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Immunological Relatedness of Ribosomes from Mycobacteria, Nocardiae and Corynebacteria, and Microorganisms in Leprosy Lesions

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Serological relatedness of ribosomes from microorganisms of the *Mycobacterium, Nocardia*, and *Corynebacterium* genera has been analyzed by the microplate immunodiffusion technique. *Mycobacterium* and *Nocardia* proved homogeneous and closely related taxa, whereas *Corynebacterium* was found to be a heterogeneous phylum connected by remote links to the others. The taxonomic position of "diphtheroid microorganisms" (non-acid-fast, gram-positive bacteria morphologically similar to corynebacteria), which were found together with *Mycobacterium leprae* in human leprosy lesions, was also investigated. Ribosomes of diphtheroid bacteria strongly cross-reacted with antisera against several mycobacteria and nocardiae but not against corynebacteria. Moreover, ribosomes from independently isolated diphtheroid strains proved serologically related and yielded strong cross-reactions with antisera against *M. leprae* as well as with sera from leprosy patients. Hence, diphtheroid microorganisms represent a homogeneous group immunologically related to mycobacteria in general and more specifically to *M. leprae*.

Taxonomy of *Actinomycetales* is a very difficult matter which hides numerous unsolved problems (2, 3, 14, 20, 21; for a review see 36). As a matter of fact, quite different microorganisms were initially included in this order, merely on the basis of morphological criteria; supposedly, they are filamentous, branching, gram-positive bacteria. On the other hand, structural and immunological relationships between some genera of actinomycetes and corynebacteria have been reported (8, 19, 22; M. Ridell, Ph.D. thesis, University of Göteborg, Göteborg, Sweden, 1977).

Different approaches have been recently attempted to classify *Actinomycetales*: studies of the morphology (vegetative structures and spores), metabolism (fermentative and respiratory pathways), biochemical structure (cell wall and DNA composition), antigenic properties (external, internal, and excreted antigens), and ecological distribution of these microorganisms have contributed data for a numerical taxonomy of this order. H. A. Lechevalier (20) has proposed an ecological-metabolic distinction between soil actinomycetes (saprophitic, aerophilic, true mycelial) and animal-associated actinomycetes (parasitic, fermentative, pseudomycelial). Moreover, H. A. Lechevalier (20) and M. P. Lechevalier (21) have recognized seven cell wall types (I to VII) and four whole sugar patterns (A to D) and proposed, on this basis, a classification of the major genera of actinomycetes. Within the IV-A section of such classification, not only are *Nocardia* and *Mycobacterium* genera included, but also two phyla, the classification of which is still controversial: *Corynebacterium* and *Rhodococcus*.

In cutaneous lesions of human leprosy, in addition to *Mycobacterium leprae*, other bacteria were found, which were called "diphtheroid microorganisms" (DM) because of their morphological resemblance to *Corynebacterium diphtheriae* (9-13). Whereas Hansen's bacilli are acid-alcohol-fast, gram-positive actinomycetes that cannot be multiplied in axenic culture, DM are non-acid-fast, gram-positive bacteria which can be grown in semisynthetic media (27).

In the present work, the immunological cross-reactivity of ribosomes from microorganisms belonging to different genera within the *Mycobacterium, Nocardia*, and *Corynebacterium* genera (CMN group) was investigated for taxonomical purpose. Among cellular antigens, ribosomes were chosen because of the possibility of isolation in a pure form and characterization by well-established biophysical methods, and because their powerful antigenic properties have been clearly recognized (see reference 34 for a review). According to our data, *Nocardia* and *Mycobacterium* genera proved by far more homogeneous and closely related than the *Corynebacterium*

genus. In addition, the serological relatedness of the ribosomes from DM and several bacteria of the CMN group has been analysed. DM were found to be more closely related to mycobacteria (to *M. leprae* in particular) than to corynebacteria. This study might contribute to the taxonomy of *Actinomycetales* as well as to the unravelling of the microbial etiology of human leprosy.

MATERIALS AND METHODS

Bacterial strains. Fifteen microorganisms were studied: five mycobacteria, two nocardiae, three corynebacteria, four DM, and one bacillus. Their sources are reported in Table 1.

Bacterial cultures. The following Difco media were used: (i) Dubos, supplemented with 5% horse serum (mycobacteria); (ii) Sabouraud (nocardiae); (iii) brain heart infusion (corynebacteria); and (iv) L broth (bacilli). Bacteria were grown at 37°C under vigorous shaking. They were collected during the exponential phase of growth, washed several times with saline, and kept frozen at −18°C. *M. leprae* was obtained from human lepromatous tissue by homogenization with saline differential centrifugation.

Ribosome preparation. Bacteria suspended in 0.15 M NaCl–0.010 M ethylenediaminetetraacetic acid, pH 8.0, were shaken for 15 min at 20°C with 1% toluene and washed repeatedly with saline. Cells suspended in NB buffer (10 mM magnesium acetate–50 mM NH_4Cl–6 mM 2-mercaptoethanol–10 mM tris-(hydroxymethyl)aminomethane-hydrochloride, pH 7.6) were disrupted by compression to 12,000 lb/in^2 in a French pressure cell (Aminco Instruments, Silver

TABLE 1. *Designation and sources of the studied microorganisms*

Designation	Origin	Source
Mycobacterium		
M. bovis	Calmette-Guérin strain 117-3-P_2	J. Weckx (Pasteur Institute, Brussels Belgium)
M. leprae	Biopsy material	R. Moris (Iyonda Hospital, Zaire) T. Godal (AHRI Center, Addis Abeba, Ethiopia)
M. nonchromogenicum	Isolated from humans	S. R. Pattijn (Institute Tropical Medicine, Antwerpen, Belgium)
M. scrofulaceum	Isolated from humans (by E. Mankievic, Montréal, Canada)	L. Kato (Institute A. Frappier, Quebec, Canada)
M. smegmatis	NCTC 334	L. Dumoulin-Brahy (Pasteur Institute, Brussels, Belgium)
M. vaccae	ATCC 15483	S. R. Pattijn (Institute of Tropical Medicine, Antwerpen, Belgium)
Nocardia		
N. asteroides	Institute of Hygiene, Mexico City, Mexico	R. Van Breusegem (Institute of Tropical Medicine, Antwerpen, Belgium)
N. brasiliensis	Institute of Hygiene, Mexico City, Mexico	R. Van Breusegem (Institute of Tropical Medicine, Antwerpen, Belgium)
Corynebacterium		
C. hofmannii	Isolated from humans	G. Wauters (University of Louvain, Brussels, Belgium)
C. kutscheri	Isolated from mice	J. Delville (University of Louvain, Brussels, Belgium)
C. xerosis	Isolated from humans	G. Wauters (University of Louvain, Brussels, Belgium)
DM		
DM-86	Biopsy material	J. Godal (AHRI Center, Addis Abeba, Ethiopia)
DM D32	Patient's blood	R. Moris (Iyonda Hospital, Zaire)
DM L3	Biopsy material	R. Moris (Iyonda Hospital, Zaire)
DL L11	Biopsy material	R. Moris (Iyonda Hospital, Zaire)
DM L2628LB	Biopsy material	L. Barksdale (New York University, New York)
Bacillus		
B. subtilis 168/2	ATCC 15563	J. Marmur (Albert Einstein College of Medicine, New York, N. Y.)

Spring, Md.). Cell debris were removed by centrifugation at 10,000 rpm for 10 min and at 15,000 rpm for 20 min (7). Ribosomes were sedimented by ultracentrifugation at 20,000 rpm for 14 h (Spinco rotor A30), suspended in NB buffer, and submitted to 1,000 rpm centrifugation for 15 min to remove aggregates; this treatment was repeated twice.

Ribosome analysis. Routinely, ribosome concentration was evaluated by the absorbance at 260 nm (1 absorbance unit at 260 nm = 25 pmol = 0.07 mg of ribosomes). Purity of the preparations was checked by ultracentrifugation for 1 h at 50,000 rpm at 4°C in 0.5 to 28.5% sucrose gradients (SW 50.1 Spinco rotor) (7). Colorimetric determinations of RNA, proteins, and carbohydrates in ribosome preparations were carried out with the orcinol, phenol, and anthrone reagents, respectively (6).

Antiserum preparation. Rabbits were injected intravenously every 3 days for 4 weeks with either 10 mg of whole-cell homogenates or with 3 mg of purified ribosomes. Goat antiserum against *Mycobacterium tuberculosis* H37 Rv was obtained from Seffort (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Sera from lepromatous and tuberculoid leprosy patients were obtained from several African hospitals.

Immunological analysis. Serological relatedness was established by the microplate immunodiffusion technique (26). The support was 1.5% agarose (E. Merck AG, Darmstadt, West Germany) in 0.02 M sodium barbital buffer (pH 8.6) containing 0.02% sodium azide. Samples (10 μl) were placed in the wells, and plates were developed at 20°C for 1 week and stained with amido black for 30 min.

RESULTS

Cross-reactivity among organisms of the same genus. Cross-reactivity of heterologous ribosomes and antisera was used to assess the homogeneity of the three CMN genera. The sensitivity of the reaction is indicated by the production of three to five major precipitinogen lines when ribosome preparations were tested against the corresponding antiserum. The absence of visible lines when *Bacillus subtilis* ribosomes were tested against all of the anti-CMN sera proves the specificity of the reaction.

Ribosomes from five mycobacteria reacted strongly (two to five major precipitinogen lines) with antisera against different mycobacteria (Table 2). The case of *M. scrofulaceum*, which reacted poorly against some antimycobacterial sera (e.g., *M. smegmatis* and *M. nonchromogenicum*) is rather exceptional. A similar conclusion was drawn for *Nocardia*; instead, very low reactivity among members of the *Corynebacterium* genus was found. It can be concluded that *Mycobacterium* and *Nocardia* are homogeneous taxa and *Corynebacterium* is a heterogeneous phylum.

Cross-reactivity among microorganisms of the CMN group. Relatedness of microorganisms belonging to different genera was similarly judged on the basis of the ribosomes-antisera cross-reactivity. Table 3 indicates that ribosomes of five mycobacteria reacted strongly with serum against *N. asteroides*. Likewise, a high number of precipitinogen lines was obtained when ribosomes from five nocardiae were tested with antisera against three mycobacteria. On the contrary, ribosomes from three corynebacteria reacted poorly against mycobacteria and slightly more against nocardiae. This points to a closer relationship between mycobacteria and nocardiae than that between corynebacteria and the other two CMN genera.

TABLE 2. *Serological relatedness of ribosomes from microorganisms in each CMN genus*

Ribosome	Antiserum[a]						
	M. vaccae	*M. smegmatis*	*M. tuberculosis*	*M. scrofulaceum*	*N. asteroides*	*C. xerosis*	*C. hofmannii*
Mycobacterium							
M. vaccae	4.1[b]	3.0	3.5	3.1			
M. nonchromogenicum		2.0	4.5	1.3			
M. smegmatis		3.0	4.0	3.3			
M. bovis	2.4	2.2	5.3	3.2			
M. scrofulaceum	2.7	1.5	4.3	5.1			
Nocardia							
N. asteroides					3.6		
N. brasiliensis					2.5		
Corynebacterium							
C. xerosis						3.9	0.7
C. hofmannii						1.0	3.3
C. kutscheri						0.3	0.4

[a] Antisera against whole-cell homogenates.
[b] Average number of major precipitinogen lines.

Relatedness of DM and bacteria of the CMN group. Ribosomes from four diphtheroid bacteria, which were independently isolated from human leprosy lesions, were tested with antisera against eight microorganisms of the CMN group. High cross-reactivity was found with mycobacteria and nocardiae (two to four lines), but there was practically no reaction with corynebacteria (Table 4). Conversely, ribosomes from mycobacteria and nocardiae yielded comparable reactions with antisera against diphtheroid strain 86 microorganisms (whole-cell homogenates or purified ribosomes), whereas ribosomes from corynebacteria reacted poorly with these antisera (Table 5 and Fig. 1). Hence, DM cells, in spite of their morphological resemblance to corynebacteria, are antigenically related to mycobacteria.

Homogeneity of immune reactions among DM. Serological cross-reactivity of independently isolated DM was checked to evaluate the homogeneity of this group of bacteria. In Table 6, ribosomes from four such strains were allowed to cross-react with homologous and heterologous antisera: comparable degrees of reactivity were observed in all cases. Therefore, all of the DM which were tested so far seem to be antigenically related.

Possible relationships between DM and *M. leprae* were explored by allowing the ribosomes of the former cell type to react with antisera against the latter cell type. Indeed, similar reactivity was found with antisera against *M. leprae* and DM (Table 6 and Fig. 2). Moreover, antisera against purified ribosomes of the diphtheroid strain 86 yielded strong immunological reactions against lepromin (Fig. 2A).

An independent confirmation of this conclu-

TABLE 3. *Immunological cross-reactivity of ribosomes from microorganisms of different CMN genera*

Ribosome	Antiserum[a]						
	N. asteroides	*C. xerosis*	*C. hofmannii*	*M. vaccae*	*M. smegmatis*	*M. tuberculosis*	*M. scrofulaceum*
Mycobacterium							
M. vaccae	2.8[b]	0	0				
M. nonchromogenicum	2.8		0				
M. smegmatis	2.0		0.5				
M. bovis	1.2	0	0.3				
M. scrofulaceum	2.6	0.5	0.1				
Nocardia							
N. asteroides	3.6		1.0		2.8	4.0	2.3
N. brasiliensis	2.5		0.7		2.5		
Corynebacterium							
C. xerosis	0.7		0.7	1.1	0.2	1.1	1.3
C. hofmannii	1.5		3.3	1.0	0.8	1.5	1.0
C. kutscheri	1.0		0.4	0	1.2	1.5	0.4
Bacillus							
B. subtilis	0		0	0	0		

[a] Antisera against whole-cell homogenates.
[b] Average number of major precipitinogen lines.

TABLE 4. *Immune reaction of ribosomes from DM with antisera against CMN bacteria*

DM ribosomes	Antiserum[a]						
	M. tuberculosis	*M. vaccae*	*M. smegmatis*	*M. scrofulaceum*	*N. asteroides*	*C. xerosis*	*C. hofmannii*
DM 86	3.1[b]	3.0	2.0	1.8	2.7	0.1	0
DM D32	3.4	4.0	1.5	1.3	3.5	0	0
DM L3	3.0	3.0	2.0	1.8	2.5	0	0
DM L2628LB	3.0	3.0	1.8	2.0	2.0	0	0

[a] Antisera against whole cell homogenates.
[b] Average number of major precipitinogens lines.

sion was sought by making ribosomes from DM to react with 17 lepers' sera: a high cross-reactivity was indeed observed in 77% of the cases (Table 7 and Fig. 2B).

TABLE 5. *Cross-reactivity of ribosomes from CMN microorganisms with antisera against DM and M. leprae*

Ribosome	Antiserum[a]			
	DM 86	DM D32	*M. leprae*	DM 86[b] ribosomes
Mycobacterium				
M. vaccae	3.5[c]	4.5	2.0	3.2
M. nonchromogenicum	2.6		1.8	2.5
M. scrofulaceum	1.4	2.1	0.8	1.5
M. smegmatis	2.2		1.9	1.5
M. bovis	1.0	1.0	1.3	1.4
Nocardia				
N. asteroides	2.4		1.8	2.0
N. brasiliensis	1.5		1.7	1.5
Corynebacterium				
C. hofmannii	0.9	1.0	1.5	0.6
C. xerosis	0.3	1.0	0.7	0.8
C. kutscheri	0.3	0.5	0.7	0.3
Bacillus				
B. subtilis	0	0	0	0

[a] Rabbit antisera against whole-cell homogenates except *b*.
[b] Rabbit antiserum against purified ribosomes of DM (strain DM 86).
[c] Average number of major precipitinogens lines.

DISCUSSION

In the present work, the immunological properties of bacterial ribosomes have been exploited for a taxonomic investigation of CMN microorganisms; preliminary studies have, in fact, recognized ribonucleoprotein particles as the major internal antigen of these bacteria. It is known that *Escherichia coli* ribosomes and subunits are more immunogenic than mixtures of the extracted ribosomal proteins, although the injection into rabbits of these ribonucleoprotein particles induces the formation of precipitating antibodies directed primarily against the protein moiety (34); a single 70S monosome can bind about 10^2 immunoglobulin G molecules. Moreover, ribosomes from *M. tuberculosis* were found able to produce delayed skin reactions in guinea pigs sensitized with attenuated human tubercle bacilli and to confer resistance to infection with

TABLE 6. *Serological relatedness of ribosomes from different DM and relationship with M. leprae*

Ribosome	Antiserum[a]					
	DM 86	DM D32	DM L3	DM L11	DM 86 ribosomes[b]	*M. leprae*
DM 86	3.3[c]	3.3	2.0	2.0	2.9	2.3
DM D32	3.4	4.5	2.5	2.0	2.8	2.6
DM L3	3.3	3.0	2.5	1.5	3.0	2.3
DM L2628LB	3.0	3.0	2.0	1.5	3.0	2.3

[a] Antisera against whole-cell homogenates except in *b*.
[b] Rabbit antiserum against ribosomes of DM 86.
[c] Average number of major precipitinogen lines.

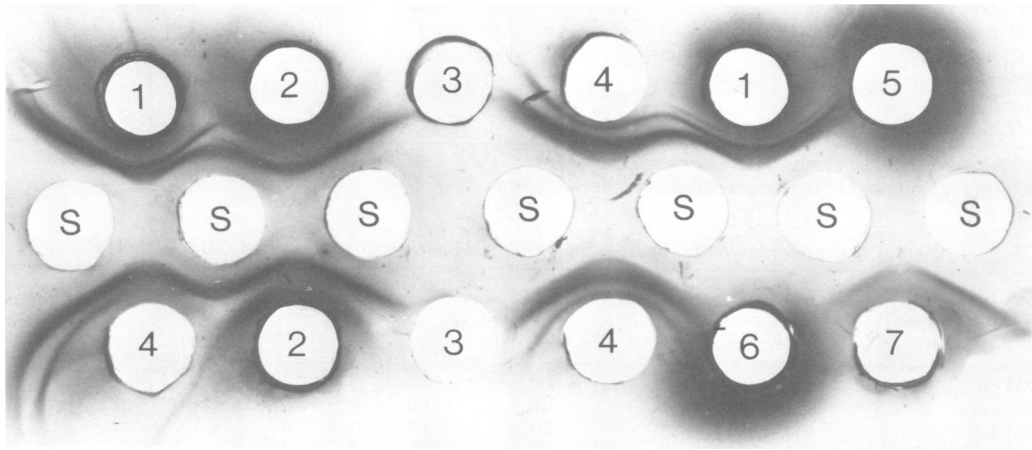

FIG. 1. *Immune reaction of ribosomes form CMN bacteria with antiserum against DM. Center wells: antiserum against homogenate of DM 86 strain; outer wells: ribosomes from different strains. 1, M. vaccae; 2, DM 86; 3, B. subtilis; 4, M. nonchromogenicum; 5, C. hofmannii; 6, C. xerosis; and 7, DM D32.*

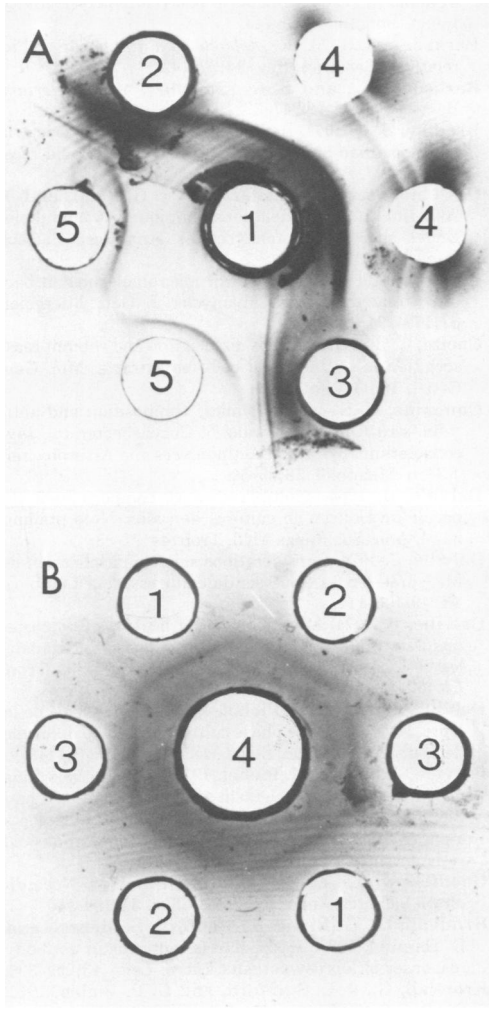

FIG. 2. *Serological reactivity of ribosomes from DM with lepromin and lepers' sera. (A) Center well: lepromin (homogenate of lepromatous tissue); outer wells: 2 and 3, ribosomes from DM 86 (2), and DM L2628LB (3); 4 and 5, antisera against ribosomes of DM 86 (4) and M. leprae (whole-cell homogenate) (5). (B) Center well: blood serum from a leprosy patients (tuberculous type); outer wells: ribosomes from DM, strain DM 86 (1), DM L3 (2), DM L2628LB (3).*

TABLE 7. *Immunological reactivity of ribosomes from DM with sera from leprosy patients*

Patient serum type	DM ribosomes		
	86	L3	L2628LB
Tuberculoid	4/7[a]	4/7	4/7
Bordeline	2/2	2/2	2/2
Lepromatous	7/8	7/8	7/8

[a] Number of sera (fraction of total tested) which presented one or two major precipitinogen lines.

virulent mycobacteria (1). The use of ribosome preparations and of the corresponding antisera for a taxonomical study of CMN microorganisms has been already reported (22, 30; Ridell, Ph.D. thesis).

Table 2 shows a greater homogeneity for the *Mycobacterium* and *Nocardia* taxa than for the *Corynebacterium* genus. This conclusion agrees with DNA composition studies, which yielded guanine plus cytosine (G+C) values of 67 to 69% for mycobacteria and nocardiae and 52 to 69% for corynebacteria (see reference 21 for a review). A further distinction was made among corynebacteria belonging or not to the rhodococcus group, the former having higher G+C content (61 to 66%) than the latter (55 to 61%; see reference 21 for a review; also 4, 23). It is worthy of note that the G+C content of DNA seems to correlate well with DNA homology test and with the chemical structure of the cell wall of several genera within the CMN group of microorganisms.

According to data in Table 3, there is a closer serological relationship between mycobacteria and nocardiae than between corynebacteria and the other two CNM genera. Similar cross-reactivity between mycobacteria and nocardiae was previously reported (17, 18, 22, 28, 35, 38; Ridell, Ph.D. thesis). Also, in 10 strains of corynebacteria, precipitinogens (X and Y) were found which cross-reacted with reference precipitation system from mycobacteria and nocardiae; the latter genera, however, possessed other precipitinogens (α and β) which were absent in corynebacteria (22, 29). In addition, genetic homologies between nocardiae and mycobacteria were disclosed by DNA reassociation studies (4) and by structural similarities revealed by chemical analysis of the cell walls (5, 8). It should be mentioned, however, that the genus *Corynebacterium* consists of at least three broad groups related to *C. diphtheriae*, *C. renale*, and *C. genitalium*, respectively. Because the strains shown in Table 1 are included within the latter group, inferences about the relatedness of the *Corynebacterium* genus to the *Mycobacterium* and *Nocardia* taxa ought to be restricted to corynebacteria related to the *C. genitalium* group.

We have previously related the presence in leprosy lesions of non-acid-fast microorganisms bearing a morphological resemblance to *C. diphtheriae* (9–13). Although the term DM is improper, as pointed out quite correctly by Barksdale (2), its replacement by the appropriate designation requires further study of the DNA and cell wall composition of serveral independently isolated strains; such study is still in progress. Serological data in Tables 4 and 5 indicate that

the ribosomes of these microorganisms are more closely related to those of mycobacteria than to those of corynebacteria. Similar conclusions were previously reached by use of immunofluorescence techniques and antisera against the external antigens of DM (27). In addition, the present work points to a serological homogeneity of different DM strains (Table 4).

The immunological relatedness of DM and *M. leprae* is shown in Table 6. Ribosomes from four independently isolated DM yielded comparable degrees of reactivity with antisera against (i) disrupted DM cells, (ii) purified DM ribosomes, and (iii) *M. leprae*. Moreover, as indicated in Table 7, ribosomes from three DM strains reacted with about ¾ of the sera from patients bearing different types of leprosy. Further increase of this already high percentage is expected upon use of fresh sera (the present work was carried out with samples received from other continents). Indeed, two groups of intradermic tests, in which the "early" (Fernandez) reactivity of leprosy patients towards heat-inactivated DM was compared with that against the lepromin, confirmed the very close relationship between DM and *M. leprae* (B. R. Chatterjee et al., unpublished data; B. Naafs and E. Touw-Langendijck, unpublished data).

In conclusion, the present work points to the presence in leprosy lesions of two types of microorganisms which differ in acid-fastness and yet are serologically related. The DM, which have a shorter generation time than *M. leprae*, might contribute to the development of the disease. As a matter of fact, the cross-reactivity of antigens from DM and mycobacteria can explain the finding of antimycobacterial antibodies in sera from leprosy patients (15, 17, 24). Moreover, the presence in lepers' sera of antibodies reacting with ribonucleoproteins from *M. tuberculosis* (38) can be accounted for by the reactivity shown in Table 7 of DM ribosomes with the blood of leprosy patients.

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