

SIMPLIFIED PRODUCTION OF ANTIMENINGOCOCCIC SERUM.

By HAROLD L. AMOSS, M.D., FREDERICK L. GATES, M.D., AND PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 10, 1920.)

Although a large amount of work in several countries has been done relative to the production of an effective antimeningococcic serum, the problems involved have not yet been wholly resolved. The increased demands arising from war conditions acted as a potent stimulus first to simplify manufacture and then to insure an effective product. This paper records primarily attempts to simplify the manufacture of an efficacious serum and deals incidentally with a number of still mooted questions regarding the antigenic properties of the meningococcus on which the production of such a serum largely rests.

It is now generally recognized that the meningococcus is not a simple, fixed antigenic entity but rather that the term meningococcus covers a class of closely related microorganisms, the distinguishing common characters of which relate to certain cultural and fermentative qualities and the power to set up in man particular forms of inflammation of the leptomeninges, while they differ markedly in their immunologic responses. Thus for identification the cultural properties are of first importance and for serum production the antigenic structure is paramount.

Ever since Dopter¹ first distinguished the immunologically distinct parameningococcus the classification of the meningococci has been under discussion and agreement has not yet been reached. Everyone admits the existence of two main groups called normal or regular meningococcus and parameningococcus; the disturbing factor is the

¹ Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxxvii, 74. Dopter and Pauron, *Compt. rend. Soc. biol.*, 1914, lxxvii, 231.

occurrence of intermediate cocci which resemble either the regular meningococcus or the parameningococcus but are immunologically less sharply defined than are the pure types of the main groups. This fact has led to the setting up of main and subsidiary types as, for example, in Gordon's classification which recognizes four types of meningococci.² But Gordon's classification has not received general acceptance and for the reason that observers cannot agree on the two subsidiary type strains.

These considerations have far more than theoretical interest, since experience has shown that a therapeutically effective antimeningococcic serum should possess wide capacities of immunologic activity as measured by the agglutinin content. Since the early work on the subject by Flexner and Jobling³ and the later studies of Amoss and Wollstein,⁴ it has been the custom to employ for the immunization of horses a large number of cultures including representatives of the regular, the para, and many intermediate strains of the meningococcus. The number of strains used in the antigen might reach 50 or more, depending upon the reaction of the serum with meningococci derived from the cerebrospinal fluid of many cases of epidemic meningitis. When the test with such a culture showed low agglutination titer the strain was added to those used for immunization. The purpose of this was to produce a serum with as wide an agglutination index as practicable.

It is obvious that this procedure implies an empirical method. In endeavoring to simplify the manufacture of antimeningococcic serum it seemed worth while to determine more precisely than had yet been done whether a wide agglutination index could be obtained from a small number of antigenically different strains. Several facts had already rendered it probable that something in this direction was achievable. For example, it had been noted that the immunity response in the horse was wider than in the rabbit and also that the longer the injections of certain fixed cultures were continued, the more inclusive the agglutinin content became. In addition to these essential data there

² Gordon, M. H., *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 3*, 1917, 10.

³ Flexner, S., and Jobling, J. W., *J. Exp. Med.*, 1908, x, 141.

⁴ Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

was a still further important fact; namely, that approximately 80 per cent of all cases of epidemic meningitis arose from infection with the two type strains; that is, the regular meningococcus and the parameningococcus. Hence the production of an effective serum for this large proportion of cases of meningitis appeared to be relatively simple; the real problem was to make the serum effective against the 20 per cent of cases due to the highly variable subsidiary strains.

Fortunately, we possessed as a standard of comparison for the sera to be produced with a limited number of strains, samples of polyvalent sera, of established therapeutic efficacy, which had been produced at The Rockefeller Institute by the injection of 51 spinal cultures of the meningococcus selected in accordance with the method outlined above.⁵ These 51 cultures were classified as follows: 10 were regular, 26 were para, and 15 were intermediate (subsidiary) strains of meningococcus.⁶

Horse Sera Produced with a Small Number of Strains.

When the experiments with horses with a smaller number of cultures of the meningococcus were begun in 1917 we had already observed that rabbits which were immunized with a single type strain over long periods of time yielded sera which contained agglutinins not only for that strain but also, in less amount, for heterologous strains including even those of the opposite type.

Use of Five Strains as Antigen.—The experiments described below were made on two horses (Nos. M 24 and M 25). Injections were begun with four cultures which were classed immunologically as

⁵ The polyvalent serum issued for therapeutic purposes was composed of serum from at least three horses. Because of the variation in the response of different horses, it has been our practice to pool serum from several horses to insure a properly balanced product.

⁶ In 1914 two strains of parameningococcus were brought from Dopter by a member of the staff of The Rockefeller Institute. It was on the basis of these strains that our classification was made. Recent comparison of our stock strains with sera and cultures lately obtained from England and France shows that our original classification is the reverse of the present accepted grouping. Conforming to general usage, we have revised our classification in this paper, so that the regular or normal group mentioned in previous papers from these laboratories is here designated as para and *vice versa*.

follows: Culture 1, para type strain; Culture 60, regular type strain; and Cultures 30 and 31 of different intermediate types. Serum obtained in early bleedings of these horses did not agglutinate Culture 38 (also an intermediate) which was then added to the antigen.

Experiment 1.—The method of immunization was as follows: After a glanders test and the subcutaneous injection of 1,500 units of antitetanic serum, Horses M 24 and M 25 were tested for sensitiveness to the meningococcus by the intravenous injection of minute doses (0.05 cc.) of a saline suspension (2.5 cc. to an agar slant culture). The immunizing injections were begun on November 29, 1917, living cultures being given in a total dose of 0.12 cc., and were continued according to the method described by Amoss and Wollstein.⁴ Trial bleedings were made with Horse M 24 after 3, 10, and 14 months, and with Horse M 25 after 2, 10, and 14 months.

TABLE I.

Number of Stock Strains Agglutinated by the Serum of Horses M 24 and M 25 Immunized with Five Strains of Meningococcus.

Horse No.	Duration of immunization. <i>mos.</i>	Agglutination test positive.	
		1:400 or higher.	1:100
M 24	3	35 of 51	50 of 51
" 24	10	43 " 60	59 " 60
" 24	14	51 " 56	56 " 56
" 25	2	35 " 58	40 " 58
" 25	10	32 " 60	58 " 60
" 25	14	47 " 56	56 " 56

In Table I the progress of the immunization is summarized according to the number of stock strains (cultures) which were agglutinated in a serum dilution of 1:100 and of 1:400 or higher. 51 of these stock strains were those used in the manufacture of the therapeutic polyvalent serum.

Table I indicates that by employing for purposes of immunization as few as five cultures, representing different strains, of the meningococcus, a serum is produced which shows considerable agglutinative capacity for as many as 51 selected stock cultures, including the

English type strains.⁷ The titer of the serum for this large number of cultures equalled that previously required for a polyvalent serum prepared with a far larger number of cultures.

Use of Three Strains as Antigen.—In this experiment the strains were reduced to three, representing regular and para types.

Experiment 2.—The procedure with Horse M 31 was similar to that already described. The injections were begun on May 1, 1918, and trial bleedings were made after 5 and 9 months of immunization. Two regular cultures (Strains 60 and 79) and one para culture (Strain 85) were employed for the injection.

Table II summarizes the results obtained, which are the equivalent, for the same period of injection, of those obtained with the five strains as shown by Table I. Although thirteen of the stock cultures did not agglutinate in the 1:400 dilution after 9 months of immunization all were agglutinated in dilutions ranging from 1:50 to 1:200.

TABLE II.

Number of Stock Strains Agglutinated by the Serum of Horse M 31 Immunized with Three Strains of Meningococcus.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
<i>mos.</i>		
5	32 of 50	47 of 50
9	43 " 56	55 " 56

Monovalent Horse Sera.

The total amount of meningococcic antigen which can be given a horse varies and is obviously limited. The use, therefore, of a single antigen might result in a greater antibody content for the homologous strain than could be obtained against the individual strains of a multiple antigen. Indeed, certain therapeutic monovalent sera have been prepared in this way by the Pasteur Institute and more recently by Gordon and others in England working under the Medical

⁷ The English type strains were kindly supplied by Dr. Gordon.

Research Council.⁸ The intent in both these instances was to fortify the therapeutic activity of the serum for treating cases of epidemic meningitis with type antisera after the type of infecting meningococcus had been determined.

It is not our intention in this place to discuss the practicability of this method of procedure or even to analyze the results thus far secured by the English investigators mentioned above. We would remark, however, that we are dubious as to the advisability of making a substitution of a monoserum for an active polyserum in view of the uncertainties and lack of uniformity which still surround the practical work of determining and distinguishing the so called types of meningococci. But as the data which follow show, the antigenic constitution of even type meningococci is such that a strictly monovalent serum is not produced when horses are injected with a type culture over a long period of time.

The experiments on monovalent sera were made in two ways: first, by injecting as many as three cultures of the same type of meningococcus, and second, by injecting a single type culture only.

Experiment 3.—Horse M 30, after the usual preliminary treatment, was first injected with suspensions of living parameningococci (Cultures 1, 4, and 36). After 6 and 10 months of immunization trial bleedings were made. The serum thus obtained was titrated against 56 stock cultures of meningococci with the results shown in Table III.

TABLE III.

Number of Stock Strains Agglutinated by the Serum of Horse M 30 Immunized with Three Strains of Parameningococcus.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
<i>mos.</i>		
6	40 of 52	50 of 52
10	56 " 56	56 " 56

The results were striking and unexpected, and yet they confirmed certain tests which we had previously made on the variations in

⁸ Hine, T. G. M., *Privy Council, Med. Research Council, Special Rep. Series. No. 50, 1920, 176.*

agglutinogenic activity exhibited by different strains of the meningococcus. It appears that in this respect separate strains of the meningococcus show a wide variation. It has been noted also that horses vary in their response (*cf.* Nos. M 24 and M 25, Table I).

The next step was to determine the effect of immunizing horses with single type strains of the regular meningococcus and the parameningococcus. For this purpose two horses were employed.

Experiment 4.—Horse M 32 was injected with a para culture (No. 1) and Horse M 33 with a regular culture (No. 60). The immunization was begun on December 5, 1918, and test bleedings were made 3 months later at a period still too early to show the final wide range of agglutinative capacities of the sera. However, the serum of Horse M 32 (para) agglutinated in dilutions of 1:400 or higher 52 of the 56 stock cultures, and the serum of Horse M 33 (regular) agglutinated in the 1:400 dilution or higher 51 of the 56 stock cultures.

These results raise certain very pertinent questions which cannot be answered offhand. We have dealt with certain aspects of these questions in a later section of this paper in connection with the changes which take place in the sera during storage and the response of the sera produced in different ways to selective absorption tests. We possess many observations which point to the greater efficacy of a truly polyvalent antimeningococcic serum in practice, and hence we do not accept, as yet, such an apparently polyvalent serum, arising from single type cultures, as being immunologically and therapeutically equivalent to the former. The results given show also that an immune horse serum cannot be used for classifying meningococci, and finally, that the so called monovalent sera prepared in France and England in the horse doubtless possessed agglutinative capacities far wider than is implied in their names.

Comparative Agglutinin Content of Monovalent and Polyvalent Sera.

The experiments on horses with single and with several strains of meningococci have shown that agglutinin formation is induced not only for the strain or strains injected but also for a wide number and diversity of other type and subsidiary strains. At first sight it may appear that the sera produced with single and with multiple strains are practically identical and could be substituted for each other in

treatment. And yet this deduction would not be justified, as the following tests show. It is also in conflict with observations on the efficacy of widely polyvalent antimeningococcic serum known to contain specific agglutinins for the main and subsidiary strains of the meningococcus.

Effects of Storage.—The first tests to be described here relate to the comparative keeping qualities of the two kinds of sera. The samples of mono- and polyvalent horse sera were kept in the refrigerator in the dark at an approximate temperature of 4°C. for about 1 year. All the sera had been preserved with 0.15 per cent tricresol. The sera tested were derived from Horses M 32 (monovalent para), M 33 (monovalent regular), M 24 (regular, para, and three intermediates), and M 31 (three regular). The polyvalent serum used for comparison was obtained by pooling the serum of six horses, each immunized over a long period of time with 50 odd strains.

The tests of keeping qualities took into account only the agglutinins, which can be quantitatively determined. The titrations were made with suspensions of killed cultures which were uniform for all the tests.

The results of the tests are shown graphically in Text-fig. 1. While the polyvalent serum has fallen off but little during a year's storage and still agglutinates all of the 41 strains of meningococci employed, the two monovalent sera have lost agglutinating power for a considerable number of the strains (sixteen in the case of Horse M 32 (para type) and ten in Horse M 33 (regular type)). On the other hand, the three strain serum (Horse M 31) and especially the five strain (Horse M 24) approach in value the polyvalent serum. The serum of Horse M 24, in a dilution of at least 1:50, agglutinated 39 of the 41 strains.

The chart brings out the important fact that the common or secondary agglutinins are the first to disappear from the serum and that specific agglutinins for homologous strains are reasonably stable during storage. This is a point of capital distinction and may well prove to be the determining factor in respect to the manufacture as well as to the therapeutic efficacy of the various sera. The striking difference as shown by the pooled polyvalent serum relates to its inclusiveness for essentially all the strains employed in the test. It

is obvious that as the monovalent sera lose their secondary (or common) agglutinins they become more suitable for use in type diagnosis, and *pari passu*, more unsuitable for therapeutic purposes. One further matter may be pointed out. The standard polyvalent serum was prepared by pooling the sera derived from six horses. The sera prepared with three and five strains respectively came each from one horse. Reasoning by analogy and taking into account the response of different horses to injections of various strains of meningococcus, for example, Horses M 24 and M 25, it is quite possible that the pooling of sera prepared from a small number of type strains might yield an enduring and still more inclusive serum.

Effects on Absorption of Agglutinin.—The differences in deterioration on storage point to a fundamental difference in the monovalent as compared with the polyvalent sera which is confirmed by the test for absorption of agglutinin as is shown graphically in Text-fig. 2.

In Columns 1 and 2 are shown the control agglutination tests made with a 14 month sample of the pooled polyvalent serum and a fresh sample of monovalent para serum (Horse M 32) before absorption with killed cultures of Strains 1 (the homologous para strain) and 5 (also of para type). After triple absorption under carefully controlled conditions, it was found that the single strains had removed all the agglutinins with which they could react, since further absorption did not reduce significantly the agglutinins remaining in the serum.

Text-fig. 2 shows that Strain 1, the homologous strain, exhausted the monovalent serum completely, but was unable to exhaust the polyvalent serum, leaving agglutinins with which 30 of the 44 test strains were able to react. Similarly, while Strain 5, another strain of the same type, removed from the monovalent serum all the agglutinins with which 27 of the 44 test strains had reacted in the control tests, a similar treatment of the polyvalent serum left available agglutinins for all but 9 of the 44 test strains. This experiment emphasizes the difference in the inherent character of the polyvalent and the monovalent serum.

Strain No.	Serum dilutions	Unabsorbed control				Absorbed 3 times with para strain (No. 1)		Absorbed 3 times with para strain (No. 5)			
		Standard polyvalent serum		Serum M32 (Strain 1; para monovalent)		Polyvalent serum	Monovalent serum	Polyvalent serum	Monovalent serum		
		1:100	1:200	1:400	1:800	1:1600	1:100	1:200	1:400	1:100	1:200
1		+	+	+	+	+	+	+	+	+	+
2		+	+	+	+	+	+	+	+	+	+
4		+	+	+	+	+	+	+	+	+	+
5		+	+	+	+	+	+	+	+	+	+
6		+	+	+	+	+	+	+	+	+	+
7		+	+	+	+	+	+	+	+	+	+
8		+	+	+	+	+	+	+	+	+	+
9		+	+	+	+	+	+	+	+	+	+
30		+	+	+	+	+	+	+	+	+	+
31		+	+	+	+	+	+	+	+	+	+
32		+	+	+	+	+	+	+	+	+	+
33		+	+	+	+	+	+	+	+	+	+
36		+	+	+	+	+	+	+	+	+	+
37		+	+	+	+	+	+	+	+	+	+
38		+	+	+	+	+	+	+	+	+	+
39		+	+	+	+	+	+	+	+	+	+
40		+	+	+	+	+	+	+	+	+	+
41		+	+	+	+	+	+	+	+	+	+
42		+	+	+	+	+	+	+	+	+	+
44		+	+	+	+	+	+	+	+	+	+
45		+	+	+	+	+	+	+	+	+	+
48		+	+	+	+	+	+	+	+	+	+
49		+	+	+	+	+	+	+	+	+	+
50		+	+	+	+	+	+	+	+	+	+
60		+	+	+	+	+	+	+	+	+	+
61		+	+	+	+	+	+	+	+	+	+
62		+	+	+	+	+	+	+	+	+	+
63		+	+	+	+	+	+	+	+	+	+
64		+	+	+	+	+	+	+	+	+	+
66		+	+	+	+	+	+	+	+	+	+
67		+	+	+	+	+	+	+	+	+	+
69		+	+	+	+	+	+	+	+	+	+
70		+	+	+	+	+	+	+	+	+	+
71		+	+	+	+	+	+	+	+	+	+
72		+	+	+	+	+	+	+	+	+	+
73		+	+	+	+	+	+	+	+	+	+
74		+	+	+	+	+	+	+	+	+	+
76		+	+	+	+	+	+	+	+	+	+
77		+	+	+	+	+	+	+	+	+	+
78		+	+	+	+	+	+	+	+	+	+
79		+	+	+	+	+	+	+	+	+	+
80		+	+	+	+	+	+	+	+	+	+
84		+	+	+	+	+	+	+	+	+	+
85		+	+	+	+	+	+	+	+	+	+

TEXT-FIG. 2. A comparison by agglutination of standard polyvalent serum and of para monovalent serum before and after absorption three times with a heavy suspension of Strain 1 or 5.

Employment of Killed Cultures.

The preparation of therapeutic meningococcic serum with living antigen is laborious and difficult. Because of the rapidity with which the meningococcus dies in artificial cultures, frequent transfers on serum media must be made, and the cultures to be used for the immunizing injections must be freshly prepared each time in a plain agar medium. The question arises, therefore, whether the practical operations cannot be simplified by the employment, at least over certain periods, of killed cultures of the meningococcus so prepared as to prevent the autolysis which tends to destroy the antigenic properties. We have not carried out an exhaustive study of this subject but from many tests on rabbits and the following experiment with Horse M 28, we are of the opinion that the subject is worthy of thorough investigation.

Experiment 5.—Because of the rapidity with which the meningococcus autolyzes in cultures, growths in plain agar in Blake bottles, incubated at 37°C. for 8 to 10 hours, were employed. The surface growths were washed off with 20 cc. of isotonic saline solution and the heavy suspensions were quickly heated in a water bath to 65°C. to destroy the autolytic ferment. Test for viability was made and 0.35 per cent tricresol added. The suspension was kept in the refrigerator.

Horse M 28, after the usual preliminary treatment, was injected, beginning January 4, 1918, with suspensions of killed cultures of the same strains that were used in the immunization of Horses M 24 and M 25; *viz.*, No. 1 (para), No. 60 (regular), and Nos. 30, 31, and 38 (intermediates). The test serum bleedings were made after 7 and 12 months of immunization with the results shown in Table IV.

TABLE IV.

Number of Stock Strains Agglutinated by the Serum of Horse M 28 Immunized with Killed Cultures of Five Strains.

Duration of immunization. <i>mos.</i>	Agglutination test positive.	
	1:400 or higher.	1:100
7	46 of 60	59 of 60
12	53 " 56	56 " 56

This single experiment may be taken merely to indicate that as far as the agglutinin response is concerned, killed cultures of me-

ningococci can be used for immunizing horses. It will be patent from all the circumstances and from what has been stated above, that this fact is not regarded as tantamount to the conclusion that a therapeutically active and efficacious antimeningococcic serum can be produced in this way. We should, indeed, be willing to go no further at the present time than to propose that the suspended killed cultures be kept on hand to be used on occasion in place of the suspended live cultures when for any reason circumstances make it impossible to inject the latter. During the war when antimeningococcic serum was being produced at The Rockefeller Institute under great pressure, we never relied on the killed cultures alone and, at most, only occasionally alternated the injection of the killed and the living cultures in the routine manufacture of antimeningococcic serum. We still believe in the practice of employing live cultures and also of cultures freshly isolated from cases of epidemic meningitis.

The power of stored killed cultures, preserved with tricresol, to induce agglutinin formation was tested in one instance in rabbits. The killed cultures had been in the refrigerator for periods of 8 and 14 months. The rabbits tolerated the usual doses. One rabbit injected with a killed regular strain gave a serum of 1:1,600 titer, and another injected with a killed para strain gave a titer of 1:800 against freshly prepared killed cultures. The stored killed suspensions were agglutinated in other immune rabbit sera in high dilutions. As a conservative routine, however, the killed cultures employed occasionally for injection and for agglutination were not used after 3 months storage.

SUMMARY AND CONCLUSIONS.

In an attempt to simplify the manufacture of an efficacious antimeningococcus serum an experimental study has been made of a number of sera produced with a few or with single strains of meningococcus, the therapeutic polyvalent serum produced at The Rockefeller Institute with more than 50 strains being used as a standard of comparison.

It was found that horses injected with an antigen limited to five, three, or even one strain yielded sera with a range of agglutinins covering in high dilution practically all the stock strains used in pro-

ducing the polyvalent serum. These sera appeared to equal the polyvalent serum in range and titer of agglutinins, but on further examination fundamental differences were found. Storage for a year had little effect upon the titer and inclusiveness of the polyvalent serum, whereas the monovalent serum had fallen off greatly, especially in regard to secondary or subsidiary agglutinins, so that only a comparatively small number of stock strains was still agglutinated. The serum made with five strains, a regular, a para, and three intermediate meningococci, approached the polyvalent serum in keeping qualities and still agglutinated at the end of this period 39 of the 41 strains tested.

Absorption tests also brought out inherent differences in the nature of the polyvalent and the monovalent sera which had appeared to be practically identical in simple agglutination tests. The homologous strain on triple absorption was able to exhaust the monovalent serum completely, but was unable to remove from the polyvalent serum agglutinins to which 30 of 44 different strains were able to react. Absorption with another single strain of the same type removed from the monovalent serum agglutinins for a majority of the test strains but left the polyvalent serum relatively unaffected.

It is comparatively easy to produce a serum effective against about 80 per cent of the spinal strains of meningococci encountered. Deficiencies in our knowledge of the antigenic capacities of the meningococcus have led to the more or less empirical use of a large number of cultures in the preparation of a serum effective against the remaining 20 per cent of the strains. How far the number of the latter in the antigen may be reduced without restricting the efficacy of the serum remains yet to be determined. However, the experimental evidence recorded here apparently does not favor the use of an antigen limited to one or too few strains. For example, three or five selected strains produced a serum which agglutinated practically all the strains against which it was tested. But in view of the many observations which point to the greater therapeutic efficacy of a serum made with a larger number of strains we would not as yet advocate a serum prepared with too limited antigens even though it contains at first a wide range of agglutinins.

It has been brought out that a monovalent serum contains, in addition to specific agglutinins, a wide range of common or secondary agglutinins which tend to disappear during storage. The difference between specific and secondary agglutinins is not apparent in simple agglutination tests, but is revealed by absorption tests. It is probable that in a serum prepared with a few strains the same condition exists, whereas in a serum produced with a large number of strains the agglutinins are mainly specific as contrasted with the fact that most of them are secondary in the serum produced with few strains. The question whether secondary agglutinins are therapeutically equivalent to primary or specific agglutinins requires further study.

We wish to acknowledge our indebtedness to Dr. J. H. Brown and Dr. R. B. Little, of the Department of Animal Pathology of The Rockefeller Institute, for their cooperation.