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# BRIEF REPORT

## FATAL INFECTION WITH A NOVEL, UNIDENTIFIED MYCOBACTERIUM IN A MAN WITH THE ACQUIRED IMMUNODEFICIENCY SYNDROME

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*MYCOBACTERIUM tuberculosis* and *Mycobacterium avium–intracellulare* are among the common pathogens that infect patients with the acquired immunodeficiency syndrome (AIDS).[1-3] In this report, we describe a patient seropositive for the human immunodeficiency virus (HIV) whose disease clinically resembled infection with *M. avium–intracellulare*. Numerous acid-fast rods were found in nearly all the tissues we examined, but the cultures remained negative. Nucleic acid analyses were negative for *M. tuberculosis*, the *M. avium* complex, and *M. leprae*, but electron microscopy and lipid analysis by chromatography suggested that the pathogen was a mycobacterium. The microorganism multiplied in congenitally athymic nude mice. Its species remains unknown.

### CASE REPORT

The patient, a man born in Geneva in 1958, injected heroin intravenously from 1982 to 1986 and was found to be HIV-seropositive in 1985. In October 1987, he was hospitalized because he had diarrhea, anorexia, fever, nausea, a 5-kg weight loss, and oral thrush. His CD4+ lymphocyte count was $0.045 \times 10^9$ per liter (45 per cubic millimeter). Acid-fast rods were found in duodenal-biopsy specimens, feces, urine, and a bone marrow–biopsy specimen, but all cultures were negative (see Results). The patient was treated for three weeks with amikacin, clofazimine, ethambutol, ciprofloxacin, and isoniazid, but treatment was interrupted because of an increase in the serum alanine aminotransferase level from 19 IU per liter to 130 IU per liter after three weeks. Despite some evidence of improvement (disappearance of fever and stabilization of weight), the patient chose not to undergo further therapy.

During the following year, the patient again began to lose weight. He received zidovudine only irregularly because of problems with neutropenia and compliance. He was hospitalized in February 1988 with pneumococcal pneumonia and again in August 1988 because of fever, anemia, and presumed esophagitis due to *Candida albicans*. At that time, his CD4+ lymphocyte count was only $0.001 \times 10^9$ per liter (1 per cubic millimeter). Acid-fast rods were observed after centrifugation in the blood (buffy coat), in ascitic fluid, and in serum. The patient had massive ascites, edema, renal failure, fever (temperature to 40.5°C), and thrombocytopenia. He died in September 1988.

### METHODS

#### Culture in Liquid and Solid Media

Specimens of the liver, spleen, and duodenum obtained at autopsy and samples of feces and urine were decontaminated,[4-6] but ascitic fluid and blood from the patient and liver from the nude mice were used without decontamination. A total of 14 samples were inoculated into the following mediums: Coletsos,[7] Ogawa,[8] Stonebrink, and Middlebrook 12B, 13A, and 12B enriched with iron citrate, ammonium, hemin, or mycobactin.[9] All cultures were incubated at 30°, 37°, and 45°C and observed for growth for six months.[10-12] Mycobacteria were detected with a radiometric procedure (Bactec, Becton Dickinson Diagnostic, Towson, Md.) according to the manufacturer's recommendations.

#### Lipid Analysis

The infected tissues were homogenized in a solution of sucrose, and the bacteria were separated from the homogenates by differential and density-gradient centrifugation in solutions of sucrose and potassium chloride.[13] The bacterial mycolic acids were transformed into methyl mycolates, which were separated by one-dimensional thin-layer chromatography on a silica-gel plate (10 cm by 10 cm) developed with petroleum ether (boiling point, 60 to 80°C) and acetone at a ratio of 95:5 (vol/vol)[14] and on another plate with dichloromethane.[15] The fatty acids were revealed by spraying the

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plates with a 5 percent solution (wt/vol) of phosphomolybdic acid in ethanol. The use of two plates developed with two different solvents was necessary to distinguish clearly the $\alpha'$-mycolates from the methoxy-mycolates. The cellular fatty acids were evaluated by gas chromotography after heating in methanolic hydrogen chloride, followed by hexane extraction.[16]

### Nucleic Acid Analysis

Assays for homology to the DNA of the *M. avium* complex and *M. tuberculosis* were performed directly in centrifugated Bactec 12B and 13A broth (Becton Dickinson) and in stool.[17] This modification of the instructions from the manufacturers of Gen-Probe assay (Gen-Probe, San Diego) gave excellent identification of *M. avium* in other HIV-positive patients (unpublished data). The polymerase chain reaction was used to detect small amounts of DNA homologous to *M. leprae*[18] and *M. tuberculosis*.[19]

### Experiments in Animals

Material prepared from the liver and spleen of the patient that contained approximately $1.5\times10^7$ acid-fast rods (in 250 $\mu$l of sterile phosphate-buffered saline) was used to inoculate congenitally athymic *nu/nu* mice by the intraperitoneal or peroral route. Control mice received only sterile saline. Two months after the intraperitoneal challenge, two of the five mice challenged with spleen homogenates died. At that time, the remaining mice were killed and examined at autopsy. In an attempt to maintain the strain under passage, nude mice were challenged with inocula prepared from mice livers and spleens containing acid-fast rods. Two months later, the mice were killed and autopsied.

Three samples from the patient (liver, duodenum, and stool) were inoculated into both hind footpads of immunocompetent outbred mice (NMRI). Groups of 10 mice received inocula of 5000, 500, 50, and 5 bacilli per footpad. The footpads were examined after 12 months, and the bacilli were counted.[20]

### Electron Microscopy

Tissue samples were thawed at room temperature and fixed in 1 percent osmium tetroxide in Palade's veronal acetate buffer (glutaraldehyde–formaldehyde–calcium) for 24 hours and postfixed in aqueous 1 percent uranyl acetate for 1 hour. All fixations were carried out at room temperature. After ethanol dehydration and embedding in Epon, ultrathin sections were stained with lead citrate[21] for five minutes.

### Studies with Mouse Peritoneal Macrophages

Spleen homogenates from the patient (kept at −70°C) were thawed, suspended in phosphate-buffered saline (pH 7.2), and centrifuged at $100\times g$ for five minutes to remove coarse tissue particles. The supernatant was removed and centrifuged at $2700\times g$ for 40 minutes to remove the bulk of soluble tissue components. The bacterial pellet was washed once with saline. Samples were taken to check for contamination in blood-agar plates. The pellet was then suspended in medium 199 (M199, Seromed, Munich, Federal Republic of Germany) supplemented with 10 percent fetal-calf serum inactivated at 56°C for 60 minutes (Seromed) and 100 U of penicillin per milliliter.

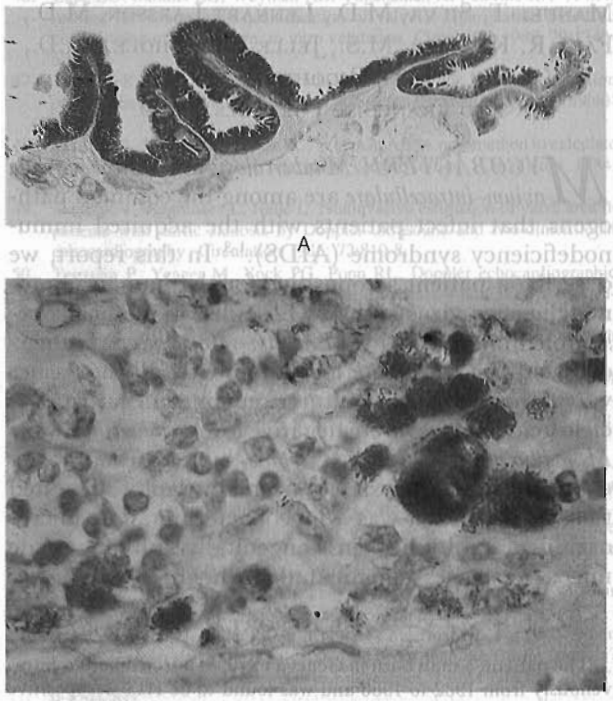
Mouse resident peritoneal macrophages were harvested by washing the peritoneal cavities of normal Swiss–Webster female mice with Hanks' balanced salt solution (HBSS, Seromed) containing 5 U of heparin per milliliter. The cells were purified and counted in a hemocytometer.

Onto each well of 96-well culture dishes (Costar, Cambridge, Mass.), $10^5$ cells were layered and allowed to adhere for three hours. Culture mediums containing the acid-fast rods ($1\times10^6$ in 100 $\mu$l) were added. Control monolayers received either heat-treated material (100°C for 30 minutes), macrophages alone, or acid-fast rods alone. After 24 hours of further incubation, the bacilli that had not undergone phagocytosis were removed by washing twice with Hanks' balanced salt solution, and 37 kBq (1 $\mu$Ci) of methyl-[$^3$H]thymidine was added (specific activity, $1.554\times10^{12}$ Bq [42 Ci] per millimole[22]). The medium and radiolabel were replaced every three to four days.

After 14 days of incubation, the culture mediums were lysed with 1 percent sodium dodecyl sulfate and treated with trichloroacetic acid (final concentration, 5 percent) for 30 minutes. Precipitates were harvested on fiberglass filters, washed, and dried. The radioactivity retained in the filters was measured.

## Results

A duodenal-biopsy specimen obtained from the patient 12 months before his death showed the infiltration of submucosal connective tissue by acid-fast rods. At autopsy, the peritoneal cavity was diffusely covered by whitish nodules ranging from 3 to 5 cm in diameter. The lymph nodes, liver, and spleen were massively enlarged. Microscopically, an acid-fast stain revealed huge quantities of acid-fast rods within the cytoplasm of histiocytes of all these tissues, and sometimes free between the cells (Fig. 1A). Grocott staining for fungi and Gram staining for bacteria were negative. There was no evidence of lymphoma, Kaposi's sarcoma, or cytomegalovirus inclusion disease. Under the electron microscope, abundant bacteria were seen (Fig. 2). The ultrastructure of the cell wall seen in the bacillary remnants was typical of mycobacteria.[23]



A

B

Figure 1. Histologic Studies.
Panel A shows a specimen from the duodenum of the patient, examined at autopsy (×0.5), and Panel B the spleen of a nude mouse. Sixty days earlier, the animal was inoculated with $10^6$ acid-fast rods from the patient's spleen (×440). In both panels, the purple areas are macrophages filled with acid-fast rods (Ziehl–Neelsen stain).

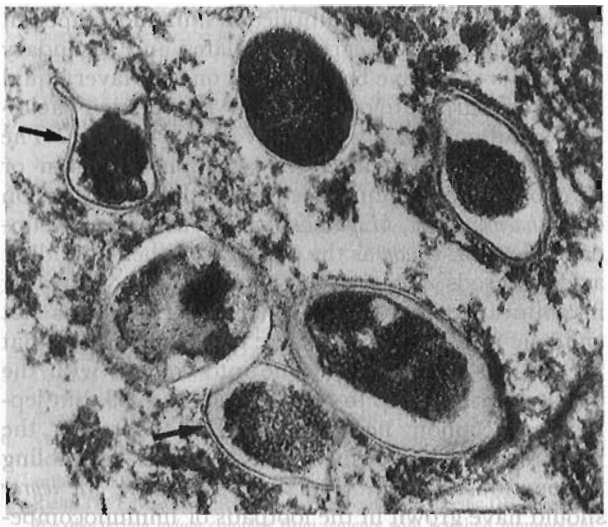


Figure 2. A Group of Bacteria in the Liver Sample from the Patient (×62,700).

The bacilli are in a state of advanced degradation, as indicated by the absence of ribosomes, the broken and symmetric membranes, and the deformed cell walls. The cell wall includes a triple-layered structure typical of mycobacteria (arrows).

All attempts to culture the putative mycobacterium on solid mediums (see Methods) failed. In liquid (Middlebrook 13A) cultures containing [$^{14}$C]palmitate (Bactec), a weak signal was detected several times after about three weeks, as recently described for *M. leprae*.[24] Subculture was attempted with use of all the methods outlined above, but without success.

Evidence for bacterial metabolism was also observed in mouse macrophages (Fig. 3), which were inoculated with spleen homogenates from the patient. After two weeks, inoculated cultures showed evidence of the incorporation of radioactive thymidine into DNA — a mean activity of 337 cpm, as compared with 161 cpm when macrophages were inoculated with heat-killed acid-fast rods.

Material obtained from feces, which resembled a nearly pure culture of mycobacteria when examined under the microscope, was used in a Gen-Probe nucleic acid–hybridization assay. The results were negative for both *M. tuberculosis* and *M. avium–intracellulare*. The polymerase chain reaction, with use of primers specific for *M. leprae*[18] and *M. tuberculosis*,[19] was also negative.

One year after the inoculation into immunocompetent NMRI mice, no multiplication of the acid-fast rods was observed in the footpads inoculated with the two specimens of liver and duodenum. One footpad inoculated with 5000 acid-fast rods from the patient's stool specimens showed evidence of multiplication, with $3.2\times10^5$ acid-fast rods present after one year. After the death of the patient, immunocompromised nude mice were inoculated with tissues containing acid-fast rods. When several of the mice that received the material intraperitoneally died after 40 to 60 days, the others were killed and examined at autopsy. The results of these studies are shown in Table 1. More than 92 percent of the mice inoculated parenterally had acid-fast rods in their livers and spleens, but control mice were negative. When acid-fast rods were given by mouth, only 1 of 10 mice was positive. Attempts to grow these acid-fast rods in artificial mediums, by the methods outlined above, also failed. However, the microorganism could be transferred to a second group of nude mice, in which it again multiplied in liver and spleen (Table 1 and Fig. 1B).

Thin-layer chromatography revealed the presence of three spots corresponding to $\alpha$-, $\alpha'$-, and keto-mycolates in the patient's duodenal specimen (Fig. 4), and gas chromatography showed tuberculostearic and hexadecanoic acid. The secondary alcohols characteristic of *M. avium–intracellulare* were absent, however.

## Discussion

This patient was severely immunodeficient as a result of HIV infection. In view of the massive quantities of acid-fast rods present in almost all the tissues we examined, there can be little doubt that this infec-

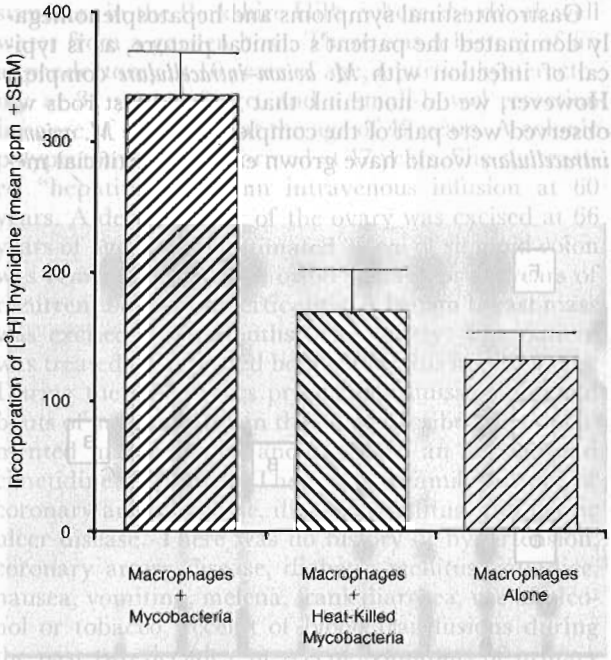



Figure 3. Incorporation of [$^3$H]Thymidine by Macrophages.

The left-hand bar represents the results for macrophages cultured with $10^6$ acid-fast rods from the patient's spleen tissue; the center bar, the results for macrophages cultured with heat-killed acid-fast rods; and the right-hand bar, the results for macrophages alone. The results shown are the means +SEM (indicated by T bars) of replicate cultures. The difference between the results for macrophages cultured with mycobacteria from the patient's spleen and for control macrophages was significant ($P<0.01$ for both controls). The difference between the results for the two controls was not significant.

Table 1. Results of Inoculation into Nude Mice.

| Material Used | Route of Administration | Positive Mice/ Total Examined |
|---|---|---|
| None | — | 0/6 |
| Patient's spleen | Intraperitoneal | 11/12* |
| Treated with sodium hydroxide | Intraperitoneal | 1/3 |
| Untreated | Oral | 1/10 |
| Spleen from positive mice (two animals) | Intraperitoneal | 8/9† |

*An additional three mice in this series died and were not autopsied.
†An additional four mice in this series died and were not autopsied.

tion was the most important factor in his death, but it was impossible to identify the microorganism. Evidence for bacterial metabolism was present in liquid Bactec cultures (since there was a transformation of radioactive palmitic acid into carbon dioxide) and after the infection of mouse peritoneal macrophages (incorporation of [$^3$H]thymidine into DNA) (Fig. 3), but all attempts to culture the microorganism on solid mediums failed. Therefore, conclusions about the nature of the microorganism must remain speculative at this time. The acid-fastness of the bacteria and the results of electron microscopy and lipid analysis all strongly suggest that it was a mycobacterium.

Gastrointestinal symptoms and hepatosplenomegaly dominated the patient's clinical picture, as is typical of infection with *M. avium–intracellulare* complex. However, we do not think that the acid-fast rods we observed were part of the complex, because *M. avium–intracellulare* would have grown easily on artificial mediums, it would have multiplied in immunocompetent mice, characteristic carbo-mycolates and secondary alcohols would have been evident on thin-layer liquid and gas chromatography, and the nucleic acid–hybridization assay would have been positive. The absence of growth after the addition of hemin or mycobactin to the liquid mediums led to the exclusion of *M. haemophilum*, *M. paratuberculosis*, and mycobactin-dependent *M. avium* as the pathogen. When abundant acid-fast rods are seen in tissue but do not grow in culture, leprosy comes to mind. Leprosy is not endemic in Switzerland. There is a small chance that the patient might have come into contact with the disease during a trip to North Africa in 1983, but leprosy is rare among people with AIDS.[25] Moreover, the patient never had skin or nerve lesions resembling lepromatous leprosy. Had it been present, *M. leprae* should have grown in the footpads of immunocompetent mice; as noted above, only 1 of 10 mice had evidence of growth on microscopical examination after one year. Rapid growth in nude mice,[26] the results of thin-layer chromatography, and the negative results of the polymerase chain reaction also do not suggest *M. leprae*.

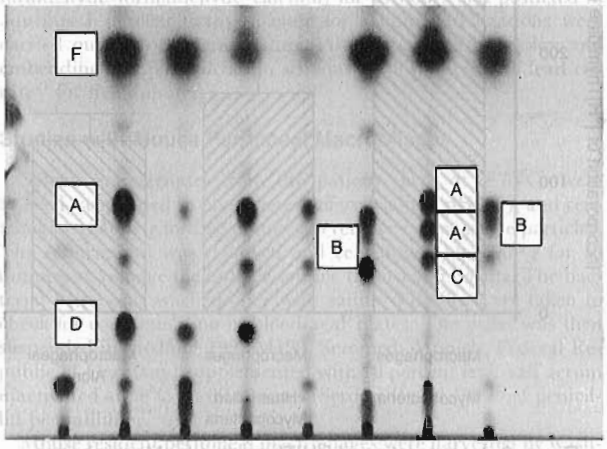



Figure 4. Results of One-Dimensional Thin-Layer Chromatography.

Akaline methanolysates are shown as follows: lanes a through c, three strains of *M. avium*; lane d, extract of a duodenal specimen from the patient; lane e, *M. gordonae*; lane f, *M. simiae*; lane g, *M. bovis* bacille Calmette–Guérin. A double development with petroleum ether–acetone (95:5 vol/vol) was performed. A indicates $\alpha$-mycolates, A′ $\alpha'$-mycolates, B methoxy-mycolates, C keto-mycolates, D $\omega$-carboxy-mycolates, and F nonhydroxylated fatty acid methyl esters.

The mycolic acid pattern of the mycobacteria extracted from the duodenal-biopsy specimen ($\alpha$-, $\alpha'$, and keto-mycolates) is similar to that of *M. simiae*, *M. malmoense*, and a new group of slow-growing mycobacteria isolated from stool of healthy persons[27] (and unpublished data). The findings of gas chromatography also indicate a relation to *M. simiae* and to our new group of slow-growing mycobacteria.

Our efforts to transmit the infection to immunocompetent NMRI mice were not successful. However, nude mice were much better hosts than normal mice. A high percentage of parenterally inoculated nude mice (11 of 12 that were injected with material from the patient's spleen homogenate) had liver and spleen lesions resembling those seen in the patient and containing abundant acid-fast rods (Fig. 1B). Spleen homogenates from these mice produced similar lesions in a second group of mice.

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# CASE RECORDS
## OF THE
# MASSACHUSETTS GENERAL HOSPITAL

## Weekly Clinicopathological Exercises



## CASE 28-1990

### PRESENTATION OF CASE

A 72-year-old woman was admitted to the hospital because of severe abdominal pain.

The patient was well and active until five days earlier, when there was the onset of severe band-like pain about the level of the umbilicus, without radiation elsewhere, accompanied by a sensation of abdominal bloating; the pain was constant and was not affected by position or antacids but increased in severity about half an hour after meals. The patient's stools became soft, with a single episode of hematochezia; after two or three days she stopped passing stools. Several days before admission the pain became more diffuse and was increased by any movement; anorexia developed. She was admitted to the hospital.

The patient was a resident of Florida and spent her summers in the Berkshire Hills, where she drank well water from a garden hose. There was a history of an appendectomy at 16 years of age, a partial hysterectomy at 32 years of age, and a small-bowel resection because of adhesions at the age of 42 years. A colonic polypectomy was performed at 47 years. She contracted "hepatitis" after an intravenous infusion at 60 years. A dermoid cyst of the ovary was excised at 66 years of age, and an estimated 30 cm of sigmoid colon was removed at the age of 68 years after 10 years of recurrent bouts of diverticulitis. A benign breast mass was excised three months before entry. The patient was treated for repeated bouts of cystitis in past years. During the three years preceding admission she had bouts of substernal pain that were ascribed to a documented hiatal hernia, and she used an antacid and cimetidine as needed. There was a family history of coronary artery disease, diabetes mellitus, and peptic ulcer disease. There was no history of hypertension, coronary artery disease, diabetes mellitus, jaundice, nausea, vomiting, melena, frank diarrhea, use of alcohol or tobacco, receipt of blood transfusions during the past two decades, or recent symptoms of urinary tract infection.

The temperature was 37.3°C, the pulse was 76, and the respirations were 14. The blood pressure was 150/70 mm Hg.

On examination the patient appeared pale, tired, and uncomfortable. No rash or lymphadenopathy was found. The head and neck were normal, and the lungs were clear. Examination of the heart and breasts was negative. The abdomen was soft, with normal bowel sounds and multiple healed surgical scars; no bruit