STUDIES ON MENINGOCOCCUS INFECTION

IX. Standardization and Concentration of Antimeningococcus Horse Serum (Type I)

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(Received for publication, January 9, 1936)

The problem of standardizing antisera intended for therapeutic purposes is an ever important one. In the last analysis, of course, the value of a serum must be decided by its therapeutic efficiency, but unless the relationship of the latter to some of the more conveniently measurable immunity reactions is determined, no criterion can be established whereby the probable therapeutic potency of an otherwise unknown serum may be estimated. The discovery of such a relationship depends primarily upon elucidation of the antigenic structure of the infective agent in question. For example, with Type I antipneumococcal sera, it has been possible to demonstrate a close correlation between various of the immunity reactions, in which the antibody to the type-specific polysaccharide is the factor of prime importance (1, 2). The situation has been less favorable in the case of the meningococcus, and as yet no satisfactory solution to the problem has been published.

Standardization of Type I Sera

Wadsworth (3) and Murray (4) have discussed the results which have been obtained in the past by applying various of the immunity reactions in attempts to establish a reliable measure of the potency of antimeningococcal sera. The agglutination reaction has been the most widely used and reliable method, and although the agglutinin

* Part of this work was completed by one of us while working in the laboratories of the Department of Medicine of the College of Physicians and Surgeons. The authors wish to thank Dr. Michael Heidelberger for the facilities placed at their disposal.

titre is not necessarily an index of therapeutic potency, there is evidence that sera of high titre possess therapeutic efficiency (3).

The precipitin reaction has not proved satisfactory in the past chiefly because the precipitinogen commonly used (a crude extract of meningococci) is not readily standardized and reproduced. Zozaya (5) has used what was probably the group-specific polysaccharide, or C substance, of the meningococcus as the precipitating antigen. The isolation from the Type I meningococcus of a highly purified, type-specific substance (6, 7) (to be referred to hereafter as S I) suggested the possibility of accurate measurement of the precipitin content of Type I antimeningococcal sera by the quantitative method developed by Heidelberger and Kendall (8). This has been done and in addition the method has been applied in following the fractionation and purification of such sera.

The sera used were prepared by immunizing a horse with vaccines prepared from recently isolated strains of Type I meningococcus according to the method already described (9). The S I samples used were preparations 8 and 18 previously described (7). These were found to be identical with respect to precipitating potency with horse sera.

The technique for the determination of nitrogen in the specific precipitates produced by the reaction between the sera and S I (to be referred to hereafter as antibody nitrogen[1]) followed closely that described by Heidelberger and Kendall. 1 or 2 cc. of serum or an equivalent amount of antibody solution (not more than 0.4 mg. antibody nitrogen) have been used. Since all of these contained preservative, sterile precautions were not observed. The sample was measured by a calibrated pipette into a Wassermann tube and 0.025 mg. of S I in solution was added for each cc. of serum. Duplicate tubes and salt control tubes were set up in all cases. The volume was adjusted to 4 cc. with saline and the tubes were closed with rubber caps. The contents were then mixed thoroughly and the tubes were allowed to stand for 1 or 2 hours at room temperature (20–25°C.). This procedure gave results identical with those obtained when the tubes were subjected to the customary 2 hour period of incubation at 37°C. It is realized that this method probably did not result in as complete precipitation of antibody as would have been obtained if all operations had been carried out at 0°C. (8), but this fact was not important for the purposes at hand. After the preliminary period of standing, the tubes were placed in the refrigerator and left for 18 to 24

[1] Since S I probably contains nitrogen as a constituent of its molecule, part of the antibody nitrogen may be attributable to the S I contained in the specific precipitate. However, the total amount of S I nitrogen added in one of these determinations (0.001 to 0.002 mg.) is less than the experimental error.

hours. They were then centrifuged in the cold. The specific precipitates formed compact discs which could not be readily broken up. For the first washing, the precipitate was suspended in 3 to 4 cc. of chilled saline, left for 1 hour at 0°C., and centrifuged off as before. For the second washing, it was found best to allow the precipitate to stand in contact with 3 to 4 cc. of saline at 0°C. for 16 to 24 hours with occasional shaking. In the first determinations a third washing was made, but this was found to be superfluous. Finally, the precipitate was centrifuged off in the cold. It was then dissolved in a little distilled water with the aid of a few drops of N/1 sodium hydroxide. The solution was rinsed into a 125 cc. Kjeldahl flask and the nitrogen determination was carried out according to a slight modification of the Pregl micro Kjeldahl method.

Heidelberger and Kendall (8), studying the reaction between the Type III pneumococcus polysaccharide and its antibody, found that when increasing amounts of polysaccharide were added to a fixed amount of antibody, the quantity of precipitate formed increased to a maximum and then remained constant over a wide range until the inhibition zone was reached. A similar relationship exists for the present system. It was found, with samples of serum containing between 0.15 and 0.20 mg. of specifically precipitable nitrogen, that identical results were obtained over the range 0.02 to 0.10 mg. of S I added. After removal of the specific precipitates, portions of the supernatant fluids were tested in each case (*a*) with an equal volume of antiserum, and (*b*) with an equal volume of a 1:50,000 solution of S I in saline. The (*a*) tests all showed strong reactions, whereas the (*b*) tests were uniformly negative, thus showing that S I was present in excess in all cases. Less than 0.02 mg. of S I added could not be relied upon to give maximum precipitation, whereas 0.3 mg. produced inhibition or prozone phenomenon. Since it was desired to characterize the sera by determining under standard conditions the maximum amount of specifically precipitable nitrogen which they contained, the procedure finally adopted was to add 0.025 mg. of S I (0.5 cc. of a 1:20,000 solution in saline) per cc. of serum (antibody nitrogen content, 0.1 to 0.2 mg. per cc.). These proportions were maintained throughout and were found sufficient to provide a definite excess of S I but at the same time to be remote from the inhibition zone. The supernatants from all determinations were, of course, tested qualitatively for the presence of excess S I and antibody.

The sera have been analyzed for both total Kjeldahl nitrogen and

antibody nitrogen. The results are shown in Table I. Each figure represents the average of at least two determinations which checked to within less than 0.01 mg. The results are therefore believed to be accurate to within ±0.005 mg.

It will be seen that the sera used (representing successive bleedings from the same horse) contained between 1 and 2 per cent of their nitrogen in the form of antibody (0.1 to 0.2 mg. antibody nitrogen per cc.). This is rather a low figure in comparison with the results found, for example, with some antipneumococcal horse sera, where the figure may be at least ten times as great. Thus Felton (2), in a study of

TABLE I

Analysis of Type I Antimeningococcal Horse Sera

Serum No.	Antibody N per cc.	Total N per cc.	Antibody N / Total N	Remarks
	mg.	*mg.*	*per cent*	
1 + 2 pooled	0.185			Preserved with tricresol
Same after 6 mos.	0.150			
3	0.135	10.78	1.25	" " "
Same after 6 mos.	0.118			
4	0.178	13.46	1.32	" " merthiolate
Same after 6 mos.	0.174			
5	0.177	10.80	1.63	" " "
Same after 6 mos.	0.181			
6	0.106			" " "
7	0.121	12.08	1.00	" " "
8	0.109			" " "

thirty-nine sera from thirty-seven horses immunized against Types I and II pneumococcus, found that the content of antibody to the Type I specific substance varied from 0.22 to 2.36 mg. nitrogen per cc. The low figures of the present series are probably due to the antibody response characteristic of the particular horse used for immunization against Type I meningococcus, for, in the case of one polyvalent antimeningococcal horse serum tested, an antibody nitrogen content (Type I) of 0.97 mg. per cc. was observed, a figure which compares more favorably with those quoted above from Felton. It was thought that the low results might be due to a loss of precipitinogenic potency of the S I solution on prolonged storage in the refrigerator. Such was not the case, for a given solution of S I precipitated identical amounts

of antibody on two occasions, 6 months apart (sera 4 and 5). It was also possible that the S I had lost part of its precipitating power because of the process used in purifying it (7). Thus, it has been shown (10) that fragments prepared by partial hydrolysis of Type III pneumococcus polysaccharide may still exhibit precipitating action when tested with homologous horse antiserum but not with rabbit antiserum. However, even at dilutions as high as 1:2,000,000, S I precipitates homologous rabbit antiserum (7) and there is no evidence that it has been isolated in a partially hydrolyzed condition, for it gives

TABLE II

Titres of Type I Antimeningococcal Horse Sera against Various Meningococcus Antigens

Date	Serum No.	Anti-S I	Anti-C	Anti-P	Agglutination Type I	Agglutination Type II
1933						
Apr. 1	1 + 2	1:8,000,000	1:100	0	1:800	1:20
Dec. 1	3	1:8,000,000	1:1,000	1:1,000	1:800	1:100
1934						
Feb. 1	4	1:8,000,000	1:10,000	1:100	1:1,600	1:100
May 15	5	1:8,000,000	1:10,000	1:1,000	1:1,600	1:100
Oct. 1	6	1:8,000,000	1:10,000	1:100	1:3,200	1:100
Oct. 15	7	1:8,000,000	1:10,000	1:100	1:3,200	1:100
1935						
Jan. 1	8	1:8,000,000	1:10,000	1:100	1:6,400	1:100

Agglutinin titres represent dilutions of serum; the other titres represent dilutions of antigens.

no test for reducing sugars until it has been subjected to acid hydrolysis.

It is of interest in connection with the problem of preserving sera that the antibody content of sera in the presence of some preservatives may remain quantitatively constant over a protracted period of time. Thus, sera 4 and 5, which contained 0.01 per cent merthiolate,[2] showed no change in antibody content after 6 months' storage at 0–4°C. On the other hand, in a similar experiment, sera 1 + 2 pooled, and 3, which contained 0.3 per cent tricresol, showed respectively a loss of 19 per cent and 13 per cent within the same period.

[2] Supplied through the kindness of Eli Lilly and Co., Indianapolis.

In Table II are shown, for comparative purposes, the titres observed when the sera were tested against S I, against the group-specific polysaccharide, C, against the group-specific protein, P, and (for agglutination) against Types I and II meningococcus.[3] The most salient feature of these data is the relatively extremely high anti-S I titre, 1:8,000,000. The anti-C and anti-P titres remained low throughout (maximum = 10^{-4} and 10^{-3} respectively) as did the Type II agglutination titre, which was never higher than 1:100. On the other hand, the Type I agglutination titre was 1:800 at the first bleeding, and rose continuously to 1:6,400. This rise was not parallelled by an increase in the anti-S I content of the sera, and for the present such lack of correlation must remain unexplained.

Purification and Concentration of Serum

The accumulated evidence of past work indicates that the antibody in antimeningococcal horse serum is associated with the water-insoluble globulin fraction in a relationship similar to that found with antipneumococcal horse serum (11). Murdick and Cohen (12) have recently reviewed the subject and they confirm this fact. By a process of dialysis and isoelectric precipitation they were able to precipitate nearly all of the antibody (as measured by the agglutinin titre and neutralizing potency in the phenomenon of local skin reactivity (13)).

The method to be presented differs from that of the above authors chiefly in the fact that the water-insoluble globulin fraction of the horse serum has been precipitated by the use of carbon dioxide. The latter procedure is not a new one. Avery (14) has used it in fractionating antipneumococcal horse serum. He found that if a Type II serum, diluted tenfold with distilled water, was saturated with carbon dioxide, somewhat more than one-half of the protective power was found in the precipitate.

In an experiment along the lines of that of Avery, a sample of serum 3 was diluted fivefold with distilled water. Carbon dioxide was bubbled slowly through the solution at room temperature until it was obvious that a precipitate had formed. The solution was then left for 1 hour in the refrigerator. The precipitate

[3] Agglutinations were carried out for 2 hours at 37°C. and overnight in the ice box.

was collected by centrifuging. It was dissolved in a volume of 0.85 per cent sodium chloride solution equal to that of the original serum. To the clear, supernatant fluid was added sufficient solid sodium chloride to make a final concentration of 0.85 per cent. Both solutions were neutralized with N/1 sodium hydroxide and then tested qualitatively with 1:10,000 and 1:50,000 dilutions of S I.

The two solutions thus prepared reacted strongly, and it was estimated that at least one-half of the antibody present had not been precipitated.

It was next found that if the serum was subjected to dialysis before being diluted, treatment with carbon dioxide precipitated all of the antibody. The results of tests on the fractions obtained in a typical experiment using a sample of serum 3 are shown in Table III.

TABLE III

Fractionation of Type I Antimeningococcal Horse Serum by Dialysis and Precipitation with Carbon Dioxide

	Dialysis precipitate (fraction A)	CO_2 precipitate (fraction B)	CO_2 supernatant	Control, original serum
1:10,000 S I	++ pd	+++ pd	−	+++ pd
1:50,000 S I	+ p	++ pd	−	+++ pd

Readings made after 2 hours at 37°C.
pd indicates formation of disc-like precipitate easily broken up by agitation.
p indicates formation of finely divided precipitate.

On dialysis, a precipitate separated out from the serum. This was collected separately and labeled fraction A. The bulk of the serum was then diluted fivefold with distilled water and treated with carbon dioxide as above. The resultant precipitate was labeled fraction B. Each precipitate was dissolved in a volume of 0.85 per cent sodium chloride equal to that of the original serum. Sodium chloride was added to the supernatant fluid from the carbon dioxide precipitation to make a final concentration of 0.85 per cent. All solutions were neutralized with N/1 sodium hydroxide and centrifuged before being tested with S I.

It will be seen that fraction A contained a considerable amount of antibody and that fraction B reacted almost as strongly as did the original serum. The most important fact was that the supernatant fluid after the removal of fraction B failed to react, indicating that the antibody had been precipitated quantitatively. This was sub-

stantiated in a similar experiment in which 100 cc. of serum 3 was fractionated. This represented 13.5 mg. of antibody nitrogen. Fraction A was found to contain 4.58 mg. (34 per cent) of the antibody nitrogen, whereas fraction B contained 8.55 mg. (63 per cent). The total recovery was therefore 97 per cent.

On the basis of these findings, the following method has been evolved for the purification and concentration of Type I antimeningococcal horse serum.

Portions of 100 to 400 cc. of serum have been used, both with and without preservative (0.3 per cent tricresol or 0.01 per cent merthiolate). The serum was placed in a bag made of either cellophane or collodion and dialyzed against cold running tap water for 24 hours. Serum which contained no preservative was protected against contamination by the use of toluene or chloroform. The precipitate which separated out as a result of dialysis was collected by centrifuging and treated as below. The supernatant was set aside for the carbon dioxide precipitation.

The dialysis precipitate was taken up in a volume of 0.85 per cent sodium chloride[4] equal to 1/15 to 1/30 the volume of the original serum. The volume chosen depended upon the extent to which it was desired to concentrate the antibody. The former quantity ensured easier manipulation and gave a final product having a volume 1/3 to 1/4 that of the original serum. The solution of the dialysis precipitate was usually very cloudy and could not be satisfactorily clarified by centrifuging or increasing the salt content of the solution. This effect was especially pronounced in those cases where tricresol had been used as preservative. It was found that the addition of 0.5 to 2 volumes of distilled water to the solution would cause the formation of a precipitate consisting mostly of inactive material. When this precipitate was centrifuged off, the supernatant was quite clear and entirely satisfactory even for precipitin ring tests, where a slight cloudiness in the serum makes reading difficult. The exact proportion of distilled water necessary to throw out this inactive material from solution varied from one preparation to another and depended particularly on the protein concentration. Thus, the smaller the volume of saline used for dissolving the dialysis precipitate, the larger had to be the proportion of distilled water added. In general, not less than 0.5 volume nor more than 2 volumes was required. Care must be exercised in carrying out this step, for the use of an excess of distilled water caused precipitation and denaturation of the antibody, which then proved to be insoluble in saline and had to be discarded.

The bulk of the dialyzed serum was diluted to five times the volume of the original serum with distilled water saturated with chloroform to prevent bacterial

[4] All solutions contained 0.01 per cent merthiolate, except the distilled water used in diluting the dialyzed serum preparatory to the carbon dioxide precipitation.

contamination. A slow current of carbon dioxide was passed through the solution until aggregates of precipitate formed which were readily visible. The carbon dioxide treatment was continued for 15 minutes to ensure the presence of an excess of the gas. The precipitate could be centrifuged off immediately without serious loss of antibody from incomplete precipitation. It separated as a compact gum which was rather difficult to manipulate.

TABLE IV

Analysis of Type I Meningococcus Antibody Preparations

Preparation No.	Volume serum taken	Volume antibody solution	Total N per cc. of antibody solution	Antibody N per cc. of antibody solution	Yield total N	Yield antibody N	Purification factor*	Remarks
	cc.	*cc.*	*mg.*	*mg.*	*per cent*	*per cent*		
7	200 No. 3 + 250 No. 4	64	5.12	0.880	5.9	79	13.5	Filtered through Seitz EK
8	400 No. 3	52	6.45	0.762	7.8	73	9.5	" " Tricresolized serum
9	200 No. 5	52	3.59	0.600	8.6	89	10.3	Not filtered
10	50 No. 3† + 50 No. 7	58.5	2.08	0.163	10.6	80	7.5	" "
11	45 pooled Nos. 1 to 8‡	33	1.34	0.144	8.7	91	10.4	" "

* Antibody nitrogen yield divided by total nitrogen yield.

† Had been stored 6 months. Antibody N content had fallen to 0.118 mg. per cc.

‡ Contained 11.24 mg. total N per cc. and 0.116 mg. antibody N per cc.

The solution of the dialysis precipitate was diluted with 1/10 its volume of M/1 phosphate buffer, pH 7.2, in order to increase the salt content to normal and to neutralize the solution. This solution was then used to dissolve the fraction precipitated by carbon dioxide. The final solution of the product was usually clear, colorless, and only slightly opalescent. On standing at 0 to 4°C., a small amount of precipitate formed slowly. This process has been accelerated by incubating the antibody solution for several hours at 37°C. and then allowing it to stand in the refrigerator for several days, after which the precipitate may be centrifuged off.

The antibody solutions have been analyzed for total Kjeldahl nitrogen and for antibody nitrogen according to the technique outlined. The analyses (Table IV) show that a 90 per cent yield of antibody was obtainable, while the corresponding purification with respect to total nitrogen averaged at least tenfold. The relatively low yields in preparations 7 and 8 are undoubtedly due to adsorption taking place during filtration through a Seitz filter. Since the purification factors remained relatively unchanged, it is probable that both specific and nonspecific proteins were alike lost. In preparation 10, the serum, after the treatment with carbon dioxide, was left in the refrigerator for 24 hours. The precipitate collected on the walls of the flask, permitting

TABLE V

Name or number	Type I serum	Type I serum concentrated	Type II serum	Type II serum concentrated	Agglutination	Notes
C.	+	++	0	±	I-III	Before intrathecal serum
W.	0	0	0	0	I-III	After intrathecal serum
505	++	+++	∓	±	I-III	
506	++	+++	0	±	I-III	
F.	0	±	±	+	II	
504	0	±	+	++	II	
498	0	±	±	+	II*	
500	0	∓	±	+	II*	
I	0	±	0	±	Pneumococcus 10	

II* = atypical Type II strain (15).

decantation of the clear supernatant, which was subsequently found to contain no antibody. It is obvious from the low purification factor (7.5) that a considerable amount of inactive protein separated out during this treatment. The low yield was found to be due to the use of too large a proportion of distilled water in the purification of the dialysis precipitate. In preparation 11, the fraction precipitated by carbon dioxide was centrifuged off immediately after it formed and the supernatant was placed in the refrigerator for 48 hours. A further precipitate formed which upon analysis was found to contain only 0.15 mg. or 3 per cent of the original antibody nitrogen and 9.74 mg. or 2 per cent of the original total nitrogen. In other words, the anti-

body purification obtained in this additional fraction was so slight in comparison with that obtained in the main fractions, that it was not considered worth while to collect it. The optimum procedure, therefore, is to collect the fraction precipitated by carbon dioxide at room temperature as soon as it is formed.

It had been hoped that sera concentrated in this manner could be used in the precipitin test with specimens of spinal fluid from cases of cerebrospinal meningitis sent into the laboratory to be typed. Such sera might be of especial value in the case of those spinal fluids whose content of precipitinogen was too low to react with the unconcentrated sera. However, even when trouble caused by the cloudiness of early preparations of the concentrated sera had been overcome, the results were confused by the occurrence of heterologous reactions. This is brought out by the data summarized in Table V, which also illustrates the degree of reaction to be expected in testing spinal fluids. The reactions with the concentrated sera were stronger throughout, and despite the cross-reactions observed, a correct diagnosis could have been made in all cases. It is probable that preliminary absorption of the sera with the group-specific antigen of the meningococcus would eliminate these troublesome cross-reactions.

DISCUSSION

The quantitative determination of precipitins is the most accurate method available for the standardization of sera. Whether the type-specific precipitin content of an antimeningococcal serum is an indication of its therapeutic value has yet to be studied by protection tests in experimental meningococcus infection in mice (16) and in possible clinical applications to man. For the present, it can be said that of the components thus far identified in the antigenic complex of the meningococcus, the type-specific substance elicits the most prompt and the greatest antibody response (9, 17), at least when the vaccines used have been made from freshly isolated strains of the organism. Contrary to what might have been expected, the agglutination titre has not run parallel to the type-specific precipitin content of the sera nor to the precipitin titre of the sera with respect to the group-specific polysaccharide and protein of the meningococcus. Whether this fact is due to partial degradation of the haptenes as isolated, or indicates the existence of haptenes as yet unidentified, cannot be said. It must

be borne in mind that the data presented represent the antibody response of a single horse and so are not subject to broad generalization.

The use of carbon dioxide as a precipitating agent in the purification and concentration of antibodies seems to offer certain advantages. All operations may be conducted at room temperature (20–25°C.). The precipitation is rapid, nearly quantitative, and not critical as to optimum conditions. There is no problem concerning removal of the precipitating agent, as is the case when salting-out methods are used. Type II antimeningococcal horse sera have been concentrated by the method outlined and it is not unlikely that the latter may find application in the purification of other types of antibody.

SUMMARY

Type I antimeningococcal horse sera have been standardized by the quantitative determination of their type-specific precipitin content.

By a method involving dialysis and precipitation by treatment with carbon dioxide, the antibody in such sera has been purified tenfold with respect to the nitrogen content.

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