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Common Antigen of *Mycobacterium leprae*, *M. lepraemurium*, *M. avium*, and *M. fortuitum* in Comparative Studies Using Two Different Types of Antisera

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No. 21 mycobacterial antigens of *Mycobacterium lepraemurium*, *M. avium*, *M. fortuitum*, and *M. leprae* were compared in crossed immunoelectrophoresis using two different antibody sources, a serum pool from lepromatous leprosy patients (LSII) and a rabbit anti-*M. smegmatis* antiserum. *M. lepraemurium*, like *M. avium*, was found to contain the 21 A and 21 C determinants. *M. fortuitum* contained in addition a new type of determinant, 21 D. *M. leprae* antigen no. 21 carried the A as well as the B determinants, the latter found so far only in the leprosy bacillus. The separate taxonomic position of *M. leprae*, suggested by earlier studies of the no. 21 antigen, is further supported by the present results, which also demonstrate the potential use of submolecular heterogeneity for such investigations.

Previous studies of antibody responses in leprosy patients revealed the existence of antigenic heterogeneity in a common mycobacterial antigen, labeled no. 21 in a *Mycobacterium smegmatis* reference system (9, 10). Individual serum samples as well as serum pools from lepromatous leprosy patients detected an antigenic determinant called 21 B on the no. 21 antigen of *M. leprae* but not on any other slow-growing or fast-growing mycobacterial species tested (10). Other antigenic constituents were either common to all mycobacteria (21 A) or shared by all mycobacteria except *M. leprae* (21 C) (10). This variation in the structure of one antigen in various mycobacteria could reflect their taxonomic positions by its measure of their evolutionary relationships.

Serological identification of mycobacterial antigens has been utilized by several investigators for taxonomic purposes (11, 15-17, 21). The introduction of crossed immunoelectrophoresis for antigen analysis has increased the number of antigens detected in mycobacterial reference systems to over 50 (3, 6, 9, 23). For *M. leprae*, however, in spite of the recent availability of larger amounts of armadillo-grown bacilli, the number of antigens detected is low, 11 antigens or less (9, 22; M. Harboe et al., submitted for publication). This is in sharp contrast to another in vivo-grown mycobacterium, *M. lepraemurium* (6). Structural variations in single antigen molecules might offer an alternative for taxonomic studies of *M. leprae*, which shows such a limited number of antigens.

In the present paper, antigen no. 21 was further analyzed in two in vivo-grown bacteria, *M. leprae* and *M. lepraemurium*, and compared with *M. avium* and *M. fortuitum*, representing so-called slow-growing and fast-growing mycobacteria, respectively. Using two types of antibody sources, we detected different antigen no. 21 determinants. This result supports the classification of *M. leprae* separately from the other mycobacteria tested (10).

MATERIALS AND METHODS

Mycobacterial antigen preparations. *M. avium* (NCTC 8551) and *M. fortuitum* (NCTC 2891) were grown on Sauton's medium solidified with 1.5% agar. The bacteria were harvested, washed three times in PBS (phosphate-buffered saline: 0.12 M NaCl, 0.03 M phosphate, pH 7.4, 0.02% sodium azide) and treated ultrasonically for 30 min (B-12 Sonifier; Branson Instruments Co., Danbury, Conn.) in 10 ml of PBS per g (wet weight). Soluble antigens were recovered by centrifugation and stored at −20°C until use. Protein determinations were performed by the modified Folin method (14).

M. leprae was harvested from infected armadillo tissues (kindly supplied by Gerald P. Walsh, G.S.R.I., New Iberia, La.) as described earlier (9).

M. lepraemurium (Douglas strain) was cultivated in vivo in C3H mice. Bacilli were purified from mouse liver tissue (6). Ultrasonic treatment was performed as described above and was followed by centrifugation. *M. leprae* and *M. lepraemurium* antigen preparations were stored at −20°C.

Antisera. Two antibody sources were utilized in the present experiments. The first was a pool of serum samples from lepromatous leprosy patients,

LSII (9). The second antibody source was a rabbit immunized with *M. smegmatis* soluble antigen, 4 mg of protein in Freund incomplete adjuvant, every third week for 4 months. The rabbit was then bled twice weekly, and the serum samples were pooled. Immunizations were continued during this time. This rabbit was selected from eight rabbits immunized at the same time because of its high titer against mycobacterial antigen no. 21. Serum samples and pools were stored at −20°C.

Crossed immunoelectrophoresis. The method of crossed immunoelectrophoresis (12) was used with modifications (2, 4, 13). A reference system consisted of the lepromatous serum pool and *M. leprae* antigen, identifying the no. 1 and no. 21 antigens. By including increasing amounts of the rabbit anti-*M. smegmatis* antiserum in the intermediate gel, antigens 1 and 21 were identified also against this second antiserum source. For comparisons with other antigens, the tandem crossed immunoelectrophoretic technique was used.

RESULTS

Antigen no. 21 studies using a lepromatous leprosy serum pool. In tandem experiments, crossed immunoelectrophoretic comparisons of no. 21 antigens from *M. lepraemurium, M. avium, M. fortuitum,* and *M. leprae,* using a lepromatous leprosy serum pool as the antibody reagent, detected a common antigenic determinant as well as an antigenic determinant specific for *M. leprae* (Fig. 1A, Table 1). There was a reaction of complete identity between antigen no. 21 of *M. lepraemurium, M. avium,* and *M. fortuitum* when this antibody reagent was used. *M. leprae* antigen no. 21, on the other hand, showed partial identity with spurring over the same antigen of the other three mycobacterial species (Fig. 1A). The two types of determinants were designated 21 A and 21 B in earlier studies (10). According to this antigenic analysis using a lepromatous leprosy pool as the reagent, *M. lepraemurium* is no more related than *M. avium* or *M. fortuitum* to *M. leprae.*

Analysis of antigen no. 21 using antiserum against *M. smegmatis*. An antiserum against *M. smegmatis* raised in a rabbit was selected for comparative studies of no. 21 mycobacterial antigens. When used at concentrations between 6 and 10% in the upper gel in crossed immunoelectrophoresis, two precipitin lines representing antigens 1 and 21 were seen against all four mycobacterial preparations (Fig. 2). Only these two antigens were detected in *M. lepraemurium* and *M. leprae* antigen preparations. A few other lines were also visible when *M. avium* was run against antiserum, and several precipitation lines were obtained when *M. fortuitum* was tested (Fig. 2). Tandem crossed immunoelectrophoresis performed to compare no. 21 antigens of *M. lepraemurium* and *M. avium* revealed a reaction of complete identity (Fig. 1B, Fig. 3A). This identity reaction was the only complete one recorded in the present studies using rabbit anti-*M. smegmatis* antiserum (Fig. 1B).

When antigen no. 21 of *M. fortuitum* was compared with the same antigen of *M. lepraemurium* and *M. avium,* these two latter no. 21 precipitation lines were always spurred over by the *M. fortuitum* line (Fig. 1B, Fig. 3B and C). This spurring, although less marked than the other ones seen during the present investigations, indicated the existence of yet another type of antigen no. 21 determinant present in one mycobacterial species but absent in two others (Table 1). This new antigen determinant on *M. fortuitum* antigen no. 21 is called 21 D (Table 1).

Comparisons between the *M. leprae* no. 21 antigen and that of *M. avium* and *M. fortuitum* (Fig. 1B, Fig. 4B and C) confirmed the presence of 21 C determinants on the latter two, as described earlier for three slow growers and six fast growers (10). No. 21 C determinants were also detected in the noncultivable bacillus *M. lepraemurium*. Therefore, the available data from the antigen analysis of mycobacterial antigen no. 21, with a mapping of submolecular heterogeneity and the definition of these determinants, suggest a close relationship between *M. lepraemurium* and *M. avium* and a solitary

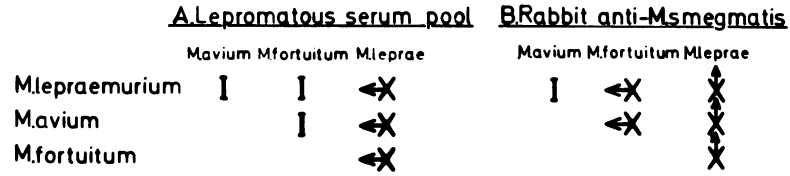

FIG. 1. *Summary of crossed immunoelectrophoretic comparisons between antigen no. 21 of M. lepraemurium, M. avium, M. fortuitum, and M. leprae. (A) Results obtained with a lepromatous serum pool, LSII, as antibody reagent. I indicates complete serological identity. X indicates a reaction of partial identity with spurring in the direction shown by the arrow. (B) Results obtained with a rabbit anti- M. smegmatis antiserum as a reagent. I and X indicate complete and partial identity, respectively. Arrows show direction of spurring.*

taxonomic status of the other noncultivable bacillus, *M. leprae*, among the mycobacteria tested.

DISCUSSION

Only a limited number of *M. leprae* antigens from armadillo-grown bacilli can be detected in immunoprecipitation systems using lepromatous serum pools as antibody reagents (9). Similar *M. leprae* antigen preparations give rise to antibodies against only a few components when injected into rabbits (22; Harboe et al., submitted for publication). More extensive analysis indicated the presence of only a few antigens in such preparations (Harboe et al., submitted for publication). Rather than choosing a conventional approach of antigen analysis which works well with a higher number of precipitation lines, a definition of submolecular antigenic heterogeneity of one antigen molecule was earlier utilized for taxonomic studies of *M. leprae* (10). The mycobacterial antigen studied, called no. 21, was present in most species tested. The results indicated a taxonomic position for *M. leprae* separate from the other mycobacterial species tested (10).

TABLE 1. *Cross-reactions of no. 21 antigens*

Species	Antigenic determinant			
	21 A	21 B	21 C	21 D
M. lepraemurium ...	+	−	+	−
M. avium	+	−	+	−
M. fortuitum	+	−	+	+
M. leprae	+	+	−	−

One major objection to the earlier studies utilizing the antigenic heterogeneity of no. 21

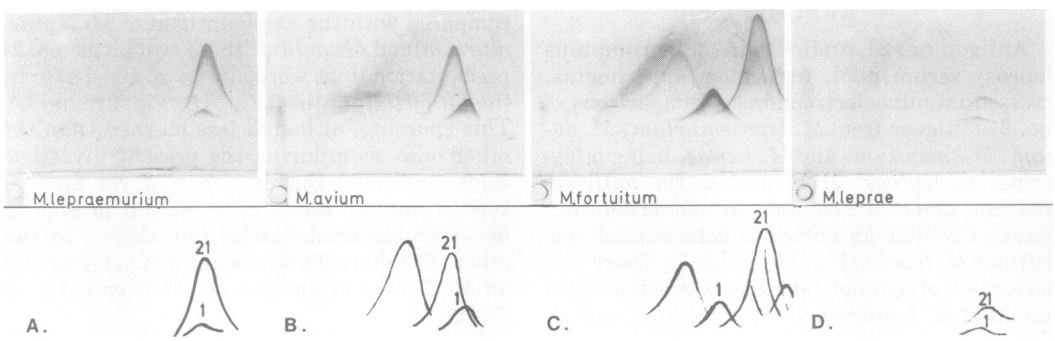

FIG. 2. *Crossed immunoelectrophoretic precipitation patterns of (A) M. lepraemurium, (B) M. avium, (C) M. fortuitum, and (D) M. leprae antigen preparations against rabbit anti-M. smegmatis antiserum. The positions of mycobacterial antigens no. 1 and no. 21 of each strain are indicated on the drawings.*

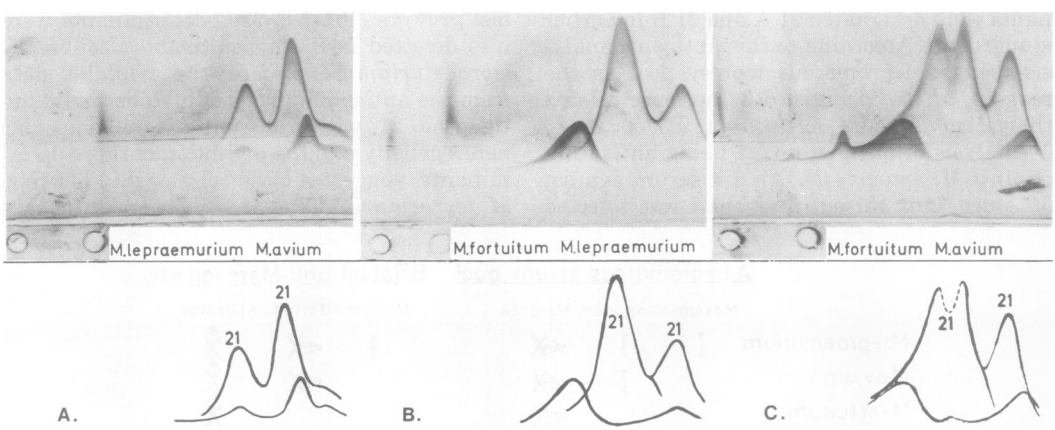

FIG. 3. *Tandem crossed immunoelectrophoretic comparisons of no. 21 antigens with rabbit anti-M. smegmatis antiserum as a reagent. (A) M. lepraemurium and M. avium; complete identity of the no. 21 antigens. (B) M. fortuitum and M. lepraemurium; partial identity with spurring. The antiserum used detects an antigenic determinant on M. fortuitum antigen no. 21 which is not present on the same antigen of M. lepraemurium or M. avium. The double peak in Fig. 3C of M. fortuitum no. 21 is a technical artifact.*

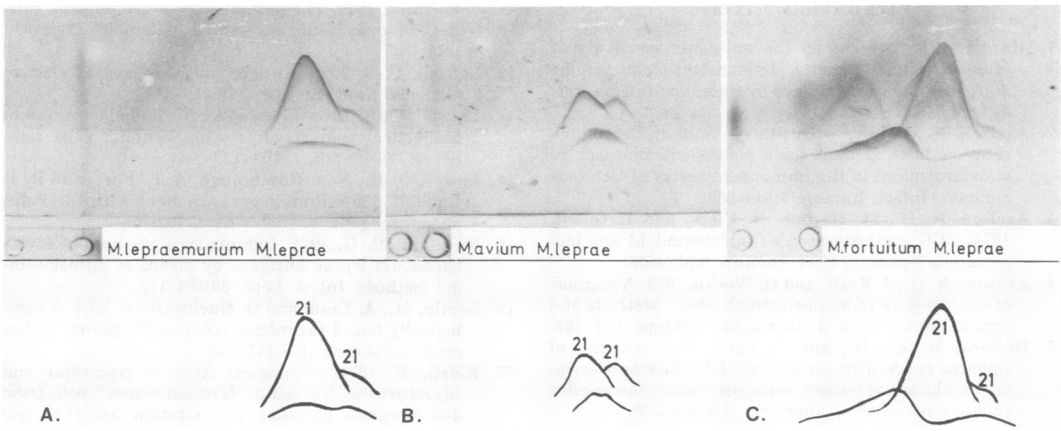

FIG. 4. *Tandem crossed immunoelectrophoretic comparisons of no. 21 antigens with rabbit anti-M. smegmatis antiserum as a reagent. (A) M. lepraemurium and M. leprae; (B) M. avium and M. leprae; (C) M. fortuitum and M. leprae. The antibody reagent used reveals a deficiency of determinants on M. leprae antigen no. 21 in comparison with the other three no. 21 mycobacterial antigens as judged by the very marked spurring.*

mycobacterial antigens for taxonomic purposes was the fact that the M. leprae bacilli were grown in vivo whereas all other mycobacterial species studied were grown in vitro. In the present studies, another in vivo-grown bacillus, *M. lepraemurium,* has been included. Conventional antigen analysis has revealed a very close relationship between this bacillus and *M. avium,* a slow-growing mycobacterium (21). In the present studies, based on the definition of antigen no. 21 submolecular determinants, no differences were noted between *M. lepraemurium* and *M. avium* (Table 1). It is therefore clear that the position suggested by similar analysis for *M. leprae* is not an artifact due to in vivo growth but might rather reflect a true taxonomic segregation (10).

A new submolecular type of antigen no. 21 determinant was found in the present studies (Table 1). This determinant, called 21 D, was present only on the no. 21 antigen of *M. fortuitum* when rabbit anti-*M. smegmatis* antiserum was used. Since these two mycobacterial species belong to the fast-growing group of mycobacteria, determinant 21 D might be a so-called fast grower-specific marker, as described by Stanford (20), but on a submolecular level. This would further emphasize the possibilities of antigen no. 21 studies for taxonomic purposes.

M. leprae components exclusively limited to this mycobacterial species have not been detected in previous studies using lepromatous serum pools as antibody reagents (9) nor in similar studies using rabbit antisera (Harboe et al., submitted for publication). The *M. leprae*-specific determinants on antigen no. 21 (Fig. 1, Table 1) are therefore of special interest. In lepromatous leprosy the patients are defective only against *M. leprae* in their cellular immune response (5, 7). Antigen no. 21 of *M. leprae* represents a defined component which should be studied in relation to cell-mediated immunity in leprosy. Its submolecular complexity with the presence of shared determinants (21 A) has to be taken into account.

The specificity of the cellular immune defect in lepromatous leprosy suggests a participation of *M. leprae*-specific antigens or submolecular determinants such as 21 B in protective immunity. However, other components of more common nature might also add to the degree of protection. Recent investigations by Shepard et al. (19) indicated that BCG gives as good protection in mouse footpad growth studies as *M. leprae* bacilli, live or killed (18). In contrast, *M. duvalii* gave a very low degree of protection (19). This fast-growing mycobacterial species has been suggested by others as a possible candidate for a vaccine against leprosy (8). *M. duvalii* seems to contain very low concentrations of antigen no. 21 (10). BCG has not been analyzed in this regard. Further experiments on the role of *M. leprae* antigen no. 21 in humoral and cellular immune responses in patients and in experimental animals are required.

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