Detection of *Chlamydia pneumoniae* in peripheral blood mononuclear cells: correlation with inflammation and atherosclerosis in haemodialysis patients

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Abstract

**Background.** *Chlamydia pneumoniae* has been implicated as an inflammatory agent in atherosclerosis. Clinical studies in this field have yielded conflicting results, which may have resulted from a lack of standardization for *C.pneumoniae* detection. We attempted to accurately estimate *C.pneumoniae* prevalence and to examine whether *C.pneumoniae* is associated with atherosclerosis and inflammation in haemodialysis (HD) patients. To do this, we assessed *C.pneumoniae* presence by a combination of methods and correlated its levels with inflammatory and atherosclerotic indexes in these patients.

**Methods.** *Chlamydia pneumoniae* was identified by polymerase chain reaction (PCR) in DNA extracted from cell cultures inoculated with patient buffy coats and by serum IgG antibodies against *C.pneumoniae* (IgGCp). Inflammation was assessed by C-reactive protein and serum amyloid A and atherosclerosis was evaluated from clinical and laboratory data.

**Results.** Of the 130 patients, only nine had viable *C.pneumoniae* in peripheral blood mononuclear cells (PBMCs) while 64 had serum IgGCp. Although patients with viable *C.pneumoniae* had higher atherosclerotic scores, seropositive and negative patients showed similar scores. Patients with atherosclerosis exhibited higher inflammatory indexes. Neither patients with detectable *C.pneumoniae* in PBMCs nor seropositive subjects had higher inflammation than negative patients.

**Conclusions.** We found that viable *C.pneumoniae* in PBMCs, assessed by cell culture and PCR, was present in a small percentage of HD patients and was correlated with atherosclerosis. Seropositivity was much higher in HD patients but was not associated with viable *C.pneumoniae* or with atherosclerosis. Further studies in HD patients using high sensitivity and specificity methods in larger populations will be necessary to clarify the relationship between *C.pneumoniae* and atherosclerosis.

**Keywords:** cell cultures; C-reactive protein; IgG antibodies against *Chlamydia pneumoniae*; peripheral blood mononuclear cells; polymerase chain reaction; serum amyloid A

Introduction

Because atherosclerosis has been recently recognized as an inflammatory process, chronic infections are now thought to play a causative role [1]. In addition, *Chlamydia pneumoniae* has been the most frequently implicated microorganism in this infection-induced atherosclerosis model [1]. Although seroepidemiological studies linking *C.pneumoniae* with atherosclerosis have yielded conflicting results [2], a lack of standardization for *C.pneumoniae* identification may have confounded these findings [3].

In renal failure patients, chronic persistent infections have been proposed as causes of chronic inflammation [4] and *C.pneumoniae* appears to be a source of such infections [5]. Moreover, the accelerated atherosclerosis in these patients [4] suggests that chronic and persistent *C.pneumoniae* infection may explain both the inflammation and atherosclerosis observed in haemodialysis (HD) patients.

In the present study, we attempted to (i) accurately assess the prevalence of *C.pneumoniae* in HD patients using a combination of cell cultures and subsequent polymerase chain reaction (PCR) for viable microorganism
Chlamydia pneumoniae, inflammation, atherosclerosis
detection in peripheral blood mononuclear cells (PBMCs) as well as by determination of serum IgG antibodies against *C. pneumoniae* (IgGCp) and (ii) examine possible contributions of *C. pneumoniae* to inflammation, assessed by C-reactive protein (CRP) and by serum amyloid A (SAA), as well as to atherosclerosis.

**Subjects and methods**

**Patients’ characteristics**

One-hundred and forty-two stable HD patients were included after having obtained informed consent. Twelve patients were excluded because of active infection and/or antibiotic treatment or surgical operation in the month before the study onset. Of the remaining 130 patients, 60% were males, with (mean ± SD) age 61.91 ± 13.72 years (range: 18–87 years); 14.6% were diabetics, 44.6% had hypertension, 18.2% had chronic bronchitis and 31 were current smokers.

The cause of end-stage renal failure (ESRF) was chronic glomerulonephritis in 52 patients, diabetic nephropathy in 14, hypertensive nephrosclerosis in 22, polycystic disease in 19, obstructive nephropathy in four, vasculitis in three, chronic interstitial nephritis in three and undetermined causes in 13 patients. The average time on HD was 72.77 ± 65.07 months (range: 1–280 months). Four patients were on haemodiafiltration and the remaining 126 were on conventional HD with bicarbonate. Eighty-three patients (63.8%) were dialysed with a modified cellulose membrane (Althane; Baxter). Dialysis filters had an ultrafiltration coefficient of 10 ml/h mmHg in 66.2% of patients, with a surface area of 1.65 ± 0.30 m² (range: 1.2–2.2 m²). The water used in the dialysate was processed from the same system for the entire group of patients.

Atherosclerotic cardiovascular disease (CVD) profiles in each patient were evaluated by a single investigator using the CVD portion of the Index of Co-Existing Diseases as applied by Cheung et al. [6] to patients in the HEMO study.

**Blood sample collection**

Using vascular accesses, we collected 15 ml blood samples before dialysis but immediately following venipuncture. These samples were taken within two consecutive days in all patients.

**CRP and SAA assays**

Quantitative determinations of CRP and SAA in serum samples were made by particle-enhanced immunonephometry on Behring Nephelometer 2. The high sensitivity CRP assay was designed to measure CRP concentrations within an overall range of 0.175–1100 mg/l and the SAA within a range of 3–200 mg/l.

**Anti-*C. pneumoniae* IgG assay**

IgGCp were determined by indirect microimmunofluorescent assay techniques (MRL Diagnostics). This assay is a two-stage sandwich procedure and uses purified elementary bodies diluted in yolk sac, which permit the qualitative detection, semi-quantitation and speciation of human serum IgG antibodies against *C. pneumoniae*. The serum screening dilution was 1/16 in phosphate-buffered saline. Endpoint titres of ≥ 1/16 were considered positive, showing evidence of infection at an undetermined time.

**Cell cultures and subsequent PCR for detection of *C. pneumoniae* in PBMCs**

*Chlamydia pneumoniae* was isolated from PBMCs after inoculation of Hep-2 cell cultures with buffy coats and subsequent detection by PCR.

**Preparation of buffy coats.** Five millilitres of ethylenediaminetetraacetic acid-treated whole blood from each patient was centrifuged at 3000 g for 15 min. The buffy coat was carefully aspirated with a sterile Pasteur pipette, transferred into 2.5 ml cryovials and stored at −160 °C (liquid nitrogen) until the day of determination.

**Cell cultures.** Cell cultures were performed using the shell vial technique with a commercially available kit (Vircell, S. L., Granada, Spain). Cells were detached after shaking with glass beads and the resulting homogenates were used for the PCR detection of *C. pneumoniae*. Controls consisted of *C. pneumoniae* ATCC VR-1355 TWAR strain 2043 suspensions which were run as the buffy coat samples.

**PCR for *C. pneumoniae*.** For the detection of *C. pneumoniae* in cell culture homogenates, a nested PCR was performed using a commercially available kit (Clonit S.r.l., Milan, Italy). The amplification product was a 193 bp fragment of the gene encoding the RNA polymerase beta of *C. pneumoniae*. Amplified products were detected by conventional agarose-gel electrophoresis. The extraction procedure of DNA from cell culture homogenates was included in the kit.

The above PCR protocol was successful in detecting 20–30 *C. pneumoniae* elementary bodies in 300 ml of cell culture medium, which was confirmed after staining air-dried suspensions with anti-*C. pneumoniae* fluorescent monoclonal antibody. This represented a very good sensitivity when compared with previous reports [7]. In the specificity control tests, PCR failed to detect DNA from a mixed suspension of ATCC reference strains of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Enterococcus faecalis*, *Candida albicans* and *Campylobacter jejuni*.

**Statistics**

Results are expressed as means ± SD. Mann–Whitney U-tests were applied for the differences in CRP and SAA between the patient groups. A stepwise linear multiple regression analysis (entry at *P*<0.05, removal at *P*>0.10) was performed on log CRP and log SAA and all patients characteristic variables, including age (patients were divided into ≥ 65 or < 65 years because median patient age was 65 years), dialysis time (patients having ≥ 5 or < 5 years on dialysis because median HD duration was 61 months), atherosclerosis, coronary disease, cerebrovascular disease, peripheral vascular disease (present or not in any severity), positivity in cell-culture–PCR and serum positivity for
IgGCp. Multiple logistic regression analysis was performed using atherosclerosis, coronary disease, cerebrovascular disease or peripheral vascular disease as the dependent variables and all variables described in ‘Patients’ characteristics’, including log CRP and log SAA, cell-culture–PCR results, serum IgGCp (patients with titres <1/16 or ≥1/16 and patients with titres <1/64 or ≥1/64) as covariates with entry factors at $P<0.05$ and with removal of factors that no longer contribute at $P>0.10$ in a forward stepwise (likelihood ratio) fashion. A significance level of 0.05 was used for all statistical tests. Analyses were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Results from cell cultures and subsequent PCR for detection of viable \textit{C. pneumoniae} in PBMCs and from determination of serum IgGCp are shown in Table 1 and Figure 1. Among patients having detectable serum IgGCp, 27 (20.8%) had an IgGCp titre of 1/16, 21 patients (16.2%) had 1/32, 12 (9.2%) had 1/64 and 4 (3.1%) had 1/128. Patients positive and negative for viable \textit{C.pneumoniae} in PBMCs were not different in sex, age, hypertension, diabetes, cause of renal failure or any of the described characteristics. They were not different for dialysis condition, except for time on HD (97.44 ± 47.02 months for positive vs 70.94 ± 66.00 for negative patients; $P = 0.052$, Mann–Whitney U-test).

The mean CRP value in the 130 patients was 8.71 mg/l (range: 0.30–56.90 mg/l) and the SAA value was 16.04 mg/l (range: 1.10–93.00 mg/l). There was a strong correlation between these two variables (Spearman coefficient of correlation, 0.6395; $P < 10^{-5}$).

The prevalence of atherosclerotic CVD is shown in Table 2.

Table 2 shows non-significant differences for CRP and SAA between those patients with positive and negative cell-culture–PCR for \textit{C. pneumoniae} in PBMCs, as well as between patients positive and negative for IgGCp. In contrast, inflammatory indexes were significantly higher in patients with atherosclerosis (Table 4).

Atherosclerosis was positively correlated with the presence of \textit{C. pneumoniae} in PBMCs, but not with the presence of serum IgGCp (Table 5). In this table, patients were also divided into groups having serum IgGCp titre <1/64 and ≥1/64, a limit used in many previous studies correlating \textit{C.pneumoniae} seropositivity with atherosclerotic CVD.

In the stepwise multiple linear regression analysis including log CRP or log SAA as dependent variables, the factors that predicted an increase in log CRP were patient age > 65 years ($\beta = 0.201$, $P = 0.025$), time on HD > 5 years ($\beta = 0.196$, $P = 0.022$) and atherosclerosis ($\beta = 0.196$, $P = 0.029$), whereas only patient age > 65 years ($\beta = 0.210$, $P = 0.017$) predicted increases in log SAA.

In multiple logistic regression analysis with atherosclerosis as the dependent factor, only male gender (odds ratio, 5.104; $P = 0.001$), increasing age (odds ratio, 1.094; $P = 0.000$) and increasing log CRP (odds ratio, 3.842; $P = 0.035$) were potential risk factors for atherosclerosis. Separate multiple logistic regression analyses for coronary disease, cerebrovascular and peripheral vascular disease revealed that the same factors remained as predictors, except for log CRP, which was significant for atherosclerosis as a whole but not for specific diseases (coronary, peripheral or cerebrovascular).

Table 1. Detection of viable \textit{C. pneumoniae} in PBMCs and serum IgG antibodies against \textit{C. pneumoniae} in 130 HD patients

<table>
<thead>
<tr>
<th>Viable \textit{C.pneumoniae} in PBMCs (titre &lt;1/16)</th>
<th>IgGCp (titre ≥1/16)</th>
<th>Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable \textit{C.pneumoniae} in PBMCs (+)</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Viable \textit{C.pneumoniae} in PBMCs (−)</td>
<td>58</td>
<td>63</td>
</tr>
<tr>
<td>Patients (%)</td>
<td>66 (50.8)</td>
<td>64 (49.2)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of patients.

<sup>b</sup>Fisher’s exact test.
Table 3. Inflammatory indexes in patients positive and negative for *C. pneumoniae* in PBMCs or serum IgG antibodies against *C. pneumoniae* (titre ≥1:16 and <1:16)

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. pneumoniae</em> in PBMCs (9 patients)</td>
<td>(121 patients)</td>
<td>8.14 ± 8.89</td>
<td>8.75 ± 9.69</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>19.60 ± 26.32</td>
<td>15.78 ± 16.11</td>
<td>0.530</td>
</tr>
<tr>
<td>SAA (mg/l)</td>
<td>9.31 ± 11.14</td>
<td>8.10 ± 7.82</td>
<td>0.821</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>15.14 ± 14.84</td>
<td>16.95 ± 18.79</td>
<td>0.917</td>
</tr>
</tbody>
</table>

Values are means ± SD.

Discussion

In the present study, a minority of HD patients showed viable *C. pneumoniae* in their PBMCs, whereas a higher percentage had serum antibodies against *C. pneumoniae*. Although inflammation indexes were higher in atherosclerotic patients, there was no correlation between the presence of *C. pneumoniae* and inflammation. Atherosclerosis was associated with patients having detectable viable *C. pneumoniae*, although this association was absent in patients positive for IgGCP.

The percentage of patients with serum IgGCCP titre ≥1/16 (49.2%) was similar but slightly lower than percentages in previous studies [3]. Only 6.9% of patients had detectable *C. pneumoniae* in PBMCs. Previous studies examining *C. pneumoniae* DNA in PBMCs revealed varying results [8,9]. In a study having the largest patient population (1205 subjects with coronary angiography), Wong et al. [9] found 8.8% positivity for *C. pneumoniae* DNA in PBMCs in 669 men with coronary artery disease. Although our findings were similar to those of Wong et al. [9], comparisons are difficult because they used PCR alone to detect *C. pneumoniae* DNA from PBMCs.

In an attempt to develop a highly reliable detection method, we performed for the first time a combination of cell culture and subsequent PCR for *C. pneumoniae* detection in PBMCs. This combination was used because (A) the cell culture remains essential to document the viability of this obligate intracellular microorganism in eukaryotic host cells [10] and (B) PCR in DNA extracted from cell cultures for the detection of a specific *C. pneumoniae* gene significantly increases the sensitivity (as a molecular amplification method) and the specificity (compared with the usually applied fluorescent antibody staining technique, which largely depends on subjective distinctions between *C. pneumoniae* inclusions and artefacts [10]). In addition, detection of *C. pneumoniae* DNA in PBMCs using PCR by itself provides a good indication of *C. pneumoniae* viability [11], mainly because DNA of dead bacteria is rapidly degraded [12]. However, this method does not differentiate between replicating and non-replicating organisms, making it difficult to arrive at conclusions about *C. pneumoniae* viability in PBMCs [13].

Table 4. Inflammatory indexes in patients with or without atherosclerosis

<table>
<thead>
<tr>
<th>Atherosclerosis</th>
<th>Positive (57 patients)</th>
<th>Negative (73 patients)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/l)</td>
<td>11.26 ± 10.45</td>
<td>6.95 ± 8.62</td>
<td>0.005</td>
</tr>
<tr>
<td>SAA (mg/l)</td>
<td>20.86 ± 21.57</td>
<td>12.73 ± 11.78</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Table 5. Prevalence of atherosclerotic CVD in patients with or without viable *C. pneumoniae* in PBMCs and in patients with different serum titres of IgG antibodies against *C. pneumoniae*

<table>
<thead>
<tr>
<th></th>
<th>Positive (57 patients)</th>
<th>Negative (73 patients)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. pneumoniae</em> in PBMCs (+)</td>
<td>7</td>
<td>2</td>
<td>0.042</td>
</tr>
<tr>
<td><em>C. pneumoniae</em> in PBMCs (−)</td>
<td>50</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>IgGCCP ≥1/16</td>
<td>31</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>IgGCCP &lt; 1/16</td>
<td>26</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>IgGCCP ≥1/64</td>
<td>7</td>
<td>9</td>
<td>0.194</td>
</tr>
<tr>
<td>IgGCCP &lt; 1/64</td>
<td>50</td>
<td>64</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Coronary or cerebrovascular or peripheral vascular disease.
Single determinations of CRP, at a given time point, may represent a weakness because acute-phase responses can vary with time in this category of patients [17]. We attempted to overcome this limitation by measuring another reliable index of inflammation (SAA) and by using strict criteria to exclude patients with recent inflammation stimulatory clinical events that might cause fluctuations in these indexes (mean CRP in the 12 excluded patients was 27.35; range: 9.90–58.5 mg/l).

In the present study, inflammation was more prominent in atherosclerotic patients than in non-atherosclerotic subjects, a finding that confirmed the inflammatory nature of this disease. Inflammatory indexes did not significantly differ between patients positive and negative for C. pneumoniae in PBMCs or in patients showing seropositivity or seronegativity.

Studies that used serology or PCR for detecting C. pneumoniae in atherosclerotic plaques to examine inflammatory systemic responses have yielded conflicting results [1,18]. Moreover, inflammation has multifactorial causes in HD patients [4]. Thus, the negative finding in our study may be due to the small number of patients positive for C. pneumoniae in PBMCs or because systemic inflammatory responses induced by viable C. pneumoniae were obscured by other sources of inflammation related to renal failure, such as consequences of oxidative stress [4] or advanced glycation end-products accumulation [19] that were not examined in this study.

Our study was limited by the semi-quantitative scoring system of atherosclerosis and because we did not directly examine the arterial walls. By using this tool, only late atherosclerotic injuries that become apparent after destabilization and rupture of an atherosclerotic plaque can be identified. Considering this limitation, the lack of correlation between seropositivity and atherosclerosis in the current study is in accordance with recent reports [2], even though a few patients with C. pneumoniae in PBMCs had higher scores for atherosclerosis. However, the multiple logistic regression analysis revealed that only male gender, increasing age and higher CRP were potential risk factors for atherosclerosis. It is possible that demographic characteristics, such as age, and the small number of patients with viable C. pneumoniae in PBMCs influenced these results.

If confirmed in larger studies, the finding that patients positive for C. pneumoniae in PBMCs had a longer HD duration may be of importance and points to the capability of this pathogen to establish a persistent infection in immunocompromised patients, such as the HD subjects in our study [5].

In conclusion, detection of viable C. pneumoniae in PBMCs using the combination of cell culture and subsequent PCR revealed that only a small number of patients were positive for this pathogen. The serum antibodies, specifically IgG, did not reflect the presence of C. pneumoniae in peripheral blood cells. Thus, we believe that findings from this study in this highly debated field may cause re-consideration of methodology used in clinical studies. Standardization of C. pneumoniae assays are urgently needed to provide answers to this interesting hypothesis linking this pathogen to atherosclerosis. Studies based in serology have lead to a ‘no way-out’ situation that has become obvious from recent serology-based antibiotic trials, wherein great expectations had been invested [20]. Our results, based on both serology and PCR, give a more accurate picture about C. pneumoniae presence in circulating monocytes, which is a crucial cell type, both for atherosclerosis and for C. pneumoniae infections. Although this methodology may provide a first step, definite answers about C. pneumoniae and atherosclerosis will require additional studies in larger populations, probably with a different design. We believe that future clinical studies should try to investigate whether there is a causal relationship between C. pneumoniae and atherosclerosis, to thereby fulfill Koch’s postulates, especially the postulate asserting that the infectious agent must be found in most, if not all, of the subjects in whom the disease process is manifested [21].

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References


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