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Cultural Studies on Two Isolates of *"Mycobacterium genavense"* from Patients with Acquired Immunodeficiency Syndrome

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*Two strains of the newly proposed species "*Mycobacterium genavense*" have been isolated, using the radiometric system (Bactec, Becton Dickinson), from the blood of two HIV-infected patients. Disseminated infections due to the new organism closely resemble those of the* Mycobacterium avium *complex, with prevalently intestinal symptomatology. We report here on the cultural behavior of the isolates, which are enhanced at pH 6, are inhibited by NAP, do not require supplements, and grow best at 37°C. We also report on the antibiotic susceptibility of the isolates.*

INTRODUCTION

Mycobacterial infections are a dominant complication in patients with acquired immunodeficiency syndrome (AIDS). *Mycobacterium avium* complex is the most common cause of mycobacterial disease in HIV-pattern-I countries (the United States, Western Europe, Japan, and Australia) whereas in HIV-pattern-II countries (Africa, Asia, and South and Central America) the most serious infectious hazard is represented by *M. tuberculosis* (Collins, 1992). Infections with many other mycobacterial species, such as *M. kansasii* (Valainis et al., 1991), *M. haemophilum* (CDC, 1991), *M. xenopi* (Ausina et al., 1988), *M. simiae* (Lévy-Frébault et al., 1987), *M. scrofulaceum* (Ewig et al., 1990), *M. fortuitum* (Sack, 1990), *M. gordonae* (Chan et al., 1984), *M. malmoense* (Claydon et al., 1991), and *M. marinum* (Lambertus et al., 1988), have also been reported in AIDS patients.

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In immunocompromised patients, mycobacterial diseases typically spread to various body districts, and acid-fast bacilli (AFB) can be isolated from blood, stools, lymph nodes, and other tissues.

A novel mycobacterial species, for which the name *Mycobacterium genavense* has been proposed, has been recently detected as the cause of infection in HIV-positive patients (Böttger et al., 1992).

We isolated two strains of such mycobacterium from seriously immunocompromised HIV-infected patients, and we report here on our experience, with particular emphasis on the microbiologic features of our *"M. genavense"* strains.

MATERIALS AND METHODS

Case Histories

The first patient (A.A.), a 34-year-old homosexual man with a history of chronic B hepatitis, had been followed at the outpatient facility of the Infectious Diseases Service at Careggi Hospital (Florence, Italy) since 1985, when he had been found to be HIV-1-antibody positive. The level of his $CD4^+$ T lymphocytes had decreased relentlessly in the years: 1324/μl in 1985, 686 in 1986, 209 in 1988, 100 in April 1991, and 27 in November of the same year. No significant symptoms, except for an occasional fever, had been recorded before 1992.

The man, continuously treated with zidovudine since 1988, was hospitalized in March 1992 with fever (>39°C), shivers, night sweats, hiccups, and weight loss; he reported mucous bowel movements, and a diffuse abdominal pain that worsened during the hospitalization; at the same time, symptoms of AIDS dementia complex appeared.

An abdominal echographic scan showed multiple peritoneal and retroperitoneal lymphadenopathy, moderate spleen and liver enlargement, and ascites; kidney and pancreas were normal. A chest radiograph did not reveal abnormalities, and the results of a computed tomography (CT) scan of his brain, performed owing to short-term memory loss, were also normal.

No opportunistic infection, other than mycobacterial, was shown by cultures from blood, stools, urine, and cerebrospinal fluid; the results of parasitologic examinations of feces were also normal. Mycobacteria were not found in cerebrospinal fluid. One of two blood cultures was positive and AFB were seen in the stools, but cultures remained negative after 8 weeks of incubation. As soon as the direct examination of the stools was found to be positive, the patient was treated using an empiric antimicrobial therapy with ciprofloxacin, rifampin, ethambutol, and clofazimine, but he died of a heart attack 4 days later; no necropsy was done.

The second patient (G.A.) was a 47-year-old homosexual man, occasional drug abuser, alcoholic from the age of 33, previously heavy smoker, and HIV-1-antibody positive since 1987. The count of his $CD4^+$, which was 313/μl in 1990, when zidovudine treatment was undertaken, decreased during 1992: 104 in March, 87 in June, 61 in August, and 35 in October, to reach 16 units in January 1993.

Before the discovery of HIV infection, he had contracted non-A–non-B hepatitis, syphilis, and gangrenous appendicitis. Several hospitalizations are reported from 1992: in June, for interstitial pneumonia that was favorably treated with trimethoprim–sulfamethoxazole (in this circumstance, zidovudine was dismissed); in October, for meningitis due to *Cryptococcus neoformans*, and, finally, in March 1993, because of fever (with peaks of >40°C), abnormal mental status, and worsening of diarrhea that had arisen 7 months before. CT scan of the brain revealed cortical atrophy without foci. A macrophagic infiltrate with AFB was discovered with a duodenal biopsy.

As with preceding patient, no infection, other than mycobacterial, was disclosed by cultures and direct examinations for parasites; once again, stools were microscopically positive for AFB that failed to grow in culture. After the isolation of AFB from one of nine blood cultures, the man was treated with amikacin, rifampin, and clofazimine, the association being chosen in the light of the susceptibility tests that had been done on our first isolate of "*M. genavense*"; he soon improved and regained his lucidity, and the fever and diarrhea disappeared.

G.A., who continued antimicrobic treatment after discharge, was still alive in August 1993.

Microbiology

All specimens for the culture of mycobacteria were inoculated both on conventional Lowenstein–Jensen medium and on radiometric Middlebrook 7H12 broths (Becton Dickinson, Towson, MD, USA): BACTEC 13A was used for blood, and BACTEC 12B for cerebrospinal fluid and stools; before the inoculation, the stools had been decontaminated with Nekal BX (Biotest, Frankfurt-Main, Germany). No growth was achieved on conventional media within 60 days of incubation. The growth index (GI) of the positive blood culture from A.A. started to rise during week 5 of incubation; the one from G.A. at the end of week 8 of incubation; 12 and 20 days, respectively, were needed for the GI to rise above 100. The Ziehl–Neelsen smears performed from the vials revealed the presence of short AFB.

All attempts to subculture the bacilli on solid media (Lowenstein–Jensen, Gottsacker, Stonebrink, Harrold, and Middlebrook 7H11) were unsuccessful after 3 months of incubation at 37°C. Liquid subcultures in BACTEC 12B were generally positive, but the GI increases were very slow and delayed; occasional growth failures were noticed with light inocula.

The lack of growth on solid media suggested that our isolates might belong to the recently proposed new species "*M. genavense*," so liquid subcultures were submitted to the Hannover (Germany) Institute for Medical Microbiology for definitive identification by means of nucleic acid sequencing of the 16S rRNA.

To find the optimal growing conditions, we used the first isolate to perform a systematic comparison of substrates, with parallel subcultures at 37°C, with identical inocula (100 μl of broth culture developed to a GI ≥100), in BACTEC 13A (with its respective enrichment), in standard BACTEC 12B, in acid (pH 6) Middlebrook 7H12 (PZA test medium, Becton Dickinson), and in nonradiometric Middlebrook 7H9 broth. Standard and acid radiometric broths were inoculated either alone or enriched with 100 μg/ml of polyoxyethylene stearate (POES), with 2 μg/ml of mycobactine J (Rhône-Mérieux, Lyon, France), or with both. The growth in the nonradiometric Middlebrook 7H9 broth was assessed indirectly by blind subculturing, after 15 days of incubation, onto BACTEC 12B, and subsequent comparison of the growth curve with that obtained with a parallel subculture from a radiometric broth; smears were also done

from the centrifuged sediment of the 15-day-old mother cultures. The temperature dependence of growth was explored by incubating cultures in standard and acid broths at 25°, 32°, 37°, 42°, and 45°C.

Susceptibility testing, according to the 1% resistance proportional method, and BACTEC NAP (*p*-nitro-α-acetylamino-β-hydroxypropiophenone) test, initially carried out on the first isolate in accordance with BACTEC system directions (Siddiqi, 1989), were successively repeated, with minimum inhibitory concentration (MIC) determination, in acid medium. For the susceptibility testing, a heavier inoculum, that is, a culture that had just exceeded a GI of 999, was used. This procedure was subsequently applied to the second isolate. For MIC estimation, all antimicrobials were tested with not less than four twofold dilutions per drug.

Hybridization tests on centrifuged (10,000 *g* for 5 min) pellets from broth cultures with GI ≥999 were performed using probes specific for *M. avium* and *M. intracellulare* (Gen Probe, San Diego, CA, USA).

RESULTS

Both our isolates were sent to Hannover, where Dr. Böttger sequenced the PCR (polymerase chain reaction)-amplified 16S rRNA and identified the unique sequence present in all the strains for which the new species "*M. genavense*" had been proposed (Böttger et al., 1992).

No hybridization was achieved with probes specific for *M. avium* and *M. intracellulare*.

From the analysis of growth characteristics under various conditions, the most striking finding is represented by the strong enhancement in the acid medium; in fact, in standard BACTEC 12B and 13A media the growth of both strains was very scanty and the GI rarely exceeded 100 at the steady state, whereas in the acid medium the GI usually exceeds the upper limit (999) of the BACTEC scale (Figure 1a).

Growth in the nonradiometric broth was very similar to that in BACTEC 12B, as assessed by the trend of GIs in the blind subcultures and by bacte-

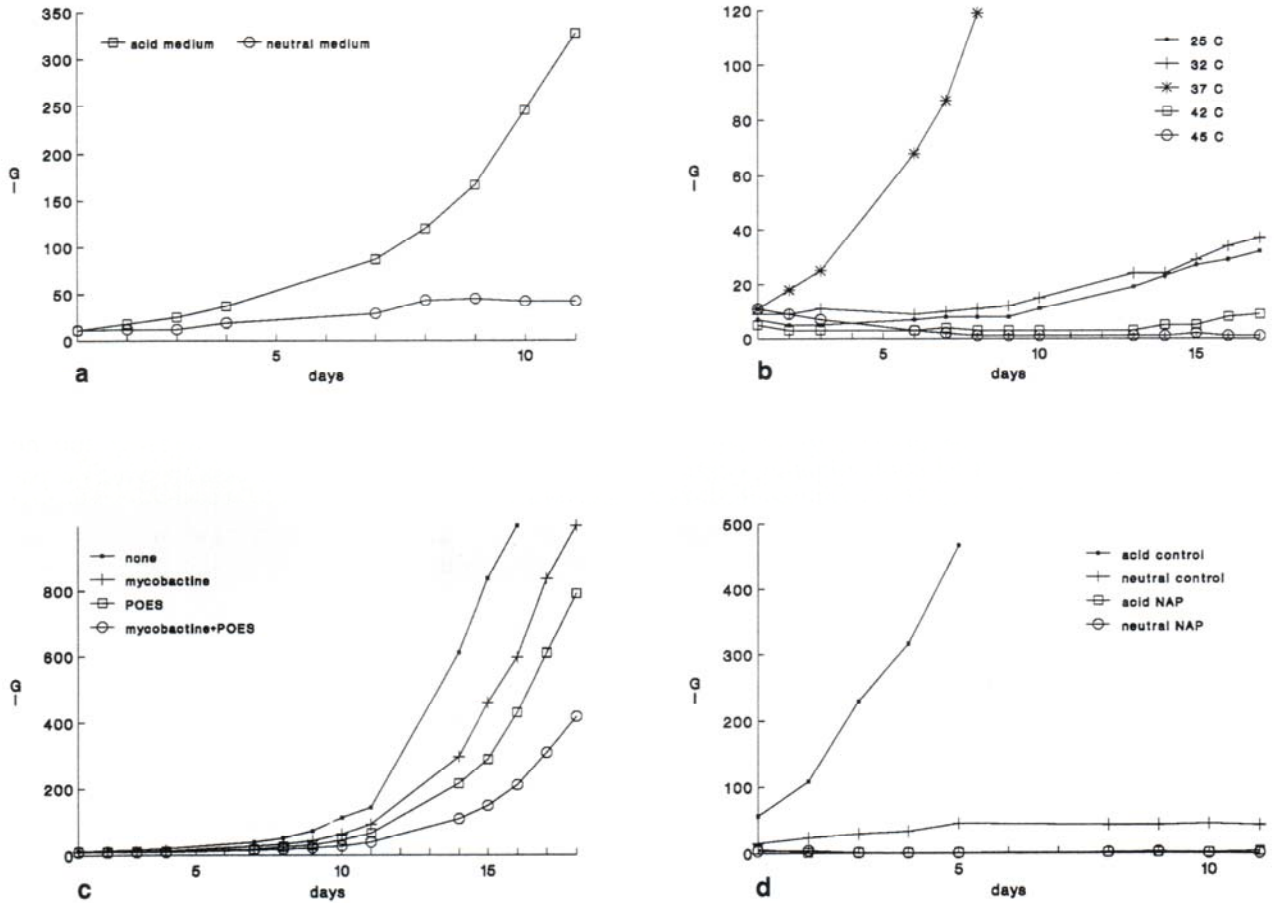

FIGURE 1 Comparison of the growth curves of "*Mycobacterium genavense*" subcultured under various conditions. **(a)** Growth rates in different BACTEC media. **(b)** Growth rates, in BACTEC acid medium, at different temperatures (on standard BACTEC 12B medium no growth was achieved at 25°C or at 32°, 42°, and 45°C). **(c)** Growth rates in BACTEC acid medium alone and supplemented with different additives (similar curves, but with lower values, were achieved in standard medium supplemented with the same drugs). **(d)** BACTEC *p*-nitro-α-acetylamino-β-hydroxypropiophenone (NAP) test in standard and acid media.

rioscopy. The optimal growth temperature was 37°C; only in the acid medium was some growth evident at 32°, 25°, and 42°C (Figure 1b). None of the supplements added to the medium was stimulatory; they rather slowed the growth curve, with an apparently additive effect (Figure 1c).

NAP, which is used in BACTEC system to distinguish nontuberculous mycobacteria, which usually are not inhibited by the drug, from the species in the *M. tuberculosis* complex, which are inhibited (Morgan et al., 1985), completely suppressed the metabolic activity of our strains, both in neutral and in acid broth (Figure 1d).

Only the susceptibility tests performed on the first isolate in acid medium were fully interpretable (MICs results are listed in Table 1). In the standard BACTEC 12B medium, the control vial did not reach the required GI value (>30) within 3 weeks, whereas with the technical adjustments introduced (a heavier inoculum into acid medium), we entered the range considered adequate for the proportional method of testing *M. tuberculosis* (the species for which the test has been standardized), and the final readings were obtained at day 10. Unfortunately, this technique was only partially applicable to the second strain, which was still slower; although less reliably, however, the test revealed a susceptibility pattern closely consistent with the former.

TABLE 1 Susceptibility Pattern of Two Strains of *"Mycobacterium genavense"*: Tests on Acid Medium Inoculated from a Subculture with Growth Index that Had Just Exceeded 999

Drugs	MICs (μg/ml)[a]	
	Strain A	Strain B[b]
Amikacin	2	2
Ciprofloxacin	2	4
Clarithromycin	0.5	0.5
Clofazimine	0.032	0.032
Ethambutol	32	32
Isoniazid	2	≥16
Kanamycin	2	2
Ofloxacin	2	2
Pyrazinamide[c]	>400	400
Rifabutin	0.062	0.062
Rifampin	0.062	0.12
Sparfloxacin	0.25	0.5
Streptomycin	2	2

[a] MIC, minimum inhibitory concentration.
[b] Tentative estimates as this isolate does not reach the threshold growth index of 30.
[c] Susceptibility testing against pyrazinamide was performed according to BACTEC directions for this drug.

DISCUSSION

It is not unusual to fail in isolating mycobacteria from samples that are positive for AFB. Apart from *M. leprae*, whose inability to grow on artificial media is well known, various explanations may exist: lack of vitality of the bacteria, particularly in the course of treatment, overgrowth by contaminating organisms or damage by a harsh decontamination, and use of inadequate media and incubation at inadequate temperatures.

Regarding special nutritional requirements, the need for hemin by *M. haemophilum* and for mycobactine by *M. paratuberculosis* and by some strains of the *M. avium* complex is well recognized. The requirement of a liquid medium, by the novel fastidious species *"M. genavense,"* shall now be added to the list of possible causes of isolation failures.

To the exquisite sensitivity and detection rapidity of the radiometric method (Middlebrook et al., 1977; Roberts, 1983; Roberts et al., 1983; Witebsky et al., 1988), which has already proved so useful for the recovery of mycobacteria from clinical specimens from AIDS patients, now should be added the exclusive ability of the system to enable growth and detection of *"M. genavense."* Although enabling some growth of *"M. genavense,"* nonradiometric liquid media have in fact, in this difficulty, limited value because they require time-consuming and low-sensitivity microscopic reading.

Susceptibility results on *"M. genavense"* strains have not been previously reported; our isolates seem to be less resistant than the average expected among *Mycobacterium avium* complex. Response to empirical therapy appeared good in patient G.A., who recovered and is still living; the other patient was critically ill when AFB were detected and he died a few days later; another case in which a patient responded well to the associated antimicrobial therapy has been reported (Nadal et al., 1993). Information about other strains would be extremely important as a guide to therapy.

The proposed species definition for *"M. genavense"* so far relies solely on its unique 16S rRNA sequence (Böttger et al., 1992); even so, the new species could be shown to be involved in serious infections in a number of immunosuppressed patients (Böttger et al., 1992). Our cases were very similar to others reported (Böttger et al., 1992; Wald et al., 1992; Nadal et al., 1993); there was multiple organ involvement with predominant gastrointestinal complaint in profoundly immunosuppressed patients; as in five of the published cases, our patients had no other life-threatening infection and it cannot be excluded that *"M. genavense"* might have been the cause of death of A.A.

The 16S rRNA sequencing is at present the only

way to identify "*M. genavense*" definitively; it is an unsuitable technique, however, for most diagnostic laboratories, while the absence of growth on solid media makes it impossible to attempt biochemical identification. The simple culture tests performed on our isolates should be taken as tentative guidelines both for obtaining further strains and for the phenotypic definition of "*M. genavense*"; in short, acid-fast bacillus, unable to grow on conventional solid media, with slow and scanty growth in unsupplemented radiometric broths, that grows best at 37°C and pH 6, and is inhibited by NAP.

In AIDS patients, when AFB are seen in biopsy specimens but are not isolated, "*M. genavense*" infection should be considered; 16S rRNA amplification would enable the rapid and specific detection of this organism (Böttger et al., 1992). A valid alternative is blood culture in radiometric liquid medium. The inoculation, besides the usual BACTEC 13A, of a second vial of the same medium whose pH had been lowered to 6 should enhance the growth of "*M. genavense*," and improve the sensitivity and shorten the time to detection in blood samples. Radiometric cultures of specimens other than blood can also be attempted even though no success has been reported so far: in our case, AFB found in the stools failed to grow in BACTEC 12B medium, possibly because they were harmed by the decontamination procedure.

The extent of infections due to "*M. genavense*" is at present unknown; it is likely that new cases will be reported in future, both because of the growing awareness of this possibility by microbiologists and because of the constantly increasing number of patients that survive in the late phases of AIDS, when the $CD4^+$ count falls to $<50/\mu l$. These patients are in fact the main target of infection due not only to *M. avium* complex, but also to "*M. genavense*."

Recently, as a result of the reports by Coyle et al. (1992) and Jackson et al. (1992), which revealed the possibility of growing "*M. genavense*" and *M. genavense*-like organisms on solid media, we tried to cultivate our strains on the media proposed by these authors. Scanty growth was achieved on 7H11-MJ (Middlebrook 7H11 with mycobactine) after 2 months of incubation (Figure 2), whereas both our strains failed to grow on 7H9/CYE (Middlebrook 7H9 with *Legionella* CYE). These observations show that the strains isolated by Jackson and colleagues do not behave exactly like ours even if other phenotypic characteristics, such as NAP inhibition, are in agreement.

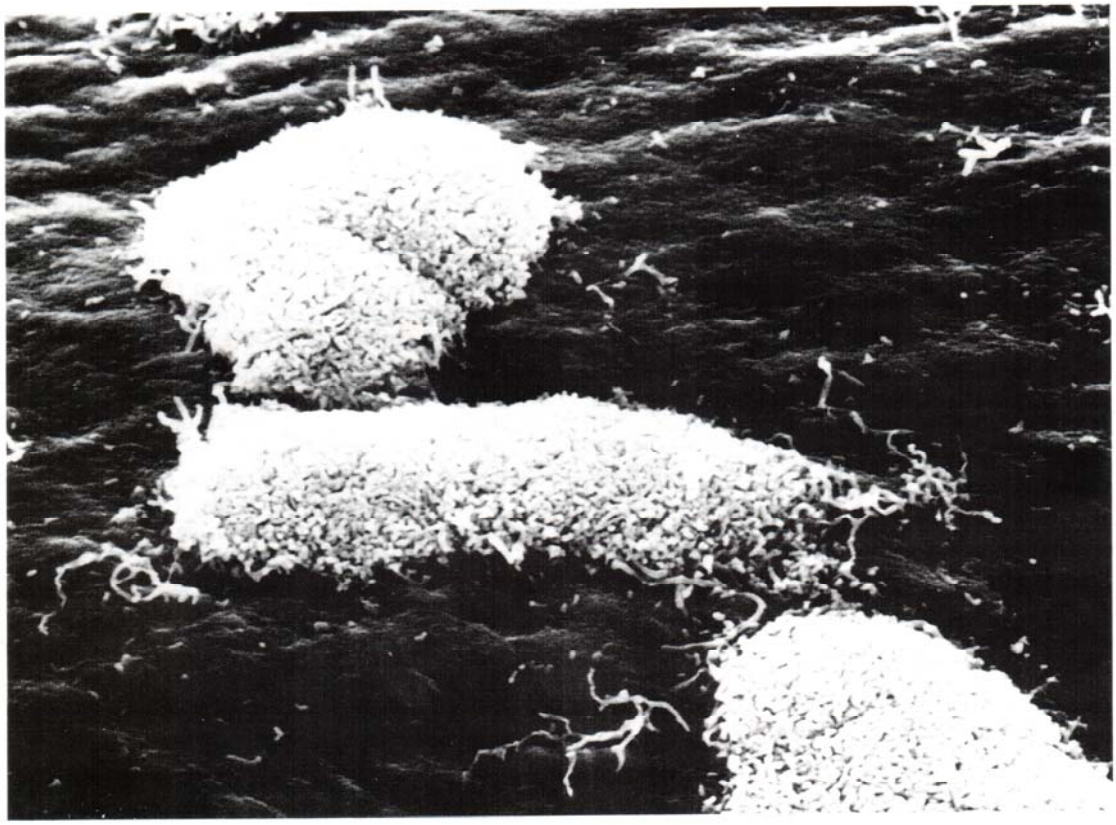

FIGURE 2 Scanning microscopy of colonies of "*Mycobacterium genavense*" grown on Middlebrook 7H11 supplemented with mycobactine: ├──────┤ = 10 μm.

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