PROPERTIES OF THE TYPE SPECIFIC PROTEINS OF ANTIPNEUMOCOCCUS SERA

I. The Mouse Protective Value of Type I Sera with Reference to the Precipitin Content

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Other factors being equal it might be expected that any specific immunological value of a given immune serum should be proportional to the amount of the specific antibody. For example it might be held that the effectiveness of a given antipneumococcus serum in protecting animals against pneumococcus infection could be judged by estimating the amount of protein specifically precipitable with the homologous capsular polysaccharide, that is, the amount of antibody protein (precipitin). This thesis is presumably tenable only if the injection of various animals with a single antigen gives rise to a single antibody, or to a mixture in fixed proportions of antibodies dominantly specific but differing in one or more chemical or immunological properties. Present evidence indicates that agglutination, precipitation, and complement fixation are due to the same substances in the serum. It has not as yet been demonstrated, however, that in a single serum there exists only one antibody substance specific for a given antigen.

This question is important from the practical as well as from the theoretical point of view. The clinician has generally held to the significance of tests for potency in which animals are used. This view has developed as a result of the fact that antipneumococcus sera of certain types afford excellent protection to the experimental animal and at the same time are effective therapeutic agents in lobar pneumonia. With other types of pneumococci, notably Type III, the immune sera may possess considerable antibody potency as judged by agglutination titer and yet be lacking in ability to protect animals against large numbers of pneumococci. Sera of these types have also proven less successful in the clinic.

On the other hand, it has long been recognized that evaluation by animal

methods is difficult and simpler means of potency estimations have been sought. In spite of many excellent studies on this subject (1–6), it is as yet by no means certain that an absolute parallelism exists between *in vitro* and *in vivo* results. It is generally held that this uncertainty is related to the lack of precise methods for determining protective potency.

The present paper consists of an experimental appraisal of the mouse protection test, its quantitative possibilities, and of the relation between the amount of antibody quantitatively determined by *in vitro* methods and the amount of demonstrable protective antibody.

EXPERIMENTAL

These experiments deal only with Type I antipneumococcus sera.

Sera.—Type I antipneumococcus horse sera were obtained through the courtesy of Dr. Augustus B. Wadsworth of the New York State Department of Health and Dr. W. E. Bunney of the Michigan State Department of Health. The standard serum used is that designated as 425 and has been described by Kirkbride, Hendry, and Murdick (7). According to titrations carried out by these workers this serum contains 1000 mouse protective units per cc. by comparison with the National Institute of Health standard serum. Type I antipneumococcus rabbit sera were produced in this laboratory.

Capsular Polysaccharide.—Viscous Type I capsular polysaccharide was prepared by the method of Heidelberger, Kendall, and Scherp (8). Analyses of this preparation showed nitrogen 5.58 per cent; acetyl 7.35 per cent; and ash calculated as sodium 3.98 per cent. In 0.1 per cent solution in 0.85 per cent NaCl the polysaccharide had a relative viscosity of 1.22.[1] For the preparation of stock solutions the polysaccharide was dried to constant weight at low temperature and dissolved in saline (containing merthiolate 1-10,000). This solution was then diluted to give a final polysaccharide concentration of 0.1 per cent.

Determination of Specifically Precipitable Nitrogen.—Specific precipitates were prepared from the various sera studied by the addition to them of varying amounts of the capsular polysaccharide. The method of Heidelberger and Kendall (9) was followed and all precipitates were prepared in the cold and washed twice with ice-cold saline. In every instance increasing amounts of polysaccharide were added to constant amounts of serum and the total volume of each reacting mixture was kept constant by the addition of cold saline. The specific precipitates were digested and their nitrogen content determined by the gasometric micro method of Van Slyke (10) as modified by Kirk, Page, and Van Slyke (11). The precise details of the application of this method have been previously described (12). Duplicate precipitates were prepared and consequently the figures given for precipitated nitrogen in the various tables are the mean of two deter-

[1] These chemical studies were kindly carried out by Dr. Walther Goebel.

minations unless otherwise indicated. Aliquots of the initial supernatant solutions decanted from the precipitates after the first centrifugation were tested separately for the presence of uncombined polysaccharide and uncombined antibody. In each instance the same serum was used for these tests as had been used in the formation of the initial precipitates. In this way it was possible to determine the position of the equivalence zone for each serum.

Inasmuch as the precipitates were prepared from constant amounts of serum in each instance and since the volume of the reacting mixtures was kept constant, there was no appreciable variation in the concentration of lipids in the individual preparations. From the results of previous work it is therefore assumed that the amount of lipid adsorbed by each precipitate within any single experiment was constant. Consequently it has not been necessary to make any correction for the very small amount of lipid nitrogen in the precipitates since this would be expected to be constant for any particular serum.

Culture.—Pneumococcus Type I, original Neufeld strain. The culture is maintained in rabbit blood broth at 37°C. with transfers every 2nd day. Under these conditions the virulence of the culture remains practically constant, that is, there is an almost constant number of virulent organisms in a given volume of a culture of a given age. The virulence is such that 0.000,000,01 cc. of culture given intraperitoneally, produces a fatal infection in mice. This amount of culture usually represents 3 microorganisms.

Mice.—Throughout this work the so called Rockefeller strain of white mice has been used. This stock is highly inbred and the host factors involved are fairly well understood. All protection tests have been carried out with mice weighing from 17 to 23 gm.

Mouse Protection Test.—Tests for the protective potencies of the various sera were carried out by the method described by Kirkbride, Hendry, and Murdick (7) and the results analyzed by the method of Muench (cited by Lloyd, Theiler, and Ricci (13)).

This protection method differs from certain others in general use by the fact that a relatively large amount of culture is used as infective inoculum. This amount of culture is so chosen as to avoid the *Schwellenwert* and yet be in the range in which the titer limit follows roughly the law of multiple proportions (14). In order to determine the value of the unknown serum a simultaneous titration is carried out with a serum of known potency which serves as a standard for comparison. In practice the following technical procedure has been observed.

1. The culture is diluted in broth so that 0.5 cc. of the dilution contains 0.1 cc. of the original 18 hour blood broth culture. (This amount of culture contains approximately 30,000,000 pneumococci and in this instance represents a corresponding number of minimum lethal doses). The usual virulence controls are carried out.

2. At least three different dilutions of each serum are used in each test. The sera are progressively diluted × 2, the precise dilutions being determined by exploratory tests.

3. For the actual test at least 5 mice are injected with each combination of serum and culture. Each mouse receives 0.5 cc. culture dilution and 0.5 cc. of the appropriate serum dilution, these being mixed in the syringe and injected into the peritoneal cavity.

4. Mice surviving for 96 hours are considered as protected by the corresponding amount of serum.

Estimation of Protective Potencies

The most difficult feature of any method involving biological variables is the accurate evaluation of the results obtained. Various workers have adopted various end-points. Thus the minimum amount of serum which will protect 60 per cent of mice can be taken as the end-point. There are three objections to this and similar criteria. First, this method gives end-points which are as widely spaced as are the dilutions of serum, that is, only rough approximations of possible numerical values can be obtained. Secondly, the result is actually dependent on very few mice, those in the group receiving the particular protecting dilution and those of the group receiving the next lower amount of serum. Thirdly, results are not always sufficiently regular to permit an adequate determination of the end-point. Thus, with 5 or even 10 mice per dilution, two amounts of serum, one being twice the other, may result in the same percentage survival. Several attempts have been made to overcome this end-point difficulty. It seemed very desirable to obtain a sensitive numerical end-point based not on the small group of mice at any particular dilution level but rather one which would take into account the fate of all mice irrespective of the amount of serum.

Since the usual data, if plotted, give curves with much sharper slopes in the region of 50 per cent survival, it must be generally agreed that in this type of biological work this is the only logical end-point. After several different methods had been tried the so called Muench 50 per cent end-point accumulation method was adopted.

This method was devised by Dr. Hugo Muench of The Rockefeller Foundation and is extremely simple in application. It has been used in the analysis of the results of protection tests in yellow fever work by Lloyd, Theiler, and Ricci (13) and in studies on the titration of vaccine virus by Parker and Rivers (15). The latter authors discuss the validity of the method and the system used for obtaining the numerical end-point. In brief, the method is as follows: The survivals and deaths for each serum dilution are separated. The figures in each column are then accumulated, each column being added beginning at the smaller end. If the resulting figures are then plotted against serum dilutions the lines will cross at a point which represents the dilution of serum which should be used to bring

about a survival rate of 50 per cent. Actually it is unnecessary to plot these data since a simple calculation will provide the figure.

An illustration of the actual application of this method is provided in Table I. It will be noted that in this titration 10 mice have been used for each dilution of serum. The results in terms of percentage survival for each dilution are shown in column 4. When these figures are directly plotted a line connecting them forms a smooth curve and the 50 per cent end-point is easily determined. Results of this order are, however, very exceptional. In the fifth and sixth columns the survivals and deaths have been accumulated as described above. The percentage survival of each serum level has been calculated and is shown in column 7. On inspection it is obvious that the desired 50 per cent end-point lies between the serum dilutions 1–40 and 1–80. The exact point is obviously 23/46 or 0.50 of the distance between 1–40 and 1–80. Since the progression in serum dilutions is geometric it is necessary either to convert the basic dilution number (40 in this

TABLE I

Specimen Mouse Protection Test with Determination of 50 Per Cent End-Point by the Muench Accumulation Method

Serum dilution	Result			Accumulation			Calculated 50 per cent end-point
	Survived	Died	Survival	Survivals	Deaths	Survivals	
			per cent			*per cent*	
1–10	10	0	100	30	0	100	
1–20	9	1	90	20	1	95	
1–40	7	3	70	11	4	73	
1–80	3	7	30	4	11	27	1–56
1–160	1	9	10	1	20	5	

instance) into a log factor for multiplication, or much simpler in operation, to convert the factor 0.50 into a proportional factor by reference to a progression chart. The calculated end-point in this example is 1–56.

For the actual estimation of the potency of a given serum it is necessary to carry out protection titrations of the unknown serum and of the standard serum at the same time. This requirement tends to overcome the disadvantages of slight variations in the number of microorganisms in a culture from day to day. The end-point for each serum is then determined and by proportional calculation the potency of the unknown serum can be estimated. An example of actual titrations on the same serum at different times is given in Table II. Five independent titrations of an unknown serum have

TABLE II

Comparative Titrations for the Estimation of the Mouse Protective Potency of Lot AA Type I Antipneumococcus Horse Serum

Date	Standard Type I antipneumococcus horse serum (No. 425) 1000 units per cc.								Type I antipneumococcus horse serum lot AA								Estimated potency of serum AA units per cc.
	Dilutions	Results			Accumulation			Calculated 50 per cent end-point	Dilutions	Results			Accumulation			Calculated 50 per cent end-point	
		Survived	Died	Survival rate	Survivors	Deaths	Survivals			Survived	Died	Survival rate	Survivors	Deaths	Survivals		
				per cent			*per cent*					*per cent*			*per cent*		
Jan. 23	1–20	5	0	*100*	11	0	*100*		1–30	2	2	*50*	8	2	*80*		
	1–40	4	1	*80*	6	1	*86*	**1–64**	1–60	4	0	*100*	6	2	*75*	**1–87**	**1360**
	1–80	2	3	*40*	2	4	*33*		1–120	1	3	*25*	2	5	*29*		
	1–160	0	5	*0*	0	9	*0*		1–240	1	3	*25*	1	8	*11*		
Mar. 13	1–40	13	2	*87*	20	2	*91*		1–32	10	0	*100*	22	0	*100*		
	1–80	7	8	*47*	7	10	*41*	**1–70**	1–64	6	4	*60*	12	4	*75*	**1–109**	**1560**
	1–160	0	10	*0*	0	20	*0*		1–128	6	4	*60*	6	8	*43*		
									1–256	0	5	*0*	0	13	*0*		
Apr. 14	1–40	4	1	*80*	8	1	*89*		1–40	5	0	*100*	11	0	*100*		
	1–80	4	1	*80*	4	2	*67*	**1–94**	1–80	3	2	*60*	6	2	*75*	**1–137**	**1460**
	1–160	0	5	*0*	0	7	*0*		1–160	3	2	*60*	3	4	*43*		
Apr. 27	1–40	5	0	*100*	7	0	*100*		1–40	4	1	*80*	8	1	*89*		
	1–80	2	3	*40*	2	3	*40*	**1–71**	1–80	3	2	*60*	4	3	*57*	**1–90**	**1270**
	1–160	0	5	*0*	0	8	*0*		1–160	1	4	*20*	1	7	*13*		
May 6	1–40	3	2	*60*	3	2	*60*		1–40	2	3	*40*	6	3	*67*		
	1–80	0	5	*0*	0	7	*0*	**1–45**	1–80	2	3	*40*	4	6	*40*	**1–62**	**1380**
	1–160	0	5	*0*	0	12	*0*		1–160	2	3	*40*	2	9	*18*		
Mean number of mouse protective units per cc. of serum AA.....																	**1405**

been carried out simultaneously with titrations of the known or standard serum.

The data presented in Table II are fairly representative of the order of results generally obtained. Some of these titrations would present an impossible problem were it necessary to judge the end-points on a simple survival basis. Furthermore the results show the great advantages of simultaneous titrations of unknown and standard for although the actual end-points for each serum vary widely the variations are generally in the same direction for both sera at the same time. Thus when the actual unitage value is calculated one finds a rather close agreement considering the number of mice used in each titration. It is believed that the

TABLE III

Mouse Protective Potencies and Specifically Precipitable Antibody of Nine Type I Antipneumococcus Horse Sera

Group	Serum lot	Character	Protective units per cc.	Maximum specifically precipitable nitrogen	Protective units per mg. of specifically precipitable nitrogen	Mean protective ratio for each group
				mg. per cc.		
A	58	Raw, single	440	0.765	575	540
	57	Raw, single	550	1.018	540	
	50	Raw, pooled	760	1.510	505	
	14	Concentrated	3145	5.798	545	
B	49	Raw, pooled	500	0.645	775	800
	59	Raw, single	870	1.081	805	
	60	Raw, single	1000	1.229	815	
	AA	Raw, pooled	1405	1.844	760	
	13	Concentrated	6100	7.125	855	

mean value, 1405 protective units per cc., is as representative of the actual protective potency of the serum as any figure obtainable by any biological method. The probable error involved in titrations such as that illustrated with five separate determinations is about 10 per cent although no accurate estimate of this factor can be made without much longer series of titrations.

Antibody Content of Type I Antipneumococcus Horse Sera

With a measure of validity having been shown for the method of estimation of protective potencies and for the analysis of the results it is now possible to proceed to a comparison of the protective potency and the amount of specific antibody (precipitin) demonstrable *in vitro*.

These two forms of experimental analysis have been carried out on nine Type I antipneumococcus horse sera and the results are shown in Table III.

For the estimation of mouse protective potencies several titrations were carried out with each serum, the average number in this series being slightly over 4. The average number of mice per serum, exclusive of those involved in the simultaneous titration of the standard serum, was 73. These repeated titrations were necessary in order to secure numerical end-points with some degree of precision. Similarly the estimations of maximum specifically precipitable nitrogen represent data secured from complete precipitin curves, each point on which represents an average of determinations on two duplicate precipitates.

The nine antipneumococcus horse sera included in Table III fall into two groups dependent upon the number of mouse protective units per mg. of specifically precipitable nitrogen (hereinafter termed the *protective ratio*). Thus group A includes four sera, both raw and concentrated, with potencies ranging from 440 to 3145 units per cc. of serum. In the number of units per mg. of specifically precipitable nitrogen (protective ratios) however, these sera differ but slightly, the range being from 505 to 575 with a mean of 540 units per mg.

In contrast are the results with the sera of group B. Although the mouse protective potencies of these sera range from 500 to 6100 units per cc., the protective ratios are rather uniform ranging from 760 to 855 units per mg. with a mean value of 800.

It is perhaps merely fortuitous that the mean protective ratios of these two groups of Type I antipneumococcus horse sera stand in the approximate relation of 2:3. It does not seem likely however that the actual protective ratios of the various sera are matters of chance. The difference between the results of the two groups is considerably greater than the outside probability of error in either protection or nitrogen estimations.

Several possible sources of error do, however, exist. The validity of the mouse protection titrations has already been discussed, and while some allowance must be made for the probability of error in each instance, this possible deviation is not sufficiently great to account for the differences between the two groups. A second possibility is that the amounts of specifically precipitable nitrogen do not accurately represent the amounts of specific antibody. These determinations were, however, very carefully carried out and the results of extensive checking show that with any given lot of pneumococcus polysaccharide the maximum amount

of specifically precipitable nitrogen as determined by the standard procedure is a fixed characteristic of a given serum. Whether one is warranted in assuming that this precipitable nitrogen represents only specific antibody is another matter. It has been shown by Heidelberger and Kendall (16), for example, that if Type II capsular polysaccharide is added to a serum containing both Type I and Type II antibodies, both will be found in the resulting precipitates. An example of a quantitative experiment designed to determine the extent of this non-specific precipitation is shown in Table IV. Type I capsular polysaccharide was added to Type I and Type II sera separately and after these had been mixed. It will be noted that while the addition of Type I capsular polysaccharide to the Type II serum gave no precipitate the addition of the carbohydrate to the mixture of the two sera gave a nitrogen result higher by 7.2 per cent than was obtained by the addition of the polysaccharide to the Type I serum. This is regarded as a sig-

TABLE IV

The Influence of Heterologous Antibodies on the Apparent Amount of Specifically Precipitable Nitrogen

Type I antipneumococcus horse serum	Type II antipneumococcus horse serum	Type I capsular polysaccharide	Precipitable nitrogen
cc.	*cc.*	*mg.*	*mg.*
1.0	—	0.25	1.490
1.0	1.0	0.25	1.597
—	1.0	0.25	0.000

Precipitation carried out at 4°C. according to the method of Heidelberger and Kendall (9).

nificant increase but the amount is not great enough even under the conditions of this experiment to affect seriously the general results shown in Table III.

Antibodies other than the type specific anticarbohydrate are present in practically every Type I antipneumococcus horse serum. Some of these, such as those directed against the pneumococcus protein and the somatic carbohydrate (C substance) are present in amounts so small as to affect the results only to a very slight extent even were a considerable proportion included in the immune precipitate. Tests as to the monovalence of the immune horse sera were carried out and it was found that only one, No. 58, gave any reaction with the capsular polysaccharides of other pneumococcus types; this serum had antibodies which reacted with the capsular polysaccharide of Type II Pneumococcus, although the reaction was faint. It would therefore appear that the quantitative results have not been seriously affected by non-specific reactions.

Since there appears to be no obvious experimental error sufficient to account for the observed differences between the two groups of

immune horse sera another explanation must be sought. In the absence of definitive evidence any explanation must, however, be of a hypothetical nature.

As a first possibility it may be that certain of these sera contain substances other than the specific antibody which largely condition the *in vivo* efficacy of these antibodies. From the results of studies on the relation of lipids to immunological reactions, one might hold that an abnormal lipid pattern would tend to inhibit the protective action of the serum (17). This possibility seems somewhat unlikely, however, in view of the fact that each group included both pooled sera and sera from single bleedings. Furthermore if some inhibitor were present in the sera of group A it would seem unlikely that the amount would be so constant with respect to the amount of precipitable antibody as to give the consistent results obtained. If the inhibitor were intimately and quantitatively associated with the antibody molecules this hypothesis might be tenable.

TABLE V

Mouse Protective Potencies and Specifically Precipitable Antibody of Seven Type I Antipneumococcus Rabbit Sera

Serum lot	Protective units per cc.	Maximum specifically precipitable nitrogen	Protective units per mg. of specifically precipitable nitrogen	Mean protective ratio
		mg. per cc.		
22	1070	0.938	1140	
28	1130	0.986	1145	
27	1190	1.108	1075	
A7	1270	1.128	1125	1170
26	1390	1.116	1245	
25	1830	1.533	1195	
24	1910	1.510	1265	

All lots represent pooled bleedings and the sera have not been concentrated.

A second explanation is that certain horses may produce antibodies having greater protective values. Here again the matter of sera from single and pooled bleedings would seem to make this possibility slight.

A third possibility is that the antibodies in a given immune horse serum, although reacting with the specific polysaccharide, may in fact represent a series of substances possessing different avidities, different degrees of specificity, different protective values, and perhaps even different chemical properties. It is possible that an immune serum may contain a mixture of specifically reactive antibodies varying in one or more of these properties.

Antibody Content of Antipneumococcus Rabbit Sera

Determinations of protective potencies and of maximum amounts of specifically precipitable nitrogen have been carried out with seven Type I antipneumococcus rabbit sera. The results of these determinations are presented in Table V.

The estimations of protective potencies are based on an average of 4.7 titrations per serum, with an average number of 77 mice per serum, exclusive of those involved in the simultaneous titration of the standard serum.

It will be noted that the protective values of the immune rabbit sera range from 1070 to 1910 units per cc. but that the number of protective units per mg. of specifically precipitable nitrogen is relatively constant, averaging 1170. Thus Type I antipneumococcus rabbit sera appear to be uniform in their protective ratios. This result stands in contrast to those obtained with antipneumococcus horse sera.

There is reason therefore to believe that in the case of immune rabbit serum the protective potency can be directly estimated from determinations of the maximum amount of specifically precipitable protein. This, however, is true only under certain definite conditions. It has been clearly pointed out by Heidelberger, Kendall, and Scherp (8) that various preparations of capsular polysaccharide vary in their capacity to precipitate the antibodies of antipneumococcus rabbit serum. This variation is so great that it is necessary to test each polysaccharide preparation. It is believed, however, that if a given polysaccharide preparation is standardized against one serum and if this serum has been fully appraised in a mouse protection test, one should be able to standardize all other immune rabbit sera of the same type on the basis of the known precipitating properties of the given lot of polysaccharide. It is known, for example, that the lot of polysaccharide used in these experiments will precipitate approximately 92 per cent of the specific protective antibody from a given antipneumococcus rabbit serum.

The general differences between the protective ratios of immune horse and immune rabbit sera can perhaps be partially explained. With Bauer (18) it has been shown by ultrafiltration that the horse antibodies are in general larger than those of immune rabbit serum. Heidelberger and Pedersen (19) have demonstrated similar differences by the use of the ultracentrifuge. Although in certain details the results by the two methods are not in complete accord, there is now little doubt but that a difference in size does exist. If the rabbit antibody is actually smaller than that of the horse there should be in a given mass of rabbit antibody a much greater reactive surface and therefore one would expect that a

greater amount of carbohydrate could be bound in proportion to the amount of antibody. At the present time it is not possible to conclude that this explanation accounts for the observed differences in protective ratios, but the facts are suggestive.

SUMMARY

The ability to carry out with some measure of precision mouse protection tests for the estimation of potency of antipneumococcus sera has made possible the correlation of the protective potency with the amount of specifically precipitable protein. With antipneumococcus rabbit sera these protective ratios are relatively constant and higher than those with immune horse serum. Type I antipneumococcus horse sera, on the other hand, show no such constancy but fall into two groups; and there is as yet no simple method for determining to which group a serum belongs.

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