

Detection of *Chlamydia pneumoniae* DNA and Antigen in the Circulating Mononuclear Cell Fractions of Humans and Koalas

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Received 20 December 1999/Returned for modification 21 January 2000/Accepted 16 February 2000

***Chlamydia pneumoniae* is a common respiratory pathogen of humans which, in addition to causing disease at the respiratory site, has recently been linked to disease at other body sites. If *C. pneumoniae* does contribute to disease at nonrespiratory sites, then it must have a mechanism by which it reaches these sites. We analyzed the peripheral blood mononuclear cell (PBMC) fractions from 60 healthy human blood donors for the presence of *C. pneumoniae* DNA (by *ompA* PCR) and chlamydial antigens (by genus- and species-specific monoclonal antibody staining). Ten of the sixty (16.7%) blood donors were *C. pneumoniae* positive by PCR, and all 10 of these PCR-positive individuals' samples demonstrated specific staining with anti-*C. pneumoniae* monoclonal antibodies. The only other host naturally infected with *C. pneumoniae* is the koala, in which the bacterium also causes respiratory infections. We demonstrated the presence of *C. pneumoniae* DNA and antigens in the PBMC fractions of 30% of 20 koalas tested. Our finding of *C. pneumoniae*-infected PBMCs in koalas as well as humans suggests that the ability to infect PBMCs and to disseminate from the respiratory site is not restricted to the human biovar of *C. pneumoniae* but is a general characteristic of this chlamydial species.**

Chlamydia (Chlamydophila) pneumoniae is a common respiratory tract pathogen in humans (6), causing disease such as mild respiratory discomfort, sinusitis, pharyngitis, bronchitis, and pneumonia. It is geographically widespread, with 50 to 70% of the adult population worldwide having serological evidence of prior exposure (1). While its pathogenic potential at the respiratory site is well established, recent studies suggest that it also disseminates from this site, probably via circulating monocytes. In vitro studies have shown that *C. pneumoniae* is able to readily infect a variety of cell types, most notably macrophages (5). Mouse studies have also shown that *C. pneumoniae* is able to disseminate from the lungs, via macrophages, to other body sites (10). Recently, Boman et al. (3) showed that in humans, *C. pneumoniae* could be detected by PCR in the peripheral blood mononuclear cell (PBMC) fractions not only of patients with cardiovascular disease but also of normal blood donors. This ability to disseminate systemically would be one of the characteristics required for *C. pneumoniae* to be a contributing factor in atherosclerosis.

While most research has focused on the human biovar of *C. pneumoniae*, recent work from Australia has shown that a second biovar of *C. pneumoniae* exists in koalas (7). The koala biovar of *C. pneumoniae*, which is genetically distinct from the human biovar, has been found in virtually every wild and captive population of koalas examined (7, 14). It can be isolated from ocular, urogenital, and respiratory sites of clinically healthy and diseased koalas.

Even though evidence linking *C. pneumoniae* to heart disease in humans is quite substantial (9, 12, 13), it has not been possible as yet to prove causation. If *C. pneumoniae*, as a species, is sufficiently different from the other chlamydiae (an additional 200 genes have been identified in comparison to

Chlamydia trachomatis) (8), then it might be unique in efficiently infecting monocytes, making them sufficiently sticky to adhere to damaged vascular endothelium, accumulate cholesterol, and contribute to the development of atherosclerosis. In this context, comparative studies on the koala biovar of *C. pneumoniae* should be informative. We therefore decided to address three key points: (i) to confirm the report of Boman et al. (3) that *C. pneumoniae* DNA can be readily found in the PBMC fractions of otherwise healthy humans, (ii) to show that whole *C. pneumoniae* organisms are present in these PBMCs by staining with specific antibodies, and (iii) to determine if the koala biovar of *C. pneumoniae* has properties similar to those of the human biovar in enabling the organism to be commonly found in the PBMC fraction of its host.

MATERIALS AND METHODS

Human and koala blood samples. Informed consent was obtained from all participants, and the Queensland University of Technology Guidelines for Human (QUT 1566H) and Animal (QUT 1413/1A) Experimentation were followed throughout. Venous blood (9-ml) samples were collected into EDTA from 60 consenting human blood donors during routine donation at the Australian Red Cross Blood Service—Queensland, Brisbane, Australia (age, 18 to 59 years; average, 39 years; male/female ratio, 30/30). The PBMC fraction was isolated by Ficoll-Paque density gradient centrifugation (3) and washed twice with phosphate-buffered saline (PBS), and the pellet was resuspended in 1 ml of PBS prior to storage at -80°C pending PCR analysis. Two to five milliliters of venous blood was collected into EDTA from each of 20 captive koalas at the Lone Pine Koala Sanctuary, Brisbane, Australia. This population of 140 koalas had experienced an outbreak of respiratory illness, presumed to be due to *C. pneumoniae* (14), approximately 12 months previously. The blood was processed to isolate PBMCs in the same manner as for the human blood samples.

Detection of *C. pneumoniae* DNA by nested PCR. A nested PCR was used which targeted the variable domain IV (VDIV) region of the *ompA* gene (outer primers Cpn5P [5' CCA ATA TGC ACA GTC CAA ACC TAA AA 3'] and Cpn3P [5' CTA GAT TTA AAC TTG TTG ATC TGA CAG 3']; nested primers Cpn5N [5' CTC TGT AAA CAA ACC GGG C 3'] and Cpn3N [5' GAT CTG ACA GGA AAC AAT TTG CAT 3']). Fifty microliters of resuspended PBMC fraction was prepared for PCR by heating to 95°C for 5 min. Two microliters of this heat-treated PBMC fraction was added to 50 μl of reaction mixture containing the following: a 1 μM concentration of each primer (Cpn5P and Cpn3P); $1\times$ Roche PCR buffer; 200 μM concentrations each of dATP, dTTP, dCTP, and dGTP (Roche); and 1.2 U of *Taq* polymerase (Roche). Cycling conditions con-

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sisted of an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension for 1 min at 72°C. For the second round of PCR, 1 µl of the first-round product was mixed with 50 µl of the above amplification mixture, using primers Cpn5N and Cpn3N, and amplified under the same cycling conditions. PCR products were visualized by ethidium bromide staining following electrophoretic separation. The presence of PCR inhibitors was determined by spiking negative PCR mixtures with 10-fold dilutions of cell culture-grown *C. pneumoniae* elementary bodies and repeating the PCR. PCR bands on gels were confirmed by high-stringency Southern hybridization (60°C in 0.1× SSC [150 mM NaCl, 15 mM sodium citrate]) with digoxigenin-labeled PCR product.

Detection of *C. pneumoniae* antigens by genus- and species-specific monoclonal antibody staining. PBMC fractions prepared by Ficoll-Paque centrifugation were analyzed by smearing 10 µl of fresh preparation onto a glass microscope slide, fixing with methanol, and staining with either a genus-specific lipopolysaccharide monoclonal antibody (ChlamydiaCel; CelLabs, Sydney, Australia) or a *C. pneumoniae*-specific monoclonal antibody (Chlamydia CelPn; CelLabs) according to the manufacturer's instructions. The presence or absence of characteristic apple-green-fluorescing chlamydial particles was determined by examining 200 high-power (×1,000) microscope fields by confocal microscopy (TCS 4D; Leica, Leitz, Germany).

Determination of antichlamydial antibody levels in plasma. (i) **Human samples.** The presence of immunoglobulin G (IgG) antibodies against *C. pneumoniae*, *C. trachomatis*, and *Chlamydia psittaci* was determined by a microimmunofluorescence (MIF) assay using the MRL Diagnostics (Cypress, Calif.) kit as per the manufacturer's instructions. Briefly, sera were diluted (1:32, 1:64, 1:128, 1:256, and 1:512) in PBS, incubated for 30 min at 37°C with the antigen slide, washed twice with PBS, and then reacted with IgG conjugate at 37°C for 30 min. After washing with PBS was done, the slides were examined by an experienced operator (who was blinded to the PCR results) at ×400 magnification using a Zeiss AXIOLAB microscope and scored for the presence of characteristic fluorescence.

(ii) **Koala samples.** The genus-specific enzyme-linked immunosorbent assay (ELISA) developed by Emmins (4) was used. This assay uses genus-specific chlamydial antigen and hence measures levels of IgG antibody to both *C. pneumoniae* and *Chlamydia pecorum* infections in koalas. The result is reported as a score between 1 and 10 ELISA units, with scores above 3 being considered positive.

Determination of plasma cholesterol levels. Total plasma cholesterol levels were determined using the Vitros 950 (Queensland Medical Laboratories, Brisbane) (human samples) and the Olympus AU600 (Veterinary Pathology Services, Brisbane) (koala samples) instrumentation.

Statistical analysis. Comparisons between groups were made using the chi-square or Fisher's exact test (if cell numbers were small) and run using SPSS 6.0 for Windows. Confidence intervals based on the normal approximation to binomial data were given where possible.

RESULTS

Human blood donors. (i) **Presence of *C. pneumoniae* DNA and antigen in the PBMC fractions of healthy human blood donors.** (a) **Detection of *C. pneumoniae* DNA by PCR.** Ten of the 60 human samples tested (16.7%) were positive by PCR for the *ompA* gene (authenticity confirmed by high-stringency Southern hybridization with a digoxigenin-labeled probe). These 10 samples were repeatedly positive, and we are confident that none of the reactions are false-positive reactions due to PCR amplicon contamination. The *ompA* VDIV genotypes for three of these *C. pneumoniae* infections were determined by sequencing of the PCR products, and all three were shown to be identical to that of the prototype strain, AR-39.

When the relationship between PBMC PCR positivity status and the ages of the blood donors was analyzed, there appeared to be a bimodal distribution, with a higher prevalence of positives in the 18-to-34-year (5 of 22; 22.7%) and over-50-year (3 of 13; 23.1%) age groups compared with the 35-to-50-year (2 of 25; 8.0%) age group. Because of the small sample size, however, these differences did not reach statistical significance ($\chi^2 = 2.318$; $P = 0.314$). There was no obvious sex bias observed, with 6 male and 4 female donors being PCR positive.

(b) **Detection of *C. pneumoniae* antigens by genus- and species-specific monoclonal antibody staining.** We randomly selected 12 of the 50 PCR-negative samples as well as all 10 PCR-positive samples for staining with genus- and *C. pneumoniae*-specific monoclonal antibodies. All 12 PCR-negative

TABLE 1. Summary of *C. pneumoniae* PBMC PCR status and its relation to characteristics of human blood donors and captive koalas

Group and characteristic [n]	% of PCR-positive subjects
Human blood donors [60]	16.7
Age (yr)	
18–34 [22]	22.7
35–50 [25]	8.0
>50 [13]	23.1
Chlamydia serology status (IgG titer)	
Positive ($\geq 1:64$) [21]	4.8
Negative ($\leq 1:32$) [39]	23.1
Plasma cholesterol level (mmol/liter)	
Elevated (>5.5) [11]	0
Average (≤ 5.5) [49]	20.4
Koalas [20]	30.0
Age (yr)	
≤ 5 [12]	41.7
5 [8]	12.5
Chlamydia serology status	
Positive [12]	25.0
Negative [8]	37.5
Plasma cholesterol level (mmol/liter)	
High (>1.92) [8]	12.5
Low (≤ 1.92) [12]	41.7

samples failed to show staining with either antibody, while all 10 PCR-positive samples contained specific, apple-green-fluorescing chlamydial elementary bodies. While no intact chlamydial inclusions were seen, approximately one PBMC per 200 cells examined contained a correctly sized and shaped, characteristic fluorescent-staining chlamydial elementary body. Similar staining patterns were observed with both the genus-specific and *C. pneumoniae*-specific antibodies.

(ii) **Relationship of PBMC PCR status to levels of antichlamydial antibody and total cholesterol in plasma of humans.** *C. pneumoniae* antibodies were detected by MIF (IgG titer of 1:64 or greater) in 40% of the human plasma samples, with a higher positivity rate in older (44.7% in the >35-year age group) than in younger (22.2% in the 18- to 34-year age group) individuals (chi-squared statistic = 2.642; $P = 0.104$). *C. trachomatis* antibodies were detected in only 1 sample, and no samples were positive for *C. psittaci* antibodies. Gender was significantly linked to positive *C. pneumoniae* serology, with men 3.06 times more likely to have circulating *C. pneumoniae* antibodies ($P = 0.057$). When the relationship between PBMC PCR status and *C. pneumoniae* serology status was analyzed, there was a strong correlation between PBMC PCR positivity and MIF-negative status, with 23.1% of MIF-negative individuals being PCR positive, compared to only 4.8% of MIF-positive individuals (Fisher's exact test; $P = 0.084$) (Table 1).

Total cholesterol levels in these 60 blood donors ranged from 3.1 to 6.9 mmol/liter (mean, 4.6 mmol/liter), with more males than females having elevated cholesterol levels (>5.5 mmol/liter) (23% versus 13%). This difference of 10% between groups had a 95% confidence interval of -9.4 to 29.4%; $\chi^2 = 1.002$; $P = 0.317$). When the relationship between PBMC PCR status and the total plasma cholesterol level was analyzed, there was a borderline significant correlation ($P = 0.10$) be-

tween elevated cholesterol levels (>5.5 mmol/liter) and PCR-negative status (all 11 individuals with elevated cholesterol levels were PCR negative). This compared with 10 of 49 (20.4%) of individuals in the low- or average-cholesterol groups being PBMC PCR positive.

Koalas. (i) Presence of *C. pneumoniae* DNA and antigens in circulating PBMC fractions from koalas. (a) Detection of *C. pneumoniae* DNA by PCR. When the PBMC fractions from 20 captive koalas were analyzed, 6 (30%) were repeatedly PCR positive. PCR positivity was markedly skewed towards the younger animals, with 41.7% (5 of 12) of animals ≤ 5 years old being PCR positive, compared to only 12.5% (1 of 8) animals >5 years old being PCR positive ($P = 0.161$). This difference between groups of 29% has a 95% confidence interval of -6.9 to 65.2%; Fisher's exact test = 1.941. More male koalas (4 of 6) were PCR positive than female koalas (2 of 6), although the very low numbers made formal statistical inference difficult. Previous testing, 12 to 18 months earlier, of these same koalas for *C. pneumoniae* showed that 74% were PCR positive at the ocular (3 of 19; 16%), urogenital (1 of 19; 5%) and/or nasal (14 of 19; 74%) sites (M. Stark, unpublished data).

(b) Detection of *C. pneumoniae* antigens by genus- and species-specific monoclonal antibody staining. Five PBMC PCR negative and five PBMC PCR positive koala samples were randomly selected for staining with genus- and *C. pneumoniae*-specific monoclonal antibodies. All five PCR positive samples contained specific, apple-green-staining elementary bodies, whereas all five PCR negative samples failed to show any staining. As with the human samples, positives were evident as individual staining elementary bodies rather than whole chlamydial inclusions and were detected at a rate of approximately one positive PBMC per 100 cells examined.

(ii) Relationship of PBMC PCR status to anti-chlamydial antibody level and total cholesterol level in plasma of koalas. The chlamydia antibody ELISA used in this work is genus specific and hence measures antibodies against both *C. pneumoniae* and *C. pecorum* infections in koalas. Sixty percent of the 20 koalas tested had antichlamydial antibodies in the ELISA, with PBMC *C. pneumoniae* PCR positives being slightly biased towards the animals with negative (3 of 8; 37.5%) rather than positive (3 of 12; 25.0%) *Chlamydia* serology (Table 1). A notable feature of the cholesterol determinations was the overall low level observed in koalas (range, 1.08 to 2.88 mmol/liter; mean, 1.92 mmol/liter). When we used the mean plasma cholesterol level of 1.92 mmol/liter to differentiate between animals with high and low cholesterol levels, there were more PBMC PCR-positive animals in the low-cholesterol group (41.7% versus 12.5%; Fisher's exact test = 1.36; $P = 0.24$) than in the high-cholesterol group. This difference between groups of 29% had a 95% confidence interval of -13.4 to 61.1%.

DISCUSSION

C. pneumoniae is now widely accepted as being a common respiratory pathogen of humans, causing symptoms ranging from very mild respiratory disease to severe pneumonia (6). It has also been strongly linked to atherosclerotic heart disease by a wide range of detection procedures, including serology, immunohistochemistry, PCR, electron microscopy, and culture, as well as studies with animal models. If *C. pneumoniae* does cause or at least contribute to disease at nonrespiratory sites, then it must have some mechanism by which to disseminate to these sites. The monocyte is a likely cell for this purpose and indeed, in vitro studies have shown that *C. pneumoniae* is able to replicate inside a variety of cell types, including macrophages (5). However, the confirmation that *C. pneu-*

moniae-infected PBMCs can be found in vivo was only recently reported (2, 3, 15). All three studies analyzed samples from patients with some form of cardiovascular disease and reported *C. pneumoniae* DNA PCR positivity levels of between 8.3 and 59%. In the only study with healthy individuals, Boman et al. (3) found a *C. pneumoniae* DNA PCR positivity level of 46% in blood donors.

Even though our sample sizes were modest, our results confirm and extend these previous observations related to the ability of *C. pneumoniae*, both the human and the koala biovars, to disseminate in the peripheral blood system via PBMCs. Our finding that samples from 16.7% of healthy Australian blood donors were positive for the presence of *C. pneumoniae* DNA by PCR confirms the earlier reports of Boman et al. (3) that this organism is commonly found in circulating PBMCs of otherwise healthy individuals. Interestingly, we found a trend opposite to that reported by others (2, 3, 15) in relation to MIF serology status. Boman et al. (3) and Blasi et al. (2) both reported a strong correlation between PBMC PCR-positive and MIF-positive cardiovascular disease patients, while Wong et al. (15) found a borderline association following the same trend in their patients receiving interventional coronary arteriography. By comparison, we found that only 10% of our PBMC PCR-positive healthy blood donors were MIF positive, compared to 40% MIF positivity in the PCR-negative group. This difference may be related to the disease state of the patient group, with healthy individuals (at least individuals with early-stage disease) having an MIF-negative and PBMC PCR-positive phenotype and diseased individuals (at least individuals with later-stage, clinically apparent disease) having progressed to an MIF-positive and PBMC PCR-positive phenotype. Relatively little is known of the possible relevance of circulating *C. pneumoniae* antibodies in individuals with systemic infection. However, if these circulating antibodies are at all protective, then an MIF-positive and PBMC PCR-negative phenotype in healthy individuals might not be unexpected. It is possible that the MIF-positive and PBMC PCR-positive subgroup consists of those that have developed a chronic *C. pneumoniae* systemic infection and are more likely to develop vascular disease. Whatever the explanation, this report further confirms the value of measuring PBMC PCR status in individuals, particularly in any attempts to use intervention therapy.

An important finding in our present study was that we were also able to confirm the presence of intact *C. pneumoniae* organisms in the PBMC fractions by antibody staining. This is the first report of the presence of *C. pneumoniae* antigens in circulating monocytes in vivo, and we found that all PCR-positive individuals examined were also antigen positive, and all PCR-negative individuals were antigen negative. This correlation between PCR and antigen detection methods supports the proposal that these PBMCs are truly infected and the PCR-positive status does not simply represent remnant pieces of nucleic acid resulting from the degradative activity of the monocyte. It will be important, however, to confirm the viability of these infections by cell culture or reverse transcriptase PCR approaches. The number of PBMCs which contained fluorescing chlamydial particles was extremely low (approximately 1 per 200 PBMC). However, this low level of antigen positivity is consistent with the level of PCR positivity, which usually was evident only with the second round of PCR testing. Preliminary studies in which we separated plastic adherent from nonadherent PBMCs suggested that PCR positivity was present in both fractions. It will be important to confirm the currently held belief, based primarily on in vitro studies, that it is the monocytes and not other cell types that are infected by *C. pneumoniae* in vivo.

Murray et al. (11) recently reported an association between *C. pneumoniae*-specific antibodies and an atherogenic lipid profile. We did not observe the same trend in our study, with 32.7% of individuals with average cholesterol levels being serology positive, versus 45.5% of individuals with elevated cholesterol levels being serology positive. In fact, our PBMC PCR data strongly suggest a relationship between *C. pneumoniae* PBMC infectivity and low-to-average total cholesterol level (all 11 individuals with elevated cholesterol levels were PBMC PCR negative).

C. pneumoniae commonly infects only two hosts, humans and koalas. The koala biovar of *C. pneumoniae* is genetically distinct from the human biovar and has sequence differences at all four gene loci studied to date (*ompA*, *ompB*, 16S rRNA, and *groESL*) (14). In koalas, *C. pneumoniae* can be readily isolated from ocular, urogenital, and respiratory sites in both wild and captive koalas (infection levels of 20 to 80% reported), although its role in causing clinical disease at these sites remains uncertain (7). Koala *C. pneumoniae* was, however, directly linked to an outbreak of respiratory disease in a captive koala colony, suggesting that it is a cause of respiratory disease in this host (14). Given the prevalence of *C. pneumoniae*-positive PBMCs in humans, we also investigated the presence of *C. pneumoniae* in the PBMC fraction of 20 captive koalas and found a similarly high percentage (30%) of animals to be PCR and antigen positive for *C. pneumoniae* in their PBMC fractions. It therefore appears that both biovars of *C. pneumoniae*, human and koala, are common causes of respiratory disease in their respective hosts and can also infect PBMCs in vivo and disseminate via the circulation to nonrespiratory sites. It is unclear at this stage whether this ability to infect PBMCs is unique to this chlamydial species; however, it does appear to be a highly efficient process in these hosts, given the relatively high proportion of individuals (both humans and koalas) that can be identified as being *C. pneumoniae* positive in their PBMC fractions. Similar trends were also observed between the human and koala hosts in relation to PBMC PCR positivity and age (with a bias towards younger individuals), *C. pneumoniae* serology status (with a bias towards negative serology status), and plasma cholesterol level (with a bias towards low rather than high levels).

Heart disease is apparently not a common occurrence in koalas and has not previously been reported. There may be several reasons for this observation. Koalas rarely live beyond 15 years, perhaps limiting the time for progression of atherosclerosis, whatever the causes. Because of their diet, koalas have low cholesterol levels (range, 1.1 to 2.9 mmol/liter), much lower than the risk level for humans of 5.5 mmol/liter. Nevertheless, the finding that *C. pneumoniae* can be commonly found infecting PBMCs in koalas, as it does in humans, suggests that this is a consistent feature of this chlamydial species, rather than a unique feature of the human biovar. The factors that might accelerate progression of PBMC infection along the pathway of atherosclerosis, however, may be unique to humans.

ACKNOWLEDGMENTS

This work was supported by grants from the Lone Pine Koala Sanctuary, The Queensland University of Technology Grants Scheme, and the CRC for Diagnostic Technologies. T.B. is supported by the Ruth Oulton Whyte koala Ph.D. scholarship.

We are grateful to the collection staff (Australian Red Cross Blood Service—Queensland) and blood donors for the human blood samples, to Galit Tzipori and Paul O'Callaghan (Lone Pine Koala Sanctuary) for the collection of koala blood samples, Charles Wan (CRC for Diagnostic Technologies) for *ompA* VDIV sequencing, Caroline Fox (Queensland Medical Laboratories) for MIF assays, and to Adrian Barnett (QUT) for statistical analyses.

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