

# Absence of DNA from Mycobacteria of the *M. tuberculosis* Complex in Sarcoidosis

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To evaluate the role of mycobacterial infection in the pathogenesis of sarcoidosis, several groups have attempted to identify mycobacterial DNA in clinical samples from these patients by polymerase chain reaction (PCR), but widely divergent results have been obtained. It has been suggested that differences in the sensitivity of the procedures used may explain these discrepant results. To test this possibility, the presence of mycobacterial DNA was sought in biopsies from patients with sarcoidosis using sequence capture-PCR, a procedure that is 100-fold more sensitive in detecting mycobacterial DNA in paucibacillary samples than standard PCR protocols. Using this approach, DNA corresponding to two different sequences specific for organisms of the *Mycobacterium tuberculosis* complex (the IS6110 insertion element and the DR region) could not be detected in any of the 15 biopsies from patients with sarcoidosis, whereas a high proportion of positive results was obtained for tissue biopsies and other clinical samples from patients with active tuberculosis, including samples that were smear-negative/culture-positive and smear-negative/culture-negative. These results support prior studies suggesting that *M. tuberculosis* does not play a pathogenic role in sarcoidosis in most patients. Vokurka M, Lecossier D, du Bois RM, Wallaert B, Kambouchner M, Tazi A, Hance AJ. Absence of DNA from mycobacteria of the *M. tuberculosis* complex in sarcoidosis.

AM J RESPIR CRIT CARE MED 1997;156:1000-1003.

Sarcoidosis is a systemic disease of unknown etiology characterized by the presence of noncaseating granulomas at sites of disease involvement (1, 2). Similarities in the clinical, pathological, and immunologic abnormalities seen in sarcoidosis and certain patients with tuberculosis have raised suspicion that mycobacterial infection could play a role in the pathogenesis of this disorder (1-3). The identification of acid-fast organisms in sarcoid tissues and the isolation of mycobacteria and mycobacteriophages from clinical specimens obtained from these patients have been reported, although these studies have generally been difficult to confirm (2, 3).

Recently, several groups have sought evidence for the presence of mycobacterial DNA in clinical samples from sarcoid patients using procedures based on the polymerase chain reaction (PCR). Techniques permitting the specific detection of *Mycobacterium tuberculosis* have been used in most studies. The results of these studies have been quite divergent. Thus, although some authors have found DNA from *M. tuberculosis* to be present in approximately half of samples from patients

with sarcoidosis (4, 5), others have reported that DNA from *M. tuberculosis* was found in only occasional samples (6-9) or could not be detected (10, 11). The reasons for these discrepancies are not known. A variety of technical factors has been identified that can strongly influence the sensitivity of PCR-based tests (3). If mycobacteria were present in small numbers in sarcoid lesions, small differences in the sensitivity of the PCR procedures used could have an important effect on the proportion of positive results obtained.

In particular, clinical samples from patients with sarcoidosis used in these studies (e.g., tissue biopsies, cells recovered by bronchoalveolar lavage) contain large numbers of immune and inflammatory cells, a source of large amounts of DNA. Because excess nontarget DNA can inhibit PCR, only a small aliquot of DNA extracted from these samples can be used in a single reaction, thereby limiting overall sensitivity. Recently, we have developed a procedure called sequence capture-PCR, in which mycobacterial DNA in clinical samples is specifically captured prior to amplification, thereby eliminating nontarget DNA and other inhibitors of the PCR reaction (12). Thus, all of the mycobacterial DNA in a given sample can be amplified in a single reaction, an approach that results in up to a 100-fold increase in sensitivity for the detection of mycobacterial DNA in paucibacillary samples. In order to determine whether the improved sensitivity offered by this technique would permit the detection of mycobacterial DNA in sarcoidosis, we have used sequence capture-PCR to evaluate tissue biopsies from patients with sarcoidosis and compared the results to those

(Received in original form December 5, 1996 and in revised form March 5, 1997)

Supported in part by a grant from the Caisse Nationale de l'Assurance Maladie des Travailleurs Salariés (CNAMTS).

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Am J Respir Crit Care Med Vol. 155. pp. 1000-1003, 1997

obtained for biopsies from patients with tuberculosis and diseases in which mycobacteria do not play a pathogenic role.

## METHODS

### Tissue Specimens

Tissue biopsies obtained in the course of diagnostic evaluation were used. Both formalin-fixed paraffin-embedded archival material and tissue fragments immediately frozen in liquid nitrogen were evaluated as indicated below. Fifteen biopsies from patients with sarcoidosis were evaluated (lung [n = 5], thoracic lymph node [n = 10]). Each biopsy was from a different patient; five biopsies had been formalin fixed. Sarcoidosis was diagnosed according to previously described criteria, which included the presence of typical noncaseating granulomas in the tissue biopsies evaluated in this study (6). Five biopsies from five different patients with tuberculosis were evaluated (all cervical, mediastinal, or subclavicular lymph nodes); four biopsies had been formalin fixed. The diagnosis of tuberculosis was established on the basis of positive culture(s) for *M. tuberculosis* in all cases. Twenty biopsies from patients undergoing evaluation for other lung pathologies were used as controls (lung [n = 15], thoracic lymph node [n = 5]). Each biopsy was from a different patient; five biopsies had been formalin fixed. Final diagnoses in these patients were: bronchogenic carcinoma (n = 13), histiocytosis X (n = 2), hypersensitivity pneumonitis (n = 2), Wegener's granulomatosis, bronchiolitis obliterans, breast carcinoma (1 each).

### Sequence Capture-PCR

Sequence capture-PCR was performed as previously described (12). Briefly, following xylene extraction (paraffin-embedded specimens only), 8 mm<sup>3</sup> tissue fragments were suspended in 0.5 ml of 100 mM TRIS-HCl containing 150 mM NaCl and 50 mM EDTA (pH 7.4), and transferred to 2-ml screw cap tubes containing 0.5 ml of 0.1-mm diameter glass microspheres (Biospec Products, Bartlesville, OK) and 50  $\mu$ l of 20 mg/ml proteinase K (Interchim, Montluçon, France). Samples were agitated (Mini-BeadBeater; Biospec Products) for 50 s, allowed to digest overnight at 50° C (Thermomixer; Eppendorf, Hamburg, Germany), and agitated again for 50 s. The supernatant was recovered by centrifugation and DNA content measured. Aliquots containing up to 750  $\mu$ g DNA were transferred to a 1.5-ml Eppendorf tube, heated to 100° C for 10 min, cooled to 4° C, and 0.2 ml 3.75 M NaCl and 2.5 pmol each biotinylated capture oligonucleotides Cap-1 and Cap-2 (12) were added (final volume 0.75 ml in 1 M NaCl). The tube was incubated with agitation at 60° C for 3 h to allow hybridization. Ten microliters of M-280 Streptavidin Dynabeads (Dyna, Oslo, Norway), washed according to the manufacturer's instructions, were then added, and the incubation was continued for 2 h at 20° C. Magnetic beads were captured (Dyna magnetic particle concentrator; Dynal), washed twice with 10 mM TRIS-HCl/0.1 mM EDTA (pH 8), and resuspended in 10  $\mu$ l of water. Capture of the DR region was performed using analogous techniques, except that the Cap-DRa and Cap-DRb oligonucleotides were used and hybridization was performed at 42° C. PCR and detection of amplified products were performed as previously described (12).

Groups of samples were processed in parallel during all steps in the procedure (solubilization of DNA, purification of DNA by sequence capture, amplification and detection of mycobacterial DNA). Each group contained a sample from one (n = 13) or two (n = 1) patients with sarcoidosis, samples from two different control subjects, a negative control sample composed of fragments of rat spleen (to ensure that samples were not being contaminated during processing), and a positive control (control sample to which the equivalent of 10 genomes of purified DNA from *M. tuberculosis* H37Rv had been added prior to processing). In some groups (n = 5), a sample from a patient with tuberculosis was included. During the amplification reaction, additional negative control (no added DNA) and positive control samples (500 ng of purified human DNA containing 0.1 and 1 genome of DNA from *M. tuberculosis* H37Rv) were added. For each sample, two PCR reactions were performed (each using half of the captured DNA); the sample was considered positive if mycobacterial DNA was amplified in one or both of the PCR reactions. In the course

of these studies, control samples to which 10 genomes of *M. tuberculosis* DNA were added were positive by sequence capture-PCR for 13/14 (IS6110) and 14/14 (DR sequence) series, respectively.

## RESULTS

The combination of mechanical disruption and proteolytic digestion was used to solubilize DNA present in tissue biopsies of granulomatous lesions from 15 different sarcoid patients. Sequence capture-PCR was then used in an attempt to identify the presence of two different sequences specifically present in mycobacteria belonging to the *M. tuberculosis* complex in two equal aliquots of this DNA ( $428 \pm 246$   $\mu$ g DNA/aliquot). In no case was DNA from the DR region of *M. tuberculosis* detected in these samples by PCR (Table 1). Similarly, all samples were negative for the IS6110 insertion element.

The sensitivity of sequence capture-PCR for the detection of mycobacterial DNA in clinical specimens from patients with tuberculosis is summarized in Table 2. Biopsies from patients with tuberculosis were positive in 5/5 cases (amplification of DR region) and 4/5 cases (amplification of IS6110), despite that acid-fast organisms were identified in Kinyoun-stained sections from only one specimen. We have also evaluated sequence capture-PCR in the detection of *M. tuberculosis* complex DNA in pleural fluid and bronchoalveolar lavage fluid obtained from patients with tuberculosis. These studies (12, 13), which were performed contemporaneously with the evaluation of the sarcoid biopsies reported here, also demonstrate that this technique is highly sensitive in detecting DNA from *M. tuberculosis*, including both smear-negative/culture-positive and smear-negative/culture-negative specimens from patients with tuberculosis (Table 2).

False-positive results were not observed during these studies. The inclusion of negative controls during all steps in the procedure demonstrated that contamination of samples was not occurring during processing. It is noteworthy that all 20 biopsy specimens (including 15 lung biopsies) from patients with diseases other than active tuberculosis were negative for *M. tuberculosis* DNA.

## DISCUSSION

Quite discrepant results have been reported for the detection of DNA from *M. tuberculosis* in specimens from patients with sarcoidosis using PCR. A variety of technical factors can influence the sensitivity of detection of mycobacterial DNA by this approach (3), and it has been suggested that such differences may explain why only some laboratories have detected mycobacterial DNA in specimens from sarcoid patients (14). Our results do not support this idea. The sequence capture proto-

TABLE 1  
DETECTION OF DNA FROM *M. tuberculosis* IN TISSUE BIOPSIES BY SEQUENCE CAPTURE-PCR

	Amplification of DR Region		Amplification of IS6110	
	Positive	Negative	Positive	Negative
Sarcoidosis*	0	18	0	18
Tuberculosis	5	0	4	1
Controls†	0	27	0	27

\* Fifteen biopsies, each from a different patient, were studied; in three cases, two samples from the same biopsy were used.

† Twenty biopsies, each from a different patient, were studied; in some cases, two (n = 3) or three samples (n = 2) from the same biopsy were used.

TABLE 2  
DETECTION OF DNA FROM *M. tuberculosis* IN SAMPLES FROM PATIENTS  
WITH TUBERCULOSIS BY SEQUENCE CAPTURE-PCR\*

Target Sequence and Source of Specimens	PCR Positive Specimens/Total Specimens		
	AFB Stain +	AFB Stain -/Culture +	AFB Stain -/Culture -
DR Region			
Tissue biopsies	1/1	4/4	—
Pleural fluids <sup>†</sup>	—	3/3	9/12
IS6110			
Tissue biopsies	1/1	3/4	—
Pleural fluids <sup>†</sup>	—	3/3	10/14
Bronchoalveolar lavage cells <sup>†</sup>	—	3/3	6/6

\* The indicated clinical specimens from patients with tuberculosis were evaluated both by standard techniques (microscopic detection of acid-fast bacteria [AFB] and mycobacterial culture) and by sequence capture-PCR.

<sup>†</sup> Results for pleural fluids and bronchoalveolar lavage cells have been previously published (references 12 and 14, respectively); the criteria used for patient selection and diagnosis are presented in the original references. For each patient, a single specimen was evaluated by sequence capture-PCR. Smear-negative/culture-negative specimens were obtained from patients with tuberculosis diagnosed on the basis of positive culture results for other specimens (n = 11), the presence of caseating granulomas in tissue biopsies (n = 8), or response to a clinical trial of anti-tuberculous therapy (n = 1) (12, 13).

col used here can detect as little as 1–10 genomes of mycobacterial DNA in samples containing 750 µg of total DNA, a finding confirmed here. This sensitivity (< 1 organism/10<sup>7</sup> human cells) is approximately 100-fold greater than obtained in our previous study (6), in which total DNA was amplified without prior enrichment for mycobacterial sequences. Nevertheless, mycobacterial DNA from organisms of the *M. tuberculosis* complex was never detected in biopsies from patients with sarcoidosis. Our ability to obtain a high proportion of positive results for clinical specimens from patients with paucibacillary forms of tuberculosis, including samples from these patients that were negative by both smear and culture, further supports the high sensitivity of sequence capture-PCR (12, 13). Using this approach, our laboratory was one of the few that obtained perfect sensitivity and specificity in the blinded evaluation of specimens containing known numbers of mycobacteria (15). Thus, it is difficult to attribute our negative results for biopsies from sarcoid patients to poor sensitivity of the methods used.

An alternative explanation for the discrepant results concerning the presence of mycobacterial DNA in sarcoid tissues is inadvertent contamination of samples in some series. It is noteworthy that the studies showing the highest proportion of positive results for patients with sarcoidosis also reported a relatively high proportion of positive results for samples from control subjects and/or individuals with prior tuberculous infections (3). In our study, numerous precautions were taken to prevent contamination, including the use of dUTP/uracil-*N*-glycosylase to prevent false-positive results due to carryover of amplified products from prior amplifications (16). The use of negative controls throughout the procedure proved that contamination was not occurring during the processing or amplification of the samples. Despite using the highly sensitive sequence capture-PCR protocol, we did not detect DNA from *M. tuberculosis* in lung or lymph node biopsies from patients other than those with active tuberculosis. We were also unable to detect mycobacterial DNA in cells recovered by bronchoalveolar lavage from patients with evidence of prior tuberculous infection (13). Thus, our findings do not support the idea that detection of *M. tuberculosis* DNA in specimens from patients' inactive tuberculosis is a frequent occurrence.

It should be remembered that although DNA from *M. tuberculosis* has not been detected in samples from most patients with sarcoidosis, our group and others occasionally have reported positive results in patients (reviewed in 3, 17). Thus,

some patients diagnosed as having sarcoidosis by current standards may indeed have a disease initiated by *M. tuberculosis* infection. It should also be stressed that the techniques used in this study only permit the detection of DNA from organisms of the *M. tuberculosis* complex. A potential role for other mycobacterial species in the pathogenesis of sarcoidosis has been invoked (3, 17); the use of PCR techniques with improved sensitivity, such as those employed here, may be useful in the further evaluation of this possibility.

In summary, DNA from mycobacteria of the *M. tuberculosis* complex was not detected in biopsies from patients with sarcoidosis, despite using the highly sensitive sequence capture-PCR protocol. These results support prior studies suggesting that *M. tuberculosis* does not play a pathogenetic role in most patients with sarcoidosis.

## References

1. Scadding, J. G., and D. N. Mitchell. 1985. Sarcoidosis, 2nd ed. Chapman and Hall, London. 546–562.
2. Thomas, P. D., and G. W. Hunninghake. 1987. Current concepts of the pathogenesis of sarcoidosis. *Am. Rev. Respir. Dis.* 135:747–760.
3. Mangiapan, G., and A. J. Hance. 1995. Mycobacteria and sarcoidosis: an overview and summary of recent molecular biological data. *Sarcoidosis* 12:20–37.
4. Saboor, S. A., N. M. Johnson, and J. McFadden. 1992. Detection of mycobacterial DNA in sarcoidosis and tuberculosis with polymerase chain reaction. *Lancet* 339:1012–1015.
5. Fidler, H. M., G. A. Rook, N. M. Johnson, and J. McFadden. 1993. Mycobacterium tuberculosis DNA in tissue affected by sarcoidosis. *B.M.J.* 306:456–549.
6. Bocart, D., D. Lecossier, A. de Lasseuse, D. Valeyre, J. P. Battesti, and A. J. Hance. 1992. A search for mycobacterial DNA in granulomatous tissues from patients with sarcoidosis using the polymerase chain reaction. *Am. Rev. Respir. Dis.* 145:1142–1148.
7. Thakker, B., M. Black, and A. K. Foulis. 1992. Mycobacterial nucleic acids in sarcoid lesions. *Lancet* 339:1537.
8. Popper, H. H., E. Winter, and G. Höfler. 1994. DNA of *Mycobacterium tuberculosis* in formalin-fixed, paraffin-embedded tissue in tuberculosis and sarcoidosis detected by polymerase chain reaction. *Am. J. Clin. Pathol.* 101:738–741.
9. Richter, E., U. Greinert, D. Kirsten, S. Rusch-Gerdes, C. Schluter, M. Duchrow, J. Galle, H. Magnussen, M. Schlaak, H. D. Flad, and J. Gerdes. 1996. Assessment of mycobacterial DNA in cells and tissues of mycobacterial and sarcoid lesions. *Am. J. Respir. Crit. Care Med.* 153:375–380.
10. Gerdes, J., E. Richter, S. Rüscher-Gerdes, U. Greinert, J. Galle, M.

- Schlaak, H.-D. Flad, D. Kirsten, and H. Magnussen. 1992. Mycobacterial nucleic acids in sarcoid lesions. *Lancet* 339:1536-1537.
11. Ghossein, R. A., D. G. Ross, R. N. Salomon, and A. R. Rabson. 1994. A search for mycobacterial DNA in sarcoidosis using the polymerase chain reaction. *Am. J. Clin. Pathol.* 101:733-737.
  12. Mangiapan, G., M. Vokurka, L. Schouls, J. Cadranel, D. Lecossier, J. van Embden, and A. J. Hance. 1996. Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens. *J. Clin. Microbiol.* 34:1209-1215.
  13. Brugière, O., M. Vokurka, D. Lecossier, G. Mangiapan, A. Amrane, B. Milleron, C. Mayaud, J. Cadranel, and A. J. Hance. 1997. Diagnosis of smear-negative pulmonary tuberculosis using sequence capture-PCR. *Am. J. Respir. Crit. Care Med.* 155:1478-1481.
  14. Fidler, H. M., G. A. W. Rook, N. M. Johnson, and J. J. McFadden. 1993. Search for mycobacterial DNA in granulomatous tissues from patients with sarcoidosis using the polymerase chain reaction. *Am. Rev. Respir. Dis.* 147:777.
  15. Noordhoek, G. T., J. D. A. van Embden, and A. H. J. Kolk. 1996. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among 30 laboratories. *J. Clin. Microbiol.* 34:2522-2525.
  16. Longo, M. C., M. S. Berninger, and J. L. Hartley. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93:125-128.
  17. Hance, A. J. 1997. The role of mycobacteria in the pathogenesis of sarcoidosis. *Semin. Respir. Infect.* (In press)