ANTIPROTEINS IN HORSE SERA

II. ANTIBODIES TO PNEUMOCOCCUS NUCLEOPROTEIN AND THEIR REACTION WITH ANTIGEN*‡

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The isolation of bacterial toxins in a state of high purity anu their identification as proteins (1) have made it evident that the long known and much studied antitoxins which appear in the sera of toxin-injected horses represent the classical form of antiprotein elaborated by this animal. The *in vitro* reactivity of antitoxins, as typified by the Ramon flocculation titration, is characterized by a narrow zone of specific precipitation with toxins. The region of insoluble compounds corresponds to the equivalence zone (2) of the precipitin reaction. This precipitin type of interaction occurs between a multitude of antigens and homologous antibodies formed in the rabbit, as well as between the specific polysaccharides of pneumococci and the corresponding anticarbohydrate in both rabbit and horse antisera to type-specific pneumococci. The reaction does not show inhibition in the zone of antibody excess.

In view of these differences it became of interest to study the reaction between a typical, non-toxic protein and the antibodies engendered by it in the horse, and an antiserum to crystalline egg albumin (Ea) was prepared (3). This was found to give the reaction pattern of an antitoxin with Ea (3, 4). Subsequently, a similar course was noted for the interaction of hemocyanin and certain samples of antihemocyanin produced in the horse (5). Since, however, the anticarbohydrate in antipneumococcus horse sera gives typical precipitin reactions with the specific polysaccharides of pneumococcus (6), it was thought desirable to characterize, if possible, the reaction of pneumococcus nucleoprotein, one of the group-specific antigens of pneumococci (7), with antinucleoprotein formed in the horse. Many potent antipneumococcus horse sera were tested, but in all of these the immune substances consisted mainly of type-specific anticarbohydrate and smaller quantities of antibodies to "C-substance" (8, 9). Finally, measurable amounts of antiprotein were found in a serum, No. 16,[1]

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‡ The first paper of this series, although not numbered as such at the time, is that given in reference 4.

[1] Kindly made available in 1939 by Dr. Alvan L. Barach, of this Department.

which had been obtained some years previously by intravenous injection of a horse with the pleural exudate resulting from the intratracheal injection of horses with virulent Type I pneumococci (10). The study of this serum is described below.

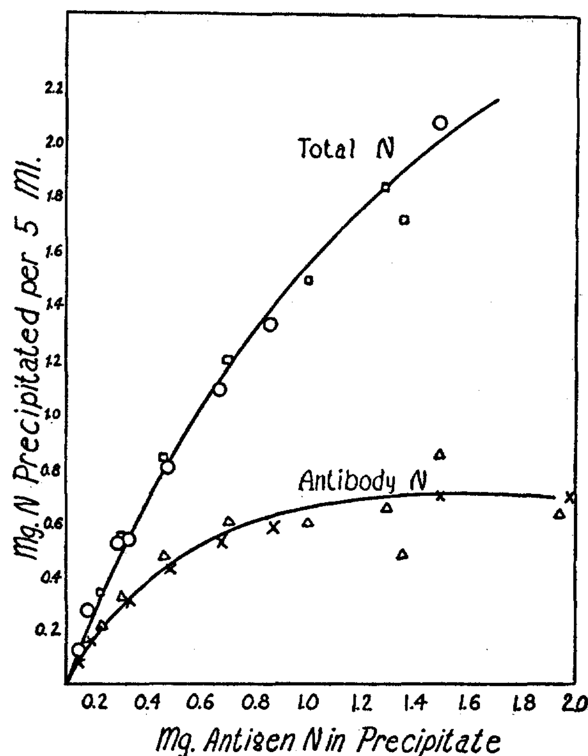

FIG. 1. Squares, circles represent total N in specific precipitate with protein from pneumococcus I R (Dawson S), II R (S), respectively.

Triangles, crosses refer to antibody N precipitated by pneumococcus I, II protein, respectively.

EXPERIMENTAL

Materials and Methods

Pneumococcus Nucleoprotein[2].—R (Dawson S) strains derived from Type I and Type II pneumococci were separately grown for 15 to 20 hours in meat-infusion-phosphate-glucose broth, centrifuged off, and, with or without a preliminary washing with saline, were extracted in the cold for 10 to 12 hours with excess 0.01 N sodium hydroxide solution. After centrifugation the clear supernatants were acidified with acetic acid to maximum flocculation. The precipitates were centrifuged off, redissolved with water and N sodium hydroxide solution until just pink to phenolphthalein, centrifuged, and reprecipitated. After several repetitions of

[2] These preparations were made by Dr. E. A. Kabat in 1933–34.

this process each solution was filtered through a Berkefeld V candle and reprecipitated by means of acetic acid. The product was washed with water and redistilled acetone and dried *in vacuo* at room temperature. Solutions were made by suspension of weighed samples in a little water, addition of a slight excess of N sodium hydroxide, neutralization after maximal solution had been attained, and centrifugation from insoluble material. The resulting solutions were diluted with 0.9 per cent saline and analyzed for nitrogen by the micro-Kjeldahl method. It was assumed, for purposes of calculation, that all of the nitrogen was contained in the nucleoprotein and was available for the precipitin reaction.

TABLE I

Precipitation of Antipneumococcus Type I Horse Serum 16 with Nucleoprotein from Type I and Type II Pneumococci

0°C., 48 to 96 hours; calculated to 5.0 ml. serum

Amount Type I pneumococcus nucleoprotein nitrogen added	Nucleoprotein N precipitated*	Total N precipitated	Antibody N precipitated	Antibody N:antigen N in precipitate*	Tests on supernatants
mg.	*mg.*	*mg.*	*mg.*		
0.123	Total	0.346	0.223	1.8	Excess A, no NP
0.205	"	0.542	0.337	1.6	" " " "
0.360	"	0.842	0.482	1.3	" " " "
0.600‡	"	1.214	0.614	1.0	" " " "
0.900§	"	1.495	0.595	0.66	Trace A, " "
1.34‖	1.10¶	1.72	0.62	0.56	Excess NP
3.60**	2.33¶	2.94	0.61	0.26	" "
Amount Type II protein N added					
0.190	Total	0.528	0.338	1.8	Excess A, no NP
0.760	"	1.34	0.58	0.76	No A or NP

NP = nucleoprotein.

* On the assumption that all of the N added is active as precipitinogen.

‡ 3.0 ml. serum and equivalent quantity of antigen actually used.

§ 2.0 ml. serum and equivalent quantity of antigen actually used.

‖ 1.5 ml. serum and equivalent quantity of antigen actually used.

¶ After deduction of the quantity of antigen N found in supernatant by analysis of aliquot portions with 5.0 ml. each of fresh serum.

** 1.0 ml. serum and equivalent amount of antigen actually used.

Antibody Determinations.—These were carried out quantitatively (6, 11) by addition of accurately measured amounts of nucleoprotein in duplicate to known volumes of the antiserum at 0°C. After the contents were mixed, the tubes containing the analyses were allowed to stand in the cold for 48 to 96 hours, with occasional mixing, and were centrifuged in the cold[3] and washed twice with cold saline. Nitrogen was estimated by a modification of the micro-Kjeldahl method. When antibody was present in excess, the amount of nucleoprotein nitrogen added was deducted from the total N precipitated and the difference was arbitrarily considered antibody N. In the region of excess antigen separate analyses were made of the amount of antigen in the supernatants by addition of these or aliquot portions to fresh por-

[3] In a refrigerated centrifuge manufactured by the International Equipment Co., Boston, Massachusetts.

tions of antiserum. From the quantity of total nitrogen precipitated, the amount of excess antigen could be read from the curve (Fig. 1) in the region of excess antibody and deducted from the total added. The two values in column 2 of Table I were obtained in this way. In the table are given selected data illustrating the course of the reaction with Type I and Type II nucleoprotein, and these and other data are plotted in the curve in the text-figure.

In addition to antiprotein, horse serum 16 contained appreciable antibody to pneumococcus C-substance. This was removed by addition of a slight excess of C-substance derived from Type II pneumococci before any of the analyses were made. The serum also contained 0.8 mg. of antibody nitrogen per ml. to Type I specific polysaccharide, considerably more than the antiprotein content. Prior removal of the type-specific anticarbohydrate led to fluctuations in the quantity of antiprotein precipitated which could not be studied owing to lack of antiserum, but the reaction still retained the characteristics of the precipitin type.

DISCUSSION

In the production of most antipneumococcus horse sera, intact Gram-positive type-specific pneumococci are injected intravenously, and relatively little antiprotein is produced. However, an exudate was used (10) in the preparation of the serum employed in this study. This presumably contained many autolyzed pneumococci and therefore free nucleoprotein, as well as intact cells, and the appreciable content of antiprotein in the serum is possibly due to this circumstance.

It is apparent from the data in the table and figure that the reaction between pneumococcus nucleoprotein and the antiprotein in this serum was of the precipitin type. It is also to be noted that the antigen had been injected intravenously. The relation of the route of injection to the antitoxin or precipitin type of antibody response in horses will be discussed more fully in the two following papers. However, antitoxic horse sera are usually produced by subcutaneous or intramuscular injections of toxin, and it was similarly found unsatisfactory to employ the intravenous route (3) in the preparation of the anti-egg albumin horse serum in which the flocculation reaction was of the toxin-antitoxin type. It was also determined in a portion dialyzed by Dr. H. P. Treffers against 0.02 M phosphate buffer at pH 6.8 that the antiprotein in antipneumococcus horse serum 16 was mainly in the water-insoluble fraction. This contrasts with the water-soluble character of the antitoxin type of antibody but resembles the anticarbohydrates in horse sera. These also give the precipitin type of reaction.

Little significance is attached to the actual magnitude of the antibody N: antigen N ratios given in Table I, since these were derived by assuming that all of the nucleoprotein nitrogen added was antigenic. This is by no means certain, nor is it likely, from what is known of other bacterial proteins, that the material used was a single substance. A rough attempt at fractionation carried out in this laboratory by Miss Graciela Leyton-Ramirez of Santiago, Chile, showed that the fraction of Type I protein precipitated at one-fifth saturation with sodium sulfate was a slightly more active precipitant of the serum than the

portion separable on acidification of the sodium sulfate supernatant, but the difference was small. Also, preparations of Type I and Type II nucleoprotein behaved remarkably alike toward the antiserum.

SUMMARY

The antiprotein in an antipneumococcus horse serum resulting from intravenous injections of infected pleural exudate showed a precipitin type of reaction with pneumococcus nucleoprotein rather than the antitoxin type of response.

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