The prevalence and clinical implications of colonization with nontuberculous mycobacteria were prospectively studied in 37 patients who had cystic fibrosis. Sputum samples were cultured on Coletsos and Löwenstein-Jensen selective media after decontamination with sodium hydroxide and oxalic acid. Oxalic acid–decontaminated fractions were also cultured in selective liquid medium. Nontuberculous mycobacteria were isolated from 6 patients (16.1%). Mycobacterium chelonae and Mycobacterium avium-intracellulare complex were the most common species. Three patients with positive results of culture had at least 1 positive result by acid-fast smear. Oxalic acid decontamination and culture in liquid medium had the lowest contamination rate (6.7%). Colonization with nontuberculous mycobacteria was associated with humoral response to mycobacteria (immunoglobulin G titers against antigen A60) in patients with samples that tested positive by acid-fast smear. An improvement in pulmonary function was observed in 2 patients after they received a course of antimycobacterial therapy. Screening for nontuberculous mycobacteria in patients with cystic fibrosis will contribute to understanding the relevance of these pathogens with regard to deterioration of pulmonary function in patients with cystic fibrosis.

Cystic fibrosis (CF) is a genetic disorder caused by alterations in the CF transmembrane conductance regulator, causing a general disarrangement of mucous secretion [1]. Chronic lung infection, mainly due to Pseudomonas aeruginosa, Staphylococcus aureus, and Haemophilus influenzae, is the major cause of morbidity and mortality in patients with CF [2]. Although these pathogens predominate in the lungs of patients with CF, emerging pathogens, such as Burkholderia cepacia and Stenotrophomonas maltophilia, are now being considered important markers of lung deterioration [3, 4]. The usual poor correlation between clinical status and microbiological findings suggests that hidden pathogens, other than those that are usually isolated, may play a role in the lung deterioration in patients with CF.

To date, the role, if any, of nontuberculous mycobacteria in the chronic lung deterioration of patients with CF remains unclear. Although nontuberculous mycobacteria have been isolated from patients with CF, major differences in prevalence of infection have been reported (range, 3%–30%) [5–7]. These differences are probably related to the high rate of contamination of sputum samples with P. aeruginosa [5, 8]. Even more controversy surrounds the distinction between actual mycobacterial infection and simple colonization, given the interpretative difficulties, found by clinicians, that result from the diffuse and complex pattern of lung deterioration in patients with CF.

The objectives of this study were to determine the prevalence of nontuberculous mycobacterial colonization or infection in patients with CF in our hospital, and, with regard to patients with CF, to compare the efficacy of different mycobacterial isolation procedures in respiratory samples, to evaluate the humoral response to mycobacteria, and to investigate a possible correlation between colonization with nontuberculous...
PATIENTS, MATERIALS, AND METHODS

Patients and samples. Two sputum samples, obtained at intervals of 2 days to 1 week, were collected from 37 patients with CF (mean age, 21 years; range, 4–48 years) who were seen in our CF unit. If a patient had a positive acid-fast smear result, additional follow-up was done, and sputum samples were obtained once per week for at least 3 months.

Culture conditions. After we performed acid-fast staining by use of the auramine method, sputum samples were semi-quantitatively cultured after undergoing 2 decontamination procedures. Sputum samples were liquefied with a known, sample characteristic–dependent volume of N-acetylcysteine. The final volume was then recorded and the sputum volume inferred. Half of the sputum sample was then decontaminated by use of the oxalic acid technique of Corper and Uyei [9], and the other half of the sample was decontaminated by use of the sodium hydroxide technique of Kubica et al. [10]. After decontamination, samples were centrifuged and resuspended in 2 mL of 0.9% saline solution. A 0.2-mL portion of each sample was plated on Coletosos medium (Biomedics) and another 0.2-mL portion was plated on Löwenstein-Jensen selective medium with nalidixic acid, cycloheximide, and lincomycin (Biomedics) [11]. In addition, 1 mL of the oxalic acid–decontaminated fraction was cultured in ESP liquid medium supplemented with ESP Myco GS and ESP Myco Tb (Accumed International). Both solid and liquid media were then incubated at 37°C for 56 days. In addition, sputum samples from all patients were quantitatively cultured [12] on Columbia sheep blood agar, bacitracin-chocolate agar, MacConkey agar, and mannitol-salt agar plates at 37°C for 48 h to evaluate the presence of other bacterial pathogens.

Identification procedures. After Ziehl-Neelsen staining was performed to discard contaminant microorganisms, cultures that tested positive for mycobacteria were identified by means of conventional biochemical tests [13] and hybridization probes for Mycobacterium avium-intracellulare complex (Gen-Probe). Ribosomal RNA amplification for Mycobacterium tuberculosis complex was done for every sample that had a positive result by means of acid-fast smear, to rule out a mixed colonization with M. tuberculosis and nontuberculous mycobacteria (Gen-Probe). Contamination of the mycobacterial cultures with nonmycobacterial organisms was also recorded to determine the accuracy of decontamination procedures.

Mycobacterial antibody detection. IgG and IgA antibody titers against mycobacterial antigen A60 (Anda Tb) [14, 15] were determined in samples from all patients with CF. Breakpoints for IgG determination were 150 U and 300 U for patients who were <16 and ≥16 years old, respectively, as described elsewhere [15]. The manufacturer’s recommended breakpoint was used for IgA determination (<200 U and ≥350 U for the negative and positive breakpoints, respectively).

Patient clinical data. Charts of patients with CF who tested positive for nontuberculous mycobacteria were reviewed. A possible correlation of colonization or infection with nontuberculous mycobacteria and pulmonary function and clinical status was studied.

RESULTS

Mycobacteria were isolated from cultures of sputum samples from 6 patients (16.1%). Three of these 6 patients had at least 1 positive acid-fast smear result. All patients with negative culture results had negative acid-fast smear results as well. Mycobacterium chelonae, M. avium-intracellulare complex, and Mycobacterium scrofulaceum were isolated from 3, 2, and 1 patient(s), respectively. The absence of M. tuberculosis was confirmed in all auramine-positive sputum samples by means of rRNA amplification, which allowed us to rule out the presence of mixed colonization with M. tuberculosis and nontuberculous mycobacteria. Results of auramine staining and semiquantitative mycobacterial culture are shown in table 1. Of note, the 3 patients with positive acid-fast smear results also had a higher number of colony-forming units in solid culture media. All patients with auramine-positive samples had ≥10⁵ cfu/mL, whereas all patients with auramine-negative samples had ≤10⁵ cfu/mL. All samples that tested positive for mycobacteria on solid media also had positive results in liquid media.

For 26 (70.2%) of the 37 patients, at least 1 culture medium was contaminated with nonmycobacterial organisms. For samples cultured on solid media, the highest rate of contamination (47.3%) was found among samples that were decontaminated in sodium hydroxide and plated on Coletosos medium, whereas the lowest rate of contamination (12.1%) was found among samples that were decontaminated in oxalic acid and plated on Löwenstein-Jensen selective medium (table 2). For 5 sputum samples (6.8%), all solid culture media were contaminated, and all solid culture media used for the 2 consecutive sputum samples obtained from 2 patients (5.4%) were also contaminated. On the other hand, samples that were cultured in liquid media after decontamination in oxalic acid had the lowest rate of contamination (6.7%).

Of the 37 patients who we investigated, 23 (62%) were colonized with P. aeruginosa, 13 (35%) with S. aureus, 7 (18.9%) with Haemophilus species, 5 (13.5%) with S. maltophilia, and 4 (10.8%) with other pathogens. We did not observe significant differences in the rates of contamination of mycobacterial culture samples by the concomitant pathogen isolated, with the exception of P. aeruginosa when it was recovered in numbers ≥10⁶ cfu/mL of sputum. In the latter case, the rate of contamin-
arterial partial pressure of oxygen \([P_{\text{a}}O_2]\), 53 mm Hg; and arterial

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mycobacterium species</th>
<th>1st sputum sample results</th>
<th>2nd sputum sample results</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>M. chelonae</td>
<td>+/+ (500/500)</td>
<td>10^4/10^4</td>
</tr>
<tr>
<td>5</td>
<td>M. avium-intracellulare complex</td>
<td>+/+ (1000/500)</td>
<td>10^3/10^3</td>
</tr>
<tr>
<td>13</td>
<td>M. avium-intracellulare complex</td>
<td>–/–</td>
<td>–/10^2</td>
</tr>
<tr>
<td>20</td>
<td>M. chelonae</td>
<td>–/+ (25)^c</td>
<td>–/10^2</td>
</tr>
<tr>
<td>25</td>
<td>M. scrofulaceum</td>
<td>–/–</td>
<td>10^4&lt;10^2</td>
</tr>
<tr>
<td>33</td>
<td>M. chelonae</td>
<td>–/–</td>
<td>10^3/–</td>
</tr>
</tbody>
</table>

NOTE. +, Positive; –, negative.

a Data in parentheses are no. of acid-fast rods per 100 fields.

b Data are no. of cfu in solid culture medium per mL of sputum.

c For 2d sputum sample only.

The patients who were colonized with mycobacteria are shown in table 3.

IgG antibody titers were greater than the breakpoint in 4 patients (10.8%; figure 1). Three of these patients (patients 4, 5, and 20) were those who had auramine-positive samples; the other patient had culture-positive but auramine-negative samples. The high IgG titers found in these patients support the diagnosis of chronic infection rather than just mere colonization. Although it was nearly as sensitive as detection of IgG, detection of IgA was less specific, because 2 of the patients who tested positive for IgA had samples that were culture and auramine negative (figure 1). The 3 patients with persistent nontuberculous mycobacterial colonization and auramine-positive smears had experienced clinical deterioration during the time prior to and during the study. Two of them (patients 4 and 5) were treated for mycobacterial infection.

Patient 4 was a 30-year-old man who had been placed on the waiting list to receive a lung transplant because he had severe and progressive lung deterioration. During the year prior to the study period, the patient had repetitive episodes of fever, anorexia, weight loss, and dyspnea. Despite having received multiple courses of iv antibiotics, his cough and pulmonary status worsened (forced expiratory volume in 1 s \([FEV_1]\), 25% of predicted value; arterial blood gases in room air, pH 7.37; arterial partial pressure of oxygen \([P_{\text{a}}O_2]\), 53 mm Hg); and arterial partial pressure of carbon dioxide \([P_{\text{a}}CO_2]\), 46.4 mm Hg), which prompted the clinicians to administer continuous oxygen therapy. Repeated mycobacterial cultures of sputum samples yielded \(M.\) chelonae, whereas the results of conventional cultures were negative for typical CF pathogens. A marked improvement in tolerance for exercise, weight gain, and general sense of well-being were noted 2 weeks after therapy with cotrimoxazole, amikacin, and clarithromycin was instituted. Four months after the commencement of therapy, his respiratory function improved to baseline levels \((FEV_1, 35%\) of predicted value; arterial blood gases in room air, pH 7.40; \(P_{\text{a}}O_2\), 59.2 mm Hg; and \(P_{\text{a}}CO_2, 45.5\) mm Hg). The results of staining and cultures of sputum samples obtained 5 and 7 months after the commencement of therapy were negative for acid-fast bacilli. At present, this patient is no longer on the waiting list to undergo lung transplantation.

Patient 5 was a 33-year-old man whose pulmonary function was stable for several years \((FEV_1, 99%\) of predicted value), but which worsened during the year prior to the study period. The patient had persistent productive cough, hemoptysis, dyspnea, and poor response to treatment with oral and iv antibiotics. Chest radiographs showed mild bronchiectasis with no infiltrates. Mycobacterial and conventional sputum cultures yielded \(M.\) avium-intracellulare complex and low bacterial counts of \(S.\) aureus \((10^3 cfu/mL)\), respectively. Treatment with clarithromycin, ethambutol, and levofloxacin markedly improved his pulmonary status. Six months after the commencement of therapy, the patient remained well and did not require treatment with iv or oral antibiotics. The results of staining and cultures of sputum samples obtained 2 and 4 months after the commencement of therapy were negative for acid-fast bacilli.

Patient 20, who had persistent nontuberculous mycobacterial colonization and auramine-positive smears, had mild pulmonary disease, and \(M.\) chelonae with low bacterial counts was...
recovered. Although, to date, his culture results have continually been positive for mycobacteria, no specific antimycobacterial therapy has been instituted because the patient’s pulmonary functions remain stable.

**DISCUSSION**

Chronic lung infection is the main cause of morbidity and mortality in patients with CF. The pathogen *P. aeruginosa* is involved most frequently, followed by *S. aureus* and *H. influenzae* [2]. The increase in life expectancy, which is mainly the result of the establishment of adequate antistaphylococcal and antipseudomonal therapy, has led us to face, if not to provoke, the presence of emerging pathogens, such as *B. cepacia* and *S. maltophilia* [3, 4]. Recent studies have suggested that nontuberculous mycobacteria may be among these emerging pathogens, although, to date, a definite conclusion has not yet been reached [16, 17].

There are major differences in the reported rate of prevalence of nontuberculous mycobacteria among patients with CF (range, 3%–30%) [5–7]; these differences probably result from differences in methodology. In our study, the rate of prevalence of nontuberculous mycobacteria in respiratory samples obtained from patients with CF was 16%. The contamination of mycobacterial culture specimens (mainly with *Pseudomonas* organisms) after they undergo the usual decontamination procedures may lead researchers to underestimate the prevalence of nontuberculous mycobacteria in patients with CF [8]. Our results show that culture on solid media and in selective liquid media after the samples undergo oxalic acid decontamination yields the lowest contamination rates, which helped us to identify patients who are infected or colonized with nontuberculous mycobacteria. On the other hand, it is notable that 8% of the patients with CF who were studied had positive results by acid-fast smear, 100% of whom had infections due to nontuberculous mycobacteria. This high proportion of infection due to nontuberculous mycobacteria is extremely unusual in the population when HIV-positive patients are excluded. In fact, in our experience, a positive acid-fast smear result for respiratory samples obtained from immunocompetent patients almost always signals infection with *M. tuberculosis*. However, an exception needs to be made when evaluating results such as those for patients with CF, because, for these patients, a positive acid-fast smear result will most probably signal infection or colonization with nontuberculous mycobacteria. This observation has therapeutic and epidemiological implications, because empirical antibiotic treatment and respiratory isolation procedures are different for patients infected with *M. tuberculosis* and those infected with nontuberculous mycobacteria. Moreover, semiquantitative mycobacterial culture may be of benefit for evaluation of the role of mycobacteria isolated from patients with CF. All patients who had positive results by auramine staining had ≥10³ cfu/mL of sputum, whereas all patients with negative results had ≤10² cfu/mL of sputum.

Most published works about the prevalence of infection with nontuberculous mycobacteria among patients with CF have not investigated the humoral immunologic reaction to these nontuberculous mycobacteria. The results of studies published elsewhere regarding delayed-type hypersensitivity are controversial. In one study, 75% of patients with positive culture results showed delayed-type hypersensitivity to mycobacteria with a high specificity (87%) [6] that suggests that most patients were infected rather than colonized. On the other hand, another study found no difference in the prevalence of delayed-type hypersensitivity to nontuberculous mycobacteria in a comparison of patients with CF and a control population [18].

In our study, we determined the humoral responses to mycobacterial antigen A60 in all patients with CF. This complex

<table>
<thead>
<tr>
<th>Decontamination method</th>
<th>Coletos medium</th>
<th>Löwenstein-Jensen selective medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contaminated</td>
<td>Positive</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>13 (17.6)</td>
<td>9 (12.1)</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>35 (47.3)</td>
<td>18 (24.3)</td>
</tr>
</tbody>
</table>

### Table 3. Bacterial pathogens, other than mycobacteria, isolated from patients who had cystic fibrosis and positive culture results for mycobacteria.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Bacterial pathogens (quantity) revealed on 1st sputum sample</th>
<th>Bacterial pathogens (quantity) revealed on 2d sputum sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Normal oropharyngeal flora</td>
<td>Normal oropharyngeal flora</td>
</tr>
<tr>
<td>5</td>
<td><em>Staphylococcus aureus</em> (10⁸ cfu/mL)</td>
<td><em>P. aeruginosa</em> (10³ cfu/mL)</td>
</tr>
<tr>
<td>13</td>
<td><em>Pseudomonas aeruginosa</em> (10⁶ cfu/mL)</td>
<td><em>P. aeruginosa</em> (10⁵ cfu/mL); <em>S. aureus</em> (10⁷ cfu/mL)</td>
</tr>
<tr>
<td>20</td>
<td><em>P. aeruginosa</em> (10⁶ cfu/mL); <em>S. aureus</em> (10⁷ cfu/mL)</td>
<td><em>S. aureus</em> (10⁵ cfu/mL); <em>S. maltophilia</em> (10⁵ cfu/mL)</td>
</tr>
<tr>
<td>25</td>
<td><em>Stenotrophomonas maltophilia</em> (10³ cfu/mL)</td>
<td><em>S. aureus</em> (10⁵ cfu/mL)</td>
</tr>
<tr>
<td>33</td>
<td><em>S. aureus</em> (10⁸ cfu/mL)</td>
<td><em>S. aureus</em> (10⁵ cfu/mL)</td>
</tr>
</tbody>
</table>
and interspecies antigen was obtained from Mycobacterium bovis (BCG) and is found in all mycobacteria, including M. tuberculosis complex, atypical mycobacteria, and Mycobacterium leprae. The level of IgG antibodies was considerably elevated in 4 patients (10.8%), including the 3 patients with positive acid-fast smear results and 1 of the patients who tested positive by means of culture alone. These results suggest that patients with repeatedly positive results of acid-fast smears are more likely to be infected than colonized. On the other hand, patients with negative results of acid-fast smears and positive culture results with low numbers of colony-forming units are more likely to be colonized than infected. In fact, a recent study has shown that only repeatedly positive results of cultures for mycobacteria were associated with granulomatous mycobacterial lung disease diagnosed at autopsy [19]. Remarkably, 2 patients with repeatedly positive results of acid-fast smears had experienced a clinical deterioration during the months prior to recovery of mycobacteria that could not be explained by the findings of conventional bacterial cultures. They did not respond to several courses of iv antibiotics or to conventional airway clearance. Nevertheless, specific antimycobacterial chemotherapy with 3 drugs was instituted, and both patients experienced a dramatic improvement in clinical status, and the results of sputum cultures became negative after 2 and 3 months of therapy, respectively. Moreover, it is of note that lung transplantation was avoided for 1 patient after mycobacterial infection was diagnosed and adequate antibiotic therapy was initiated.

Screening patients with CF for nontuberculous mycobacteria will undoubtedly contribute to our understanding of the relevance of these pathogens in the deterioration of pulmonary function seen in patients with CF.

Acknowledgments

We thank L. de Rafael for providing correction of our English.

References

9. Corper HJ, Uyei N. Oxalic acid as a reagent for isolating tubercle bacilli and study of the growth of acid-fast non pathogens on different media with their reactions to chemical reagents. J Lab Clin Med 1930; 15: 348–69.


