ORIGINAL ARTICLE 10.1111/j.1469-0691.2004.00952.x

# *Chlamydia pneumoniae* in HIV-infected patients and controls assessed by a novel whole blood interferon-γ assay, serology and PCR

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## ABSTRACT

*Chlamydia pneumoniae* seropositivity is associated with cardiovascular disease and HIV infection. Cell-mediated immune responses are important for control of *C. pneumoniae*, and such responses may be impaired in HIV-infected patients. An assay for detection of interferon (IFN)-γ in whole blood stimulated with *C. pneumoniae* antigen was developed and studied in HIV-infected patients and uninfected controls. Among 34 HIV-infected patients, none had an IFN-γ response to *C. pneumoniae* antigen, compared with five of 32 healthy controls ($p < 0.001$). Fewer HIV-infected individuals elicited a serum IgG response when tested with a commercial enzyme immunoassay (p 0.009), but this was not so for serum IgA (p 0.12). Additionally, the IFN-γ and antibody assays showed a trend towards a bivariate response in normal controls. This indicates that cellular and antibody responses against *C. pneumoniae* may be mutually exclusive, with potential implications for the role of this organism in the genesis of cardiovascular disease in both immunocompetent and HIV-infected populations.

**Keywords** *Chlamydia pneumoniae*, HIV, interferon-γ, PCR, serology

**Original Submission:** 20 July 2003; **Revised Submission:** 6 January 2004; **Accepted:** 26 January 2004

*Clin Microbiol Infect* 2004; 10: 820–825

## INTRODUCTION

*Chlamydia pneumoniae* is an intracellular organism thought to cause acute and chronic infections [1,2]. Chronic infection with this organism has been of particular interest because of a putative association with arteriosclerosis [3,4]. This association is based on significant differences in *C. pneumoniae* IgA titres between patients with arteriosclerotic disease and healthy controls [5], detection and propagation of *C. pneumoniae* from atherosclerotic plaques [6], and animal and in-vitro studies which support a role for infection in initiation and progression of arteriosclerotic disease [7,8].

Protection from infection by this intracellular pathogen has been shown in mice to depend largely on cell-mediated immunity, and particularly secretion of the cytokine interferon (IFN)-γ [9]. Human studies of cell-mediated immunity *in vitro* with lymphocyte proliferation assays have revealed that 84% of healthy volunteers respond to *C. pneumoniae* [10]. Stimulation of human peripheral blood lymphocytes with *C. pneumoniae* for 24 h induced IFN-γ protection [11]. An efficient cellular immune system thus seems to be important for eradication of *C. pneumoniae* infection.

In addition to the established link between *C. pneumoniae* and cardiovascular events, an increased risk of cardiovascular events in HIV-infected individuals has been shown recently [12–14]. Although these findings are controversial, it seems likely that at least some of this risk is unrelated to anti-retroviral treatment. One possibility is that HIV patients have a reduced cell-mediated immune response to *C. pneumoniae*, and that this could contribute to the increased risk of cardiovascular events. Therefore, the present study aimed to investigate the cellular immune response

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to *C. pneumoniae* by measuring whole blood IFN-$\gamma$ secreted in response to *C. pneumoniae* antigen in HIV-infected patients and controls.

## MATERIALS AND METHODS

### Patients and controls

Healthy volunteers ($n$ = 32; mean age 34.2 ± 9.8 years) were recruited among staff of the Alfred Hospital, Melbourne, Australia. HIV antibody-positive patients ($n$ = 34; mean age 41.6 ± 9.0 years) were recruited from among outpatients and inpatients of the Alfred Hospital during the same time period. The CD4 counts and virological status of the HIV-infected patients are shown in Table 1. Recruitment consent and all subsequent investigations were approved by the Human Research and Ethics Committee of Alfred Hospital.

### The whole blood *C. pneumoniae* IFN-$\gamma$ assay

A new method of measuring IFN-$\gamma$ release from whole blood was used. The test is rapid and simple, and demonstrates T-helper 1-type cellular immunity by detecting IFN-$\gamma$ produced in whole blood stimulated with specific antigen(s). The principle of the test takes advantage of the fact that individuals primed *in vivo* with exogenous or endogenous antigen have lymphocytes in their blood that maintain an immunological memory for the priming antigen. In-vitro addition of antigen to blood taken from such individuals results in rapid re-stimulation of antigen-specific effector (memory) T-cells, followed by release of the cytokine IFN-$\gamma$, which is then used as a specific marker for detecting a cellular immune response mounted to the antigen (recall response).

The method was modified slightly from that described for tuberculosis [15]. A QuantiFERON-CMI kit (Cellestis, Parkville, Victoria, Australia) was used in conjunction with a purified *C. pneumoniae* antigen (see below), free of cellular material, thereby reducing the risk of cross-reaction with human proteins. This antigen does not cross-react serologically with *C. trachomatis*. In brief, aliquots (1 mL) of undiluted (heparinised) whole blood were incubated overnight with *C. pneumoniae* antigen at a concentration of 10 mg/L blood, and also with phosphate-buffered saline as a negative control, and phytohaemaglutinin (PHA) as a positive control. Following overnight incubation, plasma samples were harvested for IFN-$\gamma$ quantification by a single-step sandwich-ELISA. Plasma samples were reacted simultaneously with anti-IFN-$\gamma$ antibodies bound to the solid phase and enzyme-labelled anti-IFN-$\gamma$ antibodies in solution for 60 min. Unbound material was removed by washing, followed by the addition of enzyme substrate. The reaction was terminated after 30 min and the amount of colour development was measured spectrophotometrically. The concentration of IFN-$\gamma$ in each plasma sample was then calculated by comparison with kit standards.

### Preparation of antigen

All IFN-$\gamma$ analyses were performed within 12 h of obtaining blood samples. The antigen used for the IFN-$\gamma$ assay was prepared by culturing *C. pneumoniae* (VR1310) in BHK (baby hamster kidney) cells. BHK cells were infected with one inclusion-forming unit/cell in infection medium, comprising RPMI-1640 (Gibco, Paisley, UK) 49% v/v, fetal calf serum (Biological Industries, Haemex, Israel) 2.5% v/v, phosphate-buffered saline 48.5% v/v and gentamicin 12.5 mg/L, and were then centrifuged at 700 *g* for 20 min at 35°C. After 2 h, the medium was changed to RPMI-1640 97.5% v/v, fetal calf serum 2.5% v/v, gentamicin 25 mg/L and cycloheximide 1 mg/L. Following incubation for 3 days at 35°C in $CO_2$ 5% v/v, the infection was confirmed by immunofluorescence. The elementary bodies were purified as described by Knudsen *et al.* [16] and checked for purity by electron microscopy following negative staining [16]. The amount of antigen used (10 mg/L blood) was determined by the generation of dose response curves in preliminary experiments (results not shown).

### Serological assays and PCR experiments

Commercially available enzyme immunoassays for IgA and IgG (Labsystems, Helsinki, Finland) were used for antibody detection. These assays have been shown to be the most reliable antibody assays for acute infections and do not generate false-positive reactions in the presence of a number of non-chlamydial antigens [17,18].

A nested PCR that targeted part of the MOMP gene in *C. pneumoniae* was used to assess the presence of *C. pneumoniae* in peripheral blood mononuclear cells of HIV-infected patients and controls as described previously [19,20].

**Table 1.** Characteristics of HIV-infected patients and controls, including their cellular and humoral immune responses to *Chlamydia pneumoniae* antigens

| | Age (years) | CD4 count (cells/μL) | CD4 lowest recorded count | Viral load (copies/mL) | PHA IFN-$\gamma$ (IU/mL) | *Chlamydia pneumoniae* IFN-$\gamma$ (IU/mL) | *Chlamydia pneumoniae* serum IgA (EIU) | *Chlamydia pneumoniae* serum IgG (EIU) |
|---|---|---|---|---|---|---|---|---|
| HIV-infected patients ($n$ = 34) | | | | | | | | |
| Mean | 41.6 | 397 | 169 | 14 779 | 45 | 6 | 8 | 30 |
| Median | 40.0 | 337 | 145 | 2275 | 30 | 2 | 5 | 22 |
| Minimum | 20 | 7 | 1 | 50 | 0 | 0 | 0 | 0 |
| Maximum | 63 | 1337 | 525 | 100 000 | 244 | 47 | 46 | 146 |
| SD | 9.0 | 308 | 137 | 28 085 | 52 | 11 | 10 | 31 |
| Normal controls ($n$ = 32) | | | | | | | | |
| Mean | 34.2 | N/A | N/A | N/A | 29 | 35 | 7 | 50 |
| Median | 32.0 | N/A | N/A | N/A | 21 | 16 | 6 | 46 |
| Minimum | 21 | N/A | N/A | N/A | 0 | 1 | 0 | 2 |
| Maximum | 58 | N/A | N/A | N/A | 125 | 213 | 33 | 219 |
| SD | 9.8 | N/A | N/A | N/A | 29 | 49 | 7 | 48 |

PHA, phytohaemagglutinin; N/A, not applicable; IU, international units; EIU, enzyme immunosorbent units; SD, standard deviation.

#### Statistics

Results were analysed with SPSS statistical software for Windows (SPSS Inc., Chicago, IL, USA). The Mann–Whitney *U*-test, the chi-squared test and Fisher's exact test were used to compare frequencies between the groups.

## RESULTS

### The whole blood *C. pneumoniae* IFN-γ assay

The assay was standardised with normal controls. As shown in the upper panels in Fig. 1, there was no clear cut-off level for positivity in the developed IFN-γ assay. None of the controls had both a high antibody response (Th2) and a high IFN-γ response (Th1) (Fig. 1). There appeared to be a trend towards a bivariate response in the control group; that is, no cellular response to *C. pneumoniae* was seen if there was a humoral response, and vice versa. However, this difference did not reach statistical significance (p 0.30 for IgG and p 0.14 for IgA). With an arbitrary cut-off level of 50 IU/mL being taken to define a positive reaction, none of the HIV-infected individuals was positive (Fig. 1C,D), compared with five of the healthy controls (Fig. 1A,B; p 0.02).

### Antibody response and PCR experiments

The antibody results can be analysed using either conventional cut-off values or by a comparison of means. The conventional cut-off value for a positive IgA is 1:16, and that for IgG is 1:64. By these criteria, three of the 34 HIV-infected patients had a positive IgG response, and five had a

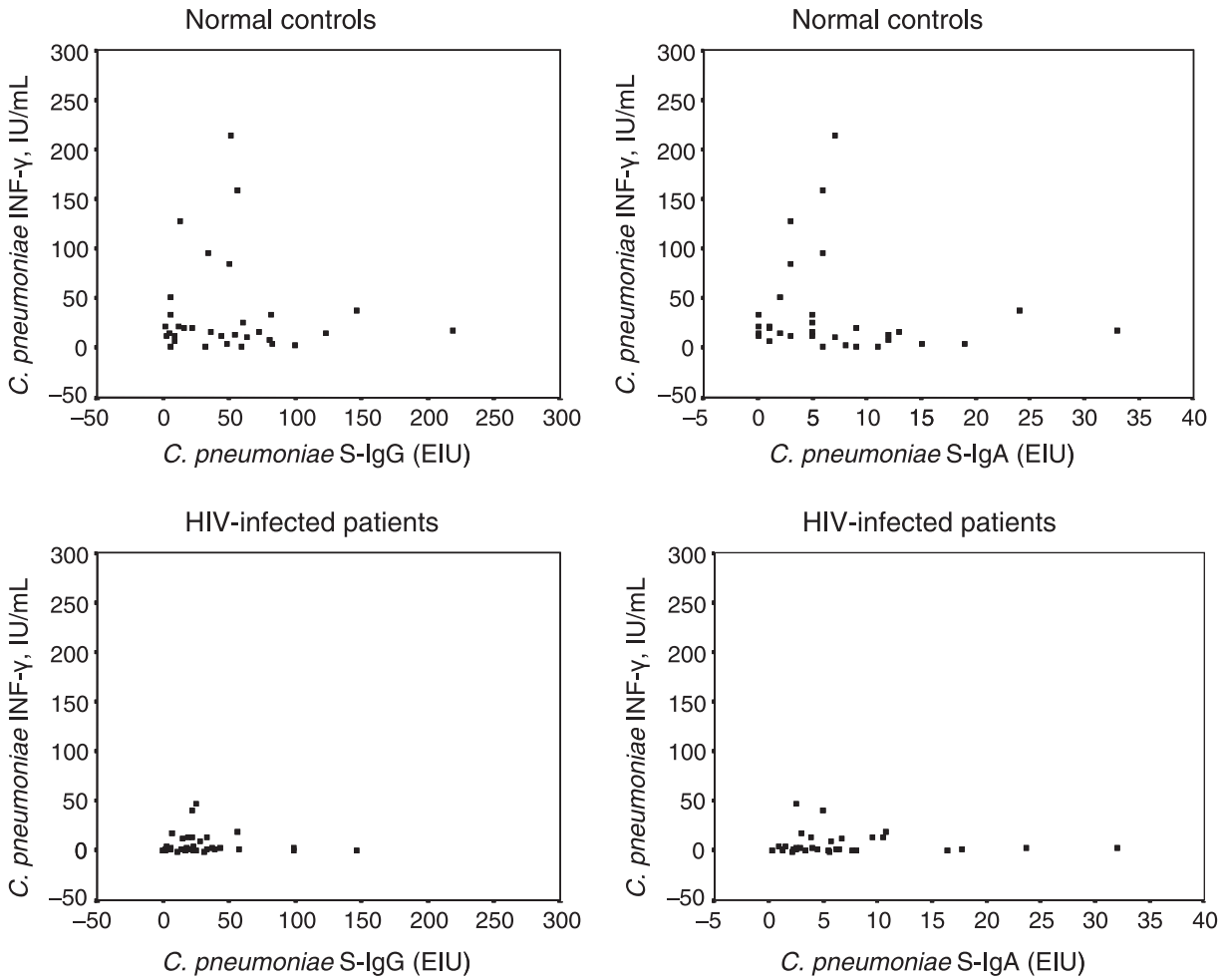


**Fig. 1.** Relationship between interferon-γ response and serum IgG and IgA response to *Chlamydia pneumoniae* in normal controls and HIV-infected patients.

positive IgA response, compared with eight of 32 controls who were positive for IgG and three of 32 who were positive for IgA (Table 1). The mean values in the HIV-positive patients were 29.6 and 7.8 EIUs for IgG and IgA, respectively, compared with 50.0 and 7.3 EIUs, respectively, for the controls. *C. pneumoniae* DNA was not detected by PCR in peripheral blood mononuclear cells from any of the HIV-infected individuals or normal controls.

### Comparison between HIV-infected patients and controls

Details of comparisons between the two groups appear in Table 1. The CD4 count at the time of study, the lowest recorded CD4 count (CD4 nadir) and the viral load at the time of the study are shown for the HIV antibody-positive patients. There was no correlation between any of these parameters and the results obtained by serology or the IFN-$\gamma$ release assay (data not shown).

Since *C. pneumoniae* serology depends on sex and age, ten controls were chosen to match the HIV antibody-positive group according to sex and age. The mean ages in this analysis were 41 years for the controls and 42 years for the HIV-positive patients. The mean IFN-$\gamma$ response was 43 IU/mL in the controls vs. 6 IU/mL in the HIV-positive patients (p 0.002). The mean PHA response was higher in the HIV-positive patients than in the matched controls (45 IU/mL vs. 35.8 IU/mL; p 0.72, Mann–Whitney *U*-test). However, the mean IgA response was 11 EIUs in controls vs. 8 EIUs in the HIV-positive patients (p 0.12), while the mean IgG response was 76 EIUs in controls vs. 30 EIUs in HIV-positive patients (p 0.009).

## DISCUSSION

This is one of the first studies to examine the cellular immune response to *C. pneumoniae*. The main findings of the study were two-fold: first, that in HIV-infected patients there was a weak cellular immune response and a lower IgG response compared with healthy controls; and second, that in healthy controls there was an apparent bivariate response to *C. pneumoniae* with regard to cellular and humoral immune response, i.e., there was a response of one or the other type, but not both.

It was notable that the IFN-$\gamma$ response to PHA in the HIV-infected patients was not reduced, but was greater than in the healthy control group. This could be a result of the direct stimulatory effect on T-lymphocytes, which is characteristic of PHA. This also indicated that a response to PHA was not an optimal measure of immunocompetence. Although the present study looked at *C. pneumoniae* immunity in a novel way and the findings were clear, there were some reservations regarding the interpretation of the study. The first is that the numbers involved were relatively small. The second is the difficulty in correlating the results with true infection, either chronic or acute, given the lack of a standard such as culture. In addition, PCR-based studies suffer from problems of reproducibility [21–23].

The finding of a decreased cellular response in HIV-infected individuals was consistent with known HIV immunology [24]. Furthermore, the findings were also consistent with previous studies which have shown that acute infection does not always induce an antibody response to *C. pneumoniae* in HIV-negative patients [25]. Such previous studies, including those linking chronic *C. pneumoniae* infection to vascular disease, have also been based largely on antibody responses [26]. Nucleic acid-based tests have been used, but are problematic [21,22]. For example, Apfalter *et al.* [23] showed that problems with false-positive reactions in negative controls tend to occur in laboratories with high detection rates of *C. pneumoniae* in monocytes by PCR.

It has been thought that the cellular immune response may be more important in clearing this intracellular organism [27]. The present study found a decreased IFN-$\gamma$ immune response to *Chlamydia* in HIV-infected patients, consistent with studies on the serologically distinct serovar *C. trachomatis* [27]. However, the previous study used the conventional technique of measuring IFN-$\gamma$ response from peripheral blood mononuclear cells rather than whole blood. A separate study examined lymphocyte proliferation rates to *Chlamydia* antigens in patients with coronary heart disease [28], and showed an association in comparison with normal controls for men but not for women. The strength of the antibody responses did not correlate with the lymphocyte proliferation rates. Furthermore, specific anti-chlamydial responses have also been seen with lymphocytes extracted from vascular lesions [29].

These studies provide additional data to support the idea that cellular responses may be important in the genesis of cardiovascular events. However, the assay used in the present study is likely to be more reproducible and less observer-dependent than the assays used previously.

It may be that the type of immune response to this organism, rather than chronic infection with it, defines the critical association with vascular disease. In the present study, there appeared to be a bivariate response; the basis of this could be predetermined genetically or might be related to the original nature or timing of exposure to a specific antigen [30,31]. This has been demonstrated in genetically modified mice, where an increased level of interleukin-10, or a Th2 response, is associated with enhanced susceptibility to infection [9]. Alternatively, the inability to mount a cellular immune response may lead to chronic infection and, eventually, to vascular disease. In the absence of positive PCR results, or other evidence of the organism, both theories are speculative at present, but the bias towards IgA production may indicate chronicity of infection. The role of such chronic infection in immunosuppressed patients, especially with respect to the possible increase in vascular events noted in association with HIV infection, remains to be elucidated.

## ACKNOWLEDGEMENTS

We thank G. Christiansen (Department of Medical Microbiology and Immunology, Aarhus University, Aarhus, Denmark) and S. Birkelund (Loke Diagnostics, Aarhus, Denmark) for providing the antigens used in the *C. pneumoniae* IFN-$\gamma$ assay. We also gratefully acknowledge the technical assistance of J. Guldberg. This work was supported by grants from The Danish National Research Council and The Scandinavian Society for Antimicrobial Chemotherapy.

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