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Comparative Studies of Antigen 21 in *Mycobacterium* and *Nocardia* Species: Possible Taxonomic Relationships with *Mycobacterium leprae*

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Studies of *Mycobacterium leprae*, *Mycobacterium tuberculosis* and *Nocardia caviae* in comparison with each other and with other *Mycobacterium* and *Nocardia* species were performed on the basis of antigen 21 intramolecular heterogeneity. Three different antisera were used: rabbit anti-*Mycobacterium smegmatis* antiserum, rabbit anti-*Nocardia asteroides* antiserum, and a lepromatous serum pool. With reference to each of the three antiserum sources used the strains were ranked in an order of relatedness or sharing of determinants. The three antisera showed distinctly different antigen 21 antibody specificities reflecting the species origin of the immunogen. The present investigations confirmed that antigen 21 of *N. caviae* shares determinants with antigens from *Mycobacterium* strains which were not present in corresponding antigens of all other *Nocardia* strains tested. *M. tuberculosis*, as judged by antigen 21 analysis, occupies a position separate from both the slow-growing and the fast-growing mycobacterial clusters in accordance with accepted taxonomic relationships. An interesting possibility of establishing a position for *M. leprae* in relation to other mycobacterial species was apparent. The order of relatedness among the strains studied went from *M. leprae* to *M. tuberculosis* to *N. caviae* to *Mycobacterium avium* to *Mycobacterium fortuitum*, the last two being representatives of the slow-growing and fast-growing mycobacteria. It can therefore be concluded that evidence from antigen 21 analysis indicates that *M. leprae* is more closely related to *M. tuberculosis* than to the other strains investigated.

Based on absence of special staining affinities, the presence of phenolase and its pyridin-extractable acid fastness, the leprosy bacillus differs from other bacterial species in the genes *Mycobacterium* (5, 11, 14, 15, 34, 35). An exact taxonomic classification of *Mycobacterium leprae* has not yet been possible. This fact is largely due to its noncultivable status in vitro which precludes the use of metabolic tests. Another mycobacterium, *Mycobacterium lepraemurium*, has been referred to the *Mycobacterium avium*-*Mycobacterium intracellulare* complex by immunological criteria (39). Similar studies of *M. leprae* failed to reveal any definite relationship to other mycobacterial species due to the fact that only a limited number of antigen molecules can be detected in *M. leprae* antigen preparations (19, 40). Conventional immunological procedures are therefore of limited value (9, 30, 39, 40; M. Ridell, Ph.D. thesis, University of Gothenburg, Gothenburg, Sweden, 1977). By using intensive immunization procedures, rabbit antisera have recently been obtained which are capable of detecting about 20 antigen components (11).

An alternative approach to taxonomic studies seemed possible by the detection of molecular heterogeneity of one antigen component (23). This antigen, called 21 in *Mycobacterium smegmatis*, cross-reacts with a similar component in *M. leprae*. In addition to cross-reacting determinants, antigen 21 of *M. leprae* contains determinants which are species specific (24, 25). The validity of antigen 21 analysis in mycobacterial taxonomy was indicated in experiments using *M. lepraemurium* (24). Results of *M. leprae* studies indicated a position separate from other mycobacteria (25). Some tests using cellular immune recognition of various antigen preparations as well as chemical analysis seems to support such a view (6, 13, 38). Similar taxonomic approaches have been used in other bacterial species (16, 17, 22, 44).

Some recent findings of mycobacterial antigen 21 cross-reactivity with *Nocardia caviae* were quite intriguing in view of earlier attempts to

establish relationships with *M. leprae* (B. Bjorvatn and G. Kronvall, Int. J. Lepr., in press). Mycobacterial antigen 21 of these two species, *N. caviae* and *M. leprae*, showed partial identity by using a lepromatous leprosy serum pool (LSP) as antibody reagent in crossed immunoelectrophoresis. All other *Nocardia* species studies were completely negative in the system used. This seemed to indicate a closer relationship of *M. leprae* with *N. caviae* than with other *Nocardia* species.

In the present investigations, other antibody sources have been included to study further antigen 21 heterogeneity in *Mycobacterium* and *Nocardia* strains. The results obtained reveal a closer relationship of *Nocardia caviae* antigen 21 to that of mycobacterial species in general, and not to antigen of *M. leprae* alone. In addition, several lines of evidence indicate that *M. leprae* is closer to *Mycobacterium tuberculosis* than to the slow-growing or the fast-growing mycobacterial strain clusters.

MATERIALS AND METHODS

Mycobacterial antigen preparations. The preparation of soluble antigens from mycobacteria followed procedures described earlier (23, 25). Strains of both slow-growing and fast-growing mycobacterial species were cultured on Sauton medium solidified with 1.5% agar (Table 1). Harvested bacteria were washed three times in phosphate-buffered saline (0.12 M NaCl, 0.03 M phosphate, pH 7.3) and suspended in 10 ml of phosphate-buffered saline per g (wet weight). Soluble antigens were prepared by ultrasonication for 30 min (6× 5 min), using a Branson Sonifier B-12 (Branson Instruments Co., Stamford, Conn.). The antigen-containing supernatant was recovered by centrifugation and stored at −20°C until use. Purified armadillo-grown *M. leprae* bacilli were kindly supplied by the Immunology of Leprosy component of the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, and antigens were extracted as described above. An alternative procedure was used for *M. tuberculosis* as bacterial extracts contained only trace amounts of antigen 21. Four-week-old cultures on liquid Sauton medium of *M. tuberculosis* strain H37Ra were harvested and centrifuged, and the supernatant was filtered through membrane filters (5-μm pore size; Millipore Corp., Bedford, Mass.). The filtrate was concentrated 10-fold by using an Amicon B15 concentrator (Amicon Corp., Lexington, Mass.). No qualitative differences have been noted between antigen 21 prepared from cells and from culture filtrates in experiments using strains from several species. Antigen preparations were stored in small samples at −20°C until use. Protein determinations were performed by the modified Folin method (29). The antiserum used was obtained from a rabbit immunized with *M. smegmatis*-soluble antigens (4 mg of protein in Freund incomplete adjuvant given subcutaneously every third week for 4 months) (23). The rabbit was bled twice weekly, and the serum samples were pooled. The rabbit was selected from among eight rabbits immunized at the same time because of its high titer against *M. smegmatis* antigen 21. The third antibody source was a rabbit anti-*Nocardia asteroides* antiserum specific for antigen 10 of strain N81 corresponding to antigen 21 in the *M. smegmatis* numbering system (19). The rabbit was immunized with precipitates cut out of gels after crossed immunoelectrophoresis and mixed with Freund incomplete adjuvant as described in detail elsewhere (19–21). Serum samples and pools were stored at −20°C.

TABLE 1. *Mycobacterium and Nocardia strains studied*

Strain		Source
M. leprae		World Health Organization
M. tuberculosis	H37Ra, TMC 201	A.L., Gbg[a]
M. avium	NCTC 8551	J.S., London[b]
M. smegmatis	NCTC 10265	J.S., London
Mycobacterium phlei	NCTC 10266	J.S., London
M. fortuitum	NCTC 2891	J.S., London
N. caviae	R. Gordon N93, 1370	A.L., Gbg
N. caviae	R. Gordon 606	M.M., Cphn[c]
N. brasiliensis	ATCC 19296	M.M., Cphn
N. brasiliensis	R. Gordon 605	A.L., Gbg
N. asteroides	Emmons 9935	M.M., Cphn
N. asteroides	ATCC 19247	A.L., Gbg
Nocardia rubra	McClung 784	M.M., Cphn

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Crossed immunoelectrophoresis. The method of crossed immunoelectrophoresis as described by Laurell (27) was used, with modifications (3, 4, 28). A reference system consisting of the LSP and an *M. leprae* antigen preparation identified antigens 1 and 21 (25). The intermediate gel technique was used to define antibody activities of serum samples in the *M. smegmatis* and *N. asteroides* reference systems. For comparisons with other antigens, the tandem crossed immunoelectrophoretic technique was used, including alternating arrangements for the confirmation of true spur formation (31).

RESULTS

Detection of mycobacterial antigen 21 analogs in *Nocardia* species. Antigen preparations of most mycobacterial strains regularly give rise to two precipitation lines in crossed immunoelectrophoretic analysis using an anti-*M. smegmatis* rabbit antiserum (24, 25). The antigens are identified as 1 and 21 according to a reference numbering system for *M. smegmatis* (23). In the present studies, we have retained these numbers to denote the corresponding antigens in other mycobacterial species as well. Antigen 21 corresponds to *M. leprae* antigen 4 in the reference system of Harboe et al. (19). Fig. 1A shows crossed immunoelectrophoresis of *Mycobacterium fortuitum* antigens against anti-

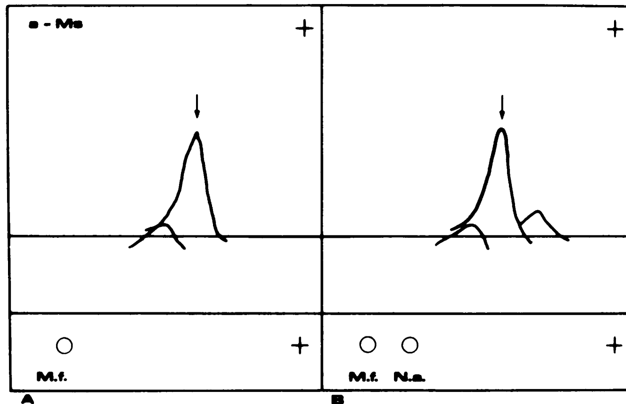

FIG. 1. *Crossed immunoelectrophoretic precipitation patterns of antigen preparations from (A) M. fortuitum (M.f.) and (B) M. fortuitum in tandem with N. asteroides (N.a.) using rabbit anti-M. smegmatis antiserum (a-Ms). Arrows indicate antigen 21 of M. fortuitum.*

M. smegmatis rabbit antiserum. With the tandem crossed immunoelectrophoretic technique, different bacterial species can be analyzed for the presence of shared determinants. Antigen 21 in *M. fortuitum* cross-reacted with the corresponding antigen of *Nocardia* strains using this antiserum (Fig. 1B). *N. asteroides* gave only one precipitation line in this system. The spurring phenomenon means that the antiserum detects antigenic determinants on *M. fortuitum* antigen 21 which are not present on the same antigen of *N. asteroides*.

Comparisons of antigen 21 in *Mycobacterium* and *Nocardia* species. Crossed immunoelectrophoretic studies were performed on strains of mycobacteria in tandem with *Nocardia* strains using the anti-*M. smegmatis* antiserum. The various patterns of partial identities are summarized in Table 2. *M. tuberculosis, M. avium*, and *M. fortuitum* showed partial identity with spurring over *N. asteroides* and *Nocardia brasiliensis. N. asteroides* and *N. brasiliensis*, on the other hand, showed spurring over *M. leprae*. These results indicate that *M. smegmatis* antigen 21 has more determinants in common with antigens of *M. avium, M. fortuitum* and *M. tuberculosis* than it has with those of *N. asteroides* and *N. brasiliensis. M. leprae* is in a different position, since it has fewer antigen 21 determinants in common with *M. smegmatis* than all the others, including *N. brasiliensis*, as judged by the anti-*M. smegmatis* antiserum used.

Comparisons were also made by using a rabbit anti-*N. asteroides* (strain N81) antiserum raised against antigen 10, e.g. its antigen 21 equivalent. The autologous system with *N. asteroides* strain N81 gave only one precipitation line in crossed immunoelectrophoresis against the antiserum

TABLE 2. *Crossed immunoelectrophoretic comparisons of determinants on antigen 21 in various Mycobacterium and Nocardia strains using anti-M. smegmatis and anti-N. asteroides antisera*[a]

Species	anti-*M. smegmatis* serum		anti-*N. asteroides* serum
	N. asteroides	*N. brasiliensis*	*N. asteroides*
M. leprae	←	←	←
M. tuberculosis	↑	↑	←
M. avium	↑	↑	←
M. fortuitum	↑	↑	←

[a] Partial identity reactions are indicated by arrows showing direction of spurring.

(Fig. 2A). The antigen 21 was compared in tandem electrophoresis with *N. brasiliensis* and *M. leprae*, respectively (Fig. 2B and C). In both cases, there was a reaction of partial identity with *N. asteroides* antigen 21 spurring over *N. brasiliensis* and *M. leprae*. The same is also true for *N. asteroides* strain N81 in tandem with *N. asteroides* strain N100 (Fig. 2D). This indicates that the serum contains antibodies both against cross-reacting determinants and determinants specific for antigen 21 of *N. asteroides* strain N81. It also illustrates both the variability of antigen 21 among different species and the importance of selecting antisera for comparative studies.

***N. caviae* antigen 21 studies.** In previous investigations of antigen 21 heterogeneity, *N. caviae* was found to be different from other *Nocardia* strains (Bjorvatn and Kronvall, in press). *N. caviae* was therefore compared in the present studies with *M. leprae, M. avium, M. fortuitum, M. tuberculosis* and *N. asteroides* in tandem crossed immunoelectrophoresis. Both

anti-*M. smegmatis* antiserum and an LSP were used. The present investigations showed complete identity between antigen 21 determinants of *N. caviae* and *M. tuberculosis*, by using the anti-*M. smegmatis* antiserum (Fig. 3A). There was partial identity between antigen 21 of *N. caviae* and that of *M. leprae*, *M. avium*, *M. fortuitum*, and *N. asteroides*. *M. avium* and *M. fortuitum* spurred over *N. caviae*, but *N. caviae* spurred over *N. asteroides* and *M. leprae*. This indicates that *M. avium* and *M. fortuitum* have more antigen 21 determinants in common with *M. smegmatis* than has *N. caviae*. *M. leprae* and *N. asteroides*, on the other hand, share fewer antigen 21 determinants with *M. smegmatis* than with *N. caviae*. When the LSP was used, antigen 21 determinants of *N. caviae* and *M. tuberculosis* were similar. Determinants of *M. avium* and *M. fortuitum* showed complete identity with *N. caviae*. *M. leprae* showed partial identity with spurring over *N. caviae*. Antigen 21 of *N. asteroides* did not give a precipitin reaction with this antiserum, as described earlier (Bjorvatn and Kronvall, in press). Thus, with respect to similarity to *M. leprae* antigen 21, *N. caviae* and *M. tuberculosis* behaved like several other mycobacteria studied earlier.

Additional studies of *N. caviae* using the anti-*N. asteroides* antiserum were also performed. This antiserum shows a high species specificity but still gives a strong precipitin reaction with most other *Nocardia* species. The one exception, *N. caviae*, indicates that the antigen 21 of this species is only distantly related to the corresponding antigen in all other *Nocardia* species studied. The mycobacterial species included in

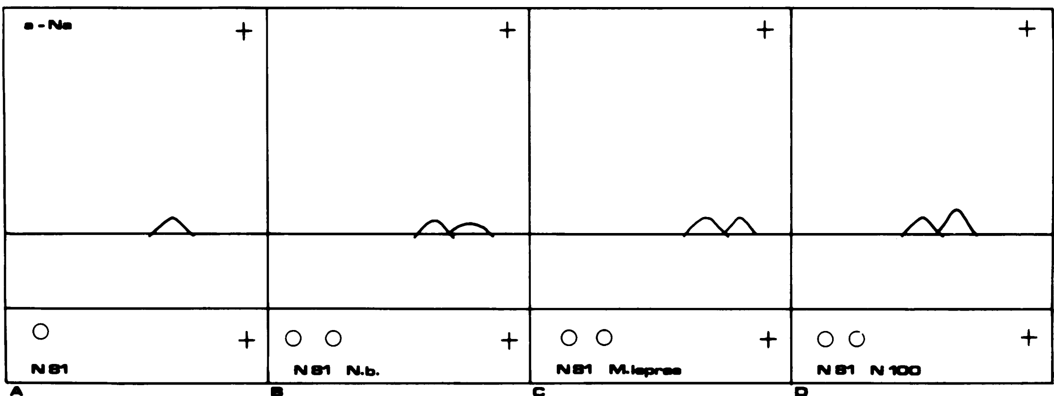

FIG. 2. *Tandem crossed immunoelectrophoretic comparisons of antigen 21 with rabbit anti-N. asteroides N81 antiserum (a-Na); (A), N. asteroides N81; (B), N. asteroides N81 and N. brasiliensis (N.b.); (C), N. asteroides N81 and M. leprae, and (D), N. asteroides N81 and N. asteroides N100.*

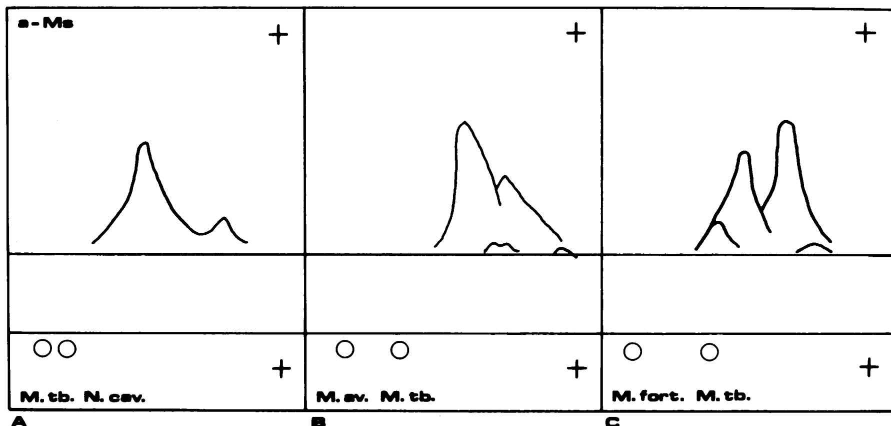

FIG. 3. *Tandem crossed immunoelectrophoretic analyses of antigen 21 in mycobacterial strains using a rabbit anti-M. smegmatis antiserum (a-Ms). (A) Complete identity between antigen 21 of M. tuberculosis (M.tb.) and N. caviae (N. cav.); (B and C) reactions of partial identity between antigen 21 of M. tuberculosis and M. avium (M.av.) and M. fortuitum (M. fort.), respectively.*

the present investigations all show the same low degree of cross-reactivity as *N. caviae*, further emphasizing the closer relationship between antigen 21 of this species and that of the mycobacteria.

***M. tuberculosis* antigen 21 studies.** Interactions of *M. tuberculosis* with the other bacterial species was tested further. *M. tuberculosis* antigen 21 was compared in tandem crossed immunoelectrophoresis with *M. leprae*, *M. avium*, *M. fortuitum*, and *N. asteroides* against anti-*M. smegmatis* antiserum and LSP (Table 3, Fig. 3B and C). When using the anti-*M. smegmatis* antiserum there was a reaction of partial identity in all cases. *M. avium* and *M. fortuitum* spurred over *M. tuberculosis*, whereas *M. tuberculosis* spurred over *M. leprae* and *N. asteroides*. This means that *M. avium* and *M. fortuitum* have more antigen 21 determinants in common with *M. smegmatis* than has *M. tuberculosis*, but *M. tuberculosis* has more antigen 21 determinants in common with *M. smegmatis* than have *M. leprae* and *N. asteroides*. Figure 3 shows some examples of the tandem crossed immunoelectrophoresis experiments using this antiserum. Alternate-well arrangements (31) confirmed the true spurring phenomena.

Tandem crossed immunoelectrophoresis against LSP gave a different picture. *M. tuberculosis* antigen 21 showed partial identity with spurring over *M. fortuitum*. The determinants of antigen 21 of *M. tuberculosis* that were detected using this antiserum were identical to those of *M. avium* and *M. leprae*. *N. asteroides* gave no antigen 21 precipitation with this antiserum.

DISCUSSION

In species of higher organisms the type and extent of amino acid sequence differences between similar protein molecules correlates well with what is known about their phylogeny. This is often true also for procaryotic cells (1, 2, 16, 44). Conventional taxonomic criteria separate the mycobacteria into several clusters (18, 26, 41, 43). In previous studies of mycobacterial antigen 21 heterogeneity the results were found to correlate with known taxonomic relationships in this group of organisms, including the noncultivable species *M. lepraemurium* (8, 23–25). In the present extension of these investigations, it was found that antigen 21 analogous proteins were present in all *Mycobacterium* and *Nocardia* strains tested. Comparative studies were therefore possible to perform. With reference to each one of the three antiserum sources used the strains can be ranked in an order of relatedness or sharing of determinants (Fig. 4 and 5).

TABLE 3. *Summary of tandem crossed immunoelectrophoretic comparisons of antigen 21 using rabbit anti-M. smegmatis antiserum and LSP as antibody reagents*

Species	*N. caviae*		*M. tuberculosis*	
	anti-*M. smegmatis*	LSP	anti-*M. smegmatis*	LSP
M. leprae	←[a]	↑[a]	NP[b]	I[c]
M. tuberculosis	I	I	I	I
M. avium	↑	I	↑	I
M. fortuitum	↑	I	↑	←
N. caviae	I	I	I	I
N. ateroides	←	NP	←	NP

[a] Partial identity reactions indicated by arrows showing direction of spurring.

[b] NP, No precipitation between anti-*M. smegmatis* and *M. leprae* and between LSP and *N. asteroides*.

[c] Indicates immunological identity.

In previous studies, antigen 21 of *N. caviae* was shown to share determinants with mycobacteria. No other *Nocardia* strains tested showed presence of antigen determinants found on mycobacterial antigen 21 components (Bjorvatn and Kronvall, in press). This closer relationship to antigen 21 of mycobacteria is confirmed in the present investigations. *N. caviae* showed complete identity with *M. tuberculosis* by using both anti-*M. smegmatis* antiserum and the LSP. *N. caviae* also gave a reaction of identity with antigen 21 determinants of *M. avium* and *M. fortuitum* using LSP. *M. leprae* and *N. asteroides*, however, showed only partial identity with *N. caviae* with respect to antigen 21 by using both antisera. The results suggest a position of relatedness of *N. caviae* antigen 21 between *M. tuberculosis* and *M. avium* (Fig. 4). The relationships revealed by using LSP as the antibody source are summarized in Fig. 5, which clearly shows the gradual increase in cross-reactivity of antigen 21 from more distant strains. The unexpected position of antigen 21 from *N. caviae* among the mycobacteria and different from all other *Nocardia* strains is difficult to reconcile with current concepts of taxonomy (42, 43). The possibilities of genetic transfer among procaryotic cells during evolution must be considered as one possible explanation for such irregularities in patterns obtained (1).

M. tuberculosis antigen 21 was included in the present studies as a representative for a separate and, in the human context, important mycobacterial cluster (26, 41, 43). *M. tuberculosis* antigen 21 showed complete identity with *M. leprae* by using LSP. This suggests that *M. tuberculosis* might be closely related to *M. leprae*. Since *M. tuberculosis* also shows identity with *M. avium* by using LPS and with *N. caviae* by using both anti-*M. smegmatis* antiserum and LSP, additional information is required for their exact

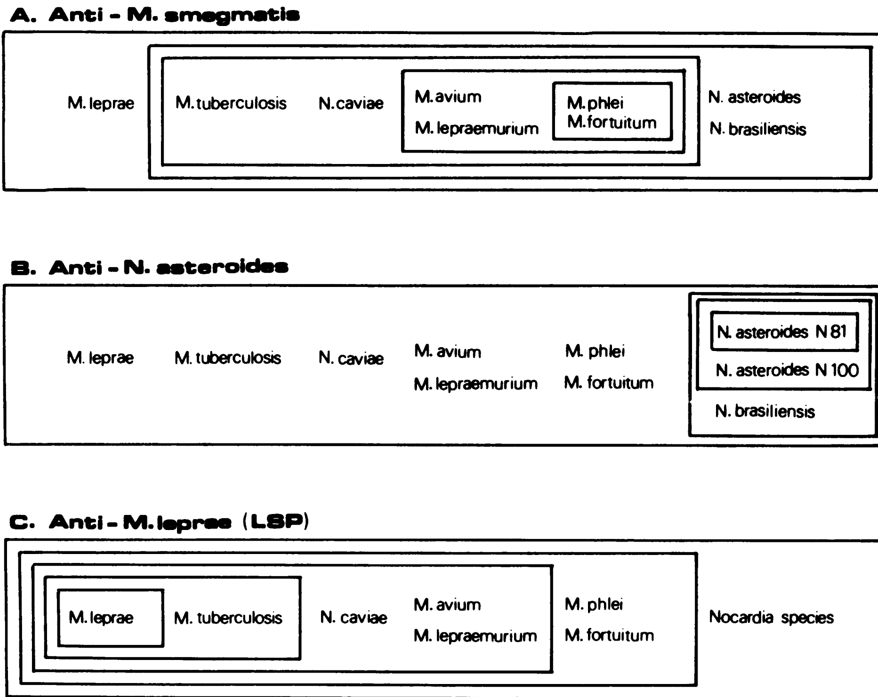

FIG. 4. *Antigen 21 relationships among Mycobacterium and Nocardia species. Boxes enclose strains showing complete immunological identity with the antiserum used as indicated. Data include results from previous studies (8, 24, 25).*

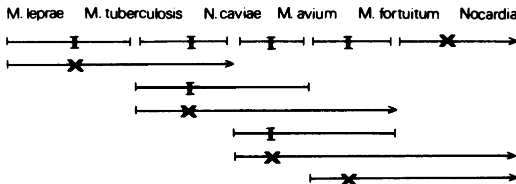

FIG. 5. *Summary of antigen 21 comparisons using an LSP as antibody reagent. Complete identity* (I) *and reactions of partial identity* (X) *with spurring (arrows) are indicated with strains arranged in an order compatible with results obtained.*

location in the comparative analysis. This is provided by experiments showing that *M. tuberculosis* spurred over *M. fortuitum* with LSP and that when anti-*M. smegmatis* antiserum was used, *M. fortuitum* spurred over *M. tuberculosis*. Also, *M. leprae* antigen 21 gave a reaction of partial identity with *N. caviae* by using LSP. This indicates that *M. tuberculosis* as judged by antigen 21 analysis occupies a position separate from both the slow-growing and the fast-growing mycobacterial clusters in accordance with accepted taxonomic relationships (26, 41, 43; Ridell, thesis). These results lend further support to the possible taxonomic value of antigen 21 comparisons (24, 25; Bjorvatn and Kronvall, in press).

The most interesting result from the present investigations is the possibility of establishing a position for *M. leprae* in relation to other mycobacterial species. As can be seen from Fig. 4 and as based on comparative considerations summarized in Fig. 5, there is a definite order of relatedness among the strains studied. This order goes from *M. leprae* to *M. tuberculosis* to *M. avium* to *M. fortuitum*, the last two being representatives of the slow-growing and the fast-growing mycobacteria studied earlier. It can therefore be concluded that evidence from antigen 21 analysis indicates that *M. leprae* is most closely related to *M. tuberculosis* of the fourteen mycobacterial strains studied so far (20, 25; Bjorvatn and Kronvall, in press). In spite of the noncultivable status of the leprosy bacillus and the low number of components detected in antigen extracts, the present approach using the intramolecular heterogeneity of one single antigen component as the basis for a comparative analysis has enabled us to add new information to answer to the question of the taxonomy of *M. leprae*.

Three different antisera were used in the present investigations. Anti-*N. asteroides* antiserum 10 was largely species specific and therefore was of no use in the present comparative studies. It even detected differences between the two *N.*

asteroides strains used, N81 and N100. Such a difference is in line with other reports which indicate that *N. asteroides* includes a large and heterogenous group of strains (42). The anti-*M. smegmatis* antiserum and the LSP showed broader reactivity patterns suitable for the studies. Further studies of antigen 21 heterogeneity aiming at a closer definition of *M. leprae-M. tuberculosis-M. avium* relationships require other antisera raised against these three mycobacterial species. A wider spectrum of antisera would also be of great value for further taxonomic studies.

False spurs are sometimes formed in tandem crossed immunoelectrophoretic analysis when immunologically identical antigens of different electrophoretic mobilities are compared. Rearrangement of the position of the antigen wells in tandem crossed immunoelectrophoresis has appeared to be a useful principle for distinguishing between such false spurs and true spurs due to partial antigenic identity (31). In these investigations each tandem crossed immunoelectrophoresis was repeated several times in different arrangements to avoid false interpretations of spurring phenomena.

The importance of utilizing LSP as a reagent in our studies is apparent from Fig. 4 and Fig. 5 showing its decisive role in establishing a sequential order of relatedness among the mycobacteria studied. The potential use of serum samples from lepromatous leprosy patients with high antibody titers needs to be emphasized. Such sera can be obtained in large quantities. They can be selected for their titer against single antigen components or for their degree of specificity. In addition, with the availability of *M. leprae* antigen preparations from the World Health Organization it is possible for more laboratories to set up reference systems in crossed immunoelectrophoresis. An LSP set up against an *M. leprae* antigen preparation will easily define antigens 1 and 21 (19, 24). These possibilities might also be further utilized for comparisons with antigen preparations from strains claimed to be identical to *M. leprae*.

The immunological relationship between antigen 21 of *M. leprae* and *M. tuberculosis* and its possible taxonomic significance is of considerable interest in view of cross-reactivities and cross-protective effects between the two as reported by several others. Closs found cross-reactivity between *M. leprae* and BCG and between *M. leprae* and *M. tuberculosis* when skin-testing apparently healthy people in a nonendemic country (10). Several investigations concerning immunization with *Mycobacterium bovis* strain BCG and irradiation-killed *M. leprae* showed cross-reactive effects on infection of *M. bovis*, *M. tuberculosis*, and *M. leprae* (31, 33, 37). Mice which were immunized with irradiation-killed *M. leprae* gave rise to significant resistant to BCG and *M. tuberculosis* strain R1Rv (32). Vaccination of mice with irradiation-killed *M. leprae* protects them against *M. leprae* infections (33). Mice have also been successfully immunized against *M. leprae* with *Mycobacterium bovis* BCG both before and up to 56 days after the *M. leprae* challenge (37). The vaccine-induced immunity persisted unchanged over a long period of time. Although the degree of efficiency of BCG vaccination in humans to protect against leprosy is still debated because of differences in the results obtained in three large well-conducted field trials, the existence of cross-protective effects cannot be disputed (7, 8, 36). All of the studies cited indicate a relationship between *M. leprae* and *M. tuberculosis* antigens in line with the results of the present investigations. Their close association on the intramolecular level of antigen 21 analysis might therefore have a bearing not only on taxonomic considerations of the strains but also on studies aiming at finding cultivable mycobacterial strains of potential use for immunoprophylactic measures in leprosy. Avirulent variants in the *M. tuberculosis* cluster might provide candidates for such purposes.

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LITERATURE CITED

1. **Ambler, R. P., M. Daniel, J. Hermoso, T. E. Meyer, R. G. Bartsch, and M. D. Kamen.** 1979. Cytochrome c_2 sequence variation among the recognised species of purple nonsulphur photosynthetic bacteria. Nature (London) **278**:659–660.
2. **Ambler, R. P., T. E. Meyer, and M. D. Kamen.** 1979. Anomalies in amino acid sequences of small cytochromes c and cytochromes c′ from two species of purple photosynthetic bacteria. Nature (London) **278**: 661–662.
3. **Axelsen, N. H.** 1973. Quantitative immunoelectrophoretic methods as tools for a polyvalent approach to standardization in the immunochemistry of *Candida albicans*. Infect. Immun. **7**:949–960.
4. **Axelsen, N. H., J. Krøll, and B. Weeke.** 1973. A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J. Immunol. **2**(Suppl. 1):1–169.
5. **Beaman, B. L., and B. Burnside.** 1973. Pyridine extraction of nocardial acid fastness. Appl. Microbiol. **26**:426–428.
6. **Beaman, B. L., K.-S. Kim, M. A. Lanéelle, and L. Barksdale.** 1974. Chemical characterization of organisms isolated from leprosy patients. J. Bacteriol. **117**: 1320–1329.

7. **Bechelli, L. M., A. Garbajosa, K. Uemura, V. Engler, V. Martinez Dominguez, L. Paredes, T. Sundaresan, G. Koch, and M. Matejka.** 1970. BCG vaccination of children in leprosy. Preliminary findings of the WHO-controlled trial in Burma. Bull. W. H. O. **42**:235-281.
8. **Brown, J. A. K., M. M. Stone, and I. Sutherland.** 1969. Trial of BCG vaccination against leprosy in Uganda. Lep. Rev. **40**:3-7.
9. **Chaparas, S. D., T. Brown, and I. Hyman.** 1978. Antigenic relationships among species of *Mycobacterium* studied by fused rocket immunoelectrophoresis. Int. J. Syst. Bacteriol. **28**:547-560.
10. **Closs, O.** 1975. In vitro lymphocyte response to purified protein derivative, BCG, and *Mycobacterium leprae* in a population not exposed to leprosy. Infect. Immun. **11**: 1163-1169.
11. **Closs, O., R. N. Mshana, and M. Harboe.** 1979. Antigenic analysis of *Mycobacterium leprae*. Scand. J. Immunol. **9**:297-302.
12. **Convit, J., and M. E. Pinardi.** 1973. A simple method for the differentiation of *Mycobacterium leprae* from other mycobacteria through routine staining technics. Int. J. Lepr. **40**:130-132.
13. **Etamadi, A. H., and J. Convit.** 1974. Mycolic acids from "noncultivable" mycobacteria. Infect. Immun. **10**:236-239.
14. **Fisher, C. A., and L. Barksdale.** 1971. Elimination of the acid fastness but not the Gram positivity of leprosy bacilli after extraction with pyridine. J. Bacteriol. **106**: 707-708.
15. **Fisher, C. A., and L. Barksdale.** 1973. Cytochemical reactions of human leprosy bacilli and mycobacteria: ultrastructural implications. J. Bacteriol. **113**:1389-1399.
16. **Gasser, F., and C. Gasser.** 1971. Immunological relationships among lactic dehydrogenases in the genera *Lactobacillus* and *Leuconostoc*. J. Bacteriol. **106**:113-125.
17. **Gasser, F., and M. Hontebeynie.** 1977. Immunological relationships of glucose-6-phosphate dehydrogenase of *Leuconostoc mesentroides* NCDO 768 (= ATCC 12291). Int. J. Syst. Bacteriol. **27**: 6-8.
18. **Goodfellow, M., A. Lind, H. Mordarska, S. Pattyn, and M. Tsukamura.** 1974. A co-operative numerical analysis of cultures considered to belong to the 'rhodochrous' taxon. J. Gen. Microbiol. **85**:291-302.
19. **Harboe, M., O. Closs, B. Bjorvatn, G. Kronvall, and N. H. Axelsen.** 1977. Antibody response in rabbit to immunization with *Mycobacterium leprae*. Infect. Immun. **18**:792-805.
20. **Harboe, M., O. Closs, and J. Deverill.** 1976. Production of monospecific antisera against antigenic components of *Mycobacterium bovis* (BCG). Scand. J. Immunol. **5**: 221-226.
21. **Harboe, M., O. Closs, K. Svindahl, and J. Deverill.** 1977. Production and assay of antibodies against one antigenic component of *Mycobacterium bovis*, BCG. Infect. Immun. **16**:662-672.
22. **Høiby, N., J. B. Hertz, and V. Andersen.** 1976. Cross-reactions between *Bordatella pertussis* and twentyeight other bacterial species. Acta Pathol. Microbiol. Scand. Sect. B **84**:395-400.
23. **Kronvall, G., G. Bjune, J. Stanford, S. Menzel, and D. Samuel.** 1975. Mycobacterial antigens in antibody responses of leprosy patients. Int. J. Lepr. **43**:299-306.
24. **Kronvall, G., O. Closs, and G. Bjune.** 1977. Common antigen of *Mycobacterium leprae*, *M. lepraemurium*, *M. avium*, and *M. fortuitum* in comparative studies using two different types of antisera. Infect. Immun. **16**: 542-546.
25. **Kronvall, G., J. L. Stanford, and G. P. Walsh.** 1976. Studies of mycobacterial antigens, with special reference to *Mycobacterium leprae*. Infect. Immun. **13**:1132-1138.
26. **Kubica, G. D., I. Baess, R. E. Gordan, P. A. Jenkins, J. B. G. Kwapinski, C. McDurmont, S. R. Pattyn, H. Saito, V. Silcox, J. L. Stanford, K. Takeya, and M. Tsukamura.** 1972. A co-operative numerical analysis of rapidly growing mycobacteria. J. Gen. Microbiol. **73**:55-70.
27. **Laurell, C.-B.** 1965. Antigen-antibody crossed electrophoresis. Anal. Biochem. **10**:358-361.
28. **Laurell, C.-B.** 1972. Electrophoretic and electro-immunochemical analysis of proteins. Scand. J. Clin. Lab. Invest. **29**(Suppl. 124):1-136.
29. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265-275.
30. **Minden, P., J. K. McClatchy, and R. S. Farr.** 1972. Shared antigens between heterologous bacterial species. Infect. Immun. **6**:574-582.
31. **Negassi, K., O. Closs, and M. Harboe.** 1978. False spurs in quantitative immunoelectrophoresis. Scand. J. Immunol. **8**:279-290.
32. **Patel, P. J., and M. J. Lefford.** 1978. Induction of cell-mediated immunity to *Mycobacterium leprae* in mice. Infect. Immun. **19**:87-93.
33. **Patel, P. J., and M. J. Lefford.** 1978. Specific and nonspecific resistance in mice immunized with irradiated *Mycobacterium leprae*. Infect. Immun. **20**:692-697.
34. **Prabhakaran, K.** 1973. Dopa metabolism by *Mycobacterium leprae*: its implications in culture of the bacillus and chemotherapy of leprosy. Lepr. Rev. **44**:112-119.
35. **Prabhakaran, K., and W. F. Kirchheimer.** 1966. Use of 3,4-dihydrophenylalanine oxidation in the identification of *Mycobacterium leprae*. J. Bacteriol. **92**:1267-1268.
36. **Russel, D. A., G. C. Scott, and S. C. Wigley.** 1968. BCG and prophylaxis. Int. J. Lepr. **36**:618.
37. **Shepard, C. C., L. L. Walker, and R. M. von Landingham.** 1978. Immunity to *Mycobacterium leprae* infections induced in mice by BCG vaccination at different times before or after challenge. Infect. Immun. **19**:391-394.
38. **Smelt, A. H. M., F. Y. Liew, and R. J. W. Rees.** 1978. Lymphocyte response of leprosy patients to human derived and purified armadillo derived *Mycobacterium leprae*, BCG and PPD. Clin. Exp. Immunol. **34**:164-169.
39. **Stanford, J. L.** 1973. An immunodiffusion analysis of *Mycobacterium lepraemurium* Marchoux and Sorel. J. Med. Microbiol. **6**:435-439.
40. **Stanford, J. L., G. A. W. Rook, J. Convit, T. Godal, G. Kronvall, R. J. W. Rees, and G. P. Walsh.** 1975. Preliminary taxonomic studies on the leprosy bacillus. Br. J. Exp. Pathol. **56**:579-585.
41. **Tsukamura, M.** 1976. Numerical classification of slowly growing Mycobacteria. Int. J. Syst. Bacteriol. **26**:409-420.
42. **Tsukamura, M.** 1977. Extended numerical taxonomy study of *Nocardia*. Int. J. Syst. Bacteriol. **27**:311-323.
43. **Tsukamura, M., S. Mizuno, S. Tsukamura, and J. Tsukamura.** 1979. Comprehensive numerical classification of 369 strains of *Mycobacterium*, *Rhodococcus*, and *Nocardia*. Int. J. Syst. Bacteriol. **29**:110-129.
44. **Wayne, L. G., and G. A. Diaz.** 1979. Reciprocal immunological distances of catalase derived from strains of *Mycobacterium avium*, *M. tuberculosis* and closely related species. Int. J. Syst. Bacteriol. **29**:19-24.