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## ISOAGGLUTININS ASSOCIATED WITH ABO ERYTHROBLASTOSIS \*

By S. KOCHWA, R. E. ROSENFELD, L. TALLAL AND L. R. WASSERMAN

(From the Department of Hematology, The Mount Sinai Hospital, New York, N. Y.)

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The occurrence and severity of erythroblastosis fetalis, due to maternal Rh isoimmunization, can be predicted through simple prenatal serological tests (1, 2). If Rh isoagglutinins are present in maternal serum, and if the incompatible Rh antigen is transmitted from the father, the newborn will have hemolytic disease, the severity of which is often proportional to the titer of the mother's serum Rh isoagglutinins (1, 2). In the event that the isoimmunized Rh negative mother delivers an Rh negative infant, the titer of Rh antibodies in the cord serum, except for saline agglutinins, is generally equal to the titer obtained in the maternal serum (3). For practical purposes, almost all maternal Rh isoagglutinins seem to cross the placental barrier freely, and their titration value affords guidance toward the effective management of hemolytic disease of the newborn. This has not been the case for erythroblastosis fetalis due to ABO incompatibility (1, 2).

ABO erythroblastosis of sufficient severity to warrant exchange transfusion therapy for the control of hyperbilirubinemia, has a frequency equal to that of erythroblastosis due to Rh incompatibility (2, 4), and yet prenatal tests have been of little value thus far in identifying those mothers who may deliver affected infants (4). Since ABO erythroblastosis occurs often in the first incompatible pregnancy (5, 6), a reliable prenatal test would be of extreme importance in anticipating the occurrence of this disease for intelligent management.

A varying proportion of maternal anti-A ( $\alpha$ ) and anti-B ( $\beta$ ) isoagglutinins can be demonstrated in the serum of the newborn (7, 8). Most ABO isoagglutinins cannot cross the placenta and therefore play no role in the pathogenesis of erythroblastosis. Fudenberg, Kunkel and Franklin (9)

suggested that 7S isoagglutinins traverse the placental barrier, thus predisposing to hemolytic disease. If so, diffusion of antibodies across the placenta may be dependent upon the molecular size of the antibody molecule, with low molecular weight (about 160,000)  $\gamma_2$  antibodies of the mother reaching the fetus (9).

Of equal importance in the pathogenesis of ABO hemolytic disease of the newborn are the protective effects of nonerythrocytic ABO blood group substances in the fetus. Whereas Rh antigens appear to be restricted to the red blood cells, ABO antigens occur in other tissues, as well as in the secreted mucopolysaccharides of most persons (10), and ABO isoagglutinins possess varying degrees of sensitivity to inhibition by specific soluble blood group substances (SSBGS) (11, 12). An excess of ABO secretors has been observed in infants with ABO erythroblastosis (5, 6, 13, 14) and in their fathers (14). This excess may reflect either a sensitivity of ABO secretors, or a resistance of ABO nonsecretors, toward ABO disease, or even a loss of ABO nonsecretors *in utero*.

Since blood group substances are readily demonstrable in the tissues of both secretors and nonsecretors (15), further quantitative and/or qualitative distinctions must exist between these two classes to explain these observations. Resistance to neutralization of some  $\alpha$  and  $\beta$  isoagglutinins might be related to the clinical problem of ABO erythroblastosis, if such antibodies were 7S  $\gamma_2$ -globulins crossing the placenta (9). Large antibody molecules (macroglobulins), because they fail to traverse the placental barrier, are not pertinent to this problem (9).

Column chromatographic separation of serum proteins on anion exchanger, diethylaminoethyl (DEAE) cellulose, first described by Sober, Gutter, Wyckoff and Peterson (16, 17), offers a relatively simple means of separating 7S  $\gamma_2$ -globulins from other serum proteins (18). Such separation of human isoagglutinins has been reported by

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Abelson and Rawson (19) and by Fahey and Morrison (20).

The present report deals with the resistance to neutralization by SSBGS of ABO isoagglutinins which can traverse the placental barrier. These isoagglutinins were obtained by a simplified DEAE cellulose column chromatographic method, designed to separate 7S  $\gamma_2$ -globulins from the remainder of the serum proteins. The results observed in the presence and the absence of ABO erythroblastosis suggest that the disease might be anticipated by suitable prenatal tests.

#### MATERIALS AND METHODS

Chromatographic separation was performed according to a modified method of Sober and Peterson (17) and of Levy and Sober (18). In general, the following procedure was used for separation of serum: DEAE cellulose<sup>1</sup> was purified according to Sober and co-workers (16) and the slurry, suspended in the initial buffer (0.02 M phosphate, pH 6.3), was poured into 5-ml serological pipets (25  $\times$  0.5 cm). The ion exchanger was packed under water pressure of 40 to 50 cm and washed with the initial buffer overnight. The dead volume of such columns was about 2.5 ml.

The serum sample, dialyzed<sup>2</sup> against 3 changes of the initial buffer in a 4° C cold room, was applied to the column, together with the slight precipitate formed during dialysis. For each separation, 1 ml serum (corrected for the increase of volume during dialysis) was applied to the column at room temperature, and washed with 10 ml of the initial buffer, followed by 10 ml of 1 M NaCl. Two-ml samples were collected at a flow rate of 6 to 8 ml per hour, at room temperature. The protein content of the separated fractions was measured by absorption at 280  $\mu\mu$  in a Beckman DU spectrophotometer.

Under these conditions, the 0.02 M phosphate buffer (pH 6.3) fraction (hereafter referred to as 0.02 M fraction) contained the first four chromatographic peaks described by Humphrey and Porter (21), and if isoagglutinins were present in this fraction, the highest titers were always found in those tubes which contained most protein. No difference was found in the elution pattern of the isoagglutinins when 0.01 M phosphate buffer was followed by 0.02 M buffer, or when more than 10 ml of buffer was used as first eluent (Figure 1). The 0.02 M fraction was not found to contain proteins other than  $\gamma_2$ -globulins when tested by paper electrophoresis and acrylamide electrophoresis (22). Ultracentrifugation of this fraction at a protein concentration of 1 per cent (courtesy of Drs. O. Singher and R. Fenichel, Ortho Research Foundation, Raritan, N. J.) revealed only a single component having a sedimentation con-

<sup>1</sup> Eastman Organic Chemicals, Rochester, N. Y.

<sup>2</sup> Cellulose casings, Visking Co., Chicago, Ill.

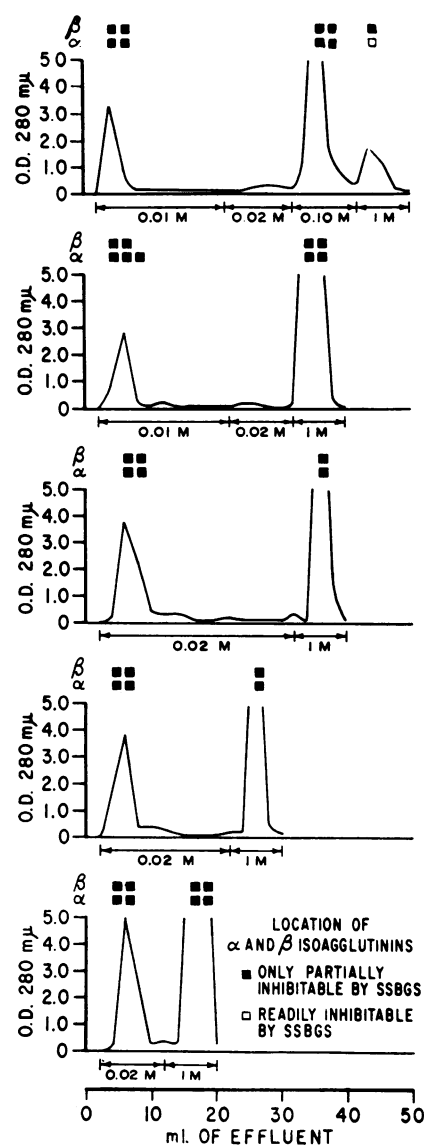


FIG. 1. SEPARATION OF ISOAGGLUTININS ON DEAE CELLULOSE COLUMNS UNDER VARYING CONDITIONS. Variations of the concentration (0.01 to 0.02 M) of the phosphate buffer, pH 6.3, and of the volume of effluent collected (10 to 30 ml), failed to alter the pattern of 7S  $\gamma_2$ -globulins or  $\alpha$  and  $\beta$  isoagglutinins emerging from the column. The  $\alpha$  and  $\beta$  isoagglutinins in the fractions eluted with 1 M NaCl could be further separated by using 0.1 M phosphate buffer, pH 6.3, prior to 1 M NaCl.

stant of 7S. Heavier components were not observed despite special attention for their detection in the earliest parts of the ultracentrifugal run.

The second fraction eluted with 1 M NaCl (hereafter referred to as 1 M fraction) contained 19S macroglobulins and all other serum proteins, but paper and acrylamide electrophoresis failed to reveal  $\gamma_2$ -globulins.

The isoagglutinin activity of this fraction could be split into two fractions using, after 0.02 M phosphate buffer, pH 6.3, first 0.1 M phosphate buffer, pH 6.3, followed by 1 M NaCl (Figure 1). The significance of this observation is under study.

The isotonicity of 0.02 M phosphate buffer fractions was restored by addition of 0.05 ml of 18 per cent NaCl per 1 ml of eluate, and of 1 M NaCl fractions by dilution with 5 vol of distilled water.

Native serum and pooled fractions, the latter concentrated to the original serum volume by dialysis at 4° C against 20 per cent polyvinylpyrrolidone (PVP), provided materials for titration of antibodies before and after separation. Parallel titrations showed a good recovery of the isoagglutinins (Table I).

The agglutination tests for  $\alpha$  and  $\beta$  isoagglutinins were done by conventional methods, usually in duplicate. Two-fold serial dilutions of serum or fractions were mixed with equal volumes of 2 per cent washed red cells from type A<sub>1</sub> and B donors. After incubation at 37° C for 1 hour and centrifugation, agglutination was recorded as the saline titer. An additional volume of 4 per cent acacia (5) was then added and the mixture reincubated for 15 minutes at 37° C, centrifuged, and 1 vol of saline added to abolish rouleaux. The agglutination then observed, usually by two observers, neither familiar with the clinical details, was recorded as the acacia titer. This concentration of acacia was chosen because it afforded results which agreed within one dilution of those observed with the  $\gamma$ -antiglobulin test.

The neutralization tests for  $\alpha$  and  $\beta$  isoagglutinins were performed by mixing serial dilutions of 0.02 M phosphate buffer fractions with suitable serial dilutions of SSBGS

TABLE I

Recovery of ABO isoagglutinins after fractionation on DEAE cellulose columns

Serum no.	Anti-body	Isoagglutinin titers (reciprocal)			
		Native	0.02 M*	1 M†	Pool‡
3	$\alpha$	80	0	80	80
	$\beta$	160	0	80	80
21	$\alpha$	1,280	160	640	1,280
	$\beta$	640	40	320	640
42	$\alpha$	640	80	320	640
	$\beta$	160	4	80	80
140	$\alpha$	160	4	80	80
	$\beta$	160	0	160	160

\* Titer of pooled fractions that were eluted with 0.02 M phosphate buffer, pH 6.3, and concentrated to original serum volume.

† Titer of pooled fractions that were eluted with 1 M NaCl and concentrated to original serum volume.

‡ Mixture of \* and † corrected for volume change due to mixing.

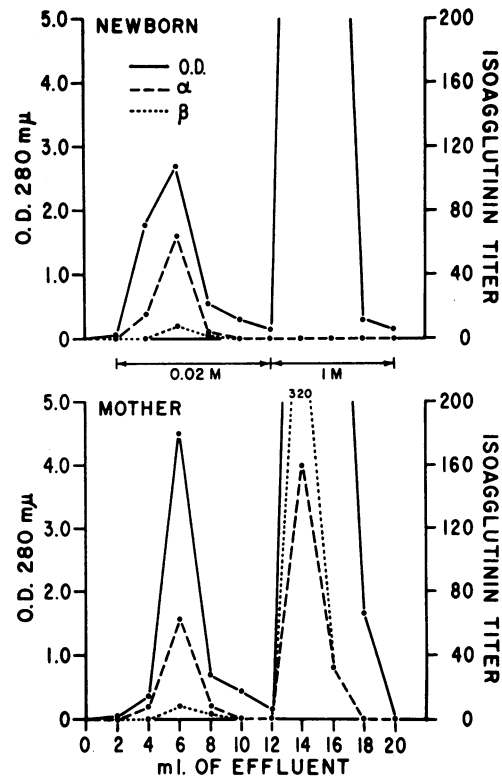


FIG. 2. THE RECOVERY OF MATERNAL AND FETAL ABO ISOAGGLUTININS FROM DEAE CELLULOSE COLUMNS. Both mother (no. 268) and newborn (no. 265) type O; 1 ml serum samples fractionated. Isoagglutinin titers of whole serum: mother,  $\alpha$  1:512 and  $\beta$  1:512; newborn,  $\alpha$  1:160 and  $\beta$  1:16. Isoagglutinin titer in figure is represented by reciprocal values.

of hog (A) and horse (B) origin.<sup>3</sup> The test cells were added after 15 minutes' incubation at room temperature. After further incubation for 1 hour at 37° C and centrifugation, agglutination was recorded as saline titer. Then 4 per cent acacia was added to the tests which were further incubated for 15 minutes at 37° C. The agglutination observed after centrifugation and addition of saline was recorded as the acacia titer. The acacia titer was always found to be more reliable and is given throughout this report. Titers with and without SSBGS were frequently checked for reproducibility at a later date and consistent results were always obtained.

## RESULTS

1. *The separation of cord serum  $\alpha$  and  $\beta$  isoagglutinins on DEAE cellulose columns.* ABO isoagglutinins have been demonstrated in cord bloods, especially those from type O offspring of type O mothers. Cord serum ABO isoagglutinins could

<sup>3</sup> Biosales Inc., 300 W. 43rd St., New York, N. Y.

TABLE II

The recovery of cord blood \* isoagglutinins in 0.02 M eluates † from DEAE cellulose columns ‡

Specimen no.	Blood type		Isoagglutinin titer (reciprocal)			
			Native cord serum		0.02 M fraction	
			Anti-A	Anti-B	Anti-A	Anti-B
44	O	O	160	10	40	<10
62	O	O	80	160	40	40
117	O	O	40	<10	20	<10
143	O	O	80	<10	40	<10
144	O	O	20	<10	20	<10
145	O	O	160	<10	80	<10
146	O	O	80	40	80	20
147	O	O	160	<10	80	<10
148	O	O	40	40	40	40
150	O	O	320	10	160	<10
152	O	O	80	10	40	<10
153	O	B	20		20	
154	O	O	20	<10	<10	<10
155	O	O	40	20	40	20
156	O	O	80	<10	80	<10
180	O	B	80		40	

\* Cord sera selected to have >1:10 anti-A.

† Cord blood isoagglutinins not observed in the 0.1 to 1.0 M eluates of these and 17 other cord sera tested.

‡ Unconcentrated single 0.02 M phosphate buffer, pH 6.3, eluates which contained the most isoagglutinins were titrated and the values adjusted for dilution over the volume of the native serum.

be of 7S character (9), because they are incomplete reflections of  $\alpha$  and  $\beta$  isoagglutinins present in the serum of the mother.

Thirty-three cord sera, each containing  $\alpha$  and  $\beta$  isoagglutinins, were separated on DEAE cellulose columns, and eluates collected as described under Methods. The isoagglutinins of these sera were recovered only in the 0.02 M fractions; the 1 M fractions, under the standard test conditions, did not reveal isoagglutinin activity (Figure 2). Rarely, and only when some cord sera contained in excess of 1:200 titer of ABO isoagglutinins, concentrations of 1 M NaCl fractions contained irregular trace amounts of isoagglutinins up to approximately 1 per cent of the native titer, due perhaps to contamination with 7S  $\gamma_2$ -globulins.

Sixteen sera were found to have anti-A in a titer of 1:20 or greater, and these sera were studied by titration of the native material and that single 0.02 M fraction which contained most of the isoagglutinin activity. There was no need to pool or to concentrate fractions, because these antibodies invariably appeared with the effluent having most of the 7S  $\gamma_2$ -globulins, and one fraction always contained most of the isoagglutinins in a titer quite similar to that of the native cord serum (Figure 2). Titrations were seldom performed in parallel, and the test red cells were sometimes varied. Table II summarizes these

results and shows the good recovery of cord serum agglutinins in single 0.02 M fractions from DEAE cellulose columns.

2. Comparison of the 7S  $\alpha$  and  $\beta$  isoagglutinin titers of maternal and cord sera. If the  $\alpha$  and  $\beta$  isoagglutinins of cord sera are 7S  $\gamma_2$ -globulins that can be recovered in 0.02 M fractions from DEAE cellulose columns, and if these isoagglutinins are of maternal origin, having diffused across the placental barrier, the 0.02 M fractions of maternal and cord serum pairs (collected at the time of delivery) should be expected to have identical titer values, if equilibrium is attained.

Eight pairs of maternal and cord sera, all type O and containing  $\alpha$  and  $\beta$  isoagglutinins, were fractionated on DEAE cellulose columns. The 0.02 M fractions and the native maternal sera were titrated for  $\alpha$  and  $\beta$  isoagglutinins, and the results (summarized in Table III) show that the 0.02 M fraction of the  $\alpha$  and  $\beta$  isoagglutinin activity in maternal serum is duplicated in the cord serum. The quantitative data of one of these maternal-cord pairs, shown in Figure 2, reveals that these isoagglutinins, as well as other 7S  $\gamma_2$ -globulins without isoagglutinin activity, exist in equilibrium on both sides of the placenta.

3. The occurrence of  $\alpha$  and  $\beta$  isoagglutinins in the 0.02 M fractions of 110 prenatal specimens. Study of the cord sera of compatible pregnancies (7) has furnished some information concerning placenta-crossing ABO isoagglutinins, but there has been no method thus far to study such antibodies in unselected pregnancies in advance of delivery. The 0.02 M fractions of DEAE cellu-

TABLE III

Comparison of the  $\alpha$  and  $\beta$  isoagglutinin titers \* of maternal and cord sera obtained at delivery †

Specimen no.	Isoagglutinin titer (reciprocal)					
	Infant 0.02 M fraction		Maternal 0.02 M fraction		Native maternal serum	
	$\alpha$	$\beta$	$\alpha$	$\beta$	$\alpha$	$\beta$
26 45	160	80	160	160	640	640
56 61	40	4	40	20	160	160
27 63	20	<2	20	<2	80	80
107 119	80	40	40	80	320	640
135 43	20	20	20	20	160	160
139 157	20	40	20	40	160	160
169 171	40	2	40	4	640	80
265 268	64	8	64	8	512	512

\* Unconcentrated single 0.02 M phosphate buffer, pH 6.3, eluates which contained the most isoagglutinins were titrated, and the values adjusted for dilution over the volume of the native serum.

† Mothers and newborn all type O.

TABLE IV  
The distribution of the ABO blood types of the newborn of 110 mothers of types O, A and B

Blood type, newborn	Blood type, mother		
	O	A	B
O	45	5	7
A	20	20	0
B	8	1	1
AB	0	2	1
Total	73	28	9

lose columns offered such an opportunity, and 110 prenatal specimens of type O, A and B patients were fractionated. Table IV shows the distribution of the blood types of the newborn subsequently delivered, and Table V reveals the distribution of the  $\alpha$  and  $\beta$  isoagglutinins in maternal 0.02 M fractions. To be detected by the screening tests employed, a titer of at least 1:2 was required.

In this series, isoagglutinins were not observed in the 0.02 M fractions of the sera of 28 type A mothers and 9 type B mothers, but were observed in the sera of 53 out of 73 type O mothers. Deviation from our earlier results in study of compatible gestation (7) may be due to the dilution at which these tests were now performed. Whereas the cord serum of compatible newborn can be tested without dilution, the unconcentrated 0.02 M fractions represented a dilution of at least 1:2 over the volume of the native serum that was placed on the cellulose columns. A further slight dilution occurred when concentrated NaCl was added for isotonicity, and some loss of antibody was expected, because only one of several antibody-containing, or potentially antibody-containing, fractions was tested. This small study of 0.02 M fractions revealed only that ABO isoagglutinins in a titer of at least 1:2 were observed in 69 per cent of 73 type O mothers and were

TABLE V  
The occurrence of  $\alpha$  and  $\beta$  isoagglutinins of at least 1:2 titer in the 0.02 M phosphate buffer fractions of 110 mothers of blood types O, A and B

	Blood type, mother		
	O	A	B
$\alpha + \beta$	24	0	0
$\alpha$ only	27	0	0
$\beta$ only	2	0	0
No isoagglutinins	20	28	9

not observed in 37 mothers of type A or B. This is consistent with the strong association of ABO erythroblastosis with mothers of type O (16).

Furthermore, it is of interest to note (Table V) that anti-B was observed in only 26 of 73 type O mothers, whereas anti-A was found in 51 of the same mothers. In addition,  $\beta$  without  $\alpha$  was observed only twice, but  $\alpha$  without  $\beta$  was encountered 27 times. This preponderance of  $\alpha$  activity might well be associated with the preponderance of erythroblastosis due to A incompatibility (7).

4. *Relative inhibition by specific soluble blood group substances of the isoagglutinins in the 0.02 M fractions of 53 type O mothers.* Table V reveals that the placenta-crossing  $\alpha$  and  $\beta$  isoagglutinins contained in 7S  $\gamma_2$ -globulin fractions occur commonly, but clinical ABO erythroblastosis is observed in only 1 of 200 newborn (23). This difference could be due to the protective effect of extraerythrocytic ABO antigens.

Witebsky (11) suggested that "non-inhibitable" ABO isoagglutinins might be related to ABO erythroblastosis, but Zuelzer and Kaplan (6) noted a poor correlation. However, the study of total serum isoagglutinins (12) would not be informative, since the bulk of  $\alpha$  and  $\beta$  antibodies are 19S (20) (Table V), which not only fail to cross the placental barrier, but could confuse the results.

The erythrocytic, tissue, and mucopolysaccharide ABO antigens, even of animal origin, share common structural groupings. On the other hand, agglutinins which are resistant to neutralization by large quantities of SSBGS permit a distinction between erythrocytic and extraerythrocytic ABO antigens. Therefore, diffusible noninhibitable maternal isoantibodies, even of low titer, could be responsible for clinical ABO erythroblastosis. The 0.02 M cellulose column fractions of the isoagglutinins of 53 type O mothers, presented an opportunity to attack this problem directly.

Titration of the ABO isoagglutinins in the 0.02 M fractions of these 53 mothers yielded values ranging from 1:4 to 1:640, and little if any correlation with the native serum titers (Table II). Of more importance were duplicate titrations to which SSBGS were added to each tube of the titration series. The concentration of the SSBGS was varied from 1:10 to 1:500 for A substance, and from 4:1 to 1:500 for B substance, to deter-

TABLE VI  
*Relative inhibition by specific soluble blood group substances of the isoagglutinins in the 0.02 M phosphate buffer fractions of 53 type O mothers*

Blood type, newborn	No. of mothers tested	No. of isoagglutinins tested	Number of mothers with noninhibitable isoagglutinins Highest dilution of SSBGS which fails to inhibit							
			$\frac{1}{500}$	$\frac{1}{100}$	$\frac{1}{40}$	$\frac{1}{20}$	$\frac{1}{10}$	$\frac{1}{1}$	$\frac{1}{4}$	
A	13	$\alpha$	11	7	3	1				
		$\beta$	5	2	3					
B	4	$\alpha$	4	3				1*		
		$\beta$	4	3						1*
O	36	$\alpha$	36	21	10		2	3†		
		$\beta$	17	2	7			5		3†
All	53	$\alpha$	51	31	13	1	2	4	0	0
		$\beta$	26	7	10	0	0	5	0	4

\* Same case; child had severe ABO erythroblastosis requiring transfusion.

† Only four sera represented; two contained both noninhibitable  $\alpha$  and  $\beta$ , one contained only  $\alpha$  and another only  $\beta$ .

mine that concentration which just failed to inhibit visible agglutination. These results, correlated with the ABO type of the newborn, are summarized in Table VI. The horse B substance used for these tests inhibited anti-B much less effectively than the hog A substance inhibited anti-A.

Five prenatal sera so studied for placenta-crossing antibodies were found to contain  $\alpha$  and/or  $\beta$  agglutinins, neither of which could be neutralized by SSBGS at a concentration of 1:10 for A and 4:1 for B. Four of these patients delivered type O newborn, but one delivered a type B child which required exchange transfusion therapy for severe ABO erythroblastosis.

In 200 deliveries, approximately 90 type O mothers can be anticipated, and in the small series shown in Tables III, IV and V, 73 type O mothers were studied. Therefore, 0.8 case of ABO disease was expected (23), and one case was encountered. Type O mothers have incompatible offspring of types A<sub>1</sub> and B in approximately 30 per cent of instances (the sum of the gene frequencies of A<sup>1</sup> and B), so that for every case of erythroblastosis, an additional two to three unselected type O women should have similar ABO isoagglutinins which are of no importance to newborn of types O and A<sub>2</sub> (23). In the present series, four such women were encountered. One of these patients happened to be primagravida, consistent with the fact that ABO disease may be observed in the first incompatible pregnancy.

Table VI also shows that incompatible infants without evidence of hemolytic disease were delivered from 17 type O mothers who had demonstrable isoagglutinins in their 0.02 M fractions. In 15 instances these antibodies had the specificity of the A or B antigen found on the cord erythrocytes, and the titration values ranged from 1:4 to 1:160. The mothers of 10 incompatible newborn were found to have such incompatible isoagglutinins with a titer of at least 1:20. However, only one infant of this entire group had clinical disease, and the isoagglutinins in the 0.02 M fraction of this particular mother were highly resistant to inhibition by SSBGS, whereas the other nine were easily neutralized. Thus the inhibitable isoagglutinins in 0.02 M fractions, regardless of titer, did not appear to cause erythroblastosis, whereas the noninhibitable isoagglutinins so separated, even of low titer, furnished the expected correlation.

5. *The nature of the isoagglutinins in the sera of mothers who have had infants with severe ABO erythroblastosis.* Isoagglutinins eluted in 0.02 M fractions and resistant to inhibition by SSBGS are not common (Table VI), having been observed in only 5 of 110 prenatal specimens of blood types O, A and B. However, if such isoagglutinins are responsible for ABO erythroblastosis, they must occur in the sera of all women who have a history of children with severe clinical disease. Seven patients with typical histories, including the case

TABLE VII

*Inhibition by hog (A) and horse (B) specific soluble blood group substances of the 0.02 M phosphate buffer fractions of maternal  $\alpha$  and  $\beta$  isoagglutinins when there is a history of severe ABO erythroblastosis\**

		Titer (reciprocal) of 0.02 M fraction in the presence of SSBGS														Titer of native serum	
ABO type of affected child	Case no.	$\alpha$ agglutinins						$\beta$ agglutinins						$\alpha$	$\beta$		
		Amount of A substance added						Amount of B substance added									
		0†	$\frac{1}{500}$	$\frac{1}{100}$	$\frac{1}{40}$	$\frac{1}{20}$	$\frac{1}{10}$	0†	$\frac{1}{500}$	$\frac{1}{100}$	$\frac{1}{40}$	$\frac{1}{20}$	$\frac{1}{10}$			$\frac{4}{1}$	
A	21	160	nd†	40	40	20	4	40	nd	40	40	20	20	0	1,280	320	
A	22	80	20	20	nd	nd	4	2	0	0	nd	nd	0	0	160	20	
A	42	160	160	80	nd	nd	20	4	2	2	nd	nd	0	0	640	160	
A	64	80	80	20	nd	nd	6	20	20	6	nd	nd	2	0	2,560	160	
A	94	160	nd	80	20	4	4	2	0	0	0	0	0	0	2,560	640	
A	184	160	nd	20	10	4	2	160	nd	80	80	40	20	0	1,280	2,560	
B	141	40	20	10	nd	nd	4	40	40	20	nd	nd	4	2	320	320	

\* All mothers, type O.

† No SSBGS added; titer of isoagglutinins.

‡ Not done.

from Table VI, were recalled for study, and the results are shown in Table VII.

The sera of all of these women with a history of severe ABO erythroblastosis in previous pregnancies revealed type-specific "noninhibitable" ABO isoagglutinins in a titer of at least 1:2 in their 0.02 M fractions. All newborn in this series required exchange transfusion treatment or developed kernicterus because treatment was not available. One mother (no. 42) had delivered her last child eight years previously, thereby revealing how persistent these antibodies may be.

6. *The nature of the isoagglutinins in the serum of unselected type O donors immunized with SSBGS.* Increasing severity of ABO erythroblastosis in later deliveries is seen occasionally. Significant increase in maternal ABO isoagglu-

tinins has not been observed during the course of an incompatible gestation nor after the delivery of a nonsecretor infant (6). On the other hand, titer increases have been noted post partum following the delivery of some ABO incompatible secretors (6), presumably due to the SSBGS content of amniotic fluid. This raises the question as to whether SSBGS can incite noninhibitable isoagglutinins that can be eluted in 0.02 M fractions.

For information on this problem, the sera of five unselected type O donors, who had been immunized with both A and B SSBGS for preparation of anti-AB reagents (high titer type O sera), were fractionated on DEAE cellulose columns. The SSBGS which had been used for the immunization of these donors were of the same source and manufacture as those employed in the

TABLE VIII

*Inhibition by hog (A) and horse (B) specific soluble blood group substances of the 0.02 M phosphate buffer fractions of the  $\alpha$  and  $\beta$  isoagglutinins of unselected donors previously immunized with the same SSBGS*

		Titer (reciprocal) of 0.02 M fraction in the presence of SSBGS														Titer of native serum	
Case no.		$\alpha$ agglutinins						$\beta$ agglutinins						$\alpha$	$\beta$		
		Amount of A substance added						Amount of B substance added									
		0*	$\frac{1}{500}$	$\frac{1}{100}$	$\frac{1}{30}$	$\frac{1}{20}$	$\frac{1}{10}$	0*	$\frac{1}{500}$	$\frac{1}{100}$	$\frac{1}{40}$	$\frac{1}{20}$	$\frac{1}{10}$			$\frac{4}{1}$	
250	40	nd†	16	12	8	4	80	nd	80	80	80	40	10	2,048	2,048		
251	40	nd	20	16	16	10	80	nd	30	30	30	20	10	1,024	1,024		
253	60	nd	20	20	10	10	80	nd	80	40	40	10	8	512	2,048		
254	>160	nd	160	40	20	20	>160	nd	>160	>160	<160	>160	8	2,048	2,048		
255	>160	nd	30	20	20	4	>160	nd	>150	80	80	30	8	>2,408	1,024		

\* No SSBGS added; titer of isoagglutinins.

† Not done.



present inhibition studies. The 0.02 M fractions of all five donor sera were found to contain non-inhibitable  $\alpha$  and  $\beta$  isoagglutinins, resembling those in the sera of the mothers of erythroblastic infants. The results are summarized in Table VIII.

#### DISCUSSION

Severe ABO erythroblastosis is familial and often observed in the first ABO incompatible child (5, 6). Although almost all subsequent incompatible infants are also affected, increasing severity of the disease is not seen regularly (12, 14). Thus ABO disease resembles Rh erythroblastosis in its familial incidence, but differs by not reflecting so much evidence of specific maternal isoimmunization by the products of conception.

Schiff (12) postulated that ABO erythroblastosis was to be expected when the maternal serum contained a special ("Type VI") pattern of antibodies highly resistant to neutralization by SSBGS; but, to explain the absence of hemolytic disease in the incompatible children of some type O mothers of this description, he proposed protection against the cross-reacting antibody described by Rosenfield and Ohno (7). Since Schiff studied whole maternal serum, his conclusions must be reviewed in the light of the properties of 7S maternal isoagglutinins.

If maternal isoagglutinins are to be responsible for erythroblastosis, they must not only cross the placental barrier but combine with fetal erythrocytes. In the case of ABO isoagglutinins, extra-erythrocytic antigens occur in many tissues independent of the ABO secretor status (15) and exert a protective effect of considerable magnitude, as evidenced by the low frequency of erythroblastosis despite the common occurrence of diffusible isoagglutinins (6, 8).

At least four components, with sedimentation constants of 7S, 19S, 28S, and 44S, have been found in the  $\gamma$ -globulin fractions of normal human serum (24), and recently Fahey and Morrison (20) showed that all of the ABO isoagglutinins have a sedimentation value of either 7S or 19S. The "non- $\gamma$ -globulin" isoagglutinins reported by Abelson and Rawson (19) appear to have been due to conditions of the separation (20). Abelson and Rawson also reported that noninhibitable ABO isoagglutinins were almost all located in 7S fractions; but in all cases, and at the same con-

centration of SSBGS that they employed, we found only a small part of the total isoagglutinins to be noninhibitable and to occur in both the 0.02 M and the 1 M fractions if isoagglutinins were present at all in the former.

Table III reveals that most ABO isoagglutinins are not  $\gamma_2$ -globulins and do not cross the placenta. Poorly inhibitable isoagglutinins eluted in 1 M fractions, although not shown in this report, were observed frequently and would contribute misleading information.

Placental permeability and resistance to inhibition by SSBGS are certainly not the sole parameters of the problem of ABO erythroblastosis, but their contribution must be of primary consideration. Other contributing factors include the expression of erythrocytic ABO antigens because  $A_2$  children tend to be spared (6); the secretor status because erythroblastotic infants tend to be secretors (5, 13, 6, 14); and the ability of the affected newborn to cope with a hemolytic syndrome (4). These and, probably, other variables must be studied. If the noninhibitable isoagglutinins of 0.02 M fractions are responsible for ABO erythroblastosis, there is now an opportunity to attack these secondary problems.

The present report raises an immediate problem concerning the nature of noninhibitable isoagglutinins shown to be associated with erythroblastosis. If these antibodies can be incited by SSBGS (Table VIII), they should not represent antigenic structural grouping differences between erythrocytes and SSBGS. Instead, these differences could reflect the heterogeneity of antibody molecules with respect to the size of their specific combining sites (25, 26). Kabat (25, 26) has demonstrated that antidextran molecules vary considerably in the size of their combining sites, with the upper limit being sites complementary to terminal nonreducing chains of six or seven glucose units in  $\alpha$ -1,6 linkage. Thus, although erythrocytes of types  $A_1$  and B might agglutinate with antibody molecules having combining sites of any size, SSBGS might form reversible complexes with some antibody molecules which could be released in sufficient amount to support visible agglutination under selected circumstances. Alternatively, nonspecific anamnestic response is required to explain the observations shown in Table VIII.

The term "immune" for agglutinins which cross the placenta, as opposed to "natural" saline agglutinins (8, 27), is not supported by the present data. All ABO agglutinins appear to be immune responses to antigenic structural groupings resembling those defined by Kabat (10) for human blood group substances.

The nature of the distinction between  $A_1$  and  $A_2$  red cells is also subject to review. Anti- $A_1$  reagents of human, animal or plant origin are highly susceptible to inhibition by SSBGS, and precipitate with SSBGS in common with anti-A (28). The distinction made by these reagents thus appears to be on a quantitative basis: there is more A on  $A_1$  cells than on  $A_2$  cells. On the other hand, anti-A inhibited with SSBGS toward  $A_2$  cells, may persist in agglutinating  $A_1$  cells. The problem of the specificity of noninhibitable anti-A, as related to the restricted occurrence of erythroblastosis in type  $A_1$  offspring, is now under investigation.

The column chromatographic isolation of isoagglutinins which can cross the placenta appears to be a valuable method for the study of all forms of maternal isoimmunization in relation to erythroblastosis. This method is now being used for its applicability to Rh problems, and the results appear promising, but not identical with the report of Abelson and Rawson (29).

The column chromatographic method requires at least two days to perform and is not feasible for rapid diagnosis in the newborn period. For prenatal tests, the method appears to be practical.

#### SUMMARY AND CONCLUSION

1. ABO isoagglutinins associated with the bulk of 7S  $\gamma_2$ -globulins were separated from other serum proteins by anion exchange column chromatography.

2. The ABO isoagglutinin activity of 33 cord blood sera was found to be restricted to 7S  $\gamma_2$ -globulin-containing fractions.

3. Maternal serum ABO isoagglutinins were encountered chiefly in macroglobulin-containing fractions, but paired specimens of maternal and cord sera obtained at the time of delivery revealed that the 7S  $\gamma_2$ -globulins as well as the associated isoagglutinins existed in equilibrium on both sides of the placenta.

4. Out of 110 unselected prenatal sera, isoagglutinins, in a titer of at least 1:2 in acacia, were

eluted in 0.02 M phosphate buffer, pH 6.3, in 53 of 73 type O mothers, but not in 28 type A and 9 type B women. Anti-A ( $\alpha$ ) was noted in 51 of these fractions, but anti-B ( $\beta$ ) in only 27. These findings were considered to be consistent with the rarity of ABO erythroblastosis in offspring of mothers not of type O, and with the excess of erythroblastosis due to anti-A.

5. When these 53 examples of chromatographically separated isoagglutinins were tested for resistance to inhibition by specific soluble blood group substances (SSBGS) (hog A and horse B), only 5 contained markedly resistant antibodies. Four of these mothers delivered type O children, but one mother delivered a type B child who had erythroblastosis and required exchange transfusion therapy. An additional nine mothers in this series also delivered ABO-incompatible newborn, and their 0.02 M fractions contained type-specific isoagglutinins in a titer ranging from 1:20 to 1:160, but in each instance these antibodies were readily inhibitable with SSBGS, and clinical erythroblastosis was not observed.

6. Six other women with a history of having had one or more children with severe ABO erythroblastosis, and five unselected donors who had been immunized previously with SSBGS were all found to have such type-specific noninhibitable isoagglutinins.

7. The use of these methods in prenatal tests is implied.

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