STUDIES ON MENINGOCOCCUS INFECTION

II. Monovalent Diagnostic Sera Prepared from "Fresh" and "Stock" Strains

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The preceding paper has dealt with certain morphological and biological differences demonstrable between fresh and stock strains of the meningococcus. These differences have further been found to be associated with differing antigenic qualities in the two strain varieties when they are used in the preparation or the testing of antimeningococcal sera and this paper deals with the preparation of sera from freshly isolated strains for use in agglutination and precipitation tests. The results of agglutination tests with such sera under different conditions are compared with those obtained by the use of standard monovalent sera prepared by immunization with stock strains of meningococci.

Material and Technique

The strains used included old stock strains, fresh spinal fluid and nasopharyngeal strains, and rough variants appearing *in vivo* or *in vitro*.[1] Standard monovalent diagnostic sera prepared with old stock strains have been procured through the courtesy of the New York State Department of Health, Albany. New sera have been prepared in this laboratory with freshly isolated strains (except for Type IV, of which no freshly isolated strain has been available), using young healthy rabbits.

Two methods of serum preparation have been used. In the first, rabbits are given four injections of gradually increasing numbers of living organisms on alternate days, the initial dose being from 1,000,000,000 to 2,000,000,000. Freshly

[1] The sources of these strains are given in Paper I of this series and will not be repeated here. It is desired, however, to express thanks again to all who have so kindly assisted in the difficult task of obtaining material for this work.

isolated strains are grown on blood agar plates and used as soon as good growth appears—that is to say in 15 to 20 hours—being washed off in normal saline and adjusted to the required dilution with the help of a Gates turbidometer. After the first series of injections, the animal is allowed to rest for 5 to 7 days and the procedure is then repeated using larger doses. A third series of injections may be given, though the titre is often satisfactory after the first two. The final dose is approximately 10,000,000,000 organisms. Serum obtained in this way has a satisfactory agglutinin titre but has few if any precipitins for the soluble specific substance. Precipitins may be evoked by allowing animals to rest for 4 weeks rather than a few days between injection series, but this method of precipitin production is not as good as the one given below. A trial bleeding is made 5 days after the end of the last series of injections and if the titre proves sufficient the animal is bled immediately up to 50 or 60 cc. With care the animal may be saved and bled again after a few injections at a later date.

While the above method has proved satisfactory in the preparation of agglutinating sera, the precipitin content is usually low. A statement by Murray (1) that an interval of 6 weeks in the course of vaccination appeared to be essential for the production of sera with a high precipitin titre was tested and the following method evolved.

The animals are injected daily for 1 week with increasing doses of organisms from an initial dose of 1,000,000,000 organisms. They are then allowed to rest for a week when the procedure is repeated with larger doses; and again a third time. 5 days after this a trial bleeding can be made, but while the agglutinin titre may be high, the precipitin titre, except in rare instances, is negligible. The animals now rest for 6 weeks when the vaccination is begun anew and two series are given. Trial bleeding now shows, as a rule, a serum with a sufficiently high precipitin titre and the animal is immediately bled. If the titre is not satisfactory, a third series of injections may raise it to the required height. Besides living organisms, both formalinized and iodized antigens have been used and give even better results.

In preparing the formalinized antigen the organisms are washed off a blood agar plate with 10 cc. of a 0.2 per cent solution of formalin. The suspension so obtained stands in the ice box overnight before it is used. In the case of the iodized antigen the organisms are washed off in 10 cc. of normal saline. To this suspension is added sufficient iodine solution to render it permanently brown (about 7 or 8 cc.). The iodine solution used consists of 1.27 gm. of iodine and 5 gm. of potassium iodide in 1,000 cc. of distilled water. When this solution is added in small amounts to the suspension of organisms, the initial brown color can be seen to fade gradually (or faster if the suspension is agitated). It appears that the iodine is removed from solution possibly by adsorption to or actual chemical combination with the bacteria.

Using these treated antigens, series of injections have been given corresponding as to time intervals and quantities with those used for the suspensions of living organisms.

All agglutinations have been carried out in water baths regulated to the required

temperature. For antigen a standard suspension has been adopted consisting of a suspension of meningococci of a sufficient density to give a reading of 4 cm. when tested with a Gates turbidometer and corresponding to about 2,000,000,000 organisms per cc. 0.3 cc. of this suspension is added to an equal amount of the serum or serum dilution to be investigated, the two fluids are mixed and the rack of tubes is placed in the appropriate water bath for the required length of time. When a temperature of 37°C. is used the tubes are removed after 2 hours and left in the ice box overnight, when a final reading is taken. The reading of the results must be largely a personal matter. In order to compensate for this, readings have been made by the same person throughout. A customary set of symbols has been used; namely, ++++ = complete agglutination; +++ = almost complete; ++ = definite; + = slight; and ± = very slight. A control tube with saline and suspension in equal parts is always inserted to detect salt sensitivity. When agglutinations are done at 56°C. for 24 hours or longer, a second control tube of normal serum diluted 1/20 is added, but this has been found unnecessary at the lower temperature.

RESULTS

In Tables I and II are shown a comparison of both standard and "anti-S" (*i.e.* prepared with recently isolated strains) sera at both 37°C. and 56°C. when tested on three stock and three freshly isolated strains of Types I, II and III. Type IV will not be included in the following observation owing to the absence of any freshly isolated strains of this type.

Employing the anti-S sera at 37°C., it will be noticed that marked agglutination is obtained with homologous serum and that no cross-agglutination occurs.[2] Stock strains do not agglutinate as satisfactorily with these sera at the low temperature. It will be seen that while the type can be ascertained in all of the stock strains, the aggluti-

[2] The cross-agglutination between Types I and III must be regarded in another light. While it seems certain that in the past these two types, although antigenically similar, have been nevertheless distinct entities, the examples occurring at the moment show so much cross-agglutination that it is well nigh impossible to separate them into two types, and the term Group I-III has been adopted for all these strains. It has also been noticed both in this laboratory and elsewhere (2, 3) that the old stock strains of these two types, though antigenically distinct when first isolated, have now become so similar as to be almost indistinguishable (*vide* Strains 123 and 57 in Table I). Finally, it may be pointed out that the soluble specific substances from either of these types have proved to be indistinguishable by any of the methods adopted (4).

nation is much less than with the fresh strains, and, moreover, that the Type II strain shows a certain amount of cross-agglutination.

The anti-S sera used at 56°C. for 24 hours prove well nigh useless with both fresh and stock strains, owing to the very marked cross-agglutination which occurs. This cross-agglutination is evident even after 3 or 4 hours, though less than at the end of 24 hours.

Standard sera at 37°C. prove of little value. The titre of agglutination in both fresh and stock strains, especially the former, is insignificant and the results are obscured by cross-agglutination.

TABLE I

Strain	Anti-S monovalent serum															Remarks
	Type I					Type II					Type III					
	1/10	1/25	1/50	1/75	1/100	1/10	1/25	1/50	1/75	1/100	1/10	1/25	1/50	1/75	1/100	
Fresh Type I No. 17.....	2	2	3	3	4	0	0	0	0	0	±	2	3	3	4	
Fresh Type II No. 31.....	±	0	0	0	0	4	4	3	3	0	0	0	0	0	0	Agglutination at 37°C. for 2 hrs. and ice box overnight
Fresh Type III No. 7.....	3	3	3	2	0	0	0	0	0	0	3	3	2	±	0	
Stock Type I No. 123.....	2	2	1	1	±	0	0	0	0	0	1	1	1	1	1	
Stock Type II No. 383.....	±	±	0	0	0	±	±	±	2	2	0	±	±	±	±	
Stock Type III No. 57.....	1	1	1	1	2	0	0	0	0	0	1	±	1	1	1	
Fresh Type I No. 17.....	4	4	3	3	3	1	2	1	1	1	3	4	3	3	3	
Fresh Type II No. 31.....	1	1	1	1	±	2	1	1	±	±	1	±	±	±	±	
Fresh Type III No. 7.....	3	3	2	2	2	3	3	2	2	2	2	2	2	2	3	Agglutination at 56°C. for 24 hrs.
Stock Type I No. 123.....	2	2	2	2	2	2	1	1	±	±	1	2	2	3	3	
Stock Type II No. 383.....	1	±	±	±	0	2	2	2	2	2	2	2	1	±	±	
Stock Type III No. 57.....	3	2	2	2	2	2	2	1	1	1	2	2	3	3	3	

When the standard sera are used at 56°C., the results are better than at lower temperatures. Cross-agglutination, however, is present in all the lower dilutions, and it is only at 1/200 or above that the results can be interpreted. Since many freshly isolated strains and even occasional stock strains fail to agglutinate in these higher dilutions, inconclusive results are not infrequent with this technique. The method, moreover, calls for the use of a series of tubes twice as large as that used with the anti-S serum and has the disadvantage of

TABLE II

Strain	Standard monovalent serum																										Remarks
	Type I									Type II									Type III								
	1/20	1/50	1/100	1/200	1/300	1/400	1/500	1/800	1/1,000	1/20	1/50	1/100	1/200	1/300	1/400	1/500	1/800	1/1,000	1/20	1/50	1/100	1/200	1/300	1/400	1/500	1/800	1/1,000	
Fresh Type I No. 17	±	±	±	±	±	±	0	0	0	±	±	±	0	0	0	0	0	0	±	±	±	±	0	0	0	0	0	Agglutination at 37°C. for 2 hrs. and ice box overnight
Fresh Type II No. 31	±	±	±	0	0	0	0	0	0	±	±	0	0	0	0	0	0	0	±	0	0	0	0	0	0	0	0	
Fresh Type III No. 7	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Stock Type I No. 123	1	2	1	1	1	1	1	1	1	±	±	±	±	±	±	0	0	0	1	1	1	2	1	±	±	±	0	
Stock Type II No. 383	±	0	0	0	0	0	0	0	0	±	±	0	0	0	0	0	0	0	±	0	0	0	0	0	0	0	0	
Stock Type III No. 57	±	±	±	1	1	±	±	±	±	0	±	0	0	0	0	0	0	0	1	±	±	±	±	±	0	0	0	
Fresh Type I No. 17	1	1	2	1	1	1	1	±	0	1	1	0	0	0	0	0	0	0	1	1	2	1	1	1	±	0	0	Agglutination at 56°C. for 24 hrs.
Fresh Type II No. 31	1	1	±	±	0	0	0	0	0	1	1	±	±	±	0	0	0	0	±	±	±	0	0	0	0	0	0	
Fresh Type III No. 7	1	2	3	2	±	±	±	0	0	1	1	±	0	0	0	0	0	0	1	2	2	2	2	±	0	0	0	
Stock Type I No. 123	1	2	3	3	3	3	3	2	1	1	1	±	0	0	0	0	0	0	1	2	2	2	2	2	2	1	1	
Stock Type II No. 383	±	±	0	0	0	0	0	0	0	1	1	±	±	±	±	0	0	0	1	1	0	0	0	0	0	0	0	
Stock Type III No. 57	1	2	3	3	3	2	2	1	1	2	1	1	±	±	0	0	0	0	1	2	3	3	3	3	2	1	1	

demanding a longer period of time before the final readings can be made.

The results given in the tables are those of a single experiment. Repeated observations in the course of routine examination of freshly isolated or stock strains have served to confirm them. The fresh strains used in this experiment were of known antigenicity and necessarily not of as recent isolation as those subsequently tested in the general routine. It has been the experience in this laboratory that the fresher strains give even better agglutinations with the anti-S sera at 37°C. than those shown in Table I. Nearly all of the really fresh strains can be confidently assigned to their type on removal from the water bath at the end of 2 hours, obviating the delay of the overnight period in the ice box. While some fresh strains have been encountered in which cross-agglutination occurs, it has been found that in all cases in which this was great enough to obscure the results, it has been possible to demonstrate rough variants in the cultures.

DISCUSSION

In comparing the method of agglutination here described with those more usually adopted, certain points call for emphasis. These have to do principally with the serum used and the conditions under which the reaction is carried out.

In connection with the serum, there are two main features. The first is the choice of strains for use in serum production, and the second is the titre of agglutinins which it is considered necessary that the serum should finally attain. It is the accepted view that, in the preparation of monovalent sera, stock strains, being of known antigenicity, are as good as, indeed better than, freshly isolated strains of which the antigenic properties and complexity are problematical. It therefore becomes necessary to inquire if the freshly isolated strains have the same antigenic complex and can give rise to sera as satisfactory as those obtained by the use of stock strains. Kirkbride and Cohen (2) believe that recently isolated strains of Type I and Type III are not superior antigenically to the 13 year old stock strains used in routine serum production, while fresh Type II strains are but little better. Many observers are agreed, on the other hand, that the antigenic complex of many organisms, including meningococci (3, 5–7) does

change with prolonged culture on artificial media. Strains tend to lose what narrow specificity they originally possess and to "broaden out" until they agglutinate markedly in heterologous as well as homologous sera. More important still is the fact that, as has been shown (5, 7), these stock strains have lost at least a large part of their specific substance on which antigenic specificity depends. The results of the work here reported strongly favor the view that freshly isolated strains give rise to sera which differ quantitatively, if not qualitatively, from those prepared with stock strains. It has not been possible for the writer to produce sera with stock strains which give results as satisfactory as those obtained with the fresh strains, whether the sera be used for agglutination or precipitation tests. In fact, no precipitins for the specific fraction of the organisms have yet been obtained in sera prepared with stock strains.

It is generally agreed that the titre of agglutinins, which a serum should attain in order to be of value, must be high, 1/1,000 or more. With the standard sera generally used and the methods of agglutination usually adopted, it is not unusual for cross-agglutination to occur at a dilution of 1/100 or higher, and high titre sera are necessary in order to obviate any confusion in typing. As has been shown in the body of the present paper, however, such high titres are unnecessary when the sera are prepared with fresh strains and the reaction is carried out at 37°C. for a short period of time. Under these circumstances, a maximum titre of 1/100 will suffice and cross-agglutination is unusual even at the lowest dilution, 1/10. Since the amended method calls for the use of sera with only a low titre, it is possible to produce these in a comparatively short time and with few injections. This fact of itself is favorable to the production of narrowly specific sera, for rabbits injected repeatedly with a given strain yield a serum of steadily increasing non-specificity.

Using standard sera prepared with stock strains, it has been found necessary to perform the agglutinations at a temperature of 56°C., and the tubes have to be left at this temperature for 24 hours or longer before a conclusive result can be obtained. That the amended method calls for the use of a temperature of only 37°C. for only 2 hours, followed by the ice box overnight, is of double advantage. In the first place, the method is more rapid since agglutinations can usually be read

and determined directly on withdrawal from the water bath and without the overnight interval; and in the second, the results at this lower temperature are, as has been pointed out already, more delicate and free to a very large extent from the confusion caused by cross-agglutination. In this connection it may be said that the experience in this laboratory on applying the amended method of agglutination to strains of meningococci isolated from the cerebrospinal fluid has been at variance with that reported elsewhere. It is not unusual to find the statement made that the grouping of such meningococcus strains is by no means sharply defined. While this may be true with agglutination tests made at 56°C., all smooth cerebrospinal fluid strains tested at 37°C., 57 in number, have shown sharply defined grouping, if no distinction between Types I and III be made (see footnote 2).

It must be emphasized here that, although the agglutination method herein described is more satisfactory than that in which the test is run for 24 hours at 56°C., yet it is not put forward with the suggestion that it become the standard diagnostic method. The rapid method described by Nicolle, Debains and Jouan (8), by which agglutination can be read within 5 minutes, has all the advantages of rapidity and simplicity. The agglutination tests and agglutinating sera mentioned in this paper have appeared as corollaries during the development of a type-specific precipitating serum and are presented here as additional evidence of the differences between fresh and stock strains.

Further uses of the monovalent sera prepared with freshly isolated strains will be developed in subsequent papers which deal with the type-specific substances of the organisms and with the presence of meningococcus precipitinogens in the cerebrospinal fluid in cases of meningococcal meningitis.

SUMMARY

The production of monovalent sera for agglutinin or precipitin reactions with freshly isolated strains of meningococci is described. Agglutination reactions with such sera can be carried out more rapidly, at lower temperatures and in lower dilutions, than with the standard monovalent sera prepared from stock cultures, while the results so obtained are more satisfactory owing to the relative absence of cross-agglutination.

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