Growth-Deficient Mycobacteria in Patients with AIDS: Diagnosis by Analysis of DNA Amplified from Blood or Tissue

Stefan Emler, Eric C. Böttger, Barbara Broers, Ignazio Cassis, Luc Perrin, and Bernard Hirschel

From the Division des Maladies Infectieuses, Hôpital Cantonal Universitaire de Genève, Geneva, Switzerland; the Institut für medizinische Mikrobiologie, Medizinische Hochschule, Hannover, Germany; and the Ufficio del Medico Cantonale, Bellinzona, Switzerland

Amplification and sequencing of mycobacterial ribosomal RNA genes (16S rDNA) may permit the detection of growth-deficient species (i.e., those exhibiting no growth or those whose growth is delayed for more than 12 weeks). Of blood samples from 26 patients with AIDS and a liver sample from one additional AIDS patient, three samples (two of blood and the one of liver) were positive by polymerase chain reaction only; cultures of these three samples remained negative for more than 12 weeks. Analysis of amplified 16S rDNA from blood revealed a sequence characteristic of *Mycobacterium genavense* in the first case, in which one of many previous blood cultures had also been positive for *M. genavense*. The sequences found in the second and third cases were characteristic of *Mycobacterium avium*. The sample from the second patient was a liver biopsy specimen in which acid-fast bacilli were visualized; the culture of this specimen yielded *M. avium* after 7 months. The third sample was a blood sample from a patient in whom a relapse of treated *M. avium* infection was suspected. These results indicate that amplification and sequencing of mycobacterial 16S rDNA may permit early diagnosis and provide a rationale for treatment of infections due to growth-deficient mycobacteria.

Conventional methods for the detection and identification of mycobacteria depend on the organisms' growth in culture and subsequent analysis by hybridization with nucleic acid probes [1] or by biochemical testing on solid media [2]. New techniques involve analysis of mycobacterial DNA and may be very useful in the identification of positive cultures [3]: with use of oligonucleotides complementary to sequences common to all mycobacteria, a part of the 16S rRNA gene (16S rDNA) is amplified by PCR, and species-specific variable regions are analyzed by sequencing [4]. By this approach, several new mycobacterial species have been detected and identified [5–7]. Such techniques are usually applied to material derived from cultures; however, growth-deficient species or isolates from antibiotic-treated patients may fail to grow in cultures.

We amplified mycobacterial DNA directly from the blood of 26 patients with AIDS and from a liver biopsy sample of one additional AIDS patient. Herein we report in detail our findings for the three patients whose PCR result was positive but whose culture either was negative or exhibited only delayed growth (i.e., growth after >12 weeks).

Methods

Patients with HIV infection and suspected mycobacteremia underwent venous blood sampling. The blood sample was divided into two 5-mL portions. One portion was directly cultured on liquid Middlebrook 7H11 medium (Bactec 13A; Becton-Dickinson, Towson, MD). The other was collected in EDTA tubes, and WBCs were prepared with a Ficoll gradient (Ficoll-Paque; Pharmacia P-L Biochemicals, Milwaukee).

For PCR, a 100-μL volume of WBCs was lysed twice with 300 μL of NP40 (0.4%) in water, and DNA was extracted as described previously [4]. In one case (patient 2; see Results), a portion of a frozen liver biopsy sample (~3 mm^3) was minced and extracted; this sample was then subjected to PCR. In order to control contamination, each step was performed under a specially equipped hood in a separate laboratory, and samples from patients were interspersed with negative control samples.

A 10-μL portion of extracted DNA was used for amplification of a 1,039-bp fragment of the mycobacterial 16S rRNA gene by PCR; one biotinylated primer (285) specific for bacterial 16S rRNA and one mycobacterial genus-specific primer (264) were employed [4]. PCR was conducted as described previously [4]. Amplification of a fragment of the correct size was monitored by agarose gel electrophoresis and ethidium bromide staining. A single-strand sequencing template was then purified by binding of the amplified DNA strand containing the biotinylated primer to streptavidin-coated magnetic beads (Dynal; Oslo) according to the manufacturer's instructions [8]. Manual sequencing of a species-specific region of the 16S rRNA gene was undertaken by the Sanger method [9]. Sequenase 2.0 (USB, Cleveland) was used as recommended by the manufacturer; a bacterium-specific internal primer (244) was also employed [4].

All samples of blood and the minced liver-biopsy speci-

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Reprints or correspondence: Dr. Bernard Hirschel, Division of Infectious Diseases, Geneva University Hospital, CH-1211 Geneva 14, Switzerland.

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Table 1. Comparison of mycobacterial culture and PCR results for samples from 27 patients with AIDS.

Culture result	No. of patients with indicated PCR result		
	Positive	Negative	Total
Positive	8	3	11
Negative	3	13	16
Total	11	16	27

NOTE. Twenty-six blood samples and one liver sample were tested.

men were cultured on liquid Middlebrook 7H11 medium (Bactec 13A). These cultures were checked twice weekly during the first 2 weeks and then weekly; after 2 months of incubation, cultures were monitored at 2-week intervals for at least 4 months. *Mycobacterium avium* isolates from liquid cultures were identified with nucleic acid probes (Accu-Probe; Gen-Probe, San Diego) in accordance with the manufacturer's instructions.

Results

Each of 27 HIV-positive patients with $CD4^+$ lymphocyte counts of $<100/\mu L$ provided one sample for examination by PCR (table 1). All patients were febrile and had lost weight. Mycobacterial infection was suspected and mycobacterial culture was attempted in all cases. Cultures yielded *M. avium* in nine cases. In two additional instances, cultures showed limited mycobacterial growth after 6 weeks; the organism was later identified as *Mycobacterium genavense* by molecular techniques [5]. Eight (73%) of the 11 culture-positive patients also had positive results of PCR, while three were positive only by culture. Conversely, three patients with positive PCR results had cultures that remained negative for more than 12 weeks.

The first of these patients (patient 1), a 28-year-old woman, was hospitalized in January 1992 with fever, anemia, and weight loss. Her $CD4^+$ lymphocyte count was $20/\mu L$. Repeated blood cultures were negative with one exception: in September 1992, mycobacterial DNA with the sequence of *M. genavense* was amplified from a single blood culture bottle showing limited growth (growth index, <400). Treatment was started but had to be discontinued because of severe intolerance. In January 1993, blood was collected in EDTA tubes and subjected to PCR, which revealed a sequence characteristic of *M. genavense* (figure 1). Cultures of blood collected at that time have remained sterile for 6 months. The patient died in January 1993; no autopsy was done.

Patient 2 was a 29-year-old HIV-positive injection drug user who had had *Pneumocystis carinii* pneumonia in 1990 and who presented in the autumn of 1991 with intermittent fever, weakness, and weight loss. Her $CD4^+$ lymphocyte count was $8/\mu L$. Bacterial pneumonia and oral candidiasis were diagnosed and treated, but weight loss and intermittent fever persisted. In October 1991 and March 1992, several blood cultures were negative. In May 1992, a liver biopsy revealed granulomatous hepatitis, with rare acid-fast rods within macrophages. A portion of this biopsy sample was processed for mycobacteria by PCR, which revealed a sequence characteristic of *M. avium* (figure 1). The patient received clarithromycin, ethambutol, rifampin, and amikacin. She became afebrile and gained 5 kg. After 7 months, a culture of her liver biopsy specimen yielded an acid-fast bacterium, which was identified as *M. avium* by both the 16S rDNA sequence and nucleic acid probes. Subcultured on solid medium (Middlebrook 7H11), this isolate took 7 weeks to attain visible growth.

Patient 3 was a 48-year-old homosexual man who had had Kaposi's sarcoma since 1988 and who developed *P. carinii* pneumonia and cytomegalovirus retinitis in the summer of 1992. His $CD4^+$ lymphocyte count was $4/\mu L$. Blood cultures were positive for *M. avium* in September 1992, when the patient presented with fever, weight loss, and hepatomegaly. Treatment with amikacin, clofazimine, clarithromycin, and ethambutol was accompanied by the disappearance of fever and hepatomegaly. In November 1992, therapy with amikacin and clofazimine was discontinued. In January 1993, while still being treated with clarithromycin and ethambutol, the patient was hospitalized because of a relapse of fever and hepatomegaly. Venous blood was collected in EDTA tubes for PCR, which revealed sequences characteristic of *M. avium* (figure 1). Treatment was adjusted, but the patient died 4 weeks later; no autopsy was conducted. Blood cultures remained negative for more than 6 months.

Discussion

Confirming suspected infection with atypical mycobacteria is often an exercise in frustration. Clinical symptoms and signs are nonspecific, and weeks pass while the results of culture are awaited. A negative culture result does not settle the issue because some mycobacteria, such as *M. genavense* [5] and *Mycobacterium ulcerans* [10], do not grow at all on most media. One study has shown that supplementation with mycobactin J may promote the growth of *M. genavense* [11]. However, in our experience, this approach is not always successful.

DNA-based diagnosis is undergoing extensive trials, most often for the detection of *Mycobacterium tuberculosis* in sputum [12]. Diagnosis based on analyses of blood and tissue, particularly when atypical mycobacteria are involved, has been hindered by difficulties with DNA extraction and by the inhibition of PCR by blood constituents [13]. Therefore, no standard protocols exist for the routine use of these techniques in laboratories. Even with perfect DNA extraction

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5' ATA GGA CCA CGG GAT GCA TGT CT- TGT GGT GGA   M. tuberculosis
5' ... T.. ... ... A.C ... ... T.- ... ... ...   M. genavense
5' ... T.. ... ... A.C ... ... T.- ... ... ...   Patient 1
5' ... ... ..T .AA ..C ... ... ..- .C. ... ...   Patient 2
5' ... ... ..T .AA ..C ... ... ..- .C. ... ...   Patient 3
5' ... ... ..T .AA ..C ... ... ..- .C. ... ...   M. avium
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Figure 1. Part of the 16S rRNA gene of various mycobacterial species and clinical strains: positions 179–215 (*E. coli* alignment). Only nucleotides different from those of *M. tuberculosis* are shown. Dashes indicate deletions.

and amplification, PCR is probably not as sensitive as culture for the detection of growth-proficient mycobacteria: mycobacteremia is of variable intensity, with <1 to >5 × 10^4 microorganisms/mL [14]. In our assay, however, the maximal amount of DNA input into the PCR reaction did not exceed 2 μg, and thus the inhibition of amplification was avoided (data not shown). This amount of DNA is extracted from <250 μg of blood or tissue. There may be no mycobacterial DNA in such a sample, while a conventional culture using 5 mL of blood is still positive [15]. This circumstance could explain the three PCR-negative but culture-positive samples (table 1).

In most studies comparing DNA-based techniques with conventional methods, some samples were DNA-positive but culture-negative. In cases of suspected tuberculosis, the proportion was 54 of 271, or 20% [13]. These samples were usually from antibiotic-treated patients and from patients who had *M. tuberculosis* cultured simultaneously from other sites. This fact suggests that the PCR results reflected true infection and not laboratory contamination. In cases of suspected *M. avium* bacteremia in patients with AIDS, 32 (14%) of 228 culture-negative specimens tested positive by PCR [16]. However, since no details were given regarding the origin of these specimens, it is difficult to judge the clinical significance of these results. The sensitivity of PCR raises difficult issues of interpretation that are particularly troublesome when DNA from facultative pathogens (such as atypical mycobacteria) is found in normally unsterile specimens, such as sputum, urine, feces, and gastric contents. Conversely, amplification of mycobacterial genes from normally sterile specimens such as blood, CSF, or liver biopsy samples is probably significant.

In the three cases presented in detail herein, the circumstances suggest that contamination was unlikely and that the laboratory findings reflect true infection. In case 1, *M. genavense*, a mycobacterium that is notoriously difficult to culture [5, 17], had grown once before in a blood culture. In case 2, the culture confirmed the PCR result after an extremely long delay. In case 3, cultures had previously been positive for *M. avium* but became negative during treatment; the results of PCR, but not of culture, became positive when the patient had a clinical relapse. Thus, as in many other infections, positive cultures may be more difficult to obtain in mycobacteremia if the patient has been treated. The diagnosis of relapse will then depend on amplification and sequencing of bacterial DNA.

Our report demonstrates that in selected cases the direct amplification of DNA from blood or biopsy samples may be the only means of diagnosing infections with growth-deficient mycobacteria. The diagnosis is valuable mainly because it justifies appropriate treatment of patients with AIDS, in whom the administration of any additional drug requires critical evaluation.

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