing 10⁴ *C. pneumoniae* organisms per g of tissue. If this reasoning holds true, PCR should be more sensitive than IHC. However, this seems not to be the case: overall IHC results were positive for 486 of 958 (50.7%) specimens and PCR results were positive for 863 of 3,551 (24.3%) specimens. The 15-kDa band was not present in negative control tissue and was identified through mass spectrometry to have 95% sequence identity to hemoglobin beta chain. Similarly, Lindholt et al. (38) using purified human anti-outer membrane protein antibodies could not detect the corresponding antigen in abdominal aortic aneurysm specimens. Neureiter et al. (49) detected *C. pneumoniae* proteins in atherosclerotic lesions by Western blot analysis in 13 of 20 cases and in 1 of 40 controls, but the procedure was done with formalin-fixed tissue, a technique that is open to criticism.

Immunohistostaining gives rise to cross-reactions between anti-*C. pneumoniae* antibodies and nonchlamydial proteins in the vessel wall, producing nonspecific signals, and results should therefore be interpreted with caution.

TREATMENT TRIALS

*C. pneumoniae* is susceptible in vitro to macrolides, tetracyclines, quinolones, and rifamycins. All reported antibiotic trials used macrolides. Several prospective controlled treatment trials with humans have been published (for a review, see reference 27). Four studies concern patients after myocardial infarction, and nine studies concern patients with coronary artery disease or acute coronary syndromes. Treatment was with azithromycin in daily doses for 3 to 6 days to 30 days or weekly doses for 11 weeks, 3 months, 6 months, or 1 year. Follow-up periods ranged from 6 months to 2.5 years. Some results are expected in the year 2005. Short-term antibiotic treatment does not significantly decrease cardiovascular events. Therefore, Higgins (27) proposed that a robust study (the Azithromycin Coronary Events Study trial) should determine whether long-term treatment is required to decrease cardiac events.

CONCLUSIONS

There are now more arguments against than for a causal relationship between *C. pneumoniae* and atherosclerosis. Seropidemologic results are largely technique dependent, PCR results show intra- and interlaboratory variability, methodological factors contribute to bias, and detection of *C. pneumoniae* fails when the specificity of the reaction is optimized. IHC detects nonspecific compounds in atherosclerotic lesions, and secondary prevention trials are unsuccessful. The evidence for an association between a novel pathogen and chronic disease should be based on concordance of evidence arising from different approaches applied by different groups, at different times, in different places, and under different circumstances.
(19). None of these conditions have been fulfilled in the present case.


