Immunodiffusion Analyses of Some Diphtheroid Organisms Isolated from Patients with Leprosy[1]

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Other organisms than *Mycobacterium leprae* are frequently found in lesions of patients with leprosy ([2, 5, 8]). These organisms can, in contrast to the *M. leprae* bacillus, be cultivated *in vitro*. Knowledge about their characteristics is, however, comparatively small and the role they may play in the etiology of leprosy is unknown but certain studies have been performed concerning, e.g., their taxonomical positions. Beaman, *et al.* ([2]) performed chemical analyses of some strains and found that they could be assigned either to *Corynebacterium, Mycobacterium,* or *Propionibacterium*. Later Laub, *et al.* ([8]) found that certain strains bearing a morphological resemblance to corynebacteria were serologically more similar to mycobacteria and nocardiae than to corynebacteria. Recent studies by Danhaive, *et al.* ([4]) have, however, demonstrated that these organisms represent corynebacteria.

Eight diphtheroid strains isolated from patients with leprosy were included in the present study. The serological relationships between these strains and 25 strains representing *Corynebacterium, Mycobacterium, Nocardia, Rhodococcus,* and related taxa were analyzed.

MATERIALS AND METHODS

Bacterial strains. Eight diptheroid strains isolated from patients with leprosy (Table 1) were serologically compared with 25 strains (here referred to as the comparison strains) representing different species of *Corynebacterium, Mycobacterium, Nocardia, Rhodococcus, Streptomyces,* and related taxa (Table 2).

Antigen preparations. Antigen preparations from the eight diphtheroid strains were made as follows: strains GB259, GB260, GB267, GB268, and GB274 were cultivated at 37°C in fluid Dubos medium enriched with 5% horse serum (shake cultures). Strains GB269, GB270, and GB272 were grown at 37°C on blood agar. Cell mass was harvested from Dubos and the blood agar cultures, respectively. The cells were washed 3 times in phosphate buffered saline (PBS) with 0.02% w/v sodium azide and sonicated for 30 min at 100 W. The 25 comparison strains were cultivated on liquid or solid Sauton media or glucose nutrient broth, and antigen preparations were made as earlier described ([17]).

Antisera. Rabbit antisera against the 25 comparison strains were produced ([12]). A monospecific antiserum against the earlier described precipitinogen β ([16]) was prepared according to a method devised by Harboe, *et al.* ([6]). The β-precipitate was cut out of the gel, washed, sonicated, and used for the production of a rabbit antiserum.

Sera from nine Ethiopian patients with lepromatous leprosy were included in the study. These sera were provided by Dr. M. Harboe. Antisera against ribosomes from *M. bovis* var. BCG (Swedish substrain) and *M. phlei* (NCTC8151) were obtained in rabbits ([16]). The preparations of ribosomes were made as earlier described ([1, 9]).

Precipitation systems. Homologous precipitation systems were established for the comparison strains. The principles for the establishment and the use of serological precipitation systems have been described previously ([12, 17]). In order to reveal the precipitinogen β, a special system was established (the β system). It was heterologous in order to exclude some irrelevant precipitates and consisted of the *M. fortuitum* (GA023) antigen preparation and the anti-*M. avium* (GA009) serum.

Immunodiffusion analyses. The analyses were carried out by means of a microplate modification ([18]) of the immunodiffusion technique according to Ouchterlony.

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TABLE 1. *Diphtheroid strains isolated from patients with leprosy.*

Lab no.	Strain designation at arrival	Source[a]
GB259	D32	Isolated from blood, Iyona Hospital, Zaire. R. Moris.
GB260	86	Isolated from biopsy material, AHRI Center, Addis Ababa, Ethiopia. T. Godal.
GB267	43	Isolated in Manila, The Philippines. L. Barksdale.
GB268	2628LB	Isolated from a case of lepromatous leprosy, Carville, U.S.A. L. Barksdale.
GB269	Kim	
GB270	L3	Isolated from biopsy material, Iyona Hospital, Zaire. R. Moris.
GB271	L11	Isolated from biopsy material, Iyona Hospital, Zaire. R. Moris.
GB274	FPSA	

[a] All strains were obtained from Dr. L. Barksdale, New York University Medical Center, New York, U.S.A.

RESULTS

Antigen preparations from the eight diphtheroid strains were analyzed by immunodiffusion employing the 25 precipitation systems. Two of the strains, GB267 and GB269, did not react with the sera. The remaining six diphtheroid strains reacted with several of the sera, forming one or more precipitates (Table 2). The number of strains in which one or more precipitates were identified is also given in Table 2. All of these six strains crossreacted with *Corynebacterium glutamicum*, forming several precipitates, and in all of them at least one precipitinogen was identified. Five of the strains reacted with *C. ulcerans*, *M. bovis* var. BCG, and "*M.*" *album*. None of the precipitinogens revealed by the latter sys-

TABLE 2. *Number of strains—among eight diphtheroid strains tested—forming one or more precipitates when analyzed by precipitation systems representing the 25 comparison strains.*

Precipitation system		No. of strains	
Lab no.	Species		
GB242	*Arthrobacter globiformis* NCIB 8907, ATCC 8010	—[a]	—[b]
GB246	*Corynebacterium bovis* NCTC 3224	2	—
GB243	*Corynebacterium glutamicum* NCIB 10025	6	6
GB252	*Corynebacterium ulcerans* NCTC 7910	5	1
GB244	*Kurthia zopfii* NCIB 11155, ATCC 10536	4	1
GA009	*Mycobacterium avium* G. Penso Ceppo Faisan IV	3	—
GA001	*Mycobacterium bovis* var. BCG A. Lind Swedish substrain	5	3
GA923	*Mycobacterium farcinogenes* NCTC 10955	1	1
GA023	*Mycobacterium fortuitum* G. Penso 456	—	—
GA120	*Mycobacterium kansasii* E. H. Runyon P16	1	—
GA010	*Mycobacterium phlei* NCTC 8151	2	—
GA924	*Mycobacterium senegalense* NCTC 10956	4	—
GA029	*Mycobacterium smegmatis* NCTC 8152	1	—
GA713	*Mycobacterium tuberculosis* H37Rv ATCC 27294	4	2
GA081	*Mycobacterium vaccae* ATCC 15483	4	3
GB144	*Nocardia amarae* M. Goodfellow N667	—	—
GA761	*Nocardia asteroides* I Juhlin M-ö 5006	3	—
GA875	*Nocardia asteroides* II ATCC 19247	4	—
GA873	*Nocardia otitidis-caviarum* ATCC 14629	3	—
GB245	*Rhodococcus bronchialis* NCTC 10667	—	—
GA785	*Rhodococcus corallinus* I Juhlin M-ö 5007	3	—
GA766	*Rhodococcus rubrus* I Juhlin 107	3	—
GB285	*Streptomyces diastaticus* S. T. Williams 496	—	—
GB202	"*Gordona*" *aurantiaca* NCTC 10741	4	—
GB205	"*Mycobacterium*" *album* ATCC 25969	5	—

[a] Number of strains forming one or more precipitates by means of the antisera.
[b] Number of strains in which one or more precipitates were identified by means of the precipitation system.

TABLE 3. *Number of precipitinogens revealed when the strain GB271 was analyzed by the precipitation systems representing the 25 comparison strains.*

Precipitation system	No. of precipitinogens	
A. globiformis	—[a]	—[b]
C. bovis	—	—
C. glutamicum	5	2
C. ulcerans	4	1
K. zopfii	1	—
M. avium	1	—
M. bovis var. BCG	1	1
M. farcinogenes	—	—
M. fortuitum	—	—
M. kansasii	—	—
M. phlei	1	—
M. senegalense	1	—
M. smegmatis	—	—
M. tuberculosis	2	1
M. vaccae	2	1
N. amarae	—	—
N. asteroides I	1	—
N. asteroides II	1	—
N. otitidis-caviarum	1	—
R. bronchialis	—	—
R. corallinus	1	—
R. rubrus	1	—
S. diastaticus	—	—
"Gordona" aurantiaca	2	—
"Mycobacterium" album	3	—

[a] Total number of revealed precipitinogens.
[b] Number of precipitinogens identified by the precipitation system.

tem, were however, identified. Four of the strains crossreacted with *Kurthia zopfii, M. senegalense, M. tuberculosis, M. vaccae, Nocardia asteroides* II, and *"Gordona" aurantiaca.* None of the strains crossreacted with *Arthrobacter globiformis, M. fortuitum, Nocardia amarae, Rhodococcus bronchialis,* and *Streptomyces diastaticus.*

Table 3 is an example of the results obtained, demonstrating the number of precipitinogens revealed when strain GB271 was analyzed. This strain shared one or more precipitinogens with most of the reference strains. The largest number of shared precipitinogens was obtained when GB271 was compared with *C. glutamicum* and *C. ulcerans.* The strains GB259, GB260, GB268, GB270, and GB274 reacted basically in the same way, i.e., they shared more precipitinogens with the corynebacteria than with the other reference organisms.

The diphtheroid organisms were, furthermore, analyzed by means of the mono-

TABLE 4. *Number of precipitinogens revealed when six of the diphtheroid strains were analyzed using antisera against ribosomes from* M. bovis *var. BCG and* M. phlei.

Strain	Antisera against ribosomes from	
	M. bovis var. BCG	*M. phlei*
GB259	1	1
GB260	—	—
GB268	2	1
GB270	—	—
GB271	2	1
GB274	—	—

specific anti-β serum as well as by the β system. None of the eight diphtheroid organisms were shown to contain β.

Six of the eight diphtheroid strains were analyzed by sera against ribosomes from *M. bovis* var. BCG and *M. phlei* (Table 4). Strains GB259, GB268, and GB271 reacted with both sera, forming one or two precipitates; while the remaining three strains tested (GB260, GB270, and GB274) did not react with either of the two sera.

Finally, nine sera from Ethiopian patients with lepromatous leprosy were analyzed, employing antigen preparations from the six diphtheroid strains which reacted with any of the comparison strains. Faint precipitates were revealed in a small number (about 15%) of the test combinations.

DISCUSSION

When six of the eight diphtheroid strains tested were analyzed by means of the 25 precipitation systems representing the comparison strains, the largest number of crossreacting antigens was revealed when the corynebacterial systems were used. The results thus indicate that these six strains either belong to *Corynebacterium* or are closely related to this genus, supporting recent genetical analyses by Danhaive, *et al.* ([4]). They demonstrated that these organisms represent a cluster within the genus *Corynebacterium.* It is of interest to note that the six diphtheroid strains shared more antigens with *C. glutamicum,* which is a saprophytic organism, than with *C. bovis* and *C. ulcerans,* which are human or animal parasites.

The present analyses showed, furthermore, that the six strains share antigens with

several of the actinomycetes, e.g., mycobacteria, nocardiae, and rhodococci. The results are thus in accordance with earlier studies, indicating that corynebacteria crossreact with mycobacteria and other actinomycetes ([3, 13, 15]).

Laub, *et al.* ([8]), also investigating diphtheroid strains isolated from leprosy patients, obtained results indicating a closer immunological relationship between these organisms and the mycobacteria than between them and the corynebacteria. The present results also demonstrate an immunological linkage between these organisms and mycobacteria, but do not support the hypothesis that they are more closely related to the mycobacteria than to the corynebacteria.

Two of the eight diphtheroid test strains did not react with any of the sera. This result might be due to low concentrations of antigens in the preparations, owing to technical errors. Another explanation might be that these two organisms are serologically different from the comparison strains.

Earlier studies have demonstrated a ribosomal precipitinogen, designated β, which exists in mycobacteria (including the *M. leprae* bacillus), rhodococci, nocardiae, and streptomycetes, but not in any of the corynebacteria tested ([10, 11, 13, 14, 15, 16, 17]).

None of the diphtheroid organisms analyzed contained the precipitinogen β, a result which supports their affiliation to the genus *Corynebacterium*. Other antigens shared by some of the diphtheroid organisms and the mycobacteria were, however, shown to be ribosomal. These results are in accordance with earlier ones, demonstrating that many intergenerically crossreacting antigens are ribosomal ([13, 16]).

Several studies ([7, 15, 19]) have shown that sera from patients with leprosy contain antibodies against various mycobacteria and related organisms to a comparatively large extent. For example, the present author recently demonstrated—using the same nine sera as in the present study—that most of these sera reacted with the tested mycobacteria and streptomycetes and that the precipitates revealed often represented the β antigen ([15]). The diphtheroid strains share antigen with mycobacteria but do not contain β, which might explain the relatively limited number of precipitates revealed. In conclusion, it can be stated that the presence of anti-β antibodies in sera from patients with lepromatous leprosy is, in all likelihood, not a result of the presence of diphtheroid organisms in the patients.

SUMMARY

Eight strains of diphtheroid bacteria isolated from patients with leprosy were analyzed by immunodiffusion, using precipitation systems representing various species of *Corynebacterium, Mycobacterium, Nocardia, Rhodococcus,* and related organisms. The analyses showed that six of the eight strains shared several antigens with representatives of these four genera. The largest number of shared precipitinogens was revealed when the corynebacterial precipitation systems were used, thus indicating that these organisms either belong to, or are closely related to the genus *Corynebacterium.* This assumption was further supported by the fact that the ribosomal precipitinogen β—earlier demonstrated in mycobacteria but not in corynebacteria—was not found in the diphtheroid strains. Other ribosomal antigens were, however, revealed to be common to the diphtheroid organisms and mycobacteria. Further, the reaction between sera from patients with lepromatous leprosy and the diphtheroid strains was analyzed, very few and faint precipitates being demonstrated. It is concluded that the presence of anti-β antibodies in leprosy sera is, most likely, not a result of the presence of diphtheroid organisms in the patients.

RESUMEN

Se analizaron, por inmunodifusión, ocho cepas de bacterias difteroides aisladas de pacientes con lepra usando los sistemas de precipitación correspondientes a varias especies de los géneros *Mycobacterium, Corynebacterium, Nocardia, Rhodococcus,* y organismos relacionados. Los resultados mostraron que 6 de las 8 cepas aisladas compartieron varios antígenos con representantes de los cuatro géneros probados. El mayor número de precipitógenos compartidos se observó cuando se usaron los sistemas de precipitación corinebacteriales, indicando así que estos microorganismos corresponden o están íntimamente relacionados con el género *Corynebacterium.* Esta suposición fue posteriormente apoyada por el hecho de que el precipitógeno β-ribosomal (previamente demostrado en micobacterias pero no en corinebacterias) no se encontró en las cepas difteroides. Sin embargo, otros antígenos

ribosomales fueron encontrados tanto en los difteroides aislados como en las micobacterias. Por otro lado, la reacción entre los sueros de los pacientes con lepra lepromatosa y preparaciones antigénicas de las cepas difteroides, sólo se manifestó en forma de precipitados escasos y tenues. Se concluye que la presencia de anticuerpos anti-β en los sueros de pacientes con lepra, no es el resultado de la presencia de organismos difteroides en los pacientes.

RÉSUMÉ

On a analysé par une méthode d'immunodiffusion huit souches de bactéries diphtéroïdes isolés chez des malades atteints de lèpre. Pour se faire, on a utilisé un des systèmes de précipitation représentant divers espèces de *Corynebactérie,* de *Mycobactérie,* de *Nocardia,* de *Rhodococcus,* et d'organismes apparentés. Les analyses ont montré que 6 des 8 souches partagaient plusieurs antigènes avec les représentants de ces quatre genres. Le plus grand nombre de précipitinogènes communs a été noté lorsqu'on utilisait le système de précipitation corynebactérien. Ces résultats montrent que les organismes étudiés font partie du genre *Corynebacterium,* où ils sont étroitement apparentés. Cette hypothèse a été renforcée par le fait que le précipitinogène β des ribosomes—démontrée précédemment chez les mycobactéries mais non chez les corynebactéries—n'a pas été trouvée dans les souches de diphtéroïdes. On a toutefois observé que d'autres antigènes des ribosomes étaient communs aux organismes diphtéroïdes et aux mycobactéries. De plus, on a étudié la réaction entre les échantillons de sérum provenant de malades atteints de lèpre lépromateuse, et les souches diphtéroïdes; il n'a été possible de démontrer qu'un très petit nombre de précipitations, très peu marquées. On en conclu qu'il est fort vraisemblable que la présence d'anticorps anti-β dans le serum de malades de la lèpre ne provient pas de la présence d'organismes diphtéroïdes chez ces malades.

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