Papers

Detection and widespread distribution of *Chlamydia pneumoniae* in the vascular system and its possible implications

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Abstract

Aims—To attempt to detect *Chlamydia pneumoniae* DNA in atheromatous vascular tissue.

Methods—A modification of an existing polymerase chain reaction (PCR) assay and immunofluorescence staining with a monoclonal antibody directed against *C pneumoniae* were used to detect *C pneumoniae*. Specimens from 32 patients undergoing abdominal aortic aneurysm repair were examined. Vascular tissue, ostensibly normal, from six liver transplant donors was also examined for comparison. Altogether, 43 vessels from these 38 subjects (age range 36–85 years) were examined.

Results—*C pneumoniae* was detected in 11 (44%) of 25 aortas, five (55%) of nine iliac arteries, two (40%) of five femoral arteries, and one of two iliac veins. Immunofluorescence staining supported positive PCR results in three of 12 cases in which it was used. Overall, *C pneumoniae* was detected in the arteries of 14 (44%) of the patients undergoing vascular surgery and three (50%) of the donors.

Conclusions—This study is the first in the UK in which *C pneumoniae* organisms have been found in atherosclerotic vessels and the tendency for the organisms to be present most often in such vessels exhibiting chronic inflammatory changes suggests that a search for them in various forms of arteritis may also be rewarding.

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Chlamydia pneumoniae is a human pathogen, one of the four species of the genus *Chlamydia*.¹ It causes a range of respiratory tract diseases, including pharyngitis, sinusitis, bronchitis, mild to severe pneumonia,² and asthma.³ Serological and epidemiological studies have shown that the organisms are widespread geographically,⁴ and that epidemics occur in four, six and 10 yearly cycles.

IgG antibody to *C pneumoniae* is detected rarely in children up to five years of age, but is present in half of the adult population at any

one time, suggesting a cycle of infection and reinfection during a lifetime.⁵ IgG antibody increases with age so that it is found in almost all subjects aged 70 years. Seroconversion usually occurs between eight and 16 years of age⁶ and there is a higher rate of infection among men than women.⁷

Apart from being a respiratory pathogen, *C pneumoniae* has been associated serologically with coronary heart disease and myocardial infarction.^{8,9} More direct evidence, based on electron microscopy, immunocytochemistry and the polymerase chain reaction (PCR), of its existence in plaques of coronary arteries and in aortic lesions has been found by one group of investigators^{10–13} but not by another who used PCR only.¹⁴ In view of the controversy, we have used PCR and immunofluorescence techniques and provide the first evidence that in the UK *C pneumoniae* exists in various atherosclerotic arteries, in many of which chronic inflammatory changes are a notable feature.

Methods

TISSUE AND DNA EXTRACTION

Atheromatous arterial tissue was obtained from 29 men and three women who were undergoing surgery at St Mary's Hospital, London, mainly for repair of aortic aneurysms. The tissues were collected within three hours from the operating theatre, washed with phosphate buffered saline (PBS), and dissected aseptically into a number of pieces. Vascular tissues were also obtained from three male and three female liver transplant donors at the Royal Free Hospital, London, on the basis that these might provide normal tissue for comparison. After excision, these specimens were kept in a Ringer's solution at 4°C for a few days before being washed and dissected aseptically. Blood samples were not obtained from any subjects in this study.

One piece of dissected tissue was processed for DNA extraction in the following way. Tissue was frozen in liquid nitrogen and pulverised rapidly while still frozen in a pre-cooled mortar and pestle. The powder was scooped into a microcentrifuge tube containing 10 mM Tris, 75 mM NaCl, 25 mM EDTA. To avoid cross-contamination, a different mortar and pestle and a different set of instruments were used

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C. pneumoniae detected by a PCR assay in the wall of blood vessels

Vessel*	No. of vessels tested	No. (%) PCR positive
Subjects undergoing vascular repair		
Aorta	25	11 (44%)
Iliac	3	2 (67%)
Femoral	5	2 (40%)
Renal	1	0
Popliteal	1	0
Liver transplant donors		
Iliac	6	3 (50%)
Iliac vein	2	1
Total	43	19 (44%)
Patients	38	17 (45%)

* Arteries except where indicated.

for each tissue. All these pieces of apparatus were washed, soaked in 4 M HCl, and finally rinsed overnight in distilled water before re-use. The tissue suspension was thoroughly mixed, sodium lauryl sulphate added to a final concentration of 0.5%, and the mixture incubated at 37°C for one hour. Proteinase K was then added to a final concentration of 20 µg per ml. After further incubation at 55°C for two hours, the debris was removed by centrifugation and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the supernatant fluid. Following a second extraction step, DNA

was precipitated at -20°C overnight with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol.

POLYMERASE CHAIN REACTION FOR *C. PNEUMONIAE*

A nested PCR assay was developed for the detection of *C. pneumoniae*. The primers were based on the DNA sequence of the major outer membrane protein (MOMP) of *C. pneumoniae*. Primers for the first round of the PCR (APN1 and APN2)¹⁵ were provided by Adam Cunningham (Department of Microbiology, Southampton University Medical School, Southampton, UK). Primers for the second round of the PCR were designed by us and gave a PCR product of 227 base pairs (bp). Although there are several regions of homology within the DNA sequence of the MOMP gene to *C. psittaci* and *C. trachomatis*, we have especially chosen the primer sequences from the variable domains of the coding region so that the primers will not amplify a product from the other two species but will have specificity only for *C. pneumoniae*. The first primer, TGCCAACAGACGCTGGCGTAGCAA, spans bases 1053 to 1076 of the MOMP gene while the second primer, TAACTGCATGGAA-CCCTTCTTTACTAG, spans bases 1254 to 1280 of the MOMP gene. We determined that use of the second round PCR assay enabled as few as 10 organisms to be detected. Amplified products were electrophoresed on a 2% agarose gel and specimens were regarded as positive when the band migrated to the expected molecular weight of the positive controls (fig 1). A positive control of *C. pneumoniae* DNA and a negative control comprising water were included in each PCR assay. Positive results were confirmed by Southern blot hybridisation with a 30 bp oligonucleotide probe. Evidence for the existence of *Mycoplasma fermentans* in the tissues was also sought by means of a PCR assay that has been described and used previously.¹⁶ To avoid DNA contamination during the assays, separate hoods were used for mixing pre-PCR reagents and for adding DNA from clinical material and a different room was used for adding amplified DNA product from the first round PCR.

IMMUNOCHEMICAL STAINING AND HISTOLOGY

A piece of tissue adjacent to the piece from which DNA was extracted for the PCR was frozen in isopentane; frozen sections were cut and examined by using an indirect immunofluorescence technique for *C. pneumoniae* elementary bodies with a specific monoclonal antibody and adjacent frozen sections by using an avidin-biotin-peroxidase method (Dako, High Wycombe, UK). Sections were also stained with haematoxylin and eosin for histological examination.

Results

The PCR assay was evaluated first for sensitivity and specificity. The primers for the first

Figure 1 Detection of *C. pneumoniae* DNA from patient samples. DNA products from the nested PCR assay were electrophoresed on an agarose gel (A) and probed by Southern hybridisation (B) with an oligonucleotide probe containing a DNA sequence internal to the primer sequences used in the nested PCR. Lanes 4, 6 and 8, *C. pneumoniae* positive; lanes 1-3, 5, 7, and 9, *C. pneumoniae* negative; lane 10, positive control of *C. pneumoniae* DNA; lane 11, negative control comprising water. Results of the gel electrophoresis agree with those obtained by the Southern blot. The arrows indicate the PCR product of 227 bp. The marker used was ϕ X174/HaeIII.

Figure 2 Atheromatous changes and chronic inflammatory cells in a section of the abdominal aorta (haematoxylin and eosin). An adjacent tissue sample was positive for *C. pneumoniae* by PCR.

round of the nested PCR gave a *C. pneumoniae* DNA product of 498 bp, and the second round gave a product of 227 bp. The detection limit of this PCR product in a Southern blot was 10 chlamydial elementary bodies. Primers for the first round of the PCR had been shown not to amplify purified genomic DNA of *C. trachomatis* and *C. psittaci* (Professor M Ward, personal communication). Furthermore, we determined that the primers for the second round PCR also did not amplify DNA from these two chlamydial species, nor that from Hep2 cells in which *C. pneumoniae* was grown.

As shown in the table, 19 (44%) of 43 vessels tested by the PCR assay were positive for *C. pneumoniae* (fig 1). The vessels came from 32 subjects undergoing reparative vascular surgery, 14 (44%) of whom had *C. pneumoniae* positive vessels. There were six liver transplant donors, three (50%) of whom had *C. pneumoniae* positive vessels (iliac arteries). Thus, 17 (45%) of 38 subjects had vessels that were positive for *C. pneumoniae*. The PCR results were confirmed either by amplifying a further sample of DNA extracted from the same piece of tissue and/or by amplifying DNA from another piece of tissue from the same vessel. In tests on specimens from subjects undergoing vascular repair, all but three initially positive results were con-

firmed in this way, as were all initially negative results. In tests on specimens from liver transplant donors, the specimens were not consistently positive on repeat testing but they were always positive more than once. Only 12 of the vascular repair specimens were examined by immunofluorescence staining. Four were unreadable because of non-specific staining, five were negative and three were positive; one of the unreadable specimens and the three fluorescence positive specimens were also positive on PCR. It is clear that *C. pneumoniae* organisms were not restricted to the aorta, being detected in about the same proportion of iliac and femoral arteries, and in one of two iliac veins examined. In one subject both the aorta and the femoral artery tested were PCR positive, whereas in another, the aorta but not a renal artery was PCR positive.

There was no correlation between age and a positive PCR result. Thus, for patients undergoing vascular surgery, the mean age of those who had PCR positive vessels was 69 years and that of subjects who had PCR negative vessels was 73 years. Liver transplant donors were younger (mean age, 50 years) but, again, there was no difference between the age of PCR positive and negative subjects.

Specimens were also tested by a PCR assay for *M. fermentans*. Of 19 specimens that were positive for *C. pneumoniae*, one was also positive for *M. fermentans*.

Histologically, all of the tissue samples from subjects undergoing reparative surgery showed marked atheromatous changes, often with infiltrates mainly of chronic inflammatory cells (lymphocytes and macrophages) (fig 2), but also occasionally of polymorphonuclear leucocytes. The cells were dispersed throughout the vessel wall and/or were grouped densely. In one case (PCR positive for *C. pneumoniae*), the presence of polymorphonuclear leucocytes was the striking feature. The tissue samples from the liver transplant donors were far less affected by atheromatous changes and only one of seven samples examined showed a moderate inflammatory infiltrate. Overall, of 23 tissue samples in which there were moderate to severe inflammatory infiltrates, 13 (56%) were positive for *C. pneumoniae* on PCR; of the 16 tissue samples in which there were no or minimal infiltrates, five (31%) were positive on PCR.

Discussion

In this study, based almost entirely on the use of PCR, we detected *C. pneumoniae* in a large proportion of various major arteries. The figures are perhaps conservative as it is possible that more specimens would be found to be *C. pneumoniae* positive if a larger amount of tissue was examined. Use of PCR raises the possibility of DNA contamination, but there are several reasons why this is unlikely to account for the results. First, specimens were processed and tested in small groups reducing the possibility of contamination; second, negative controls within each assay remained negative; third, re-amplifying the DNA from the same specimen or testing another piece of tissue from the same

vessel produced the same result for all but three of the 43 tissue samples tested; and fourth, specimens that were recorded independently as fluorescence positive were also positive for *C pneumoniae* by PCR. Considerable difficulty was experienced in examining specimens by the immunofluorescence technique because of non-specific background staining and the morphological heterogeneity of *C pneumoniae* elementary bodies in contrast to the homogeneity of *C trachomatis* bodies. As a consequence, not all specimens were examined and only those that could be regarded confidently as fluorescence positive were so recorded. Even greater difficulty was encountered with the immunoperoxidase test, in which a problem of non-specific staining, not mentioned by others,^{11,12} has not yet been resolved.

There was a tendency for more of the tissue samples that had moderate to severe chronic inflammatory changes to be positive for *C pneumoniae* than those that had few inflammatory changes. While this is consistent with the known ability of chlamydial organisms to produce chronic disease with scarring, trachoma and pelvic inflammatory disease being good examples, it would be unwise to over-interpret the observation as sections of some PCR positive specimens contained few or no inflammatory cells and some PCR negative specimens contained many. It was perhaps too much to expect that vessels from organ transplant donors would be free of atheromatous changes. They did, in fact, show some such changes histologically but little evidence of inflammation compared with the tissue samples from the patients who underwent reparative surgery. However, although about the same proportion of the donor tissue samples and the more atheromatous tissue samples were positive by PCR, there were probably fewer organisms in the former as indicated by the difficulty in finding them consistently. Whether this means that healthy non-atheromatous tissue is free of *C pneumoniae*, as Kuo *et al*¹¹ suggest, is in our opinion still a moot point and is an issue that requires further study. However, if *C pneumoniae* is found only in diseased tissue and not in healthy tissue, it could mean that it has been deposited in an atheromatous area that already exists, perhaps being transported there in chronic inflammatory cells, and has nothing to do with initiating the process. As *M fermentans*, which is known to escape from mucosal surfaces into the blood stream and is associated with lymphocytes,¹⁶ was detected on one occasion only, *C pneumoniae* by contrast would seem to be specifically associated with the atheromatous inflammatory lesions at some stage. Of course, this does not preclude a role for other micro-organisms. The difficulty in attempting to resolve the "chicken or egg" problem in the case of *C pneumoniae* is that completely atheroma-free arterial tissue from individuals old enough to have experienced a *C pneumoniae* infection is not easy to find. However, examination of postmortem tissue in which the presence or absence of overt and histological changes are correlated with the presence or absence of *C pneumoniae*, based on

the PCR, would seem a sensible way to proceed, particularly if this is done for individuals of different ages. The finding of *C pneumoniae* in a vein was surprising and invites further investigation. Easy access to venous tissue removed because of varicosities should permit rapid determination of the extent to which veins might be involved.

The detection and measurement of *C pneumoniae* antibodies in seroepidemiological surveys has been helpful in suggesting a role for this organism in coronary heart disease,^{8,9} but we do not believe that serology is useful in relatively elderly patients as a means of defining, on an individual basis, the role of the organisms in the atheromatous disease process. However, if the organisms could not be detected in the vessels of young people, the presence of specific antibody would be useful in indicating that they had been infected at some time and at least had had the opportunity for vessel involvement. Furthermore, the notion that *C pneumoniae* antibody might be protective based on the absence of organisms in the vessels of patients with high antibody titres¹¹ needs to be clarified.

We believe that attempts should be made to develop an animal model of *C pneumoniae* in atheroma, possibly murine in view of the success achieved with such a model of *C trachomatis* genital tract disease.¹⁷ In addition, the judicious use of broad-spectrum antibiotics, such as tetracyclines and erythromycin, may be worthwhile. Administration of such antibiotics to patients undergoing coronary artery by-pass surgery to discover whether they increased the time to re-stenosis would be one approach. The use of a *C pneumoniae* vaccine which if not capable of preventing colonisation in the respiratory tract might at least prevent or reduce haematogenous spread of the organisms to other sites is a possible future consideration. Finally, the presence of *C pneumoniae* organisms in atheromatous tissue, which usually contains chronic inflammatory cells, raises another question. Are these organisms involved in the various forms of arteritis in which there is a chronic inflammatory response—for example, temporal arteritis, the aetiology of which is ill-understood? Examination of stored tissue samples should expedite the answer.

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