

## THE COMPLEMENT FIXATION TEST (GAY'S MODIFICATION OF THE BESREDKA METHOD) IN THE DIFFERENTIATION OF ACIDFAST BACILLI.\*†

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The great difficulty of substantiating any culture or culture-type obtained from the leprous nodule as the Hansen bacillus has necessitated the employment of the various tests and procedures in an endeavor to find a means to serve this purpose. Among these many efforts were included various serological methods. About 5,000 agglutination tests, utilizing the sera from 20 different cases of human leprosy and the sera of rabbits injected intravenously and subcutaneously with different acidfast bacilli, were performed. As the organisms used formed the more or less representative members of the acidfast group of both the saprophytic and pathogenic varieties, these studies apply to a large extent to this species. The cultures employed were the avian type of *B. tuberculosis*, the organisms considered as *B. lepra* by Kedrowski, Levy, Clegg, Karlinski, Duval, and the butter bacillus of Rabinowitsch, grass, dung, and the Smegma bacilli of Moeller; Korn I., *B. Phlei* and Grassburger's milk bacillus. The sera of these animals were also employed for the Bordet-Gengou reaction using most of the above enumerated organisms as antigens for homologous and cross-reactions. The results of these tests indicated that they were of little value, serving only as a part aid in differentiating certain groups but not affording reactions sufficiently clear-cut to serve for the identification of any culture as *B. lepra*. The fact that the serum tests were of no avail in the human cases when employing any of the various so-called lepra cultures did not, however, invalidate any of these strains as the Hansen bacillus, since bacillary suspensions prepared directly from the tissue nodules rich in bacilli

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yielded similar results. It was hoped that by the repeated injection of heavy suspensions into rabbits a serum of sufficiently high titre to serve for differentiation purposes might be produced and altho immune bodies of a fairly strong potency were stimulated for both the agglutination and complement binding tests, not only did these fail for definite specificity but the close analogy of the readings was striking. The results of the complement fixation test with these sera are shown in Table 1.

TABLE 1.

ANTIGENS	SERA					
	Non-chrome Duval	Chromogen Clegg	Chromogen Duval	Chromogen Currie	Chromogen Bayon	T. Hay
Non-chromogen Duval . . .	+++	—	+++	—	++	+
Chromogen Clegg . . . . .	++	++	++	+	+	++
Chromogen Duval . . . . .	++	++	++	+	+	++
Chromogen Currie . . . . .	++	++	++	+	+	++
Chromogen Bayon . . . . .	++	++	+++	+	+++	++
Timothy Hay . . . . .	++	+	+	+	+	+

Reagents used: Immune serum, 0.15 c.c.; complement, 0.5 c.c. of 10 per cent dilution of fresh guinea-pig serum; antigen, 0.5 c.c. of suspension of living bacilli (about 5,000,000,000 per c.c.); hemolytic system, 0.5 c.c. of 5 per cent suspension of sheep red blood cells and 2 M.H.D. of specific hemolysin.

Controls: Sera. No hemolytic nor anticomplementary property when using double quantities; antigens.

The inference from this work was that the antigenic substances used for immunization were so similar in composition that they stimulated antibodies too closely related to serve for definite differentiations. We regarded the injected substances to have acted as antigenic agents in a manner quite similar to egg albumin, milk, fibrinogen solution and the like. Thinking it probable that, while the biochemistry was quite similar in nature, their endotoxin content might present a sufficiently wide range of variance to be of differential value, we accordingly resorted to the Besredka method as modified by Gay.<sup>1</sup>

*Manner of procedure.*—The cultures were grown upon large whiskey flasks containing slanted glycerin agar. After a period of several weeks when the cultures had become quite luxuriant the

<sup>1</sup> Cited by Edith Claypole as a personal communication, *Jour. Exp. Med.*, 1913, 17, p. 108.

TABLE

ANTIGENS	SERA																																
	Smegma				Kedrowski				Non-Chrome. Duval					Node of Leper					Timothy Hay					Avian Tuberculosis					Chrome. Duval				
	1/40	1/80	1/160	1/320	1/40	1/80	1/160	1/320	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400
Chrome. Duval.	+++	++	+	-	+++	++	+	-	+	-	-	-	-	+++	++	+	-	-	+++	++	+	-	-	+++	++	+	-	-	+++	++	+	+++	++
Clegg	+++	+++	++	+	+++	+++	++	+	+++	+++	+	-	-	+++	+++	+	-	-	+++	+++	+	-	-	+++	+++	++	+	-	+++	+++	++	+	-
Kedrowski	+++	+++	++	+	+++	+++	++	+	+++	+++	+	-	-	+++	+++	+	-	-	+++	+++	+	-	-	+++	+++	++	+	-	+++	+++	++	+	-
Smegma	+++	+++	++	-	+++	+++	++	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++	-	-
Butter	+++	++	+	-	+	-	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-
Dung	+++	+	+	-	+	-	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-	++	+	-	-	-
Avian T.B.	+++	+++	+	-	+	-	-	-	+++	++	-	-	-	+++	++	-	-	-	+++	++	-	-	-	+++	++	-	-	-	+++	++	-	-	-
Timothy Hay	-	-	-	-	-	-	-	-	++	++	-	-	-	++	++	-	-	-	++	++	-	-	-	++	++	-	-	-	++	++	-	-	-

Reagents used: Immune serum, 0.15 c.c. antigen, 0.5 c.c. of the above dilutions of the 2 per cent stock; complement, 0.5 c.c. of 10 per cent dilution of fresh guinea-pig serum; hemolytic system, 0.5 c.c. of 5 per cent suspension of sheep red blood cells and 2 M.H.D. of specific hemolysin.

Controls: Sera. No hemolytic nor anticomplementary property in double quantities; antigens showed only slight anticomplementary property when double quantities of 1-40 dilution were employed.

growths were scraped from the surface and mixed with saline solution. To these emulsions was added an equal part of absolute ethylic alcohol and after prolonged centrifugalization the supernatant fluid was decanted and the precipitate used. This was desiccated in Novy jars with a partial vacuum over sulfuric acid, and after complete drying they were then weighed and a sufficient amount of sodium chlorid added to constitute 0.85 per cent of the solution when completed. The dried precipitate was ground in combination with the salt in a sterile mortar until an impalpable powder was produced and sufficient sterile water was added to form a 2 per cent solution of the pulverized bacilli. These emulsions were used both for immunizing purposes and to serve as antigens in the tests.

The sera of the rabbits injected intravenously with heavy suspensions of the whole bacilli were tested with the antigens prepared according to the new method and the results are given in Table 2. It must be noted in this table that the rabbits receiving the non-chromogenic strain of Duval and the nodule of a leper were of necessity given injections of much lighter suspensions.

For immunizing with the Besredka antigens two procedures were followed both of which were according to Gay and Fitzgerald.<sup>1</sup> In the first instance, four rabbits were injected intravenously with the following antigen: *B. lepra* (Kedrowski), *B. Smegma*, chromogenic culture (Duval) and the bacillus of timothy hay. They were given three intravenous injections of 0.5 c.c. at 24-hour intervals and were bled the fourth day after the last injection. The results of the sera when tested against the various antigens prepared are tabulated in Table 3.

The second series of rabbits were injected intravenously with the same four acidfast organisms as the previous animals but were given four injections of 0.5 c.c. at three-day intervals and were bled eight days after the last injection. These sera were likewise tested against the antigens and the results are recorded in Table 4.

It will be noted that the anti-substances produced by the three different methods employed show that the whole bacilli emulsion yielded a lower titre than those injected with the Besredka antigen

TABLE 3.

ANTIGENS	SERA															
	Chrome. Duval				Timothy Hay				Smerma				Kedrowski			
	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/40	1/80	1/160	1/320	1/400	1/400
Chrome. Duval.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Clegg.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Kedrowski.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Smerma.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Butter.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Dung.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Avian. T.B.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Timothy Hay.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

Reagents used: Immune serum, 0.15 c.c.; antigen, 0.5 c.c. of the above dilutions of the 2 per cent stock; complement, 0.5 c.c. of 10 per cent dilution of fresh guinea-pig serum; hemolytic system, 0.5 c.c. of 5 per cent suspension of sheep red blood cells and 2 M.H.D. of specific hemolysin.

Controls: Sera. No hemolytic nor anticomplementary property in double quantities; antigens showed only slight anticomplementary property when double quantities of 1-40 dilution were employed.

TABLE 4.

ANTIGENS	SERA															
	Chrome, Duval				Timothy Hay				Smegma				Kedrowski			
	1/160	1/320	1/400	1/800	1/160	1/320	1/400	1/800	1/160	1/320	1/400	1/800	1/160	1/320	1/400	1/800
Chrome, Duval.....	++	++	+	-	++	++	++	++	++	++	++	++	++	++	++	++
Clegg.....	++	++	++	-	++	++	++	-	++	++	++	++	++	++	++	++
Kedrowski.....	++	++	++	-	++	++	++	-	++	++	++	++	++	++	++	++
Smegma.....	++	++	++	-	++	++	++	-	++	++	++	++	++	++	++	++
Butter.....	++	++	++	-	++	++	++	-	++	++	++	++	++	++	++	++
Dung.....	++	++	++	-	++	++	++	-	++	++	++	++	++	++	++	++
Avian T.B.....	++	++	++	-	++	++	++	-	++	++	++	++	++	++	++	++
Timothy Hay.....	++	++	++	-	++	++	++	-	++	++	++	++	++	++	++	++

Reagents used: Immune serum, 0.15 c.c.; antigen, 0.5 c.c. of the above dilutions of the 2 per cent stock; complement, 0.5 c.c. of 10 per cent dilution of fresh guinea-pig serum; hemolytic system, 0.5 c.c. of 5 per cent suspension of sheep red blood cells and 2 M.H.D. of specific hemolysin.

Controls: Sera. No hemolytic nor anticomplementary property in double quantities; antigens showed only slight anticomplementary property when double quantities of 1-40 dilution were employed.

at 24-hour intervals and that the animals immunized by injections of the latter at three-day intervals and bled after eight days furnish the highest titre of antibodies. The amboceptors in all instances, however, varied only in their degree of titre and showed no specificity either qualitatively or quantitatively. It is not uncommon that the homologous sera and antigen may bind complement at a less high dilution than with a heterogenous antigen.

It would seem that the acidfast organisms as a species may be considered either through their biochemical relationship or through their biological products or both to produce sensibilizers so closely related that little can be expected from this method of procedure for differentiation purposes. Whether from a defense standpoint the bodies produced by the inoculation of either feeble pathogens or saprophytic acidfasts may be of avail in therapeutic lines continues a much discussed problem. Our results have been found quite in accord with those of Gengou,<sup>1</sup> who used likewise a pulverized bacillary antigen prepared by means of dissolving the fat with a potassium hydroxid solution. He employed the following organisms: the homogeneous tubercle bacillus of Arloing, the cold-blooded types of tubercle bacilli of the fish and the blind eel, the acidfast bacillus of horse dung, the butter bacillus (strain, Rabinowitsch-Lille), also the acidfast bacillus of Tobler I, II, V, and the Korn I type and the timothy hay bacillus. Gengou found that these acidfast bacilli, whether of the saprophytic or parasitic varieties, produced, when injected into guinea-pigs, sensibilizers that were active not only for the homologous organism but also for the other members of the group tested. He included in his tests the human, avian, and bovine tubercle bacilli as antigens. His work showed only three exceptions: Tobler I serum did not bind complement in the presence of Tobler V antigen; Tobler II bacilli produced no amboceptor against the fish tuberculosis and Tobler V did not produce any amboceptor against the avian tubercle bacillus. He considered these exceptions of no value and as likely to occur for some of the other organisms used. Koch<sup>2</sup> found that the serum of animals which had received injections of attenuated

*Berl. klin. Wchnschr.*, 1906, 43, p. 1531.

<sup>2</sup> Klebs on *Tuberculosis*, 1909, p. 20.

tubercle bacilli contained antibodies common to the bacilli of avian and fish tuberculosis, the hay and the butter bacilli; and conversely, animals treated with the pseudo-tubercle bacilli yielded anti-substances which were common to the true tubercle bacilli. Much,<sup>1</sup> Wills,<sup>2</sup> and others have likewise found strong relationships of the various members of the acidfast group with the employment of the serum reactions. Claypole<sup>3</sup> in her work on the classification of streptothrices included the *B. tuberculosis* (human) and *B. lepra* (chromogenic strain of Duval). She has found no apparent specificity in these instances; for example, the animals immunized against the tubercle bacillus show no binding with 1 to 80 dilution of *B. tuberculosis* antigen whereas partial fixation did occur with *B. lepra* antigen, again a partial fixation at 1 to 40 for *B. tuberculosis* and a complete fixation 1 to 40 for *B. lepra*. Kraus and Hofer<sup>4</sup> of Vienna, who employed the Pfeiffer phenomena for members of the acidfast group, is of the opinion that specific lysins may be produced if the tests are thus carried on *in vivo*. He feels that he has demonstrated that the chromogenic culture of Duval when injected into guinea-pigs produced a specific lytic substance for the same organism when later injected into the peritoneal cavity, whereas when injecting the lepra culture described by Kedrowski into the Duval animal, such a phenomenon did not occur. He has used this method in an attempt to differentiate the various tubercle bacilli types.

Much,<sup>5</sup> Deilman,<sup>6</sup> and others have claimed for this acidfast group the production of three corresponding antibodies: a protein, fatty acids, and a neutral fat (tuberculo-nastin) with which last the most intense fixation occurs. Fitzgerald and Leathes,<sup>7</sup> however, have shown that pure lipoidal substances are incapable of producing antibodies. Claypole<sup>8</sup> has suggested that nastin is an impure substance and contains the protein that is characteristic of the acid-fast organisms producing fixation with their sera.

<sup>1</sup> *Fortschr. d. Med.*, 1912, 30, pp. 161 and 201.

<sup>2</sup> *Jour. Exper. Med.*, 1913, 17, p. 99.

<sup>3</sup> *Centralbl. f. Bakteriol., Orig.*, 1911, 61, p. 37.

<sup>4</sup> *Wien. klin. Wchnschr.*, 1912, 25, p. 6.

<sup>5</sup> *Op. cit.*, and *Beitr. z. Klin. d. Tuberk.*, 1910, 17, p. 199.

<sup>6</sup> *Ztschr. f. Immunitätsf.*, 1911, 10, p. 421.

<sup>7</sup> *University of California Publications in Pathology*, 1912, 2, p. 39.

<sup>8</sup> *Op. cit.*



Whatever may be the facts finally arrived at as to the nature of the anti-substances produced, the results in general of all workers with the complement fixation test in the acidfast group have corresponded, whether the whole bacilli or the various specially prepared antigens were used for immunizing and fixation purposes. The fact that the test serves to differentiate many closely related organisms other than those of the acidfast group would indicate that the acid-resisting bacilli, because of either their chemical analogy or the strong relationship of their intrinsic biological characteristics or both, impede the production of specific amboceptors. It is possible that further refinement of the antigenic preparations with a more detailed chemical analysis of these organisms may permit of specificity production.

#### CONCLUSIONS.

1. Rabbits injected with whole bacilli or extracts of many of the members of the acidfast group produce anti-substances of a high titre.
2. The whole bacilli produce antibodies of lower potency than that produced by the Besredka antigen. This antigen produces the most potent antibodies when injected intravenously at three-day intervals for four injections and the animal bled after eight days.
3. Regardless of the various methods used to produce these sensibilizers, no clear-cut specificity for complement fixation has been found for the acidfast bacilli made use of in these experiments.