

CHLAMYDIA PNEUMONIAE DNA IS NOT DETECTABLE WITHIN SARCOIDOSIS TISSUE

Graham D. Mills*†, Roger K.A Allen* and Peter Timms†

Thoracic Medicine Department, Prince Charles Hospital and Centre for Molecular Biotechnology, Queensland University of Technology†, Brisbane, Australia.*

Summary

Sarcoidosis is a granulomatous disease of unknown aetiology. Recent studies have suggested the possibility of a bacterial origin with *Chlamydia pneumoniae* being one of the many bacteria considered. The aim of this study was to use the polymerase chain reaction (PCR) in an attempt to identify *C. pneumoniae* within fresh/frozen sarcoidosis tissue.

Tissue from 20 sarcoidosis patients and 17 controls was evaluated. DNA was extracted from all tissue specimens and PCR amplified with primers specific for *C. pneumoniae*. All study tissues were negative for the presence of DNA sequences from *C. pneumoniae*. These findings could not be attributed to PCR inhibition or to lack of sensitivity of the PCR assay.

The negative finding suggests either that there is no involvement between *C. pneumoniae* and sarcoidosis or that, having incited granulomata formation, it is no longer present in detectable amounts.

Key words: Sarcoidosis, Chlamydia pneumoniae, PCR, aetiology.

Accepted 13 March 1998

INTRODUCTION

Sarcoidosis is a multi-system granulomatous disorder of unknown aetiology whose diagnosis is made by the exclusion of other granulomatous diseases. The heterogeneity of the manifestations of the disease, lack of a precise definition, clinical overlap with other disorders and insensitive and non-specific diagnostic tests, all lead to misclassification of the disease and have contributed to the difficulties in clarifying the cause of this condition¹.

Granulomatous inflammation is a non-specific chronic response to many infective, toxic, allergic, autoimmune and neoplastic processes. Sarcoid granulomas are morphologically and immunologically similar to granulomas due to these other causes². Many putative agents have been considered in this disease. An infective agent has been considered most likely and is supported by both epidemiological evidence (climatic factors and non-familial clustering of cases)³ and experimental evidence (the development of granulomas after injection of sarcoid lymph node-homogenates into the footpads of mice)⁴. The oligoclonal expansion of T-cells in sarcoidosis suggests that specific antigens may be associated with the disease⁵. However, despite numerous investigations over the past 30 years, the identity of the causative agent(s) remains elusive.

The aim of this study was to use the polymerase chain reaction (PCR) to test the hypothesis that *Chlamydia pneumoniae*-specific DNA is present within sarcoidosis tissue. We selected *C. pneumoniae* due to the recent suggestion from antibody studies of a possible role⁶. In addition, *C. pneumoniae* conforms to the model for agents inducing granulomatous disease⁷. This model requires an organism to both remain immunogenic and also have the potential to induce granuloma formation. The genus *Chlamydia* has these properties. *C. pneumoniae* also has the ability to spread systemically after respiratory infection⁸, and this may account for granuloma formation throughout multiple organs in sarcoidosis. A study using PCR techniques in search of *C. pneumoniae* within sarcoidosis tissue has not previously been conducted.

SUBJECTS AND METHODS

Subjects

At Prince Charles Hospital, Brisbane, Australia, all consenting patients who have undergone diagnostic mediastinal lymph node biopsies since 1992 have had tissue stored in a -70°C tissue bank. Approximately one-third of sarcoidosis patients at this hospital have had sarcoidosis confirmed using mediastinal lymph node biopsies. Biopsies from 20 sarcoidosis patients (mean age 40 ± 16 yrs), 10 lung cancer patients (mean age 60 ± 11 yrs) and 7 patients with non-sarcoidosis/non-malignant

diagnoses (mean age 45 ± 9 yrs) were selected. A diagnosis of sarcoidosis was based on the presence of non-caseating granulomas from tissue bank in association with typical clinical and radiological findings and the exclusion of alternative diagnoses. Patients consented to having tissue stored at the time of their original thoracic surgical procedure in accordance with the Prince Charles Hospital Ethics Committee (Protocol No.: EC9446).

Study design

DNA was extracted from all tissue specimens, PCR amplified with *C. pneumoniae* primers and products separated by gel electrophoresis and confirmed by Southern hybridisation with specific probes. Comparison between sarcoidosis and control tissue was performed.

Methods

Tissue sections totalling 100 μm were cut from the tissue blocks by cryostat for each of the DNA extraction procedures. The microtome blade was carefully cleaned with 95% alcohol between each block to prevent sample-to-sample contamination. The tissue was transferred with a fresh brush into Eppendorf tubes. Sections were taken at the same time for histological staining to confirm the original diagnosis and to verify the presence of granulomata.

DNA extraction: Three different DNA extraction methods were compared for adequacy, of nucleic acid extraction and evidence of PCR inhibition.

Method A: Phenol chloroform extraction with ethanol precipitation. Standard methods were used⁹. Tissue was digested in a proteinase K digestion buffer (Tris 50 mM, EDTA 1 mM, Tween 20 0.5% with proteinase K to 200 $\mu\text{g}/\text{ml}$) and incubated overnight at 50°C. One phenol/chloroform extraction followed by 2 chloroform/isoamyl alcohol extractions were performed prior to ethanol precipitation and re-suspension in 50 μl distilled water.

Method B: Guanidinium isothiocyanate with diatomaceous silica. Methods were as for Boom¹⁰, with the addition of a proteinase K digestion step followed by mechanical disruption using a Mini-Beadbeater-8 (Biospec Products, Bartlesville, Oklahoma) with 0.1 mm zirconia-silica beads (Biospec Products) for 3min. Final elution was in a 50 μl volume of Tris-EDTA (TE).

Method C: Commercial DNA extraction kit (QIAamp tissue kit, Qiagen, Chatsworth, California, USA). The assays were performed as per the manufacturers' instructions. The final elution was in 400 μl of TE.

Initial pilot studies gave greater quantitative return of DNA as well as improved PCR sensitivity by one log₁₀ dilution with Method A compared with Methods B and C (data not shown). Proteinase K digestion alone and without further DNA extraction was 3 log₁₀ dilutions inferior to Method A. Because of the possibility that inhibition may be a greater problem with phenol extraction methods¹¹, and that the cell wall of *C. pneumoniae* may be resistant to degradation, the use of the other two methods were also included in various segments of this study.

C. pneumoniae PCR: PCR was performed on a Perkin Elmer DNA Thermal Cycler 480. Extracted DNA was assayed with primer sets targeting *C. pneumoniae* genes. A number of *C. pneumoniae*-specific PCR assays are in current use with sensitivity estimations ranging between 0.05 and 40 inclusion forming units^{12,13}. Quantitation of *C. pneumoniae* PCR sensitivity is fraught with difficulties based on two factors: (i) the difficulty in making adequate serial dilutions of elementary bodies because of their adhesive property; and (ii) the presence of reticulate body nucleic acids which can be detected by PCR but are themselves non-infectious and would not result in the formation of inclusions in tissue culture. We did not attempt to perform absolute quantitation of our PCR assay, but compared 3 different published primer sets using log₁₀ dilutions of cell cultured *C. pneumoniae* IOL-207^{12,14,15}. On the basis of these results the nested PCR assay based on the Major Outer Membrane Protein (*ompH*) of *C. pneumoniae* was selected.

PCR was performed on a 5 μl DNA extract in a total volume of 50 μl . The final mixture contained 1 μM primers, 0.2 mM deoxynucleoside triphosphates, 1 x PCR buffer (10 mM Tris [pH 8.3], 50 mM KCL, 1.5 mM MgCl₂, 0.01% gelatin) and 1.0 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). Outer primers APNOU = 5'-AATTCTCTGTAAACAAACCC-3' and APNOL = 5'-ATTAAGAAGCTCTGAGCATA-3' were used in the first phase of the nested reaction. The reaction mixture was subjected to 35 cycles of denaturation (94°C, 1 min), annealing (52°C, 1 min) and extension (72°C, 90 s) in thermal cycler. Three μl of amplicon from the outer primer reaction was then used as the template for amplification with the internal primers APN1 = 5'-TGCCAACAGACGCTGGCGT-3' and APN2 = 5'-AGCCTAACATGTAGACTCTGAT-3' with an annealing temperature of 63°C, but otherwise the same conditions as above.

Dr Roger K. A. Allen

Amplification products were separated by agarose gel electrophoresis using TRIS-borate-EDTA buffer (pH 8.3). Nucleic acids were then visualised by staining with ethidium bromide and confirmation of specific amplification was performed by southern hybridisation with a digoxigenin-labelled 19 mer probe within Variable Domain Four of *ompL*.

Quality control

Positive and negative controls were used throughout the study. With each run a consistency control aliquot was also amplified with the patient samples. This positive control consisted of the specific organism in the lowest concentration that could consistently be amplified. Results of this control confirmed that maximum sensitivity was attained for each run.

Testing for the presence of PCR inhibition was performed routinely with the spiking of a second aliquot of template DNA spiked with *C. pneumoniae*. Two negative controls were used: distilled water (ddW), which had undergone the full DNA extraction process, and unprocessed ddW.

In order to prevent PCR contamination the following precautions were adopted: physical separation of different steps in the PCR procedure; the use of different pipettes and filter tips (QSP filter tips, Quantum Scientific Pty Ltd, Brisbane); and the wearing of separate coats and gloves in each laboratory. Four separate laboratories were used for PCR master mix preparation, DNA extraction, template addition and gel electrophoresis. Class two bio-safety cabinets and ultra-violet decontamination were used to decrease the risk of sample-to-sample carry over.

RESULTS

Initial studies confirmed that nested PCR of the chlamydial *omp1* gene¹⁵ was more sensitive than (i) previously used *ompL* primers¹⁴ and (ii) the 437-bp *Pst*I fragment primers of Campbell et al¹². We were routinely able to amplify a five log₁₀ dilution of *Chlamydia* stock culture with the nested primer set described by Cunningham et al¹⁵, whereas the other two primer sets could only amplify to a three to four log₁₀ dilution. An advantage of nested PCR was the intensity of positive results generated with ethidium bromide stained agarose gels without the need for Southern hybridisation to increase sensitivity. A reduction in the incidence of PCR inhibition (data not shown) was also observed. On the basis of these findings, all assays were performed with the nested PCR set.

All 37 biopsy specimens extracted by method A were negative for *C. pneumoniae*. There was no evidence of inhibition. Many of the negative specimens showed multiple banding with ethidium bromide staining on agarose gel, however these non-specific bands did not hybridise to the internal probe. Positive control samples gave single intense ethidium bromide stained products that hybridised specifically to the probe. The inability to amplify specific *C. pneumoniae* sequences was confirmed with DNA extracted by methods B and C, with all specimens analysed being negative.

As a further check on the validity of our results, a selection of sarcoidosis specimens, extracted with method A, were also amplified with the 437-bp *Pst*I fragment primer¹² and an in-house primer set based on *omp2*. All of these attempts were also negative.

DISCUSSION

This PCR-based study did not confirm the presence of DNA sequences from *C. pneumoniae* within fresh/frozen tissue samples from patients with sarcoidosis. These findings need to be put in the context of the current debate on the aetiology of sarcoidosis. Currently three popular theories on the causation of sarcoidosis predominate⁷. Firstly that a single but as yet previously unsuspected agent is involved; secondly that sarcoidosis is due to a reaction against mycobacterial antigen; and thirdly that multiple antigens are capable of inducing sarcoidosis in a susceptible host. Each hypothesis assumes that not only is exposure to the agent(s) important, but that genetic or other "host" factors cause a susceptibility to develop a "sarcoid" reaction to the given antigen.

In designing this study we chose *C. pneumoniae* as an organism that could conform to the first theory on the causation of sarcoidosis. *C. pneumoniae* was first recognised as causing human disease in 1986¹⁶. Since that time considerable interest has been generated by this organism because of its association not only with pneumonia and bronchitis, but also with atherosclerosis^{17,18} and asthma¹⁹. In addition, the possible relationship between *C. pneumoniae* and sarcoidosis has been reported on several occasions over the last decade. However, studies have not progressed beyond serological associations. This organism was therefore selected as a possible previously unsuspected agent.

The suggested relationship had arisen from four separate studies, each reporting a trend toward higher seroprevalence of *C. pneumoniae* antibodies in patients with sarcoidosis when compared to either healthy individuals or patients with other interstitial lung diseases. Gronhagen-Riska et al²⁰ found a highly significant serological association in a longitudinal study of sarcoidosis patients. In their study, 86% (19/22) of sarcoidosis patients had positive MIF serology for *C. pneumoniae* compared to 43% (217/504) of controls ($p < 0.001$). The three negative patients had subacute radiographic Stage I sarcoidosis that had regressed spontaneously within two years. A correlation existed between *C. pneumoniae* antibody titre and serum ACE values. In addition fluctuations in *C. pneumoniae* titres correlated with clinically apparent fluctuations in disease activity. Black et al²¹ confirmed these findings with seropositive rates of 71% in sarcoidosis patients (95% CI 51-87%) compared to 23% in non-granulomatous interstitial lung diseases patients (CI 5-54%) and 43% in healthy volunteers (CI 27-59%). Puolakkainen et al⁶ further showed that the profile of antigen recognition using immunoblots in sera from sarcoidosis patients was similar to that seen in acute *C. pneumoniae* infection, rather than reflecting persistent antibodies from past infection. In the final study, Suzuki et al²² found high IgG antibody titres (≥ 256) in 39% (12/29) sarcoidosis cases with endogenous uveitis.

In our study serology for *C. pneumoniae* was not collected although a seroprevalence rate of 51% has been demonstrated in the same study area of South-East Queensland (unpublished) This *C. pneumoniae* seroprevalence rate is very similar to European and American populations²³.

The only non-serological report of an association between *C. pneumoniae* and sarcoidosis is by Yang et al⁸ from The University of Washington, Seattle. They found *C. pneumoniae* DNA in spleen and lymph node samples from one adult sarcoidosis patient. Unfortunately full details on this case have not been published.

The negative findings of our study may be due to several possibilities: (i) a lack of sensitivity of the PCR assays; (ii) the granuloma-inducing bacteria are no longer present; or (iii) bacterial organisms are not involved in sarcoidosis. During the course of our current study, however, we have shown good PCR sensitivity. We chose to use fresh/frozen tissue because of its theoretical advantage in improving DNA quality when compared with formalin-fixed paraffin-embedded tissue²⁴. The primers that we have used appear to be at least as sensitive as alternative sets. We also chose to use three differing DNA extraction techniques. Yet, despite this, we could not amplify *C. pneumoniae* DNA within our study tissues.

Another possible explanation is that the serological response to *C. pneumoniae* reflects past infection, with bacteria no longer present but having activated T lymphocytes and induced a self-perpetuating granulomatous process. Unfortunately current molecular technology is not able to test this hypothesis further. The biopsy specimens that constituted our study tissue were taken at diagnosis when disease was well established and well past any latent period from the time of putative infection.

The remaining possibility is that *C. pneumoniae* is not involved in sarcoidosis. If this is correct, an alternative explanation is required to explain the serological association. One such explanation is the recognition of B-cell hyper-responsiveness and hyper-gammaglobulinemia known to occur in sarcoidosis. Other investigators have previously noted both higher antibody titres and higher seroprevalence rates to a number of viral antigens in sarcoidosis patients¹. Unfortunately our study is not able to definitively conclude which of these three possibilities might explain our negative results. At present therefore, the role of *C. pneumoniae* in sarcoidosis still remains unclear²⁵ and once again attempts to assign definitively a microbiological aetiology to sarcoidosis have failed.

Addendum: since submission of this article, Blasi et al²⁶ have published a similar study using formalin fixed tissues and an alternative *C. pneumoniae* PCR primer set. The same conclusions were reached.

ACKNOWLEDGEMENTS: This research was supported by a Prince Charles Hospital Clinical Research Fellowship. The paper was presented in part at the World Sarcoidosis Congress, London, UK, October 1995.

Address for correspondence: G.D.M., Respiratory Medicine Department, Waikato Hospital, Hamilton, New Zealand.

REFERENCES

1. Newman MD, Rose CS, Maier LA. Medical progress, sarcoidosis. *N Engl J Med* 1997; 336: 1224-34.
2. Scadding JG, Mitchell DN. *Sarcoidosis*. 2nd ed. London: Chapman and Hall, 1985.
3. Hosada Y. Consensus conference: epidemiology of sarcoidosis. *Sarcoidosis* 1994; 11 (Suppl. 1): 17-21.
4. Mitchell DN, Rees RJW. A transmissible agent from sarcoid tissue. *Lancet* 1969; (ii) 81-4.
5. Tamura N, Moller DR, Balbi B, Crystal RG. Preferential usage of the T-cell antigen receptor β -chain constant region C β 1 element by lung T-lymphocytes of patients with pulmonary sarcoidosis. *Am Rev Respir Dis* 1991; 143: 635-9.
6. Puolakkainen M, Campbell LA, Kuo CC, Lemonen M, Gronhagen-Riska C, Saikku P. Serological response to *Chlamydia pneumoniae* in patients with sarcoidosis. *J Infect* 1996; 33: 199-205.
7. Mangiapan G, Hance AJ. Mycobacteria and sarcoidosis: an overview and summary of recent molecular biological data. *Sarcoidosis* 1995; 12: 20-37.
8. Yang ZP, Kuo CC, Grayston JT. Systemic dissemination of *Chlamydia pneumoniae*, following intranasal inoculation in mice. *J Infect Dis* 1995; 171: 736-8.
9. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbour: Cold Spring Harbour Laboratory Press, 1989.
10. Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, Van Der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990; 28: 495-503.
11. Noordhoek GT, Kolk AHJ, Bjune G, Catty D, Dale JW, Fine PEM, et al. Sensitivity and specificity of PCR for detection of Mycobacterium tuberculosis: a blind comparison study among seven laboratories. *J Clin Microbiol* 1994; 32: 277-84.
12. Campbell LA, Melgosa MP, Hamilton D, Kuo C, Grayston JT. Detection of *Chlamydia pneumoniae* by polymerase chain reaction. *J Clin Microbiol* 1992; 30: 434-9.
13. Gaydos CA, Quinn TC, Eiden JJ. Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. *J Clin Microbiol* 1992; 30: 796-800.
14. Rasmussen SJ. *Molecular detection and analysis of chlamydiae*. Phd thesis, Queensland University of Technology, Brisbane, Australia, 1993; 159.
15. Cunningham A, Johnston S, Julious S, Sillis M, Ward ME. The role of *Chlamydia pneumoniae* and other pathogens in acute episodes of asthma in children. In: Orfila J, Byrne GL, Chernesky MA, editors. *Chlamydia infections*. Bologna, Italy: Societa Editrice Esculapio, 1994; 480-3.
16. Grayston JT, Kuo C, Wang S, Altman J. A new *Chlamydia psittaci* strain TWAR, isolated in acute respiratory tract infections. *N Engl J Med* 1986; 315: 161-8.
17. Kuo C, Grayston JT, Campbell LA, Goo YA, RW, Benditt EP. *Chlamydia pneumoniae* (TWAR) in coronary arteries of young adults (15-34 years old). *Proc Natl Acad Sci USA* 1995; 92: 6911-4.
18. Campbell LA, O'Brien ER, Cappuccio AL, Kuo C, Wang S, Stewart D, et al. Detection of *Chlamydia pneumoniae* TWAR in human coronary atherectomy tissues. *J Infect Dis* 1995; 172: 585-8.
19. Hahn DL, Dodge RW, Golubjatnikov R. Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthmatic bronchitis and adult-onset asthma. *JAMA* 1991; 226: 225-30.
20. Gronhagen-Riska C, Saikku P, Riska H, Froseth B, Grayston JT. Antibodies to TWAR – a novel type of Chlamydia – in sarcoidosis. In: Grassi C, Rizzato G, Pozzi E, editors. *Sarcoidosis and other granulomatous disorders*. Amsterdam. Elsevier Science Publishers, 1988; 297-301.
21. Black CM, Bullard JC, Staton GW, Hutwagner LC, Perez RL. Seroprevalence of *Chlamydia pneumoniae* antibodies in patients with pulmonary sarcoidosis in north central Georgia. In: Mardh PA, La Place M, Ward M editors, *Proceedings of the European Society for Chlamydia Research* 1992; 175.
22. Suzuki K, Isobe K, Ohno S, Aoki K, Numazaki K, Chiba S. Involvement of *Chlamydia pneumoniae* in endogenous uveitis. *Jap J Clin Ophth* 1994; 48: 1208-9.
23. Grayston JT. Infections caused by *Chlamydia pneumoniae* strain TWAR. *Clin Infect Dis* 1992; 15: 757-63.
24. Greer CE, Lund JK, Manos MM. PCR Amplification from paraffin-embedded tissues: recommendations on fixatives for long-term storage and prospective studies. *PCR Meth Applic* 1991; 1: 46-50.
25. Grayston JT. *Chlamydia pneumoniae* (TWAR) infections in children. *Pediatr Infect Dis J* 1994; 13: 675-85.
26. Blasi F, Rizzato G, Gambacorta M, Costentini R, Raccanelli R, Tarsia P et al. Failure to detect the presence of *Chlamydia pneumoniae* in sarcoid pathology specimens. *Eur Resp J* 1997; 10: 2609-11.