

STUDY OF THE CLASSIFICATION OF MENINGOCOCCI.*

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Work on the classification of meningococci was started in January, 1918, in order to determine whether it was possible to classify by their agglutination and antigenic properties a sufficient number of strains among the cultures at our disposal, so that satisfactory representative cultures could be selected for standardizing serums or immunizing horses. At that time 53 meningococcus cultures were being used in these laboratories in immunizing horses for the production of antimeningococcus serum and for the standardization of the serum by agglutination tests. By the methods of immunization then in use no attention was paid to serological differences, except that the strains of meningococci which showed the least agglutination in the serum of the horses under immunization were injected more frequently than the others. The objection to this procedure is evident, for antibodies may be produced for the strains which show very little agglutination, to the detriment of antibody production for the strains which are more agglutinable. It was thought that a better balance might be obtained by injecting representative cultures in equal amounts, if it was found that a few cultures representative of all or nearly all meningococci could be selected.

The principal methods used to differentiate meningococci into groups have been the agglutination test and the absorption of agglutinin test. Complement fixation and virulence tests have been of questionable value owing to difficulty of technique and to the slight or variable pathogenicity of the meningococcus for small laboratory animals.

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Technique for the Agglutination Tests.—The meningococcus stock cultures were kept on serum dextrose agar or semisolid ascitic fluid agar. Transfers for agglutination tests were made on dextrose serum agar slants and incubated at 37°C. for 18 hours. Suspensions were made up with 0.85 per cent saline solution to a density equal to that of barium sulfate Standard No. 3, which approximates 2,000 million cocci per cc. The serum dilutions were made up with 0.85 per cent saline solution. The agglutination tests were set up in small racks in 3 by $\frac{3}{8}$ inch tubes, equal parts (0.2 cc. each) of serum dilution and culture suspension being used for each tube. Incubation was at 55°C. for 24 hours. The tubes were shaken 3 hours before reading, as it had been found that shaking increased and made more uniform the agglutination titer of the serum.

In some of the tests the cultures were grown on plain dextrose agar for 18 hours at 37°C., the growth was suspended in 0.85 per cent saline solution, the suspensions were heated at 65°C. for 30 minutes, and 0.5 per cent phenol was added. They were then diluted to the required density.

Technique for the Absorption Tests.—For the absorption tests a method, based largely upon the observations of Tulloch¹ and of Gordon,² was devised and tested by suspending in a serum its homologous culture under different conditions. Two densities of culture suspension, both heated and unheated, and two temperatures of incubation were used. The results are shown in Table I. Satisfactory absorption of the agglutinins took place only when the heated, dense suspensions of culture, incubated at 55°C., were used.

A dense suspension containing about 30,000 million cocci per cc. was secured by suspending in 0.85 per cent saline solution the 18 hour growth on dextrose agar, which had been incubated at 37°C., then heating to 65°C. for $\frac{1}{2}$ hour, and adding 0.5 per cent phenol.

¹Tulloch, W. J., On the differentiation by means of the absorption of agglutinin test for types of meningococci (etc.), *J. Roy. Army Med. Corps*, 1917, xxix, 66.

²Gordon, M. H., Bacteriological studies in the pathology and preventive control of cerebro-spinal fever among the forces during 1915 and 1916. I.—The definition of the meningococcus, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 3*, 1917, 10.

Equal parts of the serum, diluted to 1:25 with saline solution, and the suspension of cocci to be tested were thoroughly mixed and incubated at 55°C. for 2 hours and then placed in the ice box at 5–8°C. The next morning the tubes were centrifugalized at high speed for $\frac{1}{2}$ hour, or longer if necessary, to throw down all the bacteria, and the superna-

TABLE I.

Degrees of Absorption with Heated and Unheated Suspension, Incubated at 37° and 55°C., and a Thin and a Dense Suspension.

A Type II serum was saturated with its homologous culture under varying conditions, and after incubation and centrifugalization the supernatant fluid was decanted and tested in a series of dilutions against its homologous culture according to the standard method.

Suspension.	No. of cocci per cc.	Temperature at which culture was heated for $\frac{1}{4}$ hr.	Temperature at which culture was incubated with serum for 2 hrs.	Agglutination of supernatant fluid with homologous culture, Type II.
	<i>millions</i>	°C.	°C.	
A	5,000	65	55	1:400
AA	5,000	65	37	1:400
B	30,000	65	55	0
BB	30,000	65	37	1:100
C	5,000		55	1:600
CC	5,000		37	1:600
D	30,000		55	1:300
DD	30,000		37	1:600

tant fluid was decanted and tested against the homologous culture of the serum. A sample of the same serum was incubated, centrifugalized, and tested in the same dilutions as the saturated serum.

The monovalent type serums were produced in rabbits according to the method suggested by Amoss.³

RESULTS.

Standard suspensions and serums of the four types of Gordon were sent to this laboratory by the British Medical Research Committee. Living cultures of the four types of meningococci were sent

³ Amoss, H. L., quoted by Flexner, S., Mode of infection, means of prevention and specific treatment of epidemic meningitis, *J. Am. Med. Assn.*, 1917, lxi, 639.

to us by Dr. W. H. Park, of the Department of Health of the City of New York, who had received them from Colonel Gordon. Tests were made of the standard suspensions with the standard serums and with our monovalent normal, para, and irregular serums; also the standard serums were tested with our stock cultures. The results of the tests were not altogether clear-cut and satisfactory because the standard serums and suspensions were not tested until after the expiration date recorded on them. It was very clearly indicated, however, that Types I, II, and III of Gordon corresponded respectively to the normal, para, and irregular types. The same relation was indicated by a study of the living cultures of Types I, II, and III of Gordon.

We were unable to designate any of the cultures of our series as belonging to Type IV of Gordon. The Type IV standard suspension showed only a trace of agglutination in the homologous serum, but when tested with the normal, para, and irregular serums, it was agglutinated in a 1:100 dilution by the para type serum, and in a 1:300 dilution by the irregular type serum. These reactions, together with those of the Type IV living culture from Dr. Park, seem to indicate that Type IV may not be a separate group but possibly a poorly agglutinating strain belonging to Type II or III.

Agglutination tests were made with 53 stock cultures in the monovalent rabbit serums produced against the selected strains of normal, para, and irregular meningococci. 43 of the 53 cultures were classified into three distinct groups: six as Type I, thirteen as Type II, and twenty-four as Type III. One culture was agglutinated in Serums I and III, two groups which are said to be related. Nine cultures could not be classified into these groups because they did not agglutinate in sufficiently high dilutions of the four monovalent type serums which were used to classify the other cultures. The type serums employed comprised one of Type I, two of Type II, and one of Type III. Some of the unclassified cultures might conceivably be cultures which were not readily agglutinated. Others might only have failed to react with the particular monovalent serum with which they were tested. With monovalent serums produced with other representatives of the groups they might have been agglutinated.

The three types, Nos. I, II, and III, the recognition of which was based upon the agglutination test, seemed to be distinct when the absorption test was used, in that Type I culture absorbed the specific agglutinins from Type I serum, and did not absorb them from Type II or Type III serum; likewise Type II and Type III cultures absorbed the agglutinins from their respective homologous serums and not from the other types.

Stability of the Serological Types of Meningococci.

The constancy of the reactions of meningococcus strains has at times been questioned; that is, whether a culture at one time gives reactions that would indicate its classification in one group and at a later time its classification in another group. The reactions with at least some strains may be constant for considerable periods of time, for tests at weekly intervals with six strains under cultivation on the standard media used for all strains for 2 years have shown no change so far as the agglutination reaction is concerned.

Variations in Reaction of Four Type II Cultures and One Type IV.

A study was made of the variations in reaction among the cultures of our series. We record here the differences found in some of the Type II meningococcus cultures, and in the culture which we received as a Gordon Type IV.

Tulloch⁴ and others have noted that the strains of Type II differ in their agglutination reactions, and Dopter and Pauron⁵ suggest that their group of parameningococci which corresponds to the Type II of Gordon might be divided into three subgroups. Both French and British workers have noted the relation between Types II and IV of Gordon.

Monovalent serums obtained from rabbits immunized against Type II Cultures 1 and 4 agglutinated Type II Cultures 1, 4, 46, and G2 (the Gordon Type II culture received from Dr. Park). Monovalent

⁴ Tulloch, W. J., Agglutination and absorption of agglutinin reaction, *J. Roy. Army Med. Corps*, 1918, xxx, 115.

⁵ Dopter and Pauron, Différenciation des paraméningocoques entre eux par la saturation des agglutinins, *Compt. rend. Soc. biol.*, 1914, lxxvii, 231.

serums obtained by immunization with Type II Cultures 46 and G2 agglutinated in high dilution Type II Cultures 46 and G2, but did not agglutinate Type II Cultures 1 and 4 except in low dilution.

Type II Cultures 46 and G2 absorbed the agglutinins from serum obtained by immunization with Type II Culture 4, and these cultures also absorbed the Type II agglutinins from serum obtained by immunization with Cultures 46 and G2. It is evident that the agglutination reaction alone failed to bring out the relation of these cultures, but that in absorption tests they reacted similarly. It is nevertheless evident from the agglutination reaction that Cultures 1 and 4 might be said to represent one subgroup and Cultures 46 and G2 another.

No cultures were secured which typified Gordon's Type IV save the culture which came originally from Gordon and which we designated as G4. A monovalent serum obtained from a rabbit immunized with this culture agglutinated in high dilution Type II Cultures 1, 4, 46, and G2, but did not agglutinate its homologous culture, No. G4, except in low dilution. It is, therefore, a culture which is not readily agglutinated but seems to be active as an antigen. Culture G4 did not absorb the agglutinins for Type II cultures from the serum of a rabbit immunized with Type II Culture 4, but absorbed these agglutinins from serums obtained by immunization with Nos. 46 and G2. Although not readily agglutinable, the culture was apparently active as an antigen in the development of Type II agglutinins, and was capable of absorbing agglutinins for some, but not all Type II cultures from the homologous serum. Although a relation between Culture G4 and the second group of Gordon was thus indicated, it was the only culture of our series which was sufficiently definite in its reactions to suggest the fourth group of Gordon.⁶

It seems possible that Type II is a complex group and that its members cannot be properly differentiated by the agglutination reaction, although strains which react typically may be classified in it.

These irregular reactions of agglutination and absorption suggested that the agglutination reaction is not a satisfactory basis for a final

⁶ Further study is now being made of the variations in agglutination and absorption of the cultures which have been reported to comprise the fourth group of Gordon.

classification, although it might be of value in differentiating groups so that representative strains could be selected for certain purposes such as the immunization of animals to obtain polyvalent serums. That the selection of the representative strains based upon the results of this study was of value in producing a polyvalent serum in horses is well shown by the fact that such serum agglutinated all cultures of our series in high dilution.

CONCLUSION.

The meningococcus, like some other pathogenic species, varies in its agglutination in immune serum, some strains being readily agglutinable while others agglutinate with difficulty in their homologous serum as well as in heterologous serums. The different strains appear to vary also in their action as antigens. In order to secure representative strains, therefore, it was thought necessary to consider the antigenic action as well as the agglutinability of the cultures.