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Search for the Molecular Basis of Morphological Variation in *Mycobacterium avium*

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Isolates of *Mycobacterium avium* exhibit three different colonial variations: smooth domed (SmD), smooth transparent (SmT), and rough (Rg). Because the discrimination between morphotypes is founded on morphological rather than molecular principles and because of the absence of consensus over the relevance of morphology to pathogenesis and drug sensitivity, a comparative study at the protein level was undertaken. By direct immunization of BALB/c mice with the soluble sonicate of one of the morphotypes of *M. avium* serovar 2, eight monoclonal antibodies (MAbs) were identified, of which one was *M. avium* specific. Cross immunization of syngeneic mice with serum-absorbed antigens allowed the generation of 15 further MAbs; 11 were *M. avium* or *M. avium* complex specific, but none of them was morphotype specific. Subcellular fractions analyzed by electrophoresis showed similar profiles, with the exception of a cytosolic protein with a relative molecular mass of ca. 66 kDa (protein SmT 66), which was most highly expressed in SmT variants of *M. avium* serotypes 2 and 4. Because a well-known, ubiquitous stress-heat shock protein (hsp65) has a similar molecular mass, protein SmT 66 was compared with hsp65. Western blot (immunoblot) analyses using several cross-reacting MAbs and N-terminal amino acid sequencing established that this protein was not the ubiquitous stress protein. Thus, SmT 66 is the first product to be described which might be associated with the SmT morphotype.

Before 1984, organisms belonging to the *Mycobacterium avium* complex (*M. avium* complex refers to the bacteria identified as *M. avium* and *M. intracellulare*) were not commonly identified pathogens. With the advent of AIDS, these infectious agents are now regarded as among the most common opportunistic bacterial agents affecting human immunodeficiency virus-seropositive patients: nontuberculous mycobacterial infections have been diagnosed in 14 to 30% of AIDS patients and reported in up to 50% of autopsy cases (6, 7, 17). Accordingly, there is renewed interest in molecular characterization of the infectious agents. When plated on Middlebrook 7H10 or 7H11 agar, at least three variants of *M. avium* can be identified: smooth domed (SmD), smooth transparent (SmT), and rough (Rg). The SmT morphotype is usually associated with virulence, because patient-derived strains show mostly this form in primary culture (3, 14) and SmT variants seem to be more pathogenic than the other variants when tested with animal models (24). Supporting this view, recent studies of cytokine production by human macrophages have shown that SmD, as opposed to SmT, bacilli have enhanced ability to induce interleukin-1 and tumor necrosis factor, suggesting that the SmT variant may more readily escape host defenses (15, 30). There is apparently no difference between the rates of uptake of the three morphotypes by human macrophages, although one report showed a greater propensity for internalization of the SmD variant. According to some investigators (20), Sm and Rg variants grow at the same rate inside macrophages, whereas other researchers report that SmT variants multiply more rapidly inside monocytes (3, 9, 26, 28). Since the three forms are defined solely on morphological grounds and since the contribution of a particular morphotype to virulence is subject to debate, it seemed urgent to define the chemical and molecular bases of those colonial variations (31). We have attempted to address this question using two strategies, involving, on the one hand, a search for morphotype-specific monoclonal antibodies (MAbs) and, on the other, a quest for significant differences in protein profiles.

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MATERIALS AND METHODS

Bacterial strains. Two clinical isolates of *M. avium*, serovar (ser) 2 (strain 2151; obtained from A. J. Crowle, University of Colorado Health Sciences Center, Denver, Colo.) and ser 4 (obtained from A. J. Crowle), were the principal strains used in this study. Three isogeneic colonial variants, SmD, SmT, and Rg, from each serovar were isolated. Each one was grown on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-dextrose-catalase enrichment (Remel, Lenexa, Kans.). Each plate was inoculated with 4 to 5 $\times$ 10^3 CFU and incubated at 37°C for 4 weeks. Isolated colonies were examined for morphological characteristics with a dissecting microscope (magnification, $\times$40). Other mycobacterial strains were *M. tuberculosis* Erdman (strain 107; Trudeau Mycobacterial Culture Collection), *M. tuberculosis* H37Ra, *M. leprae* (from armadillos), *M. scrofulaceum* (strain 10-419 [identical to *M. avium* complex ser 22]; from our own collection) (23), and *M. intracellulare* (strain 72-888 [identical to *M. avium* complex ser 25]; from our own collection) (23).

Subcellular fractionation. After 4 weeks of incubation, bacterial cells were harvested, heat killed at 80°C for 1 h, and washed five times with phosphate-buffered saline (PBS), pH 7.4. Washed cells were resuspended in a breaking buffer containing proteinase inhibitors, as described by Hirschfield et al. (10). *M. avium* sonic extracts were prepared by exposing the microorganism to 2-s sonication pulses with a W-385 Sonication Ultrasonic Processor (Heat Systems-Ultrasonics, Farmingdale, N.Y.) for a total of 1 h in an ice bath. The total sonic extract was digested with DNase I (0.02 mg/ml) and RNase A

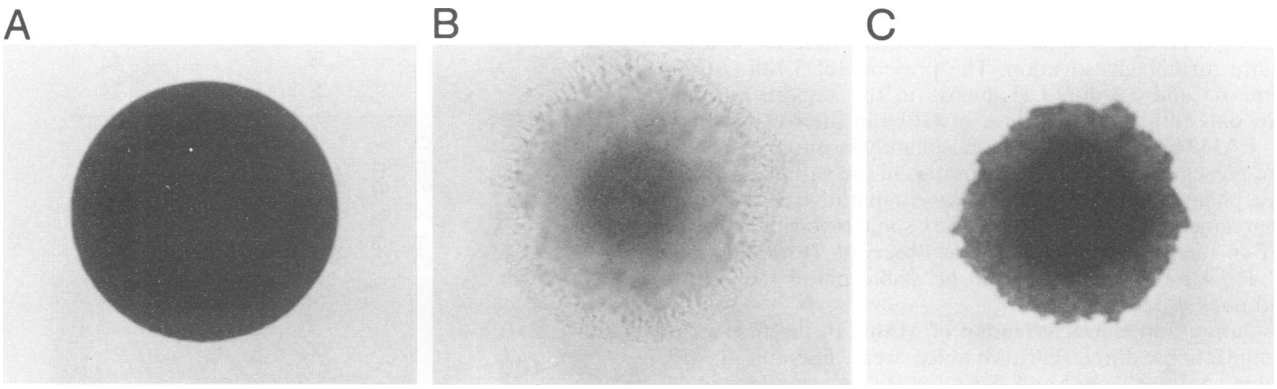

FIG. 1. Examples of the SmD (A), SmT (B), and Rg (C) colonial variants of *M. avium* ser 2 grown on 7H11 agar at 37°C for 4 weeks (magnification, ×40).

(0.02 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) in the presence of 2 mM $MgCl_2$ for 1 h at room temperature. Unbroken cells were discarded by centrifugation at 2,500 × *g* for 30 min at 4°C. Centrifugation of broken cells, performed at 27,000 × *g*, yielded a pellet enriched in insoluble cell wall. The supernatant was further centrifuged at 150,000 × *g*, for 2 h at 4°C, to separate the cytosol from the cell membrane. Protein concentrations were estimated by the method of Lowry et al. (13). Aliquots of the cytosol, cell wall, and cell membrane fractions were made and stored at −20°C.

Antigen preparation and production of MAbs. For the purpose of immunization, sonicated supernatants were precipitated at 4°C for 1 h with ammonium sulfate at 80% saturation. The precipitate was centrifuged at 14,000 × *g* for 30 min and then dialyzed against water at 4°C for 16 h. If such a complex mixture of unfractionated antigens is used for the generation of antibodies, the majority will be directed to the dominant carbohydrate antigens such as lipoarabinomannan (LAM), or the glycopeptidolipids (GPLs). Therefore, to enhance the production of MAbs to less dominant epitopes, particularly proteins, a two-step antigen selection process was devised. Firstly, the antigen preparation consisted of 50 μg of soluble sonicate and 10 μg of whole cells of either morphotype from *M. avium* ser 2 and 50 μl each of MAbs (diluted 1:1,000 in PBS) CS-35 and CS-17, which recognize LAM and the ser 2-specific GPL (21), respectively. This mixture was emulsified with 100 μl of incomplete Freund's adjuvant and incubated at 37°C for 30 min. In the second step, protein preparations from SmD, SmT, and Rg morphotypes were incubated firstly with the anti-LAM and anti-GPL MAbs and then with sera (diluted 1:1,000) raised against one of the other variants. Thus, three mixtures, anti-SmD serum–SmT antigens, anti-SmT serum–SmD antigens, and anti-SmT serum–Rg antigens, were used to immunize syngeneic BALB/c mice. Protocols for immunization, cell fusion, and selection of hybrids were performed as described by Rivoire et al. (21). The isotype and specificity of *M. avium* MAbs and other MAbs were established by using a MAb-typing kit for mouse tissue culture supernatants according to the instructions of the manufacturer (ICN Immunobiologicals, Costa Mesa, Calif.). The specificity of MAbs was determined by immunoblot analysis with either crude sonicate or proteinase K-treated antigens (8). Five MAbs to the 65-kDa heat shock protein (hsp65) and two MAbs directed against the 71-kDa stress protein were used. MAbs CS-43 and CS-44 had been raised against *M. leprae* antigens. MAbs MLIIH9, CBA1, IIC8, HAT1, and HAT3 were obtained through the World Health Organization, Immunology of Mycobacteria MAb bank (12).

Electrophoresis, immunoblotting, and chemical analysis. Proteins (5 to 7 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions (18). For immunoblotting (Western blotting), antigens (10 μg) were separated under reducing conditions by SDS-PAGE and then transferred to nitrocellulose by blotting at a constant voltage of 45 V for 3 h. The nitrocellulose strips were blocked for 1 h with 1% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween 20 (TBST) and then incubated at room temperature for 1 h with antibody in the form of either murine hybridoma culture supernatant (undiluted) or ascitic fluid (diluted in TBST). Subsequently, strips were washed with TBST and incubated with the goat anti-mouse alkaline phosphatase detection system (Sigma Chemical Co.).

Protein concentrations were determined by the method of Lowry et al. (13), with BSA as the standard. Carbohydrate content was estimated by using phenol-sulfuric acid reagent, with glucose as the standard (11). Sugars were obtained after hydrolysis of bacteria with 2 M trifluoroacetic acid at 120°C for 2 h. Relative amounts were evaluated by gas chromatography of alditol acetate derivatives (11). N-terminal amino acid sequencing was conducted at the Macromolecular Resource Facility, Colorado State University.

RESULTS

Characterization of colonial variants. For the present study, the three morphotypes of *M. avium* ser 2 and 4 (SmD, SmT, and Rg) were picked and propagated. At the macroscopic level, differences could be easily discerned: SmD colonies can be described as moist, smooth, circular, raised, and opaque (Fig. 1A); SmT colonies appeared moist, smooth to wrinkled, circular, flat, and glassy (panel B); Rg colonies were dry, wrinkled, irregularly shaped, raised and opaque (panel C). SmT colonies from both ser 2 and 4 showed a glassy, translucent aspect. Variants from ser 2 were not pigmented, whereas colonies from ser 4 were yellow to orange. A difference in the rate of growth was observed; SmT bacteria consistently multiplied more slowly. The amounts of protein and carbohydrate, based on cell weight, were comparable in the three variants. Sugar analysis by gas chromatography of the alditol acetate derivatives did not reveal grossly dissimilar amounts of arabinose, mannose, and inositol, key cell wall structural compo-

nents. However, the amounts of glucose and galactose, reflective of glucan and galactan, were noticeably different and will require further investigation. The presence of 3,4-di-*O*-CH_3-rhamnose and 2,3-di-*O*-CH_3-fucose in the smooth morphotypes only reflects the absence of GPLs in the rough variant (2). LAM, lipomannan, and phosphatidylinositolmannosides were present in comparable quantities in the variants with the three phenotypes, as established by comparative SDS-PAGE. In agreement with Rastogi et al. (20), some reversion from the SmT to the SmD morphotype was observed. However, SmD and Rg variants were found to be stable under the culture conditions used.

Isolation and characterization of MAbs. In the first set of immunizations, three BALB/c mice were immunized with soluble sonicate from SmD, SmT, or Rg morphotypes of *M. avium* ser 2. Cloned cell lines producing antibodies to *M. avium* antigen were established. MAbs obtained in tissue culture were tested by Western blot for immunoreactivity against *M. avium* ser 2 and 4 SmD, SmT, and Rg; *M. tuberculosis* Erdman; *M. tuberculosis* H37Ra; and *M. leprae*. Eight clones recognizing eight different epitopes were obtained. Five clones showed broad specificity among the mycobacterial species tested and two clones did not react with one species, whereas one recognized only *M. avium* antigen. However, this last MAb did react with all three morphotypes.

In a second set of immunizations, antisera from the previously immunized mice were adsorbed with protein antigens from another variant (29). Thus, mixtures of SmT antigens and SmD antiserum, SmD antigens and SmT antiserum, and Rg antigens and SmT antiserum were administered to syngeneic BALB/c mice. After 3 weeks, a test bleed was performed and sera against *M. avium*, *M. tuberculosis*, and *M. leprae* antigens were examined by Western blot. As a result of the immunization schedule, the immunodominant carbohydrate-containing LAM and GPL antigens were not recognized. As shown in Fig. 2, although protein-antigen profiles between *M. avium* strains or variants were very similar, differences from those of other mycobacteria were easily discerned. Fusion of splenocytes with SP2/o myeloma cells and subsequent dilution cloning allowed the identification of 15 clones which recognized epitopes on protein antigens with M_rs ranging from 17,000 to 82,000 (Table 1; Fig. 3).

Five MAbs which recognized antigens of 24, 32, 35, and 46 kDa were cross-reacting. Eleven other MAbs bound only to antigens of *M. avium* or *M. avium* complex. Interestingly, four MAbs reacted with different antigens according to the serovar tested. However, no consistent specificity for any particular morphotype was observed. As several proteinase-resistant antigens of *M. avium* have been recently described (8), all of the MAbs were assayed against proteinase K-treated sonicates. None of them bound such antigens, indicating that all of the MAbs recognized protein epitopes.

Protein profiles of different morphotypes. The subcellular fractions of *M. avium* ser 2 were extracted with Triton X-114 in order to remove major glycosylated products such as LAM, lipomannan, and phosphatidylinositolmannosides. The detergent-insoluble phases were analyzed by SDS-PAGE. A representative gel is shown in Fig. 4. Profiles were similar, although differences, some of which were not reproducible from batch to batch, were observed. Cell wall proteins were found to be remarkably conserved, showing no overt differences between the three variants. On the other hand, a cytosolic protein with an M_r of around 66,000 was observed in relatively large amounts in all batches of SmT cells but was apparently barely expressed in the other variants (Fig. 4). This protein, named SmT 66, was chosen for more extensive study. As a first step in purification, cytosolic fractions of *M. avium* ser 2 and 4 were precipitated with ammonium sulfate at 25, 50, and 80% saturation. The 80% precipitates of the SmT variants from both serovars contained SmT 66 (Fig. 5). A band of similar molecular weight was observed in SmD and Rg variants as well, but it had much less intensity. When analyzed by a two-dimensional gel, SmT 66 was the only major protein detected in this molecular weight range.

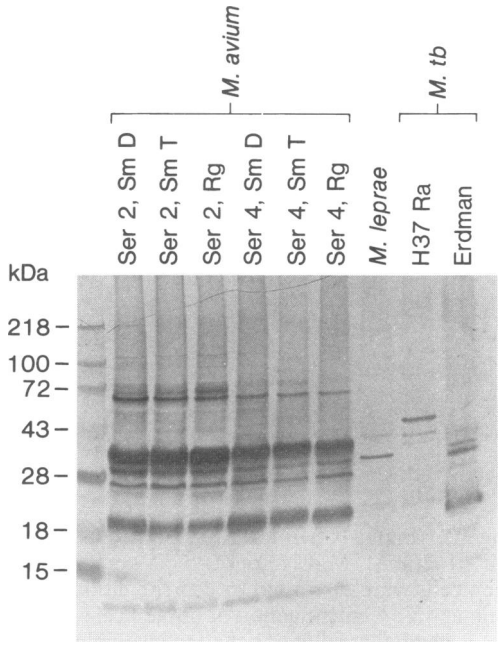

FIG. 2. Western blot illustrating the reactivity of a serum sample obtained after cross immunization of a BALB/c mouse with anti-SmD serum–SmT antigens. The serum (diluted 1:500) was reacted with nitrocellulose strips containing 8 to 10 μg of total sonicate proteins which had been separated on a 10% Tricine gel. *M. tb*, *M. tuberculosis*.

TABLE 1. Characterization of MAbs obtained by cross immunization

MAb	Mol mass(es) of reactive antigen(s)[a] (kDa)	Specificity[b]	Immunoglobulin isotype[c]
SmT 7G94	17	MAC	G1
SmT 8C53	23	MAC	G1
SmT 6H131	24	CR	G1
SmT 7C11	24	MA	G1
SmT 6H132	26[d]	MA	G1
SmT 8G12	27[d]	MA	G1
SmT 9G91	28	MA	G1
SmT 6B92	29	MA	G2a
SmT 9B54	32	CR	G1
SmD 6D92	33	MAC	G2b
SmT 6H111	34	MAC	G1
SmT 7G93	35	CR	G1
SmD 1G510	37[d]	MAC	G1
SmT 7B14	38, 82	MAC	G1
SmD 6D93	46	CR	G1

[a] Molecular masses of reactive antigens were estimated by Western blot after separation of proteins from *M. avium* ser 2 by SDS-PAGE.
[b] Specificity was tested by immunoblotting techniques. MA, *M. avium*; MAC, *M. avium* complex; CR, broad cross reactivity.
[c] Isotypes were determined by using a MAb kit.
[d] Three MAbs reacted with different antigens depending on the serovar tested.

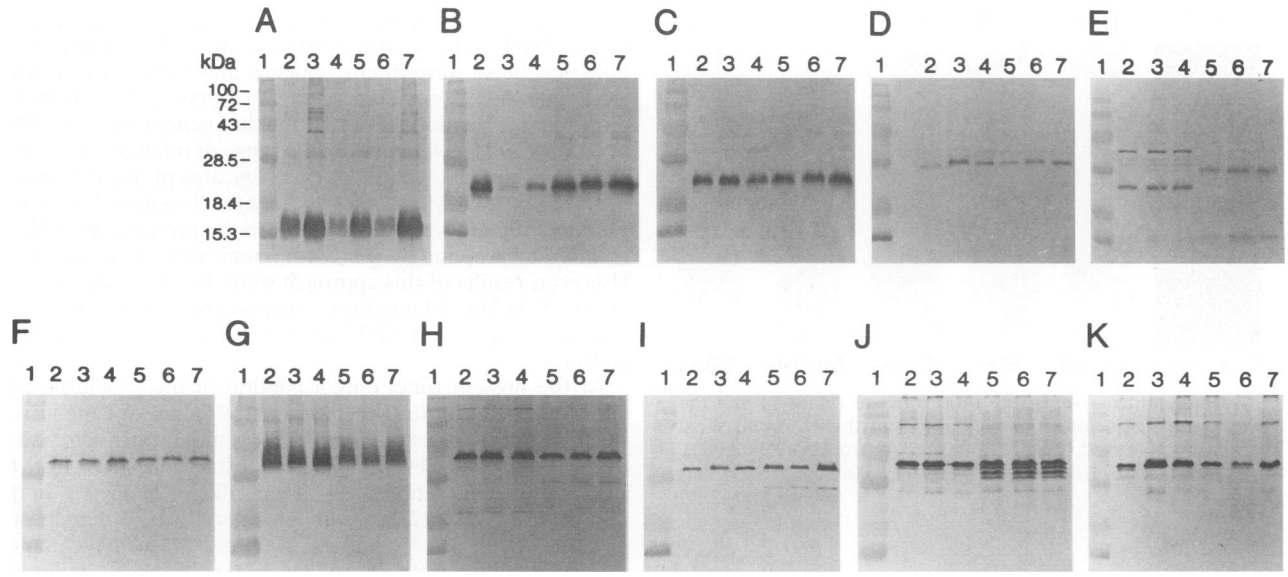

FIG. 3. Western blot analysis of MAbs recognizing *M. avium* protein antigens. MAbs SmT 7G94 (A), SmT 8C53 (B), SmT 7C11 (C), SmT 6H132 (D), SmT 8G12 (E), SmT 9G91 (F), SmT 6B92 (G), SmD 6D92 (H), SmT 6H111 (I), SmD 1G510 (J), and SmT 7B14 (K) were incubated with Western blots containing 8 to 10 μg of sonicates from *M. avium* ser 2 SmD (lane 2), SmT (lane 3), and Rg (lane 4) and ser 4 SmD (lane 5), SmT (lane 6), and Rg (lane 7). Lane 1 contained prestained molecular mass markers.

As a first order of comparison between SmT 66 and the heat shock protein hsp65, Western blot analysis was conducted. Total sonicate of *M. avium* ser 2, as a positive control, and the 80% ammonium sulfate precipitates from the same variant were blotted with cross-reacting MAbs against hsp65. The total sonicate reacted with those MAbs as expected, whereas SmT 66 was not recognized by any of them (Fig. 6). Neither did two MAbs directed against hsp70 recognize SmT 66 (data not shown). The N-terminal amino acid sequence of SmT 66 was compared with that of hsp65 (Table 2). The results confirmed that SmT 66 was not the heat shock protein. The amino acid sequence exhibited no significant similarity to protein sequences in the EuGene Database (1989 version; Baylor College of Medicine, Houston, Tex.).

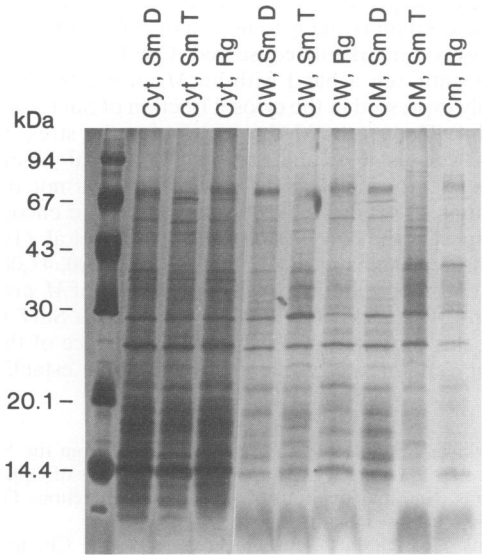

FIG. 4. Separation of proteins (5 to 7 μg) from Triton X-114-extracted subcellular fractions of *M. avium* ser 2. SDS-PAGE was conducted with 15% polyacrylamide gels. Proteins were visualized by silver staining. Cyt, cytosol; CW, cell wall; CM and Cm, cytoplasmic membrane.

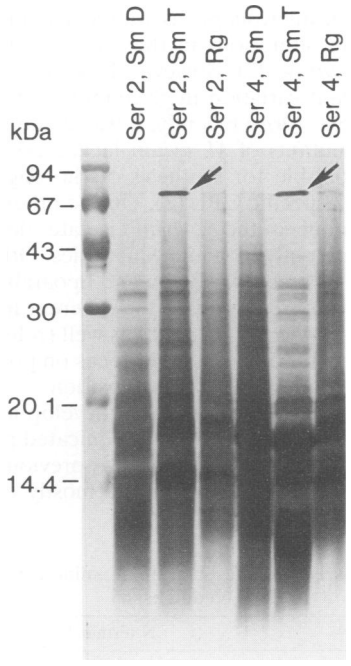

FIG. 5. Comparison of 80% ammonium sulfate-precipitated proteins from the cytosolic fractions of SmD, SmT, and Rg variants of *M. avium* ser 2 and 4. SDS-PAGE was conducted with 15% polyacrylamide gels.

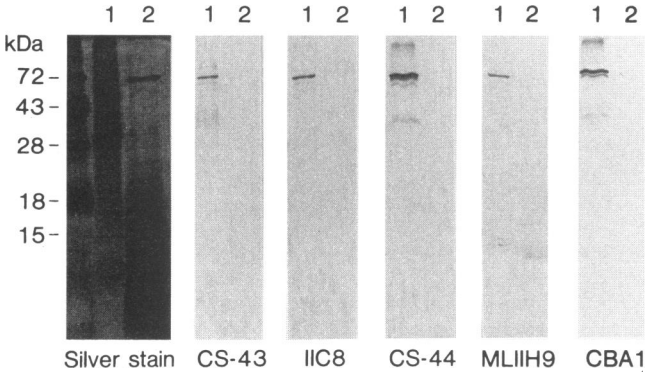

FIG. 6. Immunoreactivity of the total sonicate (lanes 1) and the 80% ammonium sulfate precipitate (lanes 2) of the *M. avium* ser 2 SmT variant. MAbs used, specific for hsp65, were CS-43 (1:8,000), IIC8 (1:1,000), CS-44 (1:10,000), MLIIH9 (1:8,000), and CBA1 (1:100).

DISCUSSION

In a recent forum on the genus *Mycobacterium* (8th Forum in Microbiology, Paris, France, 1992), as well as during a symposium dealing with *M. avium* (Frontiers in Mycobacteriology: *M. avium*, the Modern Epidemic; Vail, Colo., 1992), one major point of disagreement between participants centered on the definition and prevalence of the different colonial variations of *M. avium* isolates, especially in the context of the potential role of morphology in antimicrobial resistance. Several recent reports have also addressed the effects of morphotype on intracellular replication (3, 9, 20, 26) and on cytokine production (15, 30), demonstrating that morphology does give rise to important biological sequelae. Several earlier studies had tried to define the chemical basis for those morphological variations. Electron microscopy, as well as chemical analysis, seemed to indicate alterations in the outer layer of the cell envelope of *M. avium* (4, 27). However, David et al. (5) did not notice any significant variation in glycolipid, phospholipid, or mycolic acid content. More recently, it was demonstrated that spontaneous Rg mutants of *M. avium* lacked portions of the gene cluster responsible for synthesis of the oligosaccharide hapten of the ser 2-specific GPL (2). On the other hand, both SmD and SmT variants of the *M. avium* isolates do express the GPL antigens, apparently in similar quantities. Other cell wall components, such as arabinogalactan and lipoarabinomannan, are apparently common not only to the different morphotypes but to the whole *Mycobacterium* genus as well (reference 4 and the present study). Thus, we tended to focus on proteins which might be involved in the SmT-SmD transition.

The initial approach was designed to develop morphotype-specific MAbs by administering soluble sonicated preparations from the three variants to BALB/c mice. A previous study with rabbit polyclonal antibodies and relying mostly on immunoelectrophoresis did apparently show antigens that were present in the SmT variant and absent from SmD bacteria (27); however, those were minor antigens and were not characterized, and the observations were not pursued. In our hands, direct immunization resulted in the generation of MAbs reactive with *M. avium* and a wide range of other mycobacteria. Taking advantage of the fact that sonicates preincubated with MAbs against LAM and GPL did not react against those major protein antigens, we tried to enhance the production of MAbs to discrete proteins by a cross-immunization technique (29). However, results of this approach were disappointing, as none of the 15 MAbs obtained was morphotype specific. Nevertheless, we did pinpoint 11 MAbs specific for *M. avium* protein epitopes.

TABLE 2. Comparison of the N-terminal amino acid sequences of SmT 66 and hsp65

Protein	N-terminal amino acid sequence
hsp65 from *M. tuberculosis*[a]	MAKTIAYDEEARRGLERGLNA
hsp65 from *M. leprae*[b]	MAKTIAYDEEARRGLERGLNS
SmT 66 from *M. avium* ser 2	KPLATTLVHPNP-VA

[a] See reference 25.
[b] See reference 33.

At the present time, only a handful of mycobacterial proteins have been thoroughly described (34), most of them from *M. tuberculosis*, *M. leprae*, and *M. bovis* and mostly common to the whole genus. Several recent investigations have also focused on the generation of MAbs specific for *M. avium*. In this way, Rouse et al. (22) were able to identify *M. avium*-specific proteins of 30, 33, 35, and 36 kDa and *M. avium* complex-specific antigens (20, 30, and 34 kDa). Using a similar approach, Abe et al. (1) characterized a 27-kDa protein which, so far, is the only antigen specific for *M. avium* to have been cloned and extensively characterized (32). Morris et al. (16) mentioned four MAbs, reacting with native proteins of *M. intracellulare*, two of which were *M. avium* complex specific and reacted with 38- to 40-kDa and 43-kDa antigens, respectively. In pursuit of our present goals, we also produced several cross-reacting MAbs, most of which recognized well-known ubiquitous proteins. While our data suggest that 11 of the generated MAbs are specific for *M. avium* determinants, further studies are required to confirm this specificity and identify the determinants. Clearly, the study did not identify morphotype-specific MAbs and, in this respect, is in accord with that of Thorel and David (27) in concluding that variant-specific antigens, if they do exist, are rare or not immunodominant. Indeed, the more direct approach of comparing protein profiles by electrophoresis did point to a subtle difference. Surprisingly, the proteins of the cell envelope fractions, which were considered more likely to be responsible for the morphotype (5), were remarkably conserved. On the other hand, we did consistently see a band with an M_r of 66,000 (SmT 66), most highly expressed in the cytosol fraction of SmT variants of ser 2 and 4. The use of MAbs specific for the stress protein hsp65, as well as a comparison of N-terminal amino acid sequences, clearly established that SmT 66 was not the heat shock protein of similar molecular weight. Quite encouraging in this respect is the observation of Ramasesh et al. (19), who noticed three proteins in the M_r region of 33,000, 41,000, and 66,000 in the smooth but not the rough variant of *M. avium* ser 20. Whether the 66-kDa protein is identical to SmT 66 and what part this protein may play in the emergence of the SmT morphology and greater virulence remain to be established.

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