Molecular Analysis of Sarcoidosis Granulomas Reveals Antimicrobial Targets

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Abstract

Sarcoidosis is a granulomatous disease of unknown cause. Prior molecular and immunologic studies have confirmed the presence of mycobacterial virulence factors, such as *catalase peroxidase* and *superoxide dismutase A*, within sarcoidosis granulomas.

Molecular analysis of granulomas can identify targets of known antibiotics classes. Currently, major antibiotics are directed against DNA synthesis, protein synthesis, and cell wall formation. We conducted molecular analysis of 40 sarcoidosis diagnostic specimens and compared them with 33 disease control specimens for the presence of mycobacterial genes that encode antibiotic targets. We assessed for genes involved in DNA synthesis (*DNA gyrase A* [*gyrA*] and *DNA gyrase B*), protein synthesis (*RNA polymerase subunit β*), cell wall synthesis (*embCAB operon* and *enoyl reductase*), and *catalase peroxidase*. Immunohistochemical analysis was conducted to investigate the locale of mycobacterial genes such as *gyrA* within 12 sarcoidosis specimens and 12 disease controls. Mycobacterial DNA was detected in 33 of 39 sarcoidosis specimens by quantitative real-time polymerase chain reaction compared with 2 of 30 disease control specimens ($P < 0.001$, two-tailed Fisher's test). Twenty of 39 were positive for three or more mycobacterial genes, compared with 1 of 30 control specimens ($P < 0.001$, two-tailed Fisher's test).

Immunohistochemistry analysis localized mycobacterial *gyrA* nucleic acids to sites of granuloma formation in 9 of 12 sarcoidosis

specimens compared with 1 of 12 disease controls ($P < 0.01$). Microbial genes encoding enzymes that can be targeted by currently available antimycobacterial antibiotics are present in sarcoidosis specimens and localize to sites of granulomatous inflammation. Use of antimicrobials directed against target enzymes may be an innovative treatment alternative.

Keywords: sarcoidosis; levofloxacin; ethambutol; azithromycin; rifampin

Clinical Relevance

To our knowledge, this is the first investigation for evidence of multiple mycobacterial products within sarcoidosis granulomas that can be targeted by currently available antimicrobial therapies. Detection of genetic material encoding enzymes essential for DNA synthesis, protein synthesis, cell wall synthesis, and management of oxidative stress may influence the improvements in prognosis after 8 weeks of the concomitant levofloxacin, ethambutol, azithromycin, and rifampicin (CLEAR) regimen. Use of currently available antimicrobials to target enzymes of microbial gene products detected in >80% of sarcoidosis specimens may provide an innovative treatment alternative.

Sarcoidosis is a multisystem disorder characterized by the formation of noncaseating epithelioid granulomas. Although the cause of sarcoidosis remains

elusive, a growing body of literature from independent laboratories supports an association between pathogenic mycobacteria or their antigens and

sarcoidosis pathogenesis. Molecular analysis of sarcoidosis granulomas for mycobacterial *16S rRNA* and *RNA polymerase subunit β* (*rpoB*) revealed novel mycobacterial

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sequences consistent with members of *Mycobacterium tuberculosis* complex (MTB) (1). Sequencing has also been used to detect *Mycobacterium avium* IS1110 (2). Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Song and colleagues found MTB catalase peroxidase (KatG) peptides in 75% of sarcoidosis specimens compared with 14% of control specimens ($P = 0.0006$); *in situ* hybridization localized MTB *katG* and 16S rRNA to the inside of sarcoidosis granulomas (3). Analysis of Polish sarcoidosis lymph nodes revealed MTB complex heat-shock protein (hsp) 70, hsp65, and hsp16 (4). Mycobacterial virulence factors such as ESAT-6, KatG, superoxide dismutase A, and antigen 85A have been identified as targets of the local and systemic adaptive immune response in patients with sarcoidosis (5–10).

More recently, case reports and clinical trials from Japanese, European, and American investigators have emerged regarding the efficacy of antimicrobials against *Mycobacterium* species on pulmonary and cutaneous sarcoidosis. In a randomized trial, concomitant levofloxacin, ethambutol, azithromycin, and rifampicin (CLEAR) antimycobacterial therapy, targeting gyrase A (GyrA), arabinosyltransferase B (EmbB), 50S ribosomal subunit, and RNA polymerase, respectively, decreased chronic cutaneous sarcoidosis lesion diameter and severity when compared with placebo (11). The CLEAR regimen was associated with an FVC increase of 0.23 liters at 4 weeks and 0.42 liters at 8 weeks ($P = 0.0098$ and 0.016 , respectively); the 6-minute walk distance increased by 87 m from baseline to 8 weeks ($P = 0.0078$) (12).

To determine if the improvements in prognosis after 8 weeks of the CLEAR regimen are influenced by exposure to mycobacteria, we conducted a molecular analysis of subjects with sarcoidosis before therapeutic intervention specifically for the genes *gyrA*, gyrase B (*gyrB*), *embCAB*, enoyl reductase (*inhA*), and *rpoB*. To our knowledge, this is the first investigation into the presence of multiple mycobacterial genes within sarcoidosis granulomas that can be targeted by currently available antimicrobial therapies.

Materials and Methods

Subjects

For inclusion, the diagnosis of sarcoidosis was defined according to American

Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and Other Granulomatous Disorders diagnostic criteria (13). Fifty-two sarcoidosis diagnostic specimens and 39 disease controls were obtained from the Cooperative Human Tissue Network or from local hospitals. Disease controls were tissues obtained from patients for whom sarcoidosis was a diagnostic consideration; however, alternative diagnoses, such as chronic beryllium disease, fungal infection, and lymphoma, were obtained. There were no distinctions in the storage methods for the tissues of subjects with sarcoidosis and those of the disease control subjects. DNA from sarcoidosis or control specimens was eliminated for the following reasons: DNA quantity <365 ng; DNA spectrophotometer 260:280 ratio <1.5 because of poor quality DNA; *gapDH* CT values ≥ 25 because of the potential presence of polymerase chain reaction (PCR) inhibitors; and CT values > 40 . This resulted in further analysis of 39 sarcoidosis specimens and 30 control specimens by PCR analysis. Residual slides of 12 sarcoidosis and 12 disease control specimens reflecting four different granulomatous diseases and one nongranulomatous disease were selected on the basis of availability; *in situ* hybridization was conducted in a blinded fashion (see Table E1 in the online supplement).

Lysis of Tissues and DNA Isolation

Contamination was minimized by isolating DNA in a dedicated, sterile room, with a new blade for each subject, and by conducting the molecular analysis in a separate room. DNA was isolated using modified instructions from QIAGEN DNeasy Blood and Tissue kit (Venlo, the Netherlands), as outlined in the online supplement. DNA was quantified using a NanoDrop DN-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Real-Time PCR

Primer Express 3.0–designed single-tube primers (Life Technologies, Carlsbad, CA) for *rpoB*, *katG*, *gyrA*, *gyrB*, *inhA*, *embC*, and *GapDH* efficiencies were evaluated using *M. tuberculosis* H37Ra strain 25177 because of the conservation of our target genes among H37Ra and H37Rv (Microbiologics, St. Cloud, MN) (Table E2) (14). Primers were also validated with *Mycobacterium abscessus*, *M. avium*, and *Mycobacterium chelonae*, *chimaera*, *fortuitum*, *gordonae*,

and *kansasii*. Assessment for pathogenic microbial DNA was confirmed using TaqMan Gene Expression Assays (Life Technologies) for the *femA* gene of *Staphylococcus aureus* (pa04230918_s1) and for *HIV2-LTR* of HIV 2 (Pa03453415_s1) as positive and negative controls, respectively. Real-time PCR was performed in 20 μ l reactions on a StepOnePlus sequence detection system (Applied Biosystems, Foster City, CA) with 365 ng of genomic DNA for 50 fast-ramp amplification cycles. Nuclease-free water, or Buffer AE (QIAGEN) was run in parallel on each plate as negative controls, and *GapDH* was run in tandem with each subject as a positive control. Generation of standard curves is outlined in the online supplement.

RNAscope

RNAscope 2.0 Assay's HD Detection Kit (Red) protocol (Hayward, CA) (15) was performed on 12 sarcoidosis, 12 disease control (negative controls), and 7 acid-fast bacilli (AFB) MTB culture–positive specimens from HIV-positive subjects presenting with lymphadenitis (positive controls), with supplemental bacterial lysing steps as outlined in the online supplement.

Statistical Analysis

Differences between groups were assessed using Fisher's exact test and GraphPad Prism 6 software (La Jolla, CA) for comparative analysis, a two-tailed *t* test probability. A *P* value < 0.05 was considered statistically significant.

Results

Demographics of Study Specimens

Comparisons of race, age, and site of acquisition for pathologic diagnosis revealed no significant differences between the sarcoidosis and disease control cohorts. The majority of the specimens from both the sarcoidosis and the disease control cohorts were obtained from the lymph nodes or from the lung (Table 1). The 33 disease controls specimens' diagnoses were as follows: adenocarcinoma (12), squamous cell carcinoma (7), histoplasmosis (4), chronic beryllium disease (3), blastomycosis (1), Hodgkin's lymphoma (2), chronic granulomatous disease (1), mantle cell lymphoma (1), nonspecific interstitial pneumonia (1), and cholangiocarcinoma (1).

Table 1. Demographics of Sarcoidosis and Disease Control Populations

	Sample Distribution		P Value
	Sarcoidosis	Disease Control	
Total subjects	40	33	
qRT-PCR	39	30	
RNASeScope	12	12	
Sex			
Female	28 (70)	12 (36)	0.005
Male	12 (30)	21 (64)	0.005
Race			
African American	12 (29)	6 (18)	NS
White	27 (68)	21 (64)	NS
Unknown	1 (3)	6 (18)	
Age, yr			
Range	14 to 79	15 to 82	
Mean	48	56	NS
Biopsy			
Lymph node	24 (60)	14 (42)	NS
Lung	12 (30)	17 (52)	NS
Liver	2 (5)	1 (3)	NS
Spleen	1 (2.5)	1 (1)	NS
Placenta	1 (2.5)		

Definition of abbreviations: NS, not significant; qRT-PCR, quantitative real-time PCR.

Data are presented as No. (%) unless indicated otherwise. *P* values obtained with Fisher's two-tailed *t* test probability using sarcoidosis (*n* = 40) and disease controls (*n* = 33).

There was no significant difference in the geographic distribution of lymph nodes or lung specimens between the cohorts. In addition, 29 of 40 sarcoidosis specimens

and 20 of 33 disease control specimens were obtained either from local hospitals or from Cooperative Human Tissue Network sites that acquired specimens from the

Figure 1. Detection of pathogenic microbial DNA in sarcoidosis and disease control specimens. Detection of positive control *Staphylococcus aureus femA* gene, negative control *HIV2-LTR* gene, and *Mycobacterium species* genes by real-time polymerase chain reaction of DNA isolated from subjects with sarcoidosis and disease control subjects. There was no significance between both cohorts for the presence of staphylococcal and HIV DNA, but there was significance for mycobacterial nucleic acids (*P* < 0.001). NS, no significance; *S. aureus*, *Staphylococcus aureus*.

southeastern region, thus reflecting no significant regional difference in the sites from which the specimens were obtained. There was a significant difference according to sex in that 12 of 40 sarcoidosis specimens were obtained from males, compared with 21 of 33 disease control specimens (*P* < 0.01, two-tailed Fisher's test) (Table 1).

Real-Time PCR Reveals No Distinctions in Respiratory Microbial DNA but Distinctions in Mycobacterial DNA Are Present

To confirm that microbial DNA was present in both cohorts, an analysis for the presence of respiratory flora DNA was conducted by assessing for the presence of the staphylococcal *femA* gene. Staphylococcal DNA was present in 100% of the disease controls and sarcoidosis specimens (*P* = 1.0) (Figure 1). Investigation for nonrespiratory DNA was performed by assessing for the presence of HIV DNA, specifically *HIV2-LTR* DNA. None of the sarcoidosis or disease control specimens were positive for HIV DNA (*P* = 1.0) (Figure 1). Thus, there was no distinction between the sarcoidosis specimens and the control specimens regarding the presence of respiratory flora DNA or HIV DNA. Molecular analysis for mycobacterial DNA was conducted to a sensitivity of <10 genome copies. Distinctions for the presence of mycobacterial DNA between the sarcoidosis and the disease control specimens were present. At least one mycobacterial gene was detected in 33 of 39 sarcoidosis specimens (84%), compared with 2 of 30 disease control specimens (7%) (*P* < 0.001) (Figure 1).

Antimycobacterial Genetic Targets Are Distinctly Present within Sarcoidosis Granulomas Compared with Disease Controls

Mycobacterial *rpoB* DNA was detected in 16 of 39 sarcoidosis specimens, compared with 1 of 30 disease control specimens (*P* < 0.001) (Figure 2A). *KatG* DNA was detected in 23 of 39 sarcoidosis specimens, compared with 2 of 30 disease controls (*P* < 0.001) (Figure 2B). *GyrA* DNA was present in 20 of 39 sarcoidosis specimens, compared with 1 of 30 disease controls (*P* < 0.001) (Figure 2C); *gyrB* DNA was detected in 14 of 39 sarcoidosis specimens, compared with 1 of 30 disease controls (*P* < 0.001) (Figure 2D). *InhA* DNA was detected in 23 of 39 sarcoidosis specimens,

Figure 2. Investigation of genes that encode targets for antimycobacterial therapy. As determined by real-time polymerase chain reaction, the percentage of sarcoidosis and disease control subjects positive for (A) *rpoB*, (B) *katG*, (C) *gyrA*, (D) *gyrB*, (E) *inhA*, and (F) *embB*. P values are as indicated in figure. *embB*, arabinosyltransferase B; *gyr*, gyrase; *inhA*, enoyl reductase; *katG*, catalase peroxidase; *rpoB*, RNA polymerase subunit β .

compared with 2 of 30 disease controls ($P < 0.001$) (Figure 2E). *EmbC* DNA was present in 16 of 39 sarcoidosis specimens compared with 1 of 30 disease controls ($P < 0.001$) (Figure 2F).

Of note, most of the sarcoidosis specimens were positive for two or more mycobacterial DNA. At least two mycobacterial genes were detected in the

2 of 30 disease control specimens positive for mycobacterial DNA, compared with 24 of the 39 sarcoidosis specimens (62%) ($P < 0.001$) (Figure 3). Of the 33 sarcoidosis specimens that were positive for mycobacterial genes, one gene was detected in 9 specimens and accounted for 23% of the total sarcoidosis population. Two genes were detected in four specimens

(10%), three genes in three specimens (8%), four genes in seven specimens (18%), and five genes in one specimen (3%), and all six target genes were present in nine specimens (23%) (Figure 3). Specimens in which *katGA* and *katGB* were both detected were counted as one gene so as to not introduce bias by double counting the same gene.

Figure 3. Assessment for the presence of multiple mycobacterial genes within sarcoidosis and disease control specimens. Sarcoidosis and disease control subjects in whom mycobacterial DNA was detected were more likely to be positive for multiple genes than for a single gene when using real-time polymerase chain reaction.

In Situ Hybridization Localizes Mycobacterial DNA to Sites within and outside Sarcoidosis Granulomatous Inflammation

To localize the mycobacterial DNA that were detected by PCR analysis in the specimens, residual lung, lymph node, and liver tissues were subject to RNAscope in-situ hybridization targeting *gyrA*. Slides of lymph nodes infected with MTB served as the positive control (Figure 4A); slides of chronic granulomatous disease served as the negative control (Figure 4B). RNAscope control slides for human HeLa cell pellets were hybridized with a negative control probe corresponding to *dihydrodipicolinate reductase* for *Bacillus subtilis* and were used to assess background signals, whereas a positive control targeting the constitutively expressed *Homo sapiens Cyclophilin B* was used to assess assay optimization (data not shown). In sarcoidosis tissues, *gyrA* was detected within sites of granulomatous involvement with lymph nodes, as well as outside sites of granulomatous involvement (Figure 4C). Overall, there was a significant difference in the detection of mycobacterial nucleic acids in the sarcoidosis specimens, compared with the disease control specimens ($P < 0.01$) (Figure 4D). Detection intensity of positive sarcoidosis specimens was noted to be higher than that of the lone positive disease control, and more in line with the positivity of AFB

specimens. The intensity of all specimens was more localized to granulomatous inflammation, but not exclusively.

Discussion

To the best of our knowledge, this is the first investigation to present molecular evidence that multiple mycobacterial genes are present in sarcoidosis specimens. These genes encode enzymes that can be targeted by currently available antimicrobial therapy. Immunohistochemistry localized the gene encoding one of these targets to sites within, as well as outside, granulomatous inflammation within sarcoidosis specimens. The presence of mycobacterial DNA was demonstrated in 84% of sarcoidosis specimens compared with 7% of disease controls. In addition to the presence of mycobacterial DNA in the majority of specimens, the presence of multiple genes provides important information regarding the pathogenesis of the mycobacterial species (Figure 3). The observation of multiple mycobacterial genes within sarcoidosis specimens does not delineate active infection; however, it supports the idea that patients with sarcoidosis have at least had prior exposure to mycobacteria. The lower frequency within disease control specimens suggests that environmental contamination is a less likely contributor to the presence of mycobacterial DNA.

The primers used to target the sequences have 100% sequence homology with MTB. A limitation of the study is that we were unable to speciate which mycobacteria were present. Previous studies have identified DNA of *M. gordonae*, *M. kansasii*, and a novel *Mycobacterium* species within sarcoidosis granulomas (1). In addition, *M. avium* has been detected in sarcoidosis lesions (2, 16). Molecular determination of the mycobacterial species present within sarcoidosis granulomas has been reported previously (1). We conducted molecular analysis of DNA from multiple nontuberculous mycobacteria to determine the potential of the primers to amplify DNA from nontuberculous mycobacterial species. Although *M. abscessus*, *chimaera*, *chelonae*, and *avium* were not detected, it was possible to detect *M. kansasii* with *inhA*, *M. fortuitum* with *gyrB* and *inhA*, and *M. gordonae* with *rpoB* and *inhA* (data not shown). The current data do not delineate from which mycobacterial species the DNA is derived, but rather that multiple mycobacterial genes which products can be targeted therapeutically are present within sarcoidosis granulomas.

Although the detection of mycobacterial DNA does not prove that viable organisms are present within sarcoidosis granulomas, it does enhance our understanding of the pathogenic potential of the mycobacteria to which subjects with sarcoidosis were exposed (Figure 3). The β -subunit of RNA polymerase encoded by the *rpoB* gene is critical to mycobacterial gene transcription; rifampin is used as a first-line drug against this target. The assembly of DNA *gyrA* and *gyrB* heterotetramer provides a critical facet in maintaining the topology of mycobacterial DNA at equilibrium by opposing the relaxing capabilities of topoisomerases I and IV, thereby enhancing supercoiling (17). Fluoroquinolones are used to target these enzymes. The *embCAB* operon encodes three arabinosyl transferases essential for the synthesis of cell wall components arabinogalactan and liparabinomannan. By administering ethambutol, arabinosyl transferases are inhibited and cell death ensues because of mycolic acid accumulation (18–20). *InhA*, representing 2-trans-enoyl-acyl carrier protein reductase and the target for the front-line antituberculous drug isoniazid, is involved in the activity of

Figure 4. Localization of *gyrA* by RNAscope 2.0 HD Detection kit (Red) *in situ* hybridization. *In situ* hybridization of acid-fast bacilli-positive specimens revealed localization of mycobacterial DNA encoding *gyrase A* within sites of granulomatous inflammation (A), which were absent within granulomatous sites of involvement of disease controls, such as chronic granulomatous disease (B). Analysis of sarcoidosis specimens revealed localization primarily within sites of granulomatous involvement, although detection outside granulomas was also noted (C). Overall, there was significantly higher detection within sarcoidosis specimens compared with disease control specimens ($P < 0.01$, two-tailed Fisher's test) (D). NS, not significant.

dissociative type 2 fatty acid synthase that extends associative type 1 fatty acid synthase-derived C_{20} fatty acids to form C_{60} -to- C_{90} mycolic acids (21). Mycolic acids are major constituents of the protective layer around the pathogenic mycobacteria that contribute to virulence and resistance to acid decolorization, as well as to positive coloring on AFB stain. Prior quantitative PCR analysis reveals that the numbers of bacilli present are also below the sensitivity of the AFB stain (7).

It is notable that there was detection of *inhA* in 59% of the subjects with sarcoidosis, suggesting that the use of isoniazid would be a potential therapeutic agent in these subjects, serving as the backbone of latent and active

tuberculosis therapy. Sarcoidosis and tuberculosis can coexist within the same host (22–25). Interestingly, studies have demonstrated the appearance of sarcoidosis after completion of tuberculosis therapy (26, 27). However, the ability to detect DNA encoding potential targets of antimicrobials does not ensure that such antimicrobials would be active against the bacilli. The presence of *InhA* in many nontuberculosis mycobacteria species, such as *M. avium*, that are not susceptible to isoniazid is a case in point (28).

In situ hybridization localized *gyrA* nucleic acids within the majority of sarcoidosis specimens. There was also positive localization in one of the 12 disease

control specimens. Despite evidence of histoplasma on the fungal stain, this specimen was also positive for mycobacterial DNA by PCR analysis and *in situ* hybridization. Superinfecting mycobacteria have been shown to traffic to established granulomas (29, 30). This detection of mycobacterial nucleic acids within a significantly higher percentage of sarcoidosis specimens compared with granulomatous disease controls makes superinfection by mycobacteria less likely to be the sole reason. It further confirms the findings of a prior investigation using *in situ* hybridization tyramide signal amplification that demonstrated the presence of mycobacterial *katG* nucleic acids within sarcoidosis granulomas

when compared with granulomatous and nongranulomatous disease control tissues (3). The American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and Other

Granulomatous Disorders define sarcoidosis as negative by AFB stain and culture for bacteria; the molecular and immunologic evidence for mycobacterial involvement in pulmonary sarcoidosis continues to

strengthen, necessitating that novel methods to identify bacteria be developed. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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