A SEROLOGICAL COMPARISON OF THE PROTEINS OF VARIOUS STRAINS OF SUPPOSED LEPROSY BACILLI AND OTHER ACID-FAST BACTERIA

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The reports of different investigations into the antigenic relationships between the various cultures of mycobacteria cultivated from leprosy, and between members of that group of organisms and various acid-fast bacteria from other sources, are so contradictory that it has seemed advisable to restudy this subject by improved methods. Most of the published reports were based on studies made with either whole micro-organisms or unpurified extracts of them. Seibert (12) has shown that purified proteins obtained from the human, bovine, and avian types of tubercle bacilli, and from the timothy grass bacillus, can be differentiated by means of the precipitin test. This observation suggested that light might be thrown upon the puzzling question of the relationships of acid-fast bacteria isolated from cases of leprosy by employing similar purified specific proteins obtained from them.

To this end protein extracts were prepared from a total of twenty-seven strains of mycobacteria by the method described by Seibert (13). Antisera were obtained by immunizing rabbits with these proteins, and their antigenic relationships were determined by means of the precipitin test. The organisms dealt with comprised, as is shown in Table 1, sixteen strains of so-called leprosy bacilli,[2] three strains of tubercle bacilli, and eight other organisms.

PREPARATION OF PROTEINS

Each strain of the acid-fast bacilli studied was inoculated into approximately 50 one-liter bottles each containing 200 cc. of Long's synthetic medium, and the cultures were grown for eight weeks at 37.5° C. The bacterial mass was filtered off through Büchner funnels lined with fine China silk. The filtrate was preserved with 0.5 percent phenol, and filtered free of bacteria by passing

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[2] The claims to authenticity of the so-called leprosy bacilli dealt with are not considered in this study. However, the formal designation of *Mycobacterium leprae* is avoided.

it through a Mandler filter. Since there was a possibility that some protein from a previous filtration might have been absorbed within the pores of the filter itself, in each case the filter was boiled in a strong sodium carbonate solution for one hour, after which approximately four liters of fresh, warm, carbonate solution was passed through it by suction. The alkali remaining in the filter was then removed by passing first tap water and then distilled water through it. The final water filtrate was tested for the presence of protein before the filter was used.

The phenolized, bacteria-free filtrates were concentrated by means of ultrafiltration through alundum cups impregnated with 13 percent gun-cotton dissolved in glacial acetic acid. Substances that would pass these filters were removed by repeated additions of distilled water (with 0.5 percent phenol). The nonfilterable residue, representing about a hundred-fold concentration of the original filtrate, was passed through a Seitz filter and precipitated with trichloracetic acid. The precipitate was washed five or six times with 10 percent trichloracetic acid, partially dried in vacuo, dehydrated with anhydrous ether and dried to constant weight. Solutions of the different antigens were prepared in 5 percent concentrations by first dissolving the proteins in minimal amounts of normal sodium hydroxide, neutralizing with N/10 hydrochloric acid (litmus being used as the indicator), and diluting to volume with normal physiological saline containing 0.5 percent phenol.

Two full-grown rabbits were injected intracutaneously, at widely separate points, with 10 mgms. of each antigen, at approximately four-day intervals, until a total of 100 mgms. had been injected. The increasing degree of local sensitiveness of the rabbits was determined by observing the specific inflammatory reaction (Arthus reaction) which occurred 24 hours after the injection. Approximately eight days after the tenth injections the rabbits were bled to death. The sera were separated from the blood cells, filtered through Seitz asbestos filters, sealed in sterile, chemically cleaned test tubes, and stored in the ice-box until used.

The precipitin tests were performed by the addition of 0.1 cc. doses of the antisera to 0.1 cc. of dilutions of antigen solutions that varied in concentration from 1:200 to 1:200,000. The tubes were incubated at 37° C. for two hours, shaken, and allowed to stand in the ice-box for 24 hours, when the precipitate in each tube was noted.

The experiments presented in this paper have been repeated two times, and in some cases three times. The results obtained have been consistent.

TESTS OF ORGANISMS CULTIVATED FROM LEPROSY

With regard to attempts to differentiate serologically between different strains of mycobacteria isolated from lepers, the following previous investigations are pertinent.

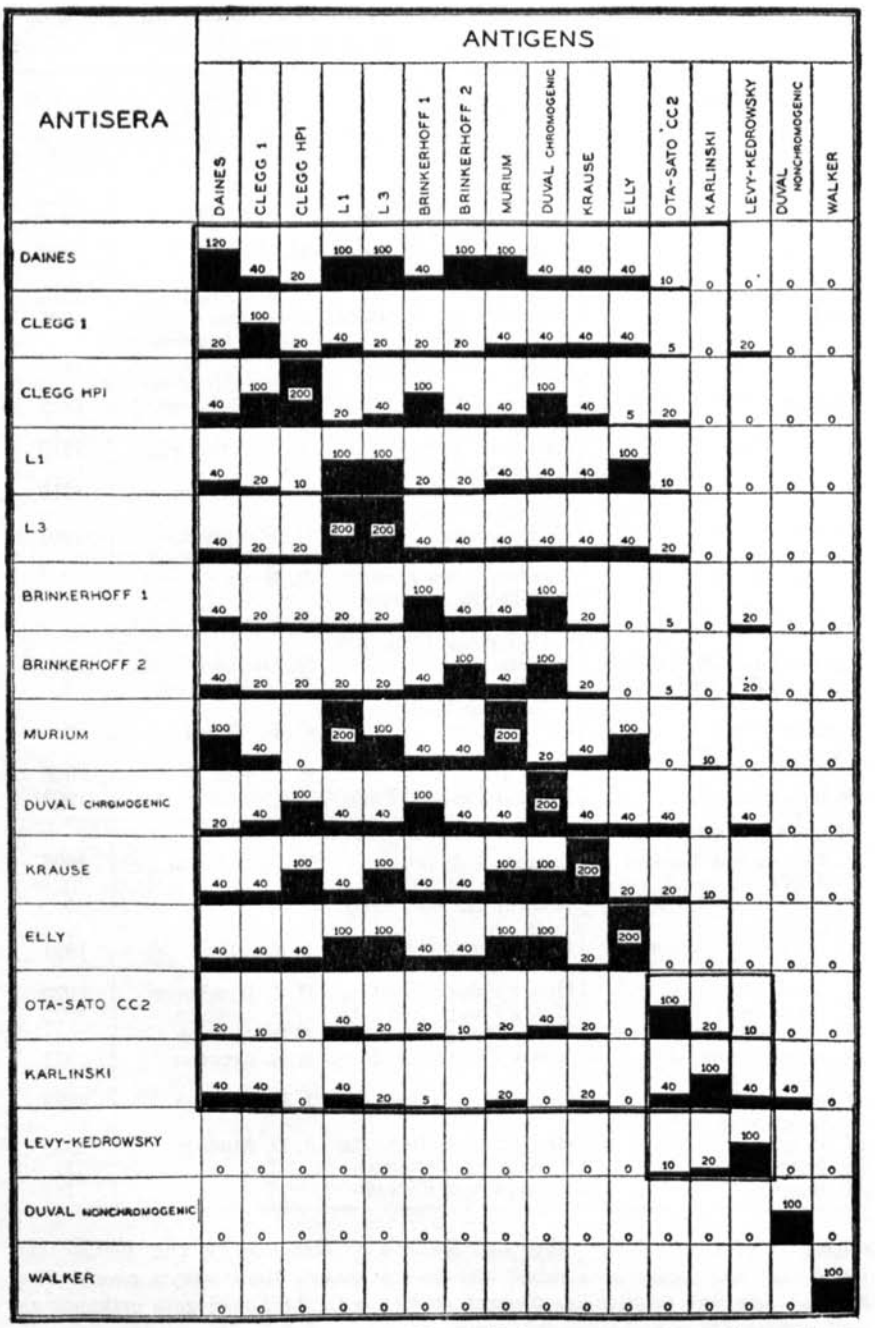
Duval and Wellman (4) found that an antiserum prepared against Clegg's chromogenic organism did not agglutinate Duval's nonchromogenic strain. Cooke (3), using the complement fixation reaction, found that both of Duval's cultures, chromogenic and nonchromogenic, gave fixation in greater dilutions with their specific immune rabbit serum than they did with antisera prepared against the

TABLE 1.—*List of mycobacteria studied, their immediate sources, investigators by whom isolated, and dates of isolation.*

Organism used	Source of stock culture	By whom isolated	Date of isolation
MYCOBACTERIA FROM LEPROSY			
Bacillus Daines	U.S.P.H.S. Hospital, Carville, La.	L. L. Daines	1933
Bacillus Clegg 1	National Type Culture Collection, London	M. T. Clegg	1909
Bacillus Clegg H. P. I.	C. W. Duval	C. W. Duval	1910
Bacillus "L 1"	Henry Phipps Institute	J. D. Aronson and H. J. Henderson	1935
Bacillus "L 3"	Henry Phipps Institute	J. D. Aronson and H. J. Henderson	1935
Bacillus Brinkerhoff 1	American Type Culture Collection	W. R. Brinkerhoff	1912
Bacillus Brinkerhoff 2	American Type Culture Collection	W. R. Brinkerhoff	1912
Bacillus murium	National Institute of Health	C. W. Chapin	1912
Bacillus Duval chromogenic	C. W. Duval	C. W. Duval	1910
Bacillus Krause	National Type Culture Collection, London	R. Krause	?
Bacillus Elly	National Type Culture Collection, London	R. Elly	?
Bacillus Ota-Sato CC2	M. Ota	M. Ota and S. Sato	1935
Bacillus Levy-Kedrowski	London School of Tropical Medicine	W. J. Kedrowski	?
Bacillus Duval nonchromogenic	C. W. Duval	C. W. Duval	?
Bacillus Walker	E. L. Walker	E. L. Walker	1926
Bacillus Karlinski	Institut Pasteur, Paris	J. Karlinski	1901
OTHER MYCOBACTERIA			
M. tuberculosis hominis (H 37)	Saranac Laboratory	E. R. Baldwin	1905
M. tuberculosis bovis (1698)	Institut Pasteur, Paris	?	?
M. tuberculosis avian (531)	University of Nebraska	L. Van Es	1921
M. phlei	Henry Phipps Institute	H. J. Henderson	1923
M. marinum 1 (fish)	Henry Phipps Institute	J. D. Aronson	1925
M. marinum 2 (fish)	Henry Phipps Institute	J. D. Aronson	1931
M. thamnopheous 1 (garter snake)	Henry Phipps Institute	J. D. Aronson	1927
M. thamnopheous 2 (garter snake)	Henry Phipps Institute	J. D. Aronson	1928
M. smegmatis	Henry Phipps Institute	J. D. Aronson	1933
M. butyricum	University of Chicago	?	?
M. ranae	University of Chicago	?	?

organisms of Brinkerhoff, Clegg and Karlinski. Kritchewsky and Bierger (9) found that the serum of a rabbit immunized against Kedrowsky's nonchromogenic culture gave positive complement fixation with the homologous organism as well as with Duval's chromogenic strain.

Lewis and Aronson (10) found that human sera from cases of leprosy gave



TEXT-FIG. 1. Precipitin tests with purified protein antigens from sixteen strain of so-called leprosy bacilli (titer 10^3) and their corresponding rabbit antisera. Lines have been drawn around those records of reaction which seem to show a group relationship. Ciphers indicate negative reactions.

positive fixation with an antigen prepared from Clegg's bacillus, but Harris and Langford (8), using the agglutination reaction, were unable to demonstrate specific antibodies in sera obtained from lepers or from rabbits immunized with the Duval, Barry, Bayon 1, and No. 18 strains of leprosy bacilli, or with *M. phlei*. These authors concluded that little reliability can be placed upon the agglutination test as a means of identifying cultures isolated from lesions of leprosy.

RESULTS OBTAINED

The results of the precipitin tests made with the proteins isolated from sixteen strains of bacilli derived from leprosy and the antisera produced with those proteins are presented in Text-figure 1. It will be seen that, with but few exceptions, the antisera gave maximum degrees of precipitation with their homologous antigens. With but one exception (the Karlinski strain), thirteen of these organisms gave definite cross reactions with one another. In view of this fact it is considered justifiable to place them in one large group, and to consider them as being antigenically related. On the other hand, the Levy-Kedrowsky organism did not react antigenically with this larger group. The Ota-Sato and Karlinski strains reacted with both the larger group and with the Levy-Kedrowsky strain, thus overlapping the larger and the smaller antigenically diverse groups. Duval's non-chromogenic and Walker's chromogenic organisms did not react with any of the other strains studied. Chromogenesis evidently plays no role in these differences, and apparently it has no relationship to antigenicity in so far as that is related to the protein element of these micro-organisms.

COMPARISON OF BACILLI FROM LEPROSY AND OTHER ACID-FAST ORGANISMS

Since purified proteins prepared from a number of strains of mycobacteria isolated from leprosy show definite group relationships, and since there have been contradictory observations by various investigators as to the relationships of these organisms to other acid-fast bacteria, it was thought advisable to determine what antigenic relationship exists between purified proteins prepared from these two classes of bacteria. Among the more significant observations on this matter in the past the following may be cited:

Duval and Wellman (4), by means of the agglutination and complement fixation reactions, found that Clegg's chromogenic organism is not related antigenically to *M. phlei* or to the acid-fast bacillus isolated from butter. They further found that Duval's nonchromogenic strain differed antigenically from Clegg's chromogenic organism, and from acid-fast bacteria isolated from timothy grass and from butter.

ANTISERA	ANTIGENS														
	LEPROSY, DAINES	M. PHLEI	LEPROSY, KARLINSKI	M. AVIUM	LEPROSY, DUVAL, NONCHROMOGENIC	M. MARINUM 1	M. TUBERCULOSIS HOMINIS	LEPROSY, LEVY-KEDROWSKY	M. THAMNOPHEOS 2	M. SMEGMATIS	M. BUTYRICUM	M. MARINUM 2	M. RANAE	M. TUBERCULOSIS BOVIS	M. THAMNOPHEOS 1
LEPROSY, DAINES	120	20	0	0	0	0	0	0	0	0	0	0	0	0	0
M. PHLEI	20	100	10	0	0	0	5	0	0	0	0	0	0	0	0
LEPROSY, KARLINSKI	40	10	100	0	40	20	40	40	0	0	0	0	0	0	0
M. AVIUM	0	0	0	100	40	10	5	0	0	0	0	0	0	0	0
LEPROSY, DUVAL, NONCHROMOGENIC	0	0	0	10	100	10	2	0	0	0	0	0	0	0	0
M. MARINUM 1	0	0	20	10	10	100	10	0	0	0	0	0	0	0	0
M. TUBERCULOSIS HOMINIS	5	5	0	10	10	20	100	10	0	20	0	10	0	0	0
LEPROSY, LEVY-KEDROWSKY	0	0	20	0	10	0	4	100	20	20	0	10	0	0	0
M. THAMNOPHEOS 2	0	0	0	0	0	0	0	20	100	0	0	0	0	0	0
M. SMEGMATIS	0	0	0	0	0	0	20	20	0	100	10	0	0	0	0
M. BUTYRICUM	0	0	0	0	0	0	0	0	0	20	50	0	0	0	0
M. MARINUM 2	0	0	0	0	0	0	20	20	0	10	0	200	0	0	0
M. RANAE	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0
M. TUBERCULOSIS BOVIS	0	0	0	0	0	0	40	0	0	0	0	0	0	100	0
M. THAMNOPHEOS 1	0	0	0	0	0	2	4	0	0	0	0	0	0	0	100

TEXT-FIG. 2. Comparison of precipitin tests with proteins isolated from so-called leprosy bacilli and other acid-fast bacteria. Lines have been drawn around those records of reactions which seem to show a group relationship. Ciphers indicate negative reactions.

On the basis of cultural characteristics, Duval and Harris (5) divided several strains of leprosy organisms into the following groups: (*a*) Duval's and Kedrowsky's nonchromogenic strains, (*b*) Clegg's, Brinkerhoff's and Duval's chromogenic strains, and (*c*) Karlinski's nonchromogenic strain.

Using alcoholic extracts and bacillary emulsions as antigens, Lewis and Aronson (10) found that human sera from cases of tuberculosis and leprosy gave positive complement fixation with *M. tuberculosis bovis*, the Duval and Kedrowsky organisms, and a chromogenic acid-fast saprophyte. They further found that human leprosy serum reacted with the Clegg strain, whereas human tuberculous serum did not. Hans Much (11) found that when human leprosy sera were tested by the complement fixation reaction with antigens prepared from *M. tuberculosis hominis* and *bovis*, or from the timothy grass bacillus, they were not specific. Kritchewsky and Bierger (9) reported that the serum of a rabbit immunized against Kedrowsky's organism gave positive fixation with an antigen obtained from the human tubercle bacillus. Gengou (7) demonstrated by means of the complement fixation reaction that both rabbit and guinea pig antiserum prepared against *M. tuberculosis hominis* and *bovis*, *M. butyricum*, *M. phlei* and *M. avium*, showed antibodies not only against their homologous antigens but also against heterologous ones.

Aronson and Lewis (2) found with the complement fixation reaction that human and bovine tubercle bacilli were similar to each other in antigenic structure, but that they differed from the avian bacillus, the nonchromogenic leprosy organisms of Duval and Kedrowsky, and various other acid-fast bacteria. By means of agglutination and agglutination-absorption experiments Wilson (14) and Furth (6) found that the antigenic structures of the human and the bovine tubercle bacilli are similar, but that the avian bacillus differs. Lewis and Aronson (10) by complement fixation, and Furth (6) by agglutination and agglutination-absorption, found that the Duval and Kedrowsky strains are antigenically identical with the avian tubercle bacillus.

Aronson (1) demonstrated by tissue culture methods that "tuberculins" prepared from the Duval and Kedrowsky nonchromogenic organisms inhibited the growth of the cells of explants of the spleen and bone marrow of tuberculous fowls, but had no effect on explants from tissues of nontuberculous fowls. He further noted that these tuberculins when injected intradermally into the wattles of tuberculous fowls produce reactions indistinguishable from the reaction induced by avian tuberculin.

RESULTS OBTAINED

The mycobacteria studied in comparison with the leprosy group are listed in the second section of Table 1. For this work the organism isolated from leprosy by Daines was chosen as a typical representative of that group because it shows a maximum degree of cross reaction with the other strains (see Text-figure 1). The results of the precipitin tests made in this comparative study are presented in Text-figure 2.

The results obtained indicate that Daines' organism has an antigenic component or components similar to those of *M. phlei* and the

Karlinski leprosy organism. Although it is true that the titer of the cross precipitin reaction is not very high, the presence of precipitins in a dilution of 1:10,000 or 1:20,000 would seem to be an indication of similarity of antigenic structure.

The cross reactions between the antigens and antisera of the Karlinski and Duval nonchromogenic organisms, *M. avium, M. marinum 1 and M. tuberculosis hominis* indicate the presence of common antigenic components in this group. Furthermore, the Karlinski strain also reacts with the Daines group of mycobacteria, thus linking the latter with the avian group.

A third group possessing common antigenic components is that represented by the Levy-Kedrowsky organism, which group includes *M. thamnopheous 2* and *M. smegmatis.* The last-named strain also reacts slightly with *M. butyricum,* indicating that the two have common antigenic components.

The last four strains of organisms shown in Text-figure 2 are composed of antigenic units which are different from those of the other strains studied. In only rare instances have antisera derived from these strains reacted with any but their homologous antigens.

As in the first series of tests (Text-figure 1), each of the antisera reacted most strongly with its specific antigen.

SUMMARY

The antigenic relationships of sixteen strains of mycobacteria isolated from leprosy, and of this group of organisms and eleven other strains of acid-fast bacteria, have been determined by means of the precipitin reaction, using purified specific proteins and sera of rabbits immunized against those proteins. The organisms employed all grew readily on Long's synthetic medium, which was used for all cultures; obviously, strains not growing on the artificial medium could not be included. With but few exceptions the antisera tested gave the highest precipitin titers with their homologous antigens. Thirteen of the sixteen leprosy strains gave strong precipitin reactions with one another (Text-figure 1). In view of this it is considered justifiable to place them in one large, antigenically related group. Of the three remaining strains, those of Duval (nonchromogenic) and Walker (chromogenic) failed to cross-react with the large group and may therefore be considered as antigenically unrelated. The one remaining strain, Levy-Kedrowsky, while it failed to give definite cross reactions with all members of the large lepra group, did react with two of them, the Ota-Sato and Karlinski strains, and

therefore has an antigenic relationship to the group.

All of the acid-fast bacteria studied, including the bacilli from leprosy, various types of tubercle bacilli, and acid-fast saprophytes, tend to fall into certain groups (Text-figure 2). A relationship between the leprosy strains and other acid-fast bacteria is indicated by their common cross reactions. Striking relationships are noted in the case of the strains of Daines, Karlinski and Duval (nonchromogenic), either directly or through linkage with *M. avium*, *M. tuberculosis*, ***hominis***, *M. marinum* and with *M. phlei*.

It is interesting to note that the avian tubercle bacillus studied has some antigenic relationship to Duval's nonchromogenic organism, while it has no direct relationship with the large leprosy group represented by Daines' strain.

The bovine tubercle bacillus strain studied apparently bears no antigenic relationship to any of the other acid-fast organisms employed except the human tubercle bacillus.

Chromogenesis apparently bears no relationship to the antigenic composition of these micro-organisms, in so far as that is related to their protein elements.

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