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SHORT REPORT

Failure to detect the presence of *Chlamydia pneumoniae* in sarcoid pathology specimens

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Failure to detect the presence of Chlamydia pneumoniae *in sarcoid pathology specimens. F. Blasi, G. Rizzato, M. Gambacorta, R. Cosentini, R. Raccanelli, P. Tarsia, C. Arosio, E. Savini, C. Cantoni, L. Fagetti, L. Allegra. ©ERS Journals Ltd 1997.*

ABSTRACT: The pathogenesis of sarcoidosis is not yet known. On the basis of seroepidemiological data, an association between *Chlamydia pneumoniae* infection and sarcoidosis has been suggested, but so far no study has addressed the direct detection of this agent in the affected tissues. The aim of the present study was to detect *C. pneumoniae* deoxyribonucleic acid (DNA) within sarcoid tissue specimens by means of a two-step polymerase chain reaction.

Lung biopsy specimens of 33 patients with histologically confirmed pulmonary sarcoidosis and 21 control lung biopsies or pathology specimens of patients with pulmonary carcinoma or emphysema were retrospectively analysed. A nested polymerase chain reaction was applied using two sets of primers designed to detect a fragment of the 16 strand ribosomal ribonucleic acid (rRNA) gene of *C. pneumoniae*. The results of the study failed to demonstrate the presence of *C. pneumoniae* in biopsy specimens of sarcoid tissue and in the control lung biopsies or pathology specimens.

Our results, therefore, tend to rule out the possibility of a direct involvement of *Chlamydia pneumoniae* in the pathogenesis of sarcoidosis.

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Sarcoidosis is a systemic, noncaseating, granulomatous disease. The pathogenesis of this disorder is not yet known, though many theories have been postulated. Numerous studies have been carried out to investigate the possible role of mycobacterial agents, given the granulomatous nature of the disease together with clinical and immunological similarities with tuberculosis [1–6]. The results both of serological and molecular biology studies are conflicting, and no definitive conclusion has been reached on the role of mycobacteria in the pathogenesis of sarcoidosis.

The search for an infective agent responsible for sarcoidosis has recently been expanded to other pathogens, including *Chlamydia pneumoniae*. The first report of an association between chlamydial antibodies and sarcoidosis dates back to 1976 [7]. A link between antibody titres specific for *C. pneumoniae* and sarcoidosis was subsequently found [8–10]. Furthermore, sarcoidosis-like clinical and radiographic findings have been associated with acute *C. pneumoniae* infection [11, 12], as with respiratory tract infections due to other chlamydial agents [13, 14]. So far, the evidence for a role of *C. pneumoniae* in the development of sarcoidosis, therefore, rests on a seroepidemiological basis alone. No study has yet addressed the direct detection of this agent in the affected tissues, which is necessary to confirm an association with sarcoidosis. The aim of the present study was to detect *C. pneumoniae* deoxyribonucleic acid (DNA) within sarcoid tissue specimens by means of a nested polymerase chain reaction (PCR).

Material and methods

Lung biopsy specimens, performed between June 1992 and June 1995, of 33 patients with histologically confirmed pulmonary sarcoidosis (14 males and 19 females, mean age±SD 34±11 yrs) were analysed retrospectively. Eighteen patients had stage I disease, 10 had stage II disease, and five had stage III disease. In addition, 21 control lung biopsies or pathology specimens of patients with emphysema (10 specimens) or pulmonary adenocarcinoma (11 specimens) were analysed.

DNA extraction

The procedure for DNA extraction from paraffin-embedded tissues was as follows: for each specimen, three 5 µm slices were immersed in 1.8 mL of xylene and agitated. The mixture was incubated at 37°C for 15 min, centrifuged at 14,000 revolutions per minute (rpm) for 3 min and the supernatant was discarded. Immersion, incubation and centrifugation were repeated three times. A 1.8 mL aliquot of 100% ethanol was added and agitated. The mixture was centrifuged at 14,000 rpm for 3 min and the supernatant was discarded. Thereafter, 1.8

mL of 95% ethanol was added and agitated. The mixture was centrifuged at 14,000 rpm for 3 min and the supernatant was discarded. These operations were repeated twice. Digestion was performed by letting the pellet dry and then incubating at 55°C for 3 h with 400 $\mu g \cdot mL^{-1}$ proteinase K, buffer, and water to reach a volume of 200 mL. Proteinase K was then heat denatured at 92°C for 7 min and at 72°C for 30 min. Following each extraction procedure, an electrophoresis on agarose gel was run for a sample of the extracted DNA.

Human lymphocyte antigen (HLA) amplification

Following extraction, the presence and integrity of DNA in the specimens was assessed by means of HLA amplification. A single-step PCR was employed using the following primers:

GH26 - 5' GTGCTGCAGGTGTAAACTTGTACCAG 3'
GH27 - 5' CACGGATCCGGTAGCAGCGGTAGAGTG 3'

Amplification was performed in an automated thermocycler (Robocycler; Stratogene, USA): one cycle at 94°C for 4 min, then 35 cycles at 94°C for 30 s, at 58°C for 1 min, and 72°C for 1 min. A final cycle was performed at 72°C for 5 min. A 229 base pair (bp) amplification product was obtained and visualized by electrophoresis in 3% agarose gel containing ethidium bromide 0.2 $\mu g \cdot mL^{-1}$.

Nested PCR

A two-step nested PCR was applied using two sets of primers designed to detect a fragment of the 16 strand (16s) ribosomal ribonucleic acid (rRNA) gene of *C. pneumoniae* reported by BLACK *et al.* [15]. This PCR technique allows the detection of 1–10 *C. pneumoniae* elementary bodies [16]. In each assay, a negative and a positive control were run. The negative control contained all of the PCR reagents and sterile distilled water. As positive control, *C. pneumoniae* purified elementary bodies were used at a concentration of 10^3 bodies$\cdot \mu L^{-1}$.

Taq inhibitors detection

The presence of Taq polymerase inhibitors in the specimens was tested. In a single test tube, 10 µL of biopsy-extracted DNA and 10 µL of DNA extracted from the positive control were denatured by heat (94°C for 3 min) followed by ice. Subsequently, 10 µL were drawn to be added to the 40 µL mixture for the first amplification of the nested PCR. The first amplification was then run, followed by the second step in accordance with the study protocol.

The amplification reaction was tested by means of gel electrophoresis by running 10 µL of amplificate on a 3% agarose gel. The absence of an amplification band indicates the presence of inhibitors in the specimen.

Results

In all cases, a gel electrophoresis was performed following the DNA extraction procedure. This showed the presence both of high and low weight DNA.

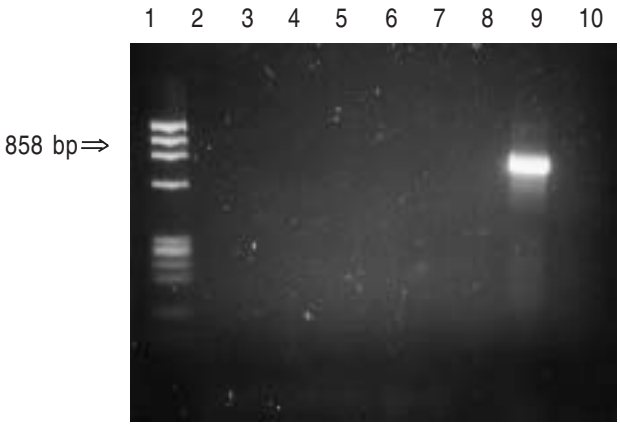

Fig. 1. – Nested polymerase chain reaction amplification results for *Chlamydia pneumoniae* deoxyribonucleic acid (DNA) (in agarose gel). Lane 1: DNA molecular weight marker IX Boehringer Mannheim (1353–1372 bp); Lanes 2–8: negative samples; Lane 9: positive control; Lane 10: negative control. bp: base pair.

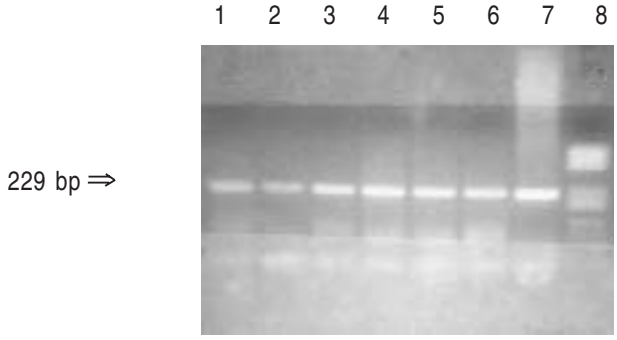

Fig. 2. – Single step polymerase chain reaction amplification of human lymphocyte antigen (HLA) as a control for the presence of deoxyribonucleic acid (DNA) in the specimens examined (in agarose gel). Lanes 1–6: positive samples; Lane 7: positive control; Lane 8: DNA molecular weight marker V Boehringer Mannheim (587–588 bp). bp: base pair.

Sarcoidosis

In all 33 lung biopsy specimens from patients with pulmonary sarcoidosis tested with nested PCR we were unable to detect *C. pneumoniae* DNA (fig. 1). In all cases, the HLA DNA amplification was achieved (fig. 2) and there was no evidence of Taq polymerase inhibitors in the biopsy samples.

Controls

Similarly, the nested PCR method showed no evidence of *C. pneumoniae* DNA in the 21 control lung biopsies. HLA DNA amplification was obtained in all specimens, and the presence of Taq polymerase inhibitors in the biopsy samples was ruled out.

Discussion

The results of this study failed to demonstrate the presence of *C. pneumoniae* DNA in biopsy specimens of sarcoid tissue. The analysis of control lung biopsies or pathology specimens of patients with pulmonary carcinoma or emphysema also failed to detect *C. pneumoniae* DNA.

The search for the causative agent(s) in sarcoidosis has so far given no definitive results. Among the hypotheses raised, it has been suggested that infective agents may initiate an immunological imbalance that then leads to the formation of granulomas. *C. pneumoniae* is now known to be a common respiratory pathogen both of upper and lower airways [17, 18]. Moreover, it has recently been shown that this agent may be involved in chronic diseases [16, 19]. In 1988, a trend toward a higher seroprevalence of antibodies toward *C. pneumoniae* was found in patients with sarcoidosis compared to healthy subjects [9]. These results were later confirmed [10], and it was shown that this higher prevalence applies to sarcoidosis alone among interstitial lung diseases [8]. It has recently been demonstrated that *C. pneumoniae* is able to survive and replicate within alveolar macrophages, and that macrophage products, including angiotensin converting enzyme and lysozyme, commonly employed as markers of activity in sarcoidosis, appear to correlate positively with antibody titres toward this pathogen [9]. Theoretically, *C. pneumoniae* could, therefore, initiate granuloma formation in sarcoidosis in a similar fashion to *C. trachomatis* granuloma formation in lymphogranuloma venereum.

The above-mentioned studies are all based on seroepidemiological data alone. Since many antibody titres are elevated in sarcoidosis, the presence of *C. pneumoniae* in affected tissue must be demonstrated before a definitive conclusion on the association between this agent and sarcoidosis is reached.

In this study, a nested polymerase chain reaction technique was applied to determine the presence of *Chlamydia pneumoniae* deoxyribonucleic acid in lung biopsy specimens from patients with histologically confirmed sarcoidosis. This technique was chosen, as we have previously shown it to be highly sensitive in detecting the presence of *Chlamydia pneumoniae* deoxyribonucleic acid in abdominal aortic artery specimens [16]. The present results, therefore, tend to rule out the possibility of a direct involvement of *Chlamydia pneumoniae* in the pathogenesis of sarcoidosis. Nevertheless, infection with this agent may trigger a self-maintaining immune response which could persist even after elimination of the organism from the lung tissue.

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