COMPLEMENT-FIXATION REACTION OF LEPERS' SERA WITH BACILLARY ANTIGENS

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INTRODUCTION

The first of our experiments on complement fixation with lepers' sera, work which is still under way, were planned simply to determine the nature of the acid-fast bacilli cultivated from leprous materials by Ota and Sato. As for that, we found that it is almost impossible to distinguish the different strains of these bacteria by this method, a conclusion reached by many earlier investigators.

At the same time we obtained results which were not at first anticipated. We found that our specific antigens give very high percentages of positive results with leprous sera, and almost negligible numbers with non-leprous sera. This is especially the case with Ota and Sato's Bg strain; like the Clegg strain of leprosy bacillus in the hands of Lewis and Aronson it gives the most valuable antigen.

Published reports of work along this line are so numerous that no attempt will be made to refer to all of them. We will chiefly give here an account of our own work.

TECHNIC

The antigen is of primary importance, the results depending upon its sensitivity. Out of Ota and Sato's fifteen strains (4) we selected strains Bg (nearly white) and Cd (orange-yellow) for use. For comparison we used: *Bacillus tuberculosis,* human and avian types, *Mycobacterium phlei,* and *Myco. smegmatis* (all from the Government Institute for Infectious Diseases), *Myco. leprae muris* (E-1684 strain, Dr. Sh. Asami), and a nearly white acid-fast organism cultured from running water by Sato. In every instance the Kolmer Wassermann antigen, which gives a low percentage of positive Wassermann reactions with lepers' sera, was also used.

TABLE 1.—*Arrangement of tubes in the complement-fixation reaction employed. Quantities in cc.*

Reagent	Test series; tubes						Controls; tubes			
	1	2	3	4	5	6	1	2	3	4
Serum	0.1	0.05	0.025	0.0125	0.00625	0.003125	—	—	0.2	—
Complement	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—
Antigen	0.5	0.5	0.5	0.5	0.5	0.5	—	1.0	—	—
Saline	0.4	0.45	0.475	0.4875	0.49375	0.496875	1.0	—	0.8	1.5
Water bath	Thirty minutes at 37°C.									
Hemolysin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sheep cells	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Water bath	Thirty minutes at 37°C.									

TABLE 2A.—*Results obtained with 28 patients' sera tested solely with antigens of bacilli cultivated from leprosy.*[1]

Antigen	T. I. (21)	S. I. (6)	H. N. (3)	K. M. (2)	T. K. (14)	G. I. (1)	H. E. (15)	16[2] cases	G. M. (20)	S. O. (25)	S. G. (26)	M. F. (27)	H. S. (28)
No. 2 Bg	+++	+++	++	++	++	−	−	−	±	−	−	−	−
No. 2 Cd	−	++	++	+	+	−	−	−	−	−	−	−	−
No. 3 Bg[3]									+++	+++	++	±	−
Wassermann	−	−	−	−	++	++	+	−	−	−	−	−	−

[1] In this and the other tables − signifies negative, ± very weakly positive, + weak, ++ moderately strong and +++ strong.
[2] These negative cases comprise all those from 1 to 28 that are not listed individually.
[3] Dilution 1:99.

TABLE 2B.—*Results obtained with 17 patients' sera tested with antigens of two other bacilli as well as those of bacilli from leprosy.*

	Y. M. (41)	Y. K. (43)	M. C. (44)	I. N. (36)	T. H. (39)	K. C. (40)	M. K. (42)	Y. N (45)	B. H. (29)	S. N. (30)	S. I. (31)	S. M. (32)	T. O. (34)	4 [a] cases
No. 2. Bg	+++	+++	+++	+++	++	++	++	++	++	−	−	+++	+	−
No. 2. Cd	+++	++	++	++	++	++	+	+	−	−	−	+++	−	−
No. 3. Bg	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++			
No. 2. Rat leprosy ..	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 2. Tb. human ...	−	−	+++	++	−	−	++	++	+	−	−	+	−	−
Wassermann	−	−	−	++	−	−	−	±	++	−	−	+	−	−

[a] Cases 33, 35, 37 and 38.

TABLE 2C.—*Results obtained with 17 patients' sera tested with the No. 3 Bg antigen (two dilutions), and four or six other antigens.*

Antigen [a]	S.U. (67)	U. S. (68)	J. M. (72)	M. T. (77)	G. O. (71)	T. O. (66)	H. T. (73)	M. Y. (75)	K. O. (80)	S. M. (82)	K. O. (79)	S. O. (76)	T.Y. (74)	K. O. (65)	N. W. (69)	M. K. (81)	K. I. (70)
No. 3. Bg (1:49)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	−
No. 3. Bg (1:99)	+++	+++	+++	+++	+++	++	++	++	++	++	++	+	++	+	+	+	−
No. 3. Rat lepros	+++	+++	++	+	++	++	++	+	++	+	±	−	+	+	+	−	−
No. 3. Tb. human	+++	+++	+++	+++	++	+++	+++	+++	++	++	+	±	++	++	++	−	−
No. 3. Tb. avian		+++				−	−		++	−	±				+	−	−
No. 9. Hay	+++	+++	+++	±	+	+++	++	+	++	+	+	±	±	++	+	−	−
No. 3. Water	+++	+++	+++	+++	+++	+++	+++	+	++	++	+	±	+	+	++	−	−
No. 3. Smegma .		++				−	−		++	−	±				+	−	−
Wassermann	+++	+++	−	−	−	−	−	−	−	−	−	−	++	−	−	−	−

[a] Except where otherwise stated, the antigen dilutions were 1:49.

Antigens 1a (BG *strain*) *and 1b* (CD *strain*).—Watery extracts of the bacteria grown on 3 per cent glycerol agar for two or three months. A uniform suspension was made in salt solution, an ordinary platinum loopful to 5 cc.; this was filtered through paper and heated at 60°C for one hour.

Antigens 2a (BG *strain*) *and 2b* (CD *strain*).—These suspensions were made in 70 per cent alcohol, filtered, but not heated. In antigenic value they proved superior to the first variety. After diluting ten times with saline the minimal antihemolytic dose was 0.5 or 0.6 cc. Less than half of this was used in the tests.

Antigen 3.—The BG strain was used chiefly for this. The growth on 3 per cent glycerol bouillon, two months or so old, was heated at 80°C. for half an hour, separated by centrifugation, washed twice in sterile distilled water, dried over sulphuric acid in a desiccator for a week, extracted with ether for 48 hours in a Soxhlet apparatus, and the bacterial residue dried. Of this powder 0.1 gram was ground in an agate mortar for a half hour, then mixed with one or two drops of sterile saline and ground for 10 minutes more, after which enough saline was added to make it fluid. Of a 1 per cent solution of cholesterin (Merck) in absolute alcohol, as prescribed by the Japanese pharmacopoeia, 3 to 5 drops were slowly added, and then a little more saline, stirring the while. This was repeated about ten times, until a total of 2 cc. of the cholesterin solution and 8.0 cc. of saline was used. To this preparation 1 cc. of 5 per cent phenol was added. The cholesterinization took about half an hour, and from the time the dried bacterial mass was put into the mortar the process required about one hour. For use this original suspension was diluted 1 part to 99 parts of saline, this being added slowly, and 0.5 cc. was used as the test dose, 1.0 cc. proving not anti-complementary. Control tests with cholesterin in the amount actually used were always negative with sera from lepers, non-lepers and immunized rabbits.

Antigens of human tubercle bacillus.—Since this bacillus grows slowly on 3 per cent glycerol-bouillon, we used a culture over three months old. Methods Nos. 1 and 2 were used in preparing these antigens and they were numbered accordingly.

Antigens of other acid-fast bacilli.—These were made up by Method No. 3.

Other reagents.—The *complement* was mixed guinea-pig serum, diluted 10 times; 0.2 to 0.25 cc. gave complete hemolysis, and 0.5 cc. (about 2 units) was used. Of rabbit anti-sheep *hemolysin*, inactivated at 56°C. for 30 minutes, twice the minimal hemolytic dose was used, diluted to 0.5 cc. Sheep's *red blood corpuscles* were washed, diluted to 5 per cent and 0.5 cc. used. *Patients' sera* were usually used within one or two days, but some were tested after five to seven days, these having been both inactivated and phenolized (0.5 per cent). Lepers' sera are often strongly anti-complementary, and some of those taken from 16 patients in the Zensei Hospital (Tokyo) showed this with 0.2 cc., but never with 0.1 cc. From 0.1 down to 0.003125 was used in the test.

The test.—The set-up is shown in Table 1. The doses of complement and antigen were kept always the same, the amount of anti-body (inactivated serum) being reduced gradually. Control tubes Nos. 1 to 3 give complete hemolysis, control tube No. 4 gives none. With a strongly positive serum as little as $0.1 \times (1/2)^3$ cc. (0.0125 cc.) may still give a positive reaction.

RESULTS WITH LEPERS' SERA

Each of the 82 sera dealt with has been tested with several antigens. The results obtained with 62 of them are shown in Tables 2A, 2B and 2C, and those of the entire 82 are summarized in Table 3.

The first of these (Table 2A) covers the earlier tests, with leprosy-bacillus antigens alone. In the second (Table 2B) two other antigens have been introduced, and the third (Table 2C) shows the reactions with six antigens other than those of the leprosy cultures. In all three the superior sensitiveness of the No. 3 BG antigen is evident. Of twenty other sera, tested with only one bacillary antigen (No. 3 BG), four gave +++, four ++, three +, and two ±, while seven were negative. In Table 3 are summarized all the reactions made.

TABLE 3.—*Summary of results obtained with various antigens in sera from 82 cases of leprosy.*

Antigen	Number tested	Number positive	Per cent positive
No. 2 BG	45	16	35.6
No. 2 CD	45	13	28.9
No. 2 Tuberculosis human	17	6	35.3
No. 2 Rat leprosy	17	0	0.0
No. 3 BG (1:49)	23	20	86.9
No. 3 BG (1:99)	53	41	77.4
No. 3 Tuberculosis human	17	14	82.4
No. 3 Tuberculosis avian	9	3	33.3
No. 3 Rat leprosy	17	13	76.5
No. 3 Timothy hay	17	12	70.6
No. 3 Running water	17	14	82.4
No. 3 Smegma	9	3	33.3
Wassermann reaction	82	13	15.8

It would be of interest could the results be analyzed in relation to the type and duration of the disease, but this would be difficult since the greater part of the sera were obtained from others. Of the last 23 cases tested with the No. 3 BG antigen 11 were nodular and 12 anesthetic (including macular); of the former 10 (90.9 per cent) and of the latter 10 (83.3 per cent) reacted positively.

EXPERIMENTS WITH NON-LEPROUS SERA

For a control we tested 358 sera of non-lepers treated in our clinic. The No. 2 (alcoholic) antigens, both BG and CD, were used with 181 sera, of which 29 were Wassermann positive. None gave positive fixation with either antigen. Antigen No. 3 BG (1 to 49 as well as 1 to 99) was used with 177 sera, of which 27 were Wassermann-

positive. Two of these Wassermann-positive sera gave positive reactions with both dilutions of the bacillary antigen, but the other 25 gave no such reactions, though some were strongly Wassermann-positive. Thus only 7.4 per cent of the Wassermann-positive non-lepers' sera, or 1.1 per cent of the entire group, were positive with this test. This, it may be said, is quite low.

EXPERIMENTS WITH IMMUNE RABBIT SERA

ANTIGENS NOS. 1 AND 2

The systematic study of immunizing animals with *Myco. leprae* and other acid-fast bacilli, and of the complement fixation reaction with their sera, is still in progress and will be reported in due course. Here will be described only the results of preliminary experiments that were made on animal sera before trying the method on lepers' sera.

Immune sera.—(1) A supension of the orange-yellow CD variety (Tanaka strain), 1 loopful to 5 cc., was boiled for 10 minutes. Rabbits were injected intravenously, the dose increasing gradually from 1 to 2 cc. This was done with four animals, six times at intervals of a week. (2) The white BG variety (Sekiguchi strain) was used with four rabbits in the same way, but as two of them died during the treatment sera were gotten from only two. (3) and (4) Suspensions of the same strains, heated at 60°C. for half an hour and not boiled, were used to immunize two other groups of four rabbits each. We made four injections, but several animals died so we only got CD serum from two, and BG serum from one. Blood was obtained from the rabbits by heart puncture, and phenol was added in the concentration of 0.5 per cent.

The tests.—With the first two immune sera antigen No. 1 (saline suspension) was used. With the last two sera the alcoholic No. 2 antigen was used. The set-up was as in Table 1.

Results of tests.—Using the saline antigens, BG serum caused complement fixation only with BG antigen, but not with CD antigen, and CD serum fixed only CD antigen, but not BG antigen. In other words, complement fixation occurred only in the homologous series.

Using the alcoholic antigens with the (3) and (4) sera: (a) CD antigen fixed with CD serum, but not BG serum; (b) on the other hand BG antigen fixed with both BG and CD sera. Neither antigen reacted with normal rabbit's serum, and Kolmer's Wassermann reaction gave no positive reaction with any of the sera.

ANTIGEN NO. 3 (BG STRAIN)

Group A sera.—Three rabbits were immunized with the whole bacterial mass. A heavy suspension (1 loopful of growth from a glycerol agar slant to 1 cc.

of saline) was heated at 70°C. for half an hour and injected intravenously 3 times at weekly intervals, the dose being increased gradually. Blood was taken a week from the day on which the last injection was made.

Group B sera.—Into 10 cc. of salt solution was put 0.1 gm. of the ether extracted bacterial powder of the Bg strain, and this was heated at 70°C. for half an hour. Four rabbits were injected in the same manner as those of Group A.

TABLE 4.—*Results obtained with sera of rabbits immunized with Bg strain.*

Antigen	Amount of serum	Test sera							Control
		A group			B group				10 normal rabbits
		No. 1404	No. 1406	No. 1407	No. 1562	No. 1563	No. 1564	No. 1565	
No. 3 Bg (1:49) ...	0.10	+++	+++	+++	+++	+++	+++	+++	−
	0.05	+++	+++	+++	+++	+++	+++	+++	−
No. 3 Bg (1:99) ...	0.10	+++	+++	+++	+++	+++	+++	+++	−
	0.05	+++	+++	+++	+++	+++	+++	+++	−
No. 1 Bg (1:99) ...	0.10	+	+	+	+++	+++	++	+	−
	0.05	−	−	−	+++	+++	++	+	−
No. 3 Cd (1:99) ...	0.10	−	−	−	−	±	−	+	−
	0.05	−	−	−	−	−	−	−	−
No. 3 Tb. human [a] ..	0.10	−	−	−	++	++	+	+	−
	0.05	−	−	−	++	+	−	+	−
No. 3 Rat leprosy [a].	0.10	−	−	−	+	+	+	−	−
	0.05	−	−	−	+	+	−	−	−
No. 3 Water [a]	0.10	−	−	−	+	+	−	−	−
	0.05	−	−	−	−	−	−	−	−
No. 3 Timothy hay [a]	0.10	−	−	−	+	++	−	+	−
	0.05	−	−	−	±	+	−	+	−

[a] Dilutions 1:99.

Results of tests.—The results obtained are shown in Table 4, the controls being sera taken from the seven test rabbits before immunization, and of three other normal rabbits. The sera of the rabbits immunized with the whole bacterial mass gave positive reactions only with antigens of the homologous strain. On the other hand the sera of rabbits immunized with the ether-extracted bacteria had no such specific property. This being the case we are brought to the very interesting conclusion that the ether-soluble elements have a specific antigenic property.

DISCUSSION

The results of our experiments are so fragmentary that it is difficult to draw definite conclusions from them. From the liter-

ature it appears that not a few investigators have gotten results quite similar to ours, but the conditions are too varied to permit making a satisfactory comparison. The greatest variations have been in the kinds of antigens used, the materials from which they were prepared including: (a) leprous tissues, especially extracts of nodules; (b) cultivated organisms supposed to be the leprosy bacillus; (c) other acid-fast organisms not related to leprosy, especially the tubercle bacillus; (d) non-acid-fast bacteria of various kinds, as the typhoid bacillus, diphtheroids, the meningococcus, etc.; (e) Wassermann-reaction antigens of various kinds, and (f) human tissues, normal and pathological, including heart, liver, skin, carcinoma, sarcoma, etc.

Results with antigens of acid-fast bacteria depend in part upon the method of preparation. However, though many investigators since the early work of Gengou (1906) and of Much (1909) have devoted themselves to the study of antigens made by fractioning these organisms in various ways, the problem of specificity remains unsolved. Those workers and others, including Kritchewsky and Bierger (1913), Harris and Lanford (1913), and Cooke (1919), etc., showed that immunization of an animal with one kind of acid-fast organism results in the production of complement-fixing antibodies not only for the organism used (homologous), but also for other kinds (heterologous). And there is no doubt that this is the condition in the sera of lepers, for judging from the results of various workers—as Wills (1912), Cooke (1919), Aoki and Murao (1933)—it seems impossible to distinguish the different bacteria of this group by reactions with lepers' sera, or with sera from immunized rabbits. This was essentially true of our experiments in which antigens of the No. 3 type were used.

There is, however, another distinctly different problem, namely, to obtain an antigen which will give positive reactions in leprosy and negative in other conditions, including tuberculosis and syphilis. In connection with this question, and of our own investigation of it, the results obtained by Lewis and Aronson are of special interest.

They used alcoholic extracts of growths from glycerol bouillon, collected on filter paper, washed with saline, dried in a vacuum, and extracted with alcohol. Before being used the extracts were heated to boiling and mixed with saline. The antigen from the Clegg bacillus gave 93.2 per cent positive reactions with 41 sera from 39 cases of leprosy, and none with 152 non-lepers' sera. The human tubercle bacillus antigen gave even more positives with the

lepers' sera (97.7 per cent), but at the same time it gave 31.6 per cent positive with control sera. Positive results with the controls were also given by antigens of several other strains of acid-fast bacteria, none of which excelled the Clegg antigen as regards positive results with leper sera.

These results, in part, bear a close similarity to ours. With one of their antigens these authors obtained 93.2 per cent positive results with patients' sera, though their controls cannot be said to be entirely satisfactory. With one of our antigens we have obtained 87 per cent positive results with patients' sera, while of 177 unselected non-leprous controls only 2 gave positive reactions.

SUMMARY

In our experiments certain acid-fast bacteria, whether in alcoholic or salt-solution suspensions of the entire bacterial mass, gave the complement fixation only with lepers' sera, not with control sera. Our best antigen of this type was made from the BG strain obtained from leprous blood, but the percentage of positive results with it was not as high as with other types of antigen. A similar antigen made from the human tubercle bacillus often gave positive reactions with lepers' sera, but not in a high percentage. One of a rat leprosy bacillus culture gave no positive reactions.

An antigen of the BG strain made by extracting the ether-soluble elements and adding a little cholesterin gave a much higher percentage of positive reactions with lepers' sera. However, the sensitiveness of this type of antigen is not limited to those made with the organisms from leprosy; those from other acid-fast bacteria give fairly many positive reactions, though there was more or less disparity among them as regards percentages. With control sera these antigens gave few positive reactions.

Though the BG antigen of this type is the best, those made similarly from the CD strain, the human tubercle bacillus, the timothy hay bacillus, and one isolated from running water have nearly the same value. Those of the rat leprosy bacillus used, and of the avian tubercle and smegma bacilli, are of less value.

Though the antigens named have a strong specific capacity for fixation with lepers' sera, it is on the other hand impossible to distinguish between the various kinds of bacilli by means of lepers' sera.

CONCLUSIONS

1. There are two quite different requirements with respect to serological work with acid-fast bacilli in leprosy, and these have differences as regards likelihood of fulfillment. They are (a) to make antigens for the discrimination between different kinds of the acid-fast bacteria, and (b) to obtain a high-value antigen for the diagnosis of leprosy.

2. While at present it is impossible to distinguish between the different kinds of acid-fast bacteria because of heterologous reactions, it may nevertheless be possible to subdivide them to a certain extent into groups according to the intensity of the reactions. It may be expected that the isolation of the chemical elements of the bacteria concerned will ultimately permit making finer distinctions. Our experience indicates that for this purpose the ether-soluble elements are most concerned in specificity.

3. There would seem to be more promise of making antigens which will give high percentages of positive reactions in leprosy but not in other conditions. Such antigens can be obtained from certain acid-fast bacteria, especially our Bg strain, from which the ether-soluble elements have been removed. It is most probable that good antigens will be had by adding some suitable substance to such preparations as these.

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