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Monoclonal Infection Involving *Mycobacterium avium* Presenting with Three Distinct Colony Morphotypes

ESTHER L. WRIGHT,[1] SABRINA ZYWNO-VAN GINKEL,[2] NALIN RASTOGI,[3] AND WILLIAM W. BARROW[1]*

Unité de la Tuberculose et des Mycobactéries, Institut Pasteur de la Guadeloupe, 97165 Pointe-à-Pitre, Guadeloupe,[3] and Molecular Pharmacology[2] and Mycobacteriology Research Unit,[1] Southern Research Institute, Birmingham, Alabama 35205

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Recent reports indicate that polyclonal infections may play an important role in multiple drug resistance in *Mycobacterium avium* infections. We report here on the isolation of a single *M. avium* strain that appeared to have smooth colony morphology upon initial isolation on a Lowenstein-Jensen slant. Primary subculture onto Middlebrook 7H10, however, revealed three distinct morphotypes representing smooth opaque (SmO), smooth transparent (SmT), and rough (Rg) colony morphologies. All three morphotypes were identified as *M. avium* by standard biochemical procedures, Genprobe analysis, and mycolic acid patterns. Subsequent restriction fragment length polymorphism analysis, using *Sal*I- and *Pvu*II-digested genomic DNA, revealed identical patterns for hybridization with the IS*1245* probe. Thin-layer chromatographic analysis of lipids from the three morphotypes revealed that only the SmT morphotype possessed what appeared to be lipid components similar to, but unlike, previously described serovar-specific glycopeptidolipid antigens. Further analysis of internally radiolabeled deacylated lipids from the SmT morphotype, by high-performance liquid chromatography and thin-layer chromatography, disclosed that some of these components can be internally radiolabeled with [^{14}C]phenylalanine and [^{14}C]mannose. These results suggest that these components are structurally similar to previously described glycopeptidolipid antigens. This is apparently the first report of a monoclonal infection involving a single strain of *M. avium* presenting with all three colony morphotypes, SmO, SmT, and Rg.

Clinical management of AIDS is more formidable because of opportunistic infections, particularly those involving the *Mycobacterium avium* complex and *Mycobacterium tuberculosis* (2, 7). The *M. avium* complex is more difficult to treat because of a diversified drug resistance pattern (11), which in the case of *M. avium* has generally been associated with an inability of antimycobacterial drugs to cross the cell envelope barrier (6, 13). As our understanding of *M. avium* pathogenesis progresses, it appears that other things might also restrict efficient clinical management of these opportunistic pathogens.

Polyclonal infections, involving multiple strains of *M. avium*, have been reported previously (1, 16, 18, 19). Polyclonal infections involve a simultaneous infection of two genotypically distinct strains and can cause problems if those strains differ in antimicrobial sensitivity (19). Monoclonal infections are those that derive from the clonal expansion of a single microorganism (19) and appear to be a more common finding with *M. avium*. To our knowledge, no one has reported a monoclonal infection involving a single strain of *M. avium* existing as three individual morphotypes in one patient. We report here on the isolation and characterization of a single *M. avium* strain that does. Analysis by Genprobe and mycolic acid patterns had revealed that these three morphotypes were *M. avium* (15). To establish that the three morphotypes were genotypically the same and to determine the serotype, we have conducted more extensive analysis using restriction fragment length polymorphisms (RFLP), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC). Results reveal that the three morphotypes have identical RFLP patterns, indicating clonal expansion of a single strain. In addition, only the SmT morphotype appears to possess components analogous to serovar-specific glycopeptidolipids (GPL), even though they do not correspond to any of the known GPL from *M. avium.*

* Corresponding author. Mailing address: Mycobacteriology Research Unit, Birmingham, Alabama 35205. Phone: (205) 581-2139. Fax: (205) 581-2877.

MATERIALS AND METHODS

Mycobacterial isolates. The *M. avium* strain was originally obtained from a male patient on at least three different occasions. The strain was initially isolated on a Lowenstein-Jensen slant and appeared to have a smooth colony morphology. Upon serotyping the isolate by means of TLC (17), it was observed that it did not contain serovar-specific GPL components, characteristic of *M. avium* serovars (15). After the third isolation of strain 373 on Lowenstein-Jensen, it was subcultured onto Middlebrook 7H10. Three distinct colony morphotypes were observed, an SmO, an SmT, and an Rg. Strain 373 was initially identified as *M. avium* by biochemical results, mycolic acid patterns, and Genprobe analysis (15). The three morphotypes of strain 373 were later characterized individually as *M. avium* by the same techniques (15). Other mycobacteria used in this study were SmO strains of *M. avium* serovars 4, 8, and 20 (4, 5, 20) and their Rg variants developed as described previously (3).

Radiolabeling and lipid analysis by HPLC and TLC. Using techniques previously described, the lipids of the three morphotypes were internally radiolabeled for further analysis by HPLC and TLC using [^{14}C]phenylalanine or [^{14}C]mannose (ICN Radiochemicals, Inc., Irvine, Calif.) (20). Resulting radiolabeled lipids were extracted from the lyophilized mycobacteria. Lipid samples were separated on silica gel TLC plates (Whatman) by using chloroform-methanol-water (60:12:1) (solvent A), chloroform-methanol-water (65:25:4) (solvent B), and chloroform-methanol (11:1) (solvent C) and assayed for radioactivity as described previously (20). The GPL were detected on TLC plates by their characteristic yellow-gold color (9). Analysis of radiolabeled lipids by HPLC was conducted as previously described, by using Beckman System Gold (15, 20). One-milliliter fractions were collected and analyzed by TLC, using the orcinol-sulfuric acid reagent, in order to verify the presence of GPL. For serotype analysis, individual deacylated lipids from *M. avium* strain 373 (SmD, SmT, and Rg) were then examined by TLC in solvents A, B, and C and compared with deacylated lipids from known *M. avium* serotype stock cultures kindly provided by Anna Tsang (17).

Southern blot hybridization. Strains of mycobacteria were grown in 100 ml of Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-glucose-catalase (Difco Laboratories, Detroit, Mich.) and 0.2% glycerin at 37°C. Glycine

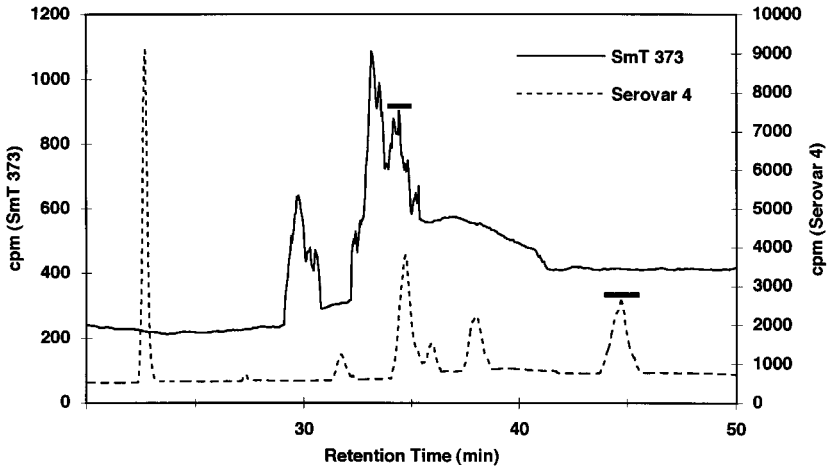

FIG. 1. HPLC distribution of radioactivity in deacylated lipid from *M. avium* SmT 373 and serovar 4, radiolabeled with [^{14}C]phenylalanine. Samples were separated in a mobile phase of 100% chloroform for 10 min and then a 40-min gradient of 0 to 10% methanol in chloroform at a flow rate of 1.0 ml/min; radioactivity was detected by a solid-cell detector (20). Bars, fractions associated with GPL components.

was added to a 2% final concentration, and the cultures were incubated at 37°C for another 48 h. The cells were harvested by centrifugation at 4°C, resuspended in 6 ml of P1 buffer (10 mM Tris [pH 7], 100 mM NaCl, 5 mM EDTA), and incubated with 5 mg of lysozyme per ml at 37°C for 30 min. Three milliliters of buffer P2 (10 mM Tris [pH 8.0], 250 mM NaCl, 1.2% Triton X-100, 100 μg of RNase A per ml, 12 mM EDTA, 0.5 M guanidine-HCl) was added to the solution, and the cells were incubated on ice for 20 min. Proteinase K was added to a final concentration of 6.6 mg/ml, and the solution was incubated at 50°C for 2 h. The cell lysate was clarified twice by centrifugation at 20,000 × *g* for 15 min. The genomic DNA was purified with the Qiagen Tip 500 column (Qiagen, Chatsworth, Calif.).

Genomic DNA was digested with either *Sal*I (Promega, Madison, Wis.) or *Pvu*II (New England BioLabs), electrophoresed in 1% agarose gel, and transferred to a Hybond-N membrane (Amersham Life Sciences, Arlington Heights, Ill.). The IS*1245* probe was made by PCR (10) and labeled by random priming with the Genius 2 kit (Boehringer Mannheim, Indianapolis, Ind.). The blot was hybridized with the probe at 65°C, developed with the luminescent kit Genius 7 (Genius system user's guide for membrane hybridization, version 3.0; Boehringer Mannheim), and exposed to XAR 5 film (Eastman Kodak Co., New Haven, Conn.).

RESULTS

Serotype analysis. Upon examination of deacylated lipids from *M. avium* 373 SmO and Rg by TLC, no characteristic GPL were observed when plates were developed in solvent A, B, or C (not shown). When the deacylated lipid pattern from *M. avium* 373 SmT was examined, a characteristic GPL component was observed with an R_f value more closely aligned to that of the GPL from SmO *M. avium* serovars 1, 2, 4, and 20 (solvent B). Presence of the GPL was detected by the characteristic yellow-gold color which appears following treatment of the TLC plate with orcinol sulfuric acid (results not shown) (17).

Examination of radiolabeled lipids by HPLC and TLC. Deacylated radiolabeled lipid was analyzed by TLC and HPLC to verify that the yellow-gold staining component was a GPL. Following internal radiolabeling of individual cultures with [^{14}C]phenylalanine, extracted radiolabeled lipids were first deacylated and then examined by HPLC and TLC. Examination of the deacylated [^{14}C]phenylalanine radiolabeled lipid from strain 373 SmT by HPLC revealed a "GPL" component that eluted at approximately 35 min (Fig. 1). This component was also radiolabeled with [^{14}C]mannose (data not shown). Fractions were collected, applied to a TLC plate, and developed in solvent A (not shown). The fraction collected at 35 min was stained with the characteristic yellow-gold color when reacted with the orcinol-sulfuric acid reagent. By comparison, the major deacylated GPL component from *M. avium* serovar 4 has a retention time of approximately 45 min under the same column conditions (Fig. 1). The deacylated GPL component from *M. avium* serovar 20 also is eluted at approximately 45 min (data not shown).

These results indicate that the GPL component has a structure composed of a phenylalanine-containing lipopeptide core and sugar moieties suggestive of those found in the previously identified serovar-specific GPL components. In addition, its R_f value in TLC and elution pattern with HPLC indicate that it is more apolar than the GPL of serovars 4 and 20. More sophisticated structural analysis is required to verify that the component has a structure identical to that of the previously identified serovar-specific GPL components.

RFLP analysis of strain 373 morphotypes. To verify that the three colony morphotypes of *M. avium* strain 373 had originated from the same strain, genomic DNA from each morphotype was examined by RFLP. Genomic DNA was digested with either *Sal*I or *Pvu*II and hybridized with IS*1245*, chemiluminescence labeled with the Genius system (Boehringer Mannheim). The RFLP patterns obtained from all three of the morphotypes are presented in Fig. 2 (patterns resulting from *Sal*I digestion) and Fig. 3 (patterns resulting from *Pvu*II digestion). The patterns obtained for all three isolates, with both restriction enzymes, appear to be identical in all respects. At

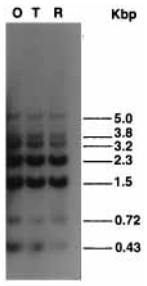

FIG. 2. IS*1245* RFLP patterns of *M. avium* 373 SmO (O), SmT (T), and Rg (R) colony morphotypes. Genomic DNA was digested with the *Sal*I restriction enzyme and hybridized with IS*1245*, nonradioactively labeled by using the Genius system. Sizes of bands were determined from known standards.

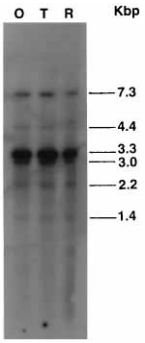

FIG. 3. IS*1245* RFLP patterns of *M. avium* 373 SmO (O), SmT (T), and Rg (R) colony morphotypes. Genomic DNA was digested with the *Pvu*II restriction enzyme and hybridized with IS*1245*, nonradioactively labeled by using the Genius system. Sizes of bands were determined from known standards.

least seven major bands are common to all three morphotypes when the *Sal*I restriction enzyme is used (Fig. 2), and at least six major bands are common to all three with the *Pvu*II restriction enzyme (Fig. 3).

For comparison, the RFLP patterns for *M. avium* serovar 4 and 8 SmO and Rg morphotypes are given in Fig. 4. With the exceptions of perhaps one or two bands, the RFLP patterns for SmO and Rg morphotypes for both serovars are virtually identical, indicating that rough and smooth morphotypes of the same strain possess indistinguishable RFLP patterns when analyzed under these conditions. To verify the specificity of the probe, the same procedure was conducted with genomic DNA extracted from SmO and Rg morphotypes of *Mycobacterium intracellulare* serovar 20 (3); no bands were observed. This is to be expected because IS*1245* is specific for *M. avium* and not for *M. intracellulare* (10).

DISCUSSION

Results reported here further substantiate that *M. avium* infections can occur as multiple morphotypes originating from the same strain, in this case as three different morphotypes. Although it is impossible to know for certain, it is likely that this patient was infected initially with one morphotype which subsequently converted to three different morphotypes during the infectious-disease process. As far as we can determine, this is the first report of three colony morphotypes (SmT, SmO, and Rg) of the same strain being isolated from one patient. Others have reported polyclonal *M. avium* bacteremia in patients with AIDS; however, those reports apparently involved only transparent and opaque colony morphotypes (1, 16, 18). As with the other reports, we agree that this is important and may be a contributing factor to some of the variable resistance patterns observed with *M. avium*-infected patients.

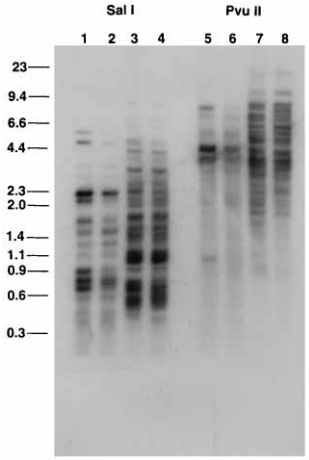

FIG. 4. IS*1245* RFLP patterns of *M. avium* serovar 4 Rg (lanes 1 and 5) and SmO (lanes 2 and 6) and serovar 8 Rg (lanes 3 and 7) and SmO (lanes 4 and 8) colony morphotypes. Genomic DNA was digested with *Sal*I or *Pvu*II and hybridized with IS*1245*, nonradioactively labeled by using the Genius system. The sizes of known standards (in kilobases) are indicated.

As reported previously, the three morphotypes of strain 373 possess different patterns of susceptibility to various antimycobacterial drugs and inhibitory drug combinations (14). In that study, four antimicrobial agents were tested: ethambutol, amikacin, sparfloxacin, and clarithromycin (14). The MIC of ethambutol was the same for all three isolates (5 μg/ml), but the MICs of the other antimicrobial agents varied from 1 to 2 μg/ml for amikacin, 8 to 16 μg/ml for sparfloxacin, and <1 to 2 for clarithromycin (14). In addition, combination studies using the antimicrobial agents listed above and drugs that inhibit GPL biosynthesis (*x/y* quotient calculation method) revealed an even wider range of susceptibility, ranging from a 1+ to a 3+ enhancement (14). As discussed previously (14), we feel that those results can be explained by the variability of cell envelope architecture inherent in these three distinct morphological variants (14). It is known, for example, that the rough morphotype characteristically lacks GPL components which are found in the outer cell envelope of the smooth morphotypes (3).

A likely explanation for the failure to observe multiple colonial morphotypes upon initial isolation was the fact that the initial culture was grown on Lowenstein-Jensen medium. It is known that the SmT colony morphotype was not observed until the introduction of Middlebrook 7H10 medium, with only SmO and Rg being observed prior to that (8, 12). The three different morphotypes in this study were observed only after the first subculture onto Middlebrook medium.

Another interesting point concerning this case is the fact that the SmO colony morphotype lacked the characteristic serovar-specific GPL components. It is generally assumed that the SmO and SmT morphotypes of *M. avium* both contain serovar-specific GPL and should thus be serotypeable by TLC techniques. This particular SmO variant does not contain these components, even though it has the characteristic appearance of a smooth opaque (dome-shaped) colony. The SmO morphotype does apparently contain the apolar GPL components, as suggested by previously described HPLC patterns of [^{14}C] phenylalanine-radiolabeled lipids (14). It is probable that initial serotyping was unsuccessful for two reasons: (i) predominance of two morphotypes lacking GPL components (i.e., SmO and Rg) and (ii) presence of a noncharacteristic GPL component in one of the morphotypes (SmT).

Interesting questions, which cannot be answered now, are how did these three different morphotypes arise, how frequently does this occur clinically, and to what degree does this occurrence influence the management of *M. avium* infections? Further studies should provide insight as to the role this phenomenon may play in the overall drug resistance patterns of *M. avium* isolates.

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