Recovery of Cell Wall-Deficient Organisms From Blood Does Not Distinguish Between Patients With Sarcoidosis and Control Subjects*

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Study objective: To determine if cell wall-deficient forms (CWDF) of mycobacteria can be grown in culture of blood from subjects with sarcoidosis.

Design: A special multicenter study of sarcoidosis (A Case Control Etiologic Study of Sarcoidosis), supported by the National Heart, Lung, and Blood Institute.

Patients and control subjects: Patients and control subjects were recruited at 10 institutions in the United States. Control subjects (controls) were of the same gender and race, and within 5 years of age as matching patients with sarcoidosis (cases).

Results: Cultures were incubated from 347 blood specimens (197 cases, 150 controls). Two investigators trained to recognize CWDF mycobacteria examined material obtained from culture tubes after 3 weeks. Structures thought to be CWDF were seen with equal frequency in cases (38%) and controls (41%). Thirty-nine percent of cases and 37% of controls were read as negative for CWDF.

Conclusion: This study fails to confirm earlier reports that CWDF mycobacteria can be grown from the blood of patients with sarcoidosis, but not from control subjects.

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Key words: A Case Control Etiologic Study of Sarcoidosis; cell wall-deficient forms; mycobacterium; sarcoidosis

Abbreviations: ACCESS = A Case Control Etiologic Study of Sarcoidosis; C-TASC = Clinical Trials and Surveys Corporation; CWDF = cell wall-deficient form; NHLBI = National Heart, Lung, and Blood Institute; PCR = polymerase chain reaction; VIM = veal infusion medium

Cumulative findings have implicated mycobacterial species or mycobacterial cell wall-deficient forms (CWDFs) as causative factors in the etiology of sarcoidosis. By light microscopy, investigators have detected acid-fast organisms and variably acid-fast spindle-shaped or yeast-like structures, termed pleomorphic chromogens, in lymph node tissue from subjects with sarcoidosis.1–7 Moscovic7 suggested that pleomorphic chromogens represent CWDFs (L-forms) of mycobacterial organisms based on morphologic appearance, acid-fastness, and the prevailing occurrence of lipid analogs of Much granules. In subsequent studies, Ang and Moscovic,8 using antimycobacterial polyclonal and monoclonal antibodies, concluded that laminated inclusion bodies in multinucleated giant cells (Schaumann bodies) are distinctive residual bodies of heterophagic mycobacterial derivation, and that pleomorphic chromogens are CWDFs that express specific reactivity for Mycobacterium tuberculosis complex.9 Cantwell10,11 also suggested that acid-fast organisms found in skin, lymph nodes, and lung tissue from patients with sarcoidosis were mycobacterial CWDFs.

Judge and Mattman12–14 grew an organism from the blood of approximately 80 patients with sarcoidosis in a series of studies. They concluded that the structures were mycobacterial CWDFs based on findings of variable size, predominantly coccoid

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forms, larger L forms, and short acid-fast rods. In a subsequent study, the organisms were further characterized with a monoclonal antibody with affinity for \textit{M tuberculosis} whole-wall antigen.\textsuperscript{15} Growth of organisms from the blood of 19 of 20 patients with sarcoidosis and from the blood of 0 of 20 control subjects were identified by the microscopic presence of microcolonies of CWDFs after staining culture material with a modified Kinyoun stain. All isolates reacted positively with the monoclonal antibody, suggesting that the organisms were mycobacteria or closely related species. The two investigators who assessed the antibody reactivity in that study were blinded to the clinical data, but the method of blinding, method of randomization, and degree of interobserver variability were not described.

The current study was performed as part of A Case Control Etiologic Study of Sarcoidosis (ACCESS), a National Heart, Lung and Blood Institute (NHLBI)-supported multicenter collaborative effort. The goal of ACCESS was to identify the cause of sarcoidosis by means of a rigorous, multicenter, prospective, case-controlled epidemiologic and special laboratory studies. ACCESS enrolled 736 patients with newly diagnosed sarcoidosis and 706 control subjects (resulting in 706 matched case and control pairs) between November of 1996 and June 1999. Epidemiologic, immunologic, and genetic information from patients with sarcoidosis was compared with data from control subjects matched for age, gender, and race. This report describes the special laboratory study designed to evaluate the association of mycobacterial CWDFs with sarcoidosis. The purpose was to determine if CWDFs could be prospectively identified in a blinded fashion from blood culture samples obtained from a large number of patients with newly diagnosed sarcoidosis as compared to control subjects.

**Materials and Methods**

**Patient Selection**

ACCESS was supported from 1995 to 2002 by the NHLBI. The diagnosis of sarcoidosis was established by tissue biopsy (or by a positive Kveim-Siltzbach test result among patients with erythema nodosum) within 6 months of entry. Patients with evidence of granulomatous diseases other than sarcoidosis were ineligible for enrollment in ACCESS. Control subjects were identified by random-digit telephone dialing to find persons of the same self-declared race and gender, and who were within 5 years of age of the matched patient. ACCESS enrolled patients from 10 centers that follow a large number of patients with sarcoidosis: Beth Israel Deaconess Medical Center (Boston, MA), Georgetown University (Washington, DC), Henry Ford Hospital (Detroit, MI), Johns Hopkins University (Baltimore, MD), Medical University of South Carolina (Charleston, SC), The Mount Sinai Medical Center (New York, NY), National Jewish Medical and Research Center (Denver, CO), University of Cincinnati Medical Center (Cincinnati, OH), University of Iowa, Hospital and Clinics (Iowa City, IA), and the University of Pennsylvania (Philadelphia, PA). Details of the ACCESS study design have been previously described.\textsuperscript{16} All clinical centers participating in ACCESS enrolled patients into the CWDF special laboratory study.

**Human Subjects Assurances**

Informed consent for this special laboratory study was obtained at the time patients with sarcoidosis (cases) and control subjects (controls) were enrolled in ACCESS. The primary protocol, the CWDF substudy, and all other ACCESS studies were reviewed and approved by local institutional review boards at each of the participating centers.

**Submission of Samples**

Phlebotomists were instructed to prepare skin by liberal application of polyvinylpyrrolidone iodine in an expanding concentric motion and then to allow the area to air dry. A second application of iodine was similarly applied and allowed to dry. Blood was collected in a Vacutainer tube (Becton Dickinson; Franklin Lakes, NJ) containing potassium ethylenediaminetetraacetic acid that was labeled with a code number that did not reveal the patient’s age, gender, race, or status as case or control. Tubes containing a minimum of 4 mL of blood were shipped by overnight mail at ambient temperature to the Bronx VA Medical Center. Specimens were processed for culture of CWDFs and for quality control on the day they were received. Processing of specimens for this special laboratory study continued until adequate numbers of matched case and control specimens for statistical analysis had been submitted as determined by the ACCESS clinical coordinating center at Clinical Trials and Surveys Corporation (C-TASC).

**Training of Personnel**

Cultures of mycobacterial CWDFs in previous investigations were performed in the laboratory of Dr. L. H. Mattman at Wayne State University. Before beginning ACCESS, two investigators and a technical assistant visited the laboratory of Dr. Mattman. A large number of slides previously prepared by Dr. Mattman were reviewed to acquaint the investigators with the morphologic characteristics of stained CWDF. Unstained archived slides from known cases and controls were then prepared according to protocol, and Dr. Mattman verified the investigators’ interpretations. Fresh specimens from several known sarcoidosis cases and controls were simultaneously cultured under her direction. Slides were then prepared from these fresh cultures of sarcoidosis cases and controls, and the investigators’ interpretations were again verified by Dr. Mattman. The investigators retained stained slides from the training session to use as comparative standards. Unstained slides from several known positive and control cultures were retained to use as internal process controls in future analyses.

**Media Preparation**

Modified veal infusion medium (VIM) was prepared by mixing veal infusion broth (Difco; Detroit, MI) with glycerol (2%; Fisher Scientific; Springfield, NJ) and Noble agar (0.09%; Difco). Prior to addition of Noble agar, the veal infusion broth-glycerol mixture was adjusted to a pH of 5.5. A 20% solution of yeast
Culture and Staining Procedures

Blood (1 mL) was mixed in sterile tubes with 18 mL of VIM and 1 mL of yeast extract and incubated at 36°C with constant gentle rotation. After 3 weeks, the media became cloudy. Dried smears of this material were fixed for 3 min with methanol. The methanol was then decanted, and the smears were allowed to air dry. The slides were flooded with the primary stain of modified Kinyoun carbol fuchsin for 5 min. Kinyoun carbol fuchsin stain was modified by adding 0.1 mL of 5% sodium bicarbonate to 9.9 mL of primary Kinyoun stain (Harle; Gibbston, NJ), mixing and filtering just before use. After the primary stain was decanted, the slides were flooded with 3% HCl in 95% ethanol and rocked and filtering just before use. The stained smears were placed in a slide holder (0.05% aqueous solution; Harle) for 1 min and allowed to air dry on a staining rack for 1 min to remove unbound stain. After decolorization, the slides were gently washed with cold water and drained. The slides were counterstained with metanil yellow (0.05% aqueous solution; Harle) for 1 min and allowed to air dry without blotting. The stained smears were placed in a slide holder at 45° for draining and drying.

Interpretation of Smears

All laboratory personnel were blinded to the case or control status of individual slides. Two trained investigators who were blinded to each other’s readings independently examined smears from CWDF cultures. Low magnification (original × 20) was used to locate acid-fast microcolonies. Apparent microcolonies were further evaluated at original × 400 and under oil (original × 1,000). Each investigator labeled the sample as positive or negative for the presence of microcolonies.

Quality Control Procedures

A 100-μL aliquot of each blood specimen was spread on sheep blood agar plates (BBL; BD Diagnostic Systems; Sparks, MD) to check for gross bacterial contamination. Plates were incubated and regularly examined for 2 weeks before being judged free of bacteria. Morphologically distinct visible colonies underwent Gram staining and subculturing, and were sent to the hospital clinical microbiology laboratory for speciation. Absence of iodine staining on Vacutainer vial caps (Becton, Dickinson and Co.; Franklin Lakes, NJ) was recorded as evidence of a possible departure from protocol procedures when blood specimens were being drawn. Gram stains were performed on smears of all broth cell wall-deficient subcultures to check for secondary contamination.

Statistical Methods

Sample sizes for this special laboratory substudy of ACCESS were based on the following statistical considerations. Based on published literature, we anticipated that we could find evidence of CWDFs in 50% of cases and 5 to 10% of control blood specimens. With a total of 50 cases and 50 controls, there would be statistical power at least 0.95 at α = 0.05 against an alternative hypothesis that the odds ratio is > 6 when the percentage of controls with evidence of CWDF in blood specimens is as large as 10%. Results of slide interpretation were submitted to the ACCESS clinical coordinating center at C-TASC. Readings from cases vs controls were compared with χ² tests for comparison of proportions. The degree of interobserver agreement was measured using the κ statistic. All statistical analyses were performed using SAS statistical software (Version 8; SAS Institute; Cary, NC).

Results

A total of 353 blood samples from patients with sarcoidosis and control subjects were evaluated in the research laboratory for the presence or absence of CWDFs. The results from six specimens were discarded because of gross contamination. The summary readings for the two independent viewers for the remaining 347 cultures, 197 cases and 150 controls, are shown in Table 1. Both readers agreed that the culture tubes of 38% of subjects with sarcoidosis exhibited stained structures that resembled CWDFs of mycobacterium and were classified as positive. Controls were similarly positive from 41% of cultures. Both readers labeled 39% of sarcoidosis and 37% of control samples as negative. The two readers disagreed in interpretation of slides from 23% of patients and 22% of controls (ie, one positive/one negative). Overall agreement between the two observers was 77% (95% confidence interval, 72 to 81%). The κ of 0.54 (95% confidence interval, 0.45 to 0.63) was consistent with a moderate degree of statistical agreement between reviewers.

There were 80 matched pairs of cases and controls identified among specimens submitted for CWDF culture. Setting aside the specimens with reader disagreement, the proportion of cases reported as positive was 0.53 and the proportion of positive controls was 0.54. Among 35 informative pairs (differing between case and control readings), the proportion of positive specimens was 0.49 with an odds ratio of positivity of 0.94 (95% confidence interval, 0.46 to 1.94; p = 1.00).

Discussion

The investigators of the current study did not find a difference in the frequency of reading mycobacterial CWDFs as present in blood samples of patients with recently diagnosed sarcoidosis compared with control subjects using culture methods previously demonstrated to be favorable for the growth of

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<th>Table 1—Kinyoun Stain for CWDFs*</th>
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*Data are presented as No. (%).
CWDFs. The findings contrast with those of a previous study published in 1996, reporting that CWDF had been successfully grown from the blood of 19 of 20 patients with sarcoidosis, but not from the blood of 20 control subjects. The findings also contrast with those obtained in previous studies demonstrating the presence of CWDFs in the blood of a larger number of patients with sarcoidosis but not in the blood of control subjects. One possible explanation for the divergent findings is that the current study was performed in a different laboratory by different investigators, even though two of the current investigators were trained in culture and recognition methods by one of the earlier investigators. Furthermore, several of our slides interpreted as positive for CWDFs were reviewed and Dr. Mattman concurred in the interpretation. Nonetheless, it is possible that unrecognized differences in methods of culture or interpretation affected the discrimination of microcolonies of CWDFs in the current investigation. With the exception of one report from Russia, all reports of isolation of CWDFs from the blood of patients with sarcoidosis have come from the laboratory of Dr. Mattman. It seems unlikely that our negative findings can be attributed to the fact that subjects used in the current study differed in some unidentified aspect from previous studies since the clinical criteria used the case definitions for ACCESS were similar to those of Almenoff et al. Of note, McLennan et al, employing polymerase chain reaction (PCR) with genus-specific mycobacterial 16s primer pairs, and species-specific M tuberculosis and M avium primer pairs, in a parallel ACCESS special laboratory study, did not find evidence of mycobacterial DNA in cultures of blood samples from five of the same patients with sarcoidosis (unpublished data; G. McLennan, MBBS, PhD; March 2001). Also, studies in our laboratory failed to identify mycobacterial DNA in multiple cultures interpreted as positive for CWDFs of mycobacteria.

We did not attempt to search for microcolonies of mycobacterial CWDFs in smears of culture material by use of the antimycobacterial monoclonal antibody developed and used by Almenoff et al. We do not believe that the failure to use the antibody explains our negative findings because, in the study by Almenoff et al, mycobacterial CWDF microcolonies were primarily identified by review of modified Kinyoun-stained slides. Kinyoun-stained slides then served as a standard for comparison to validate this preliminary study using fluorescent-labeled monoclonal antibody techniques. Additionally, no well-characterized microorganism has been consistently associated with the CWDF isolated from cases of sarcoidosis. The identification of these CWDF therefore rests on their characteristic microscopic appearance and staining properties and heuristically precedes analysis by other methods. Nonetheless, several attempts were made to amplify DNA from cultures using PCR primer sets having specificities for M tuberculosis, M avium, Mycobacterium genus, Actinomycetales, and Eubacterium (unpublished data; March 1998). Amplification products homologous to diverse species were obtained, but no consistent or dominant species identification emerged. These efforts were abandoned in the CWDFs project because of these inconsistent results and because another special laboratory project of the ACCESS study was designed to examine the application of PCR to blood of patients (unpublished data; G. McLennan, MBBS, PhD; March 2001). Our investigators were unable to detect the presence of any confirmed infectious agent despite undergoing training in the same laboratory that previously reported successful culture of CWDF of mycobacterium in sarcoidosis. The material that caused the cloudy cultures and stained for organisms thought to be CWDFs of mycobacterium could not be identified and may have been artifactual.

We did not attempt to culture mycobacterial CWDFs from tissue samples of patients with sarcoidosis, where presumably organisms might reside for protracted periods of time. Graham et al isolated cell wall-defective bacteria, which later reverted to acid-fast bacilli, from skin biopsy samples of several patients with sarcoidosis. The organisms were maintained in long-term cultures and were very slow growing. In a subsequent study using PCR, that group of investigators observed positive amplification and hybridization using probes specific for M avium complex and/or M avium subsp paratuberculosis in five of the six cultured isolates and two fresh skin biopsy samples. There was no amplification or hybridization with M tuberculosis or M avium subsp silvaticum probes. It may be that the search for mycobacterial growth in sarcoidosis should focus on culture of granulomatous tissue rather than blood. Efforts to identify distinctive microbial signatures from sarcoidosis tissues by amplification of DNA have been inconclusive to date.

This study did not confirm previous reports of distinctive bacteria with CWDFs recovered from blood of patients with active sarcoidosis. The results call into question the widespread applicability of these described techniques in the search for bacterium in the etiology of sarcoidosis.

APPENDIX

In addition to the authors, the members of the NHLBI ACCESS research group included study participants from Beth Israel...
Deaconess Medical Center (S.E. Weinberger), Georgetown University (H. Yeager), Henry Ford Hospital (M.C. Iannuzzi), Johns Hopkins University (D. Moller), Medical University of South Carolina (M.A. Judson), Mount Sinai Medical Center (A.S. Teirstein), National Jewish Medical and Research Center (L.S. Newman), University of Iowa Hospitals and Clinics (G. McLennan), University of Pennsylvania (M.D. Rossman), and University of Cincinnati Medical Center (R.P. Baughman). Study Chair was R.M.S. Cherniack. The Clinical Coordinating Center was the C-TASC (G.L. Knatterud). NHLBI staff were R.A. Musson, M.R.M.S. Cherniack. The Data and Safety Monitoring Board included W.J. Martin, T. Ashikaga, D.B. Coultas, G.S. Davis, F. Gifford, J.J. Schlesselman, and D. Stover.

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