Mycobacterium tuberculosis DNA in tissues affected by sarcoidosis

M L Wilsher, R E Menzies, M C Croxson

Abstract

Background—Although some studies have reported the presence of Mycobacterium tuberculosis (MTb) DNA in tissues affected by sarcoidosis, the data are conflicting. The aim of this study was to collect prospectively tissue from patients with sarcoidosis in whom tuberculosis had been excluded, and to use polymerase chain reaction (PCR) to search for DNA sequences specific for MTb.

Methods—Fresh tissue samples (node or lung biopsy) taken from 23 patients with newly diagnosed sarcoidosis, 10 with other respiratory disease, and four patients with culture positive tuberculosis were analysed using PCR to amplify a 123 bp fragment of IS6110, the insertion element present in MTb, and nested PCR to further amplify an 85 bp sequence within the 123 bp product. DNA was also extracted from formalin fixed tissue from eight additional patients with sarcoidosis.

Results—MTb DNA was not detected in any of the tissue samples from patients with sarcoidosis or other respiratory disease but was found in all four patients with tuberculosis.

Conclusions—This study has shown the absence of MTb DNA in lymph node and lung biopsy samples from patients with sarcoidosis. MTb is therefore unlikely to be a factor in the pathogenesis of this disease.

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Keywords: sarcoidosis; mycobacteria; tuberculosis

The aetiology of sarcoidosis remains unclear 100 years after its first description, although ample evidence supports an antigen driven immunopathogenic process. There is oligoclonal expansion of lung T cells bearing certain V beta transcripts and the T cell receptor (TCR) is internalised with upregulation of TCR mRNA transcription suggesting recent occupancy by a specific antigen.1 2 In addition, mRNA encoding the cytokines interleukin 2 (IL-2) and granulocyte macrophage colony stimulating factor (GM-CSF) are increased at sites of disease activity, suggesting local activation of antigen specific T cells.3

Mycobacterium tuberculosis (MTb) has long been a likely contender as the inciting antigen in sarcoidosis, although Koch’s postulates have never been fulfilled.4 Where the organism is found in sarcoid-like granulomas, the diagnosis is usually considered to be tuberculosis. However, the tubercle bacillus may be present in a form without a cell wall and thus not detectable by conventional microbiological methods. Renewed interest in such organisms follows their detection in the blood of patients with sarcoidosis.5

Some investigators have found evidence of MTb in tissues using the polymerase chain reaction (PCR) to amplify mycobacterial DNA or rRNA6 7 but more recent studies have failed to identify such organisms in the majority of tissue samples from patients with sarcoidosis.9 10 However, the reports to date have not always provided clear clinical evidence supporting the diagnosis of sarcoidosis nor have they excluded patients with prior tuberculous disease or infection.

The aim of this study was to collect prospectively tissue from a carefully characterised cohort of patients with newly diagnosed sarcoidosis in whom tuberculous infection or prior disease had been excluded and to use PCR to search for DNA sequences specific for MTb.

Methods

PATIENTS AND CONTROLS

Fresh tissue samples were prospectively collected from a cohort of 23 patients with newly diagnosed sarcoidosis all seen at one outpatient clinic at one hospital. Patients were enrolled in the study if they fulfilled previously accepted clinical diagnostic criteria,11 had evidence of non-caseating granulomas on biopsy specimens, a negative Mantoux test, and negative MTb cultures with no prior history of tuberculosis. In addition, formalin fixed paraffin embedded tissue was obtained from eight patients with an earlier diagnosis of sarcoidosis who were still being followed up. Ten patients with other respiratory disease undergoing lung biopsy or mediastinoscopy acted as negative controls, and four patients with tuberculosis and culture positive nodes acted as positive controls. Details of the 31 patients with sarcoidosis, the 10 negative controls, and the four positive controls are shown in table 1.

The patients with sarcoidosis had one or more of the following disease manifestations: hilar and mediastinal lymphadenopathy (27
patients), pulmonary (9), splenic (1), ocular (1), cervical node (1), hypercalcaemia (1). The chest radiographic appearances were as follows: stage I (22), stage II (7), stage III (2). Tissue for analysis was obtained from the following sites: mediastinal node (25), cervical node (1), open lung biopsy (2), transbronchial biopsy (3). Specimens from each patient were cultured for MTb and all were negative.

The diagnoses of the negative controls were as follows: carcinoma of the lung (4), metastatic seminoma (1), lymphoma (1), Castleman’s disease (1), plasma cell granuloma (1), bronchiectasis (1), reactive lymphadenopathy (1). Tissue for analysis was collected prospectively from the following sites: mediastinal node (7), cervical node (1), open lung biopsy (2). Five patients had tissue cultured for MTb and all were negative. The four patients with tuberculosis all had lymph nodes sampled (three mediastinal, one supraclavicular) and all were culture positive. Tissue collected prospectively was stored in sealed vials at –70°C in liquid nitrogen until analysis. Specimens were analysed in randomly selected groups, the Scientific Officer being blind to the diagnostic category of individual specimens. This study had the approval of the North Health ethics committee.

extraction of DNA

When sufficient frozen specimen was available four aliquots from each specimen were processed and assayed. Each sample was suspended in 500 µl of lysis buffer, final concentration 20 mM Tris HCl (pH 7.6), 20 mM ethylene diamine tetraacetic acid (EDTA), 20 mM sodium chloride, 1% sodium dodecyl sulphate (SDS), and 600 µg/ml proteinase K (Boehringer Mannheim).

Samples were incubated at 55°C with shaking for 48–72 hours. DNA was purified by phenol/chloroform extraction and precipitated with ethanol. Precipitated DNA was washed once with 70% ethanol and dissolved in PCR clean double distilled water. The quantity and quality of DNA was calculated from absorbance measurements made with a Beckman spectrophotometer DU-64. Paraffin embedded samples were deparaffinised by two extractions with xylene followed by two absolute ethanol washes. Samples were then dried in a 65°C heat block and lysis buffer was added. Their subsequent treatment was identical to that for frozen samples. One negative extraction control (PCR clean double distilled water) for every specimen was processed to detect contamination. Positive extraction controls equivalent to 1 colony forming unit (CFU) and 10 CFU of MTb H37RV were processed with each batch of specimens. The positive controls acted as checks for adequate cell lysis, DNA purification, and activity of PCR reagents.

amplification of DNA

PCR was performed on a Perkin Elmer Cetus DNA thermal cycler 480 according to standard methods. Sample DNA equal to 1.0 µg was added to each 50 µl PCR reaction. All reactions contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 200 µM each of dATP, dCTP, dGTP and dTTP, 1.5 mM magnesium chloride, 1 unit Taq DNA polymerase (Gibco, BRL, Life Technologies) and 0.1 µg of each primer. Primers IS1 and IS2 amplified a 123 bp fragment of IS6110, the insertion element present in mycobacteria of the MTb complex. An aliquot of the first round amplification products (2 µl) was further amplified using nested primers N1 (5'-ACC ACCAGACCTAACC) and N2 (5'-TGAC AAAGGCCACGTAGGCG) which recognise an 85 bp sequence within the 123 bp product. As an alternative to the above primer sets TB1 (5'-CGCAAAGTGTGGCTAACCCTG AA) and TB2 (5'-GGTCGAGTACGCCTT CTTGTTGG) which amplify a 376 bp fragment were used in conjunction with nested primers NT1 (5'-CGTTAGGGCATCGAGGG TGCC) and NT2 (5'-CGCGTCGAGGACCATGGAG) which produce a 306 bp product.

PCR conditions for primers IS1 and IS2 were 95°C for three minutes followed by 50 cycles of 95°C for 40 seconds, 68°C for 40 seconds, and 72°C for 15 seconds. Alternative primers TB1 and TB2 had an initial denaturation at 94°C for three minutes followed by 50 cycles of 94°C for 50 seconds and 70°C for 50 seconds. Nested PCR with primers N1 and N2 had an initial denaturation of 95°C for three minutes followed by 30 cycles of 95°C for 40 seconds, 61°C for 40 seconds, and 72°C for 40 seconds. When nested primers NT1 and NT2 were used, the annealing temperature of the nested PCR was increased to 68°C.

A PCR reagent blank was included with each PCR assay.

Determination of PCR sensitivity and specificity

To determine the sensitivity of the combined DNA extraction and PCR methods, cultures of MTb H37RV grown in Middlebrook 7M broth were diluted. Five identical aliquots of each dilution were made, three of which were cultured and two were assayed by PCR. For culture, aliquots were spread over Lowenstein-Jensen slopes, incubated at 37°C for six weeks, and the number of CFUs of MTb in each dilution was determined. For PCR, total DNA was extracted from each aliquot and assayed. DNA extracted from one CFU could be detected by first round PCR.

The sensitivity and specificity of PCR of MTb DNA in the presence of human DNA was also determined. When logarithmic dilutions of DNA purified from MTb H37RV were
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Each assay was in the presence of 1 µg of pooled human white blood cell (WBC) DNA, 5 fg of DNA (equivalent to one MTb bacillus) could be detected by first round PCR. The A₂₆₀/A₃₃₀ ratio of the purified MTb DNA was 1.77.

The specificity of PCR primers IS1 and IS2 was determined previously. To determine the specificity of primers TB1 and TB2, 1 µg aliquots of DNA prepared from bacterial and mycobacterial cultures were assayed by PCR. No amplified product was detected for clinical isolates of Staphylococcus aureus, Escherichia coli, M fortuitum, M simiae, M thermoresistible, M scrofulaceum, M chelonii (three isolates) and M avium complex (three isolates). DNA from MTB H₃₇RV and M bovis BCG and clinical isolates MTb (African) amplified to give a 376 base pair product. The identity of the product was confirmed by blotting and hybridization with the oligonucleotide probe IS3.

DETERMINATION OF SPECIMEN DNA
FRAGMENTATION AND PCR INHIBITION
DNA extracted from paraffin embedded specimens may be fragmented as a result of formalin fixation. To confirm the integrity of DNA in these specimens, human DNA sequences of similar size to target mycobacterial DNA was amplified. Primers β2M1 (5’-TTAGCGCTGT CTGCGCTACTCTCTC) and β2M2 (5’-CG CTCTGCGCGGCTTAACCCGT) which amplify a 317 bp sequence in the human β₂-microglobulin gene were used. Samples of DNA extracted from paraffin embedded specimens equal to 1.0 µg were added to each 50 µl PCR reaction which contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 200 µM each of dATP, dCTP, dGTP and dTTP, 1.5 mM magnesium chloride, 2 units of Taq DNA polymerase, and 0.2 µg of each primer. PCR conditions were 95°C for three minutes followed by 40 cycles of 95°C for 40 seconds, 61°C for 40 seconds, and 72°C for 40 seconds. Aliquots of amplification products were electrophoresed and compared with the product obtained by amplification of a positive control of 1 µg of pooled human WBC DNA. Decrease in signal strength of the specimen sample compared with the control sample was deemed to indicate fragmentation of specimen DNA.

To test for inhibition of the MTb PCR 5 fg and 50 fg of DNA purified from MTb H₃₇RV were added to two MTB PCR reactions each containing 1 µg of sample DNA and PCR assays for MTb were run as previously described. The absence of MTB amplicons was taken as evidence of inhibition.

Results
The results of PCR analysis are shown in table 2. Twenty six patients with sarcoidosis had intact DNA for analysis and an absence of inhibitory factors. No patients had MTb DNA detected in their tissue samples. The four tissue samples which yielded fragmented DNA were all wax embedded formalin fixed specimens. All nine negative controls with intact DNA had a negative PCR result. The four patients with tuberculosis were PCR positive.

Table 2 Results of MTb DNA PCR analysis

<table>
<thead>
<tr>
<th>Specimen</th>
<th>PCR</th>
<th>Specimen DNA</th>
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</thead>
<tbody>
<tr>
<td>Sarcoidosis</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>4</td>
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Positive extraction controls (1 and 10 CFU) processed with each batch of specimens were always positive. All blank extraction controls were PCR negative.

Replicate tests yielded consistent results when answers of negative, inhibitory or fragmented DNA were obtained. Replicate tests for patients with tuberculosis were positive for one of four extractions (two patients), three of four extractions (one patient), and four of four extractions (one patient).

Discussion
In this study MTb DNA was not detected in tissue from patients with sarcoidosis. The majority of the specimens were lymph nodes sampled from patients with newly diagnosed untreated sarcoidosis which, on histological analysis, revealed abundant non-necrotising granulomas. We expected that, if MTb was involved in the pathogenesis of sarcoidosis, then these would be the samples most likely to yield positive MTb DNA. The validity of our assay was confirmed by finding MTb DNA in all samples from culture positive tuberculosis and from none of the negative controls.

The sensitivity of each assay was demonstrated by consistent extraction and detection of MTb DNA from the one CFU (extraction control) and by the ability of the PCR to detect DNA equivalent to a single MTb bacillus in the presence of 1 µg of human DNA. Sensitivity was in part dependent on the presence of multiple copies of IS6110 in the MTb genome. However, IS6110 may not be present or may be present as a single copy in 20% of MTb strains from patients of Vietnamese origin. As far as we could ascertain, none of our patients had associations with Vietnam. Sensitivity of PCR reactions may also be affected by large amounts of DNA so we routinely restricted the total amount of DNA in the assay to 1.0 µg, equivalent to 1.4 × 10⁹ human cells per PCR reaction. This PCR cannot therefore detect MTb at a concentration of less than one bacillus per 1.4 × 10⁹ human cells. The results for the positive control specimens indicate that MTb bacilli are not evenly distributed throughout the specimen. Thus, in our study the absence of positive results for patients with sarcoidosis could mean that sites containing bacilli have not been sampled or the organisms involved do not carry IS6110.

Ghossein et al also failed to detect mycobacterial DNA in formaldehyde fixed paraffin embedded tissues from 10 patients with sarcoidosis. Thakker et al, using fixed tissue from 14 patients, identified mycobacterial DNA in one sarcoid lymph node but concluded that this was likely to be a contaminant because the node was preprocessed as a cryostat section and no mycobacterial DNA

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PCR enables tissue samples to be analysed with considerable sensitivity and a single organism can be detected. Unfortunately MTb rarely reside after infection and, unless the Mantoux response is known, prior infection status cannot be assumed. In New Zealand BCG vaccination of adolescents was routine until 1986 so the Mantoux response is less helpful in determining infection. The detection of mycobacterial DNA in lung or mediastinal nodes may simply reflect earlier infection by MTb rather than implicate the organism in the pathogenesis of sarcoidosis. Very careful clinical documentation and screening for tuberculous disease is necessary before concluding that the diagnosis is sarcoidosis and in our study we have done this.

In conclusion, our study has demonstrated the absence of MTb DNA in lymph node and lung samples from patients with sarcoidosis. We believe these findings contribute significantly to the evidence against MTb as a factor in the pathogenesis of sarcoidosis.

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