IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

I. Estimation of A and B Isoantibodies in Human Serum by the Quantitative Precipitin Method*

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Purified blood group A and B substances have not been known to give precipitin reactions with human sera containing homologous isoagglutinins although they are capable of combining with antibody as evidenced by their capacity to neutralize or inhibit isohemagglutination (1–3). The A and B substances have, however, been found to be antigenic in man (4) and have been used to produce sera of high titer for blood grouping. The recent successful development and application of micro precipitin methods (5) for the quantitative estimation of the small amounts of antibody present in human sera during convalescence from pneumonia (6) or meningococcal meningitis (7), suggested the use of these procedures for detecting the precipitation of isohemagglutinins by homologous blood group substances. With a sample of A substance obtained by the phenol method of Morgan and King (8) it was possible to demonstrate small but significant amounts of precipitin in sera of individuals of blood groups O and B, whereas none or only traces were present in A or AB sera. Injection of human beings of blood group O with A and B substances resulted in increases in isohemagglutinin titer accompanied by striking increases in the amounts of precipitin. That these specific precipitates actually contained the isoagglutinins was conclusively demonstrated by recovering anti-A by dissociation of the specific precipitate with 15 per cent sodium chloride solution (9).

EXPERIMENTAL

Blood samples were obtained from a number of normal individuals of each blood group, preferably several hours after meals, to avoid large amounts of lipid. Two samples of serum were usually obtained 1 to 2 weeks apart and are designated by subscripts X and XX. A number of these individuals were given two subcutaneous injections of 1 ml. each of the A and B preparation distributed by Eli Lilly and Company (containing about 10 mg. A and B substances in 10 ml.). Injections were given 1 day apart. Ten days after the second injection another blood sample was obtained and designated by subscript 1. Subsequent samples after immunization were numbered in order. Sera were preserved by addition of 1:10,000 merthiolate.[1]

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[1] Manufactured by the Eli Lilly Co., Indianapolis.

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The hemagglutinin titer of the sera for A or B cells was measured by adding twofold serial dilutions of serum to an equal volume of a 4 per cent suspension of washed A or B erythrocytes. After incubation for 1 hour at 37° C. the tubes were centrifuged lightly and read. The titer was expressed as the reciprocal of the highest dilution of serum in which detectable agglutination is noted.

The relative potency of sera containing anti-A was also assayed by measuring the minimum amount of a given preparation of A substance required to inhibit completely the hemagglutination of 0.1 ml. of a 4 per cent suspension of washed A erythrocytes by 0.1 ml. of serum. Neutralizing capacity for B substance was not measured since only mixtures of A and B substances were available.

The blood group A substance was prepared as described by Morgan and King from hog gastric mucin by extraction with 90 per cent phenol and precipitation by addition of alcohol to a final concentration of 10 per cent by volume (8). Three phenol extractions and precipitations were carried out. This product, preparation 1A, was used for hemagglutination inhibition tests. It was further purified by shaking a solution in dilute sodium acetate with chloroform and butyl alcohol in order to remove a portion of the accompanying proteins (Sevag). The A substance was precipitated from the aqueous phase with alcohol after separation of the chloroform emulsion by centrifugation (10). This product, 2A, was used for the quantitative precipitin tests. Precipitin tests were also carried out with the mixture of A and B substances used for immunization.

Microquantitative precipitin tests were carried out using the technic developed by Heidelberger and MacPherson (5) and described in detail in reference 6. Sterile glassware were used and all solutions contained preservative. Before setting up tests, sera in 15 ml. conical pyrex centrifuge tubes were centrifuged in the refrigerated centrifuge at 2000 R.P.M. for 2 to 6 hours. Any fat at the surface of the serum was sucked off with a pipette and the serum was decanted, taking care to avoid dislodging any precipitate at the bottom of the tube. One to 3 ml. portions of serum were pipetted into three 8 ml. conical centrifuge tubes. To two of these 0.05 mg. of A substance (2A) was added in a volume of 0.5 ml., the third tube serving as a blank. The contents of the tubes were mixed by rapid twirling and capped with sterile rubber caps. The tubes were then incubated at 37°C. for 1 hour and placed in the ice box for 1 week, with daily mixing. With group 0 sera small amounts of precipitate were usually noted after several days; with sera of immunized individuals of group 0, smaller volumes of serum (0.5 to 1.5 ml.) may be used and in some instances precipitation may be noted after 48 hours. After 1 week the tubes were centrifuged in the cold, and the precipitates and blanks washed twice in the cold with 3.0 ml. portions of chilled saline. Precipitates were finally dissolved with a few drops of 0.5 N NaOH and made up to 2.5 ml. with water. The 8 ml. conical centrifuge tubes were calibrated and marked at 2.5 ml. prior to use. One or 2 ml. aliquot portions of the dissolved precipitates were analyzed for nitrogen with the Folin-Ciocalteau tyrosine reagent (5), using a calibration curve with human gamma globulin as a standard. Since the A substance used gives no color with the Folin-Ciocalteau tyrosine reagent, the values obtained represent antibody N directly.

The supernatant serum can be tested for anti-A by hemagglutination tests or for the presence of excess A substance by assaying its capacity to inhibit hemagglutination of A erythrocytes when added to 0 serum of known neutralizing capacity for A substance.

The anti-B content can also be determined by addition of the A and B mixture to the supernatant serum after removal of the anti-A with A substance, or as the difference between total anti-A + anti-B N and anti-A N, measured with the Lilly A + B mixture and preparations such as 2A respectively.

With the sera drawn after immunization quantitative precipitin curves (*cf.* 11, 12) were obtained by the addition of increasing amounts of preparation 2A to a series of tubes con-

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taining a measured volume of antiserum. Analyses and tests on supernatants were carried out as described above.

RESULTS

Table I summarizes data on the amounts of nitrogen specifically precipitable by A and B substances from sera of individuals of the four blood groups before and after immunization with A and B substances. The hemagglutination titers of the various sera for A and B erythrocytes are also given, as well as data on the neutralizing capacity of the sera for A substance as measured by inhibition of hemagglutination of A erythrocytes. With the samples obtained before immunization, it is apparent that five of the six group O sera showed more than 2 μg. of precipitable anti-A nitrogen per ml. whereas the A and AB sera all contained less than 1 μg. of anti-A N per ml. Two of the three B sera contained a significant amount of precipitable anti-A N. Values of 0.7 to 1 μg. of N per ml. are about at the limit of experimental error. Data in the table are given to the tenths of micrograms per milliliter of serum because 3 ml. samples of serum were usually used.

Sera obtained after immunization of O individuals showed a striking increase in the amount of N precipitable by A and by B substances accompanied by parallel increases in hemagglutination titer with A and B cells and in neutralizing capacity for A substance. For example, after immunization the N precipitable specifically by A substance from the serum of E. K. increased from 2.4 or 3.2 to 54 μg./ml. while the anti-A agglutinin titer increased from 8 to 16 to 512 and the neutralizing capacity of the serum for the A substance used increased from 0.5 to 90 μg. per 0.1 ml. serum. Similarly, the increase in anti-B agglutinin titer from 16 to 128 was accompanied by an increase in anti-B specifically precipitable N from 3.0 to over 28 μg. per ml. The minimum amount of antibody N giving agglutination of A or B erythrocytes, obtained by dividing the antibody N content of the serum by the titer, ranged from 0.1 to 0.2 μg. for all the sera studied from immunized individuals.

On immunization of A individuals with the Lilly A and B substances, slight increases in the amounts of N precipitable with A substance were found, 2.5 and 5.8 μg. N per ml. being obtained in two instances. However, these sera neither agglutinated A erythrocytes nor did they neutralize A substance. With serum M. $S._1$ the supernatant, after removal of the 5.8 μg. N of precipitate, still contained all of the added A substance activity as assayed by agglutination inhibition tests. On addition of this supernatant to 0.5 ml. E. $K._2$ (*cf.* Table II) it could be shown to possess 89 per cent of the precipitating power of the A substance added.

By the addition of increasing amounts of A substance to a given volume of serum, typical quantitative precipitin curves were obtained (Table II). Tests on the supernatants after removal of the precipitate indicated that A sub-

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TABLE I

Relation between Precipitable Nitrogen, Isoagglutinin Titer, and Neutralizing Capacity of Sera from Normal and Immunized Individuals of Different Blood Groups

	Anti-A				Anti-B		
Individual	Antibody N specifically precipitable per ml. serum	Titer	Minimum amount of anti-A N giving agglutination	A neutralized per 0.1 ml. serum	Antibody N specifically precipitable per ml. serum*	Titer	Minimum amount of anti-B N giving agglutination
	μg.		μg.	μg.	μg.		μg.
Blood group O							
E. K. x	2.4	8–16		0.5		16	
xx	3.2	4–8		0.5	3.0	16	
1	54	512	0.1	90	28	128	0.2
2	55	512	0.1	100		128	
F. P. x	2.5	16–32		2.1		8	
xx	2.2	8		2.1		8	
1	13.6‡	128	0.1	15		64	0.2
M. C. x	5.5	16		6		16	
xx	4.2	16–32		6			
1	10	64	0.2	10		16	
A. N. x	3.5	8		0.5		4	
xx	2.6	4		0.4		4	
S. K. x	0.8	8		0.6		4	
A. D. x	3.3	4		1.5	1.6	8	
1	63	256	0.2		16.4	128	0.1
Blood group A							
M. S. x	0.4	0				4	
xx	1.0	0		0	0.6	4	
1	5.8	0		0	5.0	64	0.1
A. B. x	0.7						
xx	0.0	0			0.7	2–4	
1	2.5	0		0	2.6	32	0.1

* Determined either on the supernatant after removal of anti-A, or as the difference between the total anti-A+B and anti-A using separate portions of serum.

‡ 13.2 μg. N/ml. was obtained after prior removal of the complement from another portion of serum by means of the precipitate formed by addition of egg albumin and rabbit anti-egg albumin (6).

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TABLE I—*Concluded*

	Anti-A				Anti-B		
Individual	Antibody N specifically precipitable per ml. serum	Titer	Minimum amount of anti-A N giving agglutination	A neutralized per 0.1 ml. serum	Antibody N specifically precipitable per ml. serum*	Titer	Minimum amount of Anti-B N giving agglutination
	μg.		μg.	μg.	μg.		μg.
Blood group A—*continued*							
Gl.$_x$	0.7					16	
xx	0.5	0			0.9	8	
1	1.5	0			9.8	64	0.2
Fr.$_x$	0.5					4	
Blood group B							
A. K.$_x$	1.1	2–4		0.4	0.4	0	
1	9.5	64	0.2	7.5	0.1	0	
M. M.$_x$	0.8	8		0.5			
S. E.$_x$	1.4	16–32		0.7			
Blood group AB							
T. P.$_x$	0.2	0				0	
H. S.$_x$	0.3	0			0.9	0	
xx	1.0	0				0	
1	0.6	0			1.4	0	

stance, as measured by agglutination inhibition tests, and anti-A, as measured by agglutination tests, did not coexist in the same supernatant fluid. As increasing amounts of antibody N were precipitated, the agglutinin titer of the supernatant decreased progressively. Appearance of A substance did not occur in the supernatant until all of the antibody had been precipitated.

It was also possible to demonstrate that the precipitate formed on addition of A substance to E. K.$_2$ serum actually contained anti-A. Ten ml. of E. K.$_2$ were treated with 0.5 mg. A substance (preparation 2A). After 3 days in the ice box, the precipitate was washed 4 times with 0.9 per cent saline. No agglutinins for A cells could be detected in the fourth washing. The precipitate was suspended in 1 ml. of 15 per cent NaCl solution and warmed at 37° C.

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for 1 hour. After centrifugation samples of 0.01 and 0.0007 ml. diluted to 0.5 ml. and made isotonic, agglutinated 0.1 ml. of a 4 per cent suspension of A erythrocytes.

TABLE II

Addition of Increasing Amounts of A Substance to a Given Volume of Serum from Individuals of Blood Group O after Immunization with A and B Substances

Amount A added (preparation 2A)	Antibody N precipitated	Tests on supernatant	
		Hemagglutination for A cells	A substance in entire supernatant*
μg.	μg.		μg.
1.0 ml. E. K.$_{1}$ serum used—original titer 512, total volume 3 ml.			
25	18.1	32	0
50	37.2	8	0
75	43.6	2	0
100	47.6	1	0
150	55.8	0	1–2
200	58.5	0	15
0.5 ml. E. K.$_{2}$ serum used—original titer 512, total volume 2.5 ml.			
10	9.2	16	0
30	21.5	4	0
50	25.3	2	0
75	27.4	0	0
100	26.8	0	2
150	28.2	0	6
1000	9.6		
Salt	0.0	64	
1.0 ml. F. P.$_{1}$ serum used—original titer 128, total volume 2.5 ml.			
10	2.8	4	0
30	8.0	0	0
50	9.9	0	6
75	10.7	0	>12
100	10.8	0	>12
Salt	0.0	32	

* By inhibition of hemagglutination (p. 208).

DISCUSSION

Chemical studies on the blood group specific substances have been hampered by the lack of precise methods of assay, so that alterations in activity as a result of the process of preparation have been difficult to detect. In addition, the standardization of sera and solutions containing isoagglutinins A and B has been restricted to the usual serological determinations of titer, procedures

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which are subject to errors of ±50 to 100 per cent. The lack of absolute methods of assay has also created difficulties in comparing the results of different investigators.

By taking advantage of the newer microquantitative precipitin technics (5, 6), it has been possible in the present investigation not only to demonstrate that precipitin reactions occur between suitable samples of A substance and anti-A, and between B substance and anti-B, but also to use these methods for the measurement of the amounts of anti-A and anti-B in the sera of normal individuals and of persons injected with A and B substances.

The specificity of the method, that is, that the isoagglutinins are actually precipitated, is indicated by the following observations. Purified A substance precipitates significant amounts of antibody from sera of most normal individuals of group O and some of group B whereas negligible amounts of antibody are precipitated from sera of normal individuals of groups A or AB. After immunization with A and B substances, the increase in amount of antibody N precipitable by A substance from sera of individuals of groups O and B is roughly in proportion to the increase in titer (Table I). Similarly, increases in amounts of antibody precipitable by B substance (from the supernatants after removal of the anti-A) are found in the sera of persons of groups O and A. Within experimental error, the minimum amounts of anti-A and anti-B nitrogen giving homologous isoagglutination are similar in the sera studied both before and after immunization. Dissociation with 15 per cent salt of a washed specific precipitate formed by adding A substance to group O immune serum yielded a solution containing a high titer of anti-A agglutinins.

The sera of some individuals of group A who were immunized with A and B substances showed small amounts of precipitin when tested with A substance although they did not agglutinate A erythrocytes. These antibodies were probably formed to some antigenic impurity present in the A and B substances used for immunization, since little or none of the added A was found to be removed with the precipitate within experimental error as tested both by agglutination inhibition tests and by quantitative precipitin tests on the supernatants with group O serum. It is also possible that such non-specific antibodies were also formed in the immunized individuals of groups O and B. The proportion of antibodies to heterologous contaminating species-specific substances present is as yet unknown. The antibody nitrogen values thus represent a trend rather than the actual amounts of anti-A and anti-B. Determination of the actual amounts of anti-A must await the preparation of the A substance in purer form or its isolation from human sources.

Traces of precipitate may sometimes be observed on addition of A substance to sera of immunized individuals of blood groups A and AB. These traces of precipitate are below the limits of measurement of the quantitative precipitin technic (less than 1 μg. N per ml.). Whether they represent non-specific reactions or are related to antibodies of the A subgroups is as yet unknown.

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The precipitin curves obtained with A substance and the anti-A formed by immunization of group O individuals appear to be of the same general type as those obtained with the type-specific pneumococcal carbohydrates or with purified proteins (*cf.* 6, 11, 12). They are characterized by a region in which some antibody remains in the supernatant, an equivalence zone in which neither antibody or A substance can be detected in the supernatant, a region of antigen excess, and an inhibition zone (Table II).

From the amounts of A substance and antibody N precipitated in the region of excess antibody (Table II), the ratio of antibody N/A substance in the precipitate can be calculated, assuming that all of the A substance added is contained in the precipitate. The ratios varied from 0.7 in the antibody excess region to about 0.3 at the point of maximum precipitation. These ratios are much lower than those obtained in most other antigen-antibody systems, with materials of similar chemical nature. For example, the ratios of antibody N/polysaccharide are of the order of 5 for the type-specific pneumococcal polysaccharides and their homologous human antibodies. If the true antibody N/A substance ratios are of this order, it would indicate that only about 10 per cent of the weight of the A preparation added actually consisted of true A substance. This would not be unexpected, since the hog stomach, stomach lining, or gastric mucin used as a source for the preparation of the A substance might well contain other inert materials.

The quantitative precipitin method should be of considerable value as a guide to chemical fractionation and purification of the blood group specific substances, should make possible precise standardization of sera containing isoagglutinins, and should render feasible accurate studies of the variations in isoagglutinin levels with time and under various experimental conditions. It should also make it possible to determine whether or not the A and B substances from animal and human sources are immunochemically identical.

SUMMARY

1. With purified blood group A and B specific substances from the gastric mucin of pigs precipitin reactions can be obtained in sera containing homologous isoagglutinins. By microquantitative precipitin technics the anti-A and anti-B content of normal and immune isoagglutinin-containing sera can be estimated on a weight basis.

2. There was a general correlation between antibody N precipitable from the sera of groups O and B by A substance and the agglutinin titer for A erythrocytes before and after immunization with blood group substances.

3. The reaction between A substance and its homologous isoagglutinin present in the sera of immunized individuals was of the same type as in other antigen-antibody systems.

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