INVESTIGATIONS ON THE OCCURRENCE OF Rh SUBSTANCES IN AMNIOTIC FLUID

By ERNEST WITEBSKY, M.D., and JAMES F. MOHN, M.D.

With the Technical Assistance of Lucille F. Collins and Doris J. Howles

(From the Laboratories of The Buffalo General Hospital, and the Department of Bacteriology and Immunology, University of Buffalo School of Medicine, Buffalo)

(Received for publication, April 9, 1945)

Investigations on the distribution of the blood group specific substances A and B within the human body revealed the fact that the entire body has group specific stigmata (1–3). There is an interesting quantitative difference in the content of various organs in regard to group specific properties (4–6). Saliva, gastric juice, and amniotic fluid contain group specific substances in high concentration as demonstrated by Putkonen (7) and Hartmann (8). The secretion of group specific substances is a constitutional property which is inherited; the gene of secretion being dominant over the gene of non-secretion (9). Roughly about six out of seven human beings are secretors. At the present time it appears that two different chemical substances carry group specific characteristics; a water-soluble substance, carbohydrate-like in nature and an alcohol-soluble substance, lipoid-like in nature (5). Only the carbohydrate form is found in the secretions such as saliva.

Results of studies on the distribution of the Rh factor within the human body seemed to limit the Rh factor to blood cells only. However, Boorman and Dodd (10) report the occurrence of the Rh factor in tissue cells although this factor proved to be insoluble in water. According to the same authors, saliva specimens from Rh-positive individuals in a dilution of 1:2 exert a weak inhibitory effect on Rh agglutination in about one-half of the cases studied. Yet they feel that the Rh substances are almost entirely absent from body fluids. On the other hand, Levine (11) did not find the Rh factor in saliva. Wiener and Forer (12), apparently being convinced of the absence of Rh substance in saliva, recommend the use of saliva for the neutralization of the isoagglutinins anti-A and anti-B in anti-Rh serum.

Previous investigations (13–15) have shown that the decidua contains the blood group specific factor of the mother, and the amnion contains that of the baby. The blood group of the child and not that of the mother determined the blood group specific substances occurring in amniotic fluid (7). In the investigations to be reported the amniotic fluid has been chosen as the secretion to be studied because of its possible bearing on the pathogenesis of erythroblastosis and because of the relative ease with which it is obtainable.

Materials

Anti-Rh Sera.—The greatest difficulty in investigating problems in connection with the Rh factor is the lack of suitable and sufficiently large amounts of anti-Rh serum. We were

fortunate in having at our disposal a potent anti-Rh serum (Kru) obtained from a patient of the blood group O who had received multiple transfusions of group O blood. The last transfusion had resulted in a severe hemolytic transfusion reaction whose cause could be traced to the formation of Rh antibodies in the patient who was found to be Rh-negative. The agglutination of Rh-positive cells with this serum was stronger at room temperature or in the ice box than at body temperature. In this respect our serum differs from the vast majority of Rh antisera obtained from patients with erythroblastotic children. This type of serum has been observed previously (16, 17). The serum (Kru) was of the 87 per cent variety (anti-Rh_0'). In addition to that, smaller amounts of anti-Rh sera of the 87 per cent variety (anti-Rh_0', the 70 per cent (anti-Rh'), and the 30 per cent (anti-Rh'') varieties were at our disposal.[1]

Amniotic Fluid.—Care was taken to avoid as far as possible contamination of the amniotic fluids with maternal blood; however, in a few instances amniotic fluids containing traces of blood were included in our series. Frankly bloody amniotic fluids were excluded from this investigation. The amounts obtained varied from 10 ml. to 1000 ml. The majority of fluids were collected by artificial rupturing of the fetal membranes at the time of delivery, although some were obtained from Caesarean sections or from cases with spontaneous rupture of their membranes.[2] The amniotic fluids were spun down as soon as received, and the supernatants stored at −20°C.

A few milliliters of placental blood were collected in a Wassermann tube and examined for the blood group and the Rh factor. In some instances in which no placental blood was obtainable, a few drops of blood from the baby's heel were collected in citrate solution. In many instances the mother's blood was examined also.

Methods

The determinations of the blood group and the Rh type of the maternal bloods and placental bloods were carried out in test tubes using potent test sera for that purpose. Our 87 per cent anti-Rh serum (Kru) was used as routine for the determination of the Rh factor. As far as available anti-Rh' and anti-Rh'', as well as other anti-Rh sera, were used for the determinations of the subtypes of the Rh factor.

Inhibition of agglutination was used as the basic experimental test in the investigations to be reported. The underlying principle consists in the neutralization of antibodies by corresponding antigenic substances without leading to visible precipitation. This test was used for the determination of the blood group specific substances A and B and the Rh substances occurring in amniotic fluid. In both instances attention had to be paid to the optimal quantitative conditions requiring frequent preliminary titrations.

EXPERIMENTAL

The first experiment to be reported deals with the principal issue of whether or not Rh substances are secreted into the amniotic fluid. Apparently the Rh factors are present even in blood cells in a much lower concentration than the blood group specific substances A and B (18). Obviously only relatively small amounts, if any at all, could be expected in body fluids.

A typical experiment done in order to find out the possible content of Rh

[1] We are indebted to Dr. L. Diamond, Dr. P. Levine, and to Dr. A. S. Wiener for having made these sera available to us.

[2] We acknowledge with thanks the cooperation of the obstetrical departments of The Buffalo General Hospital, the Children's Hospital, and the Millard Fillmore Hospital of Buffalo, New York, and the Brooks Memorial Hospital of Dunkirk, New York, for supplying us with the material.

substance in amniotic fluid is recorded in Table I. The experiment itself was carried out in the following way:—

Decreasing amounts, volume 0.05 cc., of two amniotic fluids (A) originating from an Rh-positive baby; (B) originating from an Rh-negative baby, were mixed with 0.05 cc. of 1:4 diluted anti-Rh serum (Kru, 87 per cent variety). After being kept for 2 hours in the ice box (4° C.) and 10 minutes at room temperature, 0.05 cc. of a 3 per cent suspension of Rh_1 cells belonging to blood group O was added. The mixtures were thoroughly shaken and kept for 1 hour at room temperature. The tubes were then spun down in a centrifuge at medium speed for 1 minute. The resulting agglutinations, read macroscopically are shown in Table I.

The amniotic fluid obtained from an Rh-positive baby definitely inhibits the agglutination of Rh_1 cells. The amniotic fluid from an Rh-negative baby

TABLE I

Agglutination of Rh_1 Cells by Anti-Rh Serum (87 Per Cent) after Treatment of the Latter with Amniotic Fluids

Liquor amnii supernatants	A Amniotic fluid from an Rh-positive baby	B Amniotic fluid from an Rh-negative baby
(1) Undiluted	−	++
(2) 1:2	−	+++
(3) 1:4	−	+++
(4) 1:8	±	+++
(5) 1:16	+	+++
(6) 1:32	++	+++
(7) 1:64	++	+++
(8) 0	+++	+++

− = no agglutination. ++ = marked agglutination.
± = faint agglutination. +++ = strong agglutination.
+ = slight agglutination. ++++ = very strong agglutination.

fails to do so. The experiment shows, therefore, that Rh substances do occur in amniotic fluid.[3]

The scarcity of Rh antisera prevented us from doing comparative studies on a larger scale. However, it was felt that the degree of inhibition as well as the specificity of the reaction was increased by keeping the antiserum-amniotic fluid mixtures in the ice box instead of at body temperature during the first phase of the experiment.

Our anti-Rh serum (Kru, 87 per cent variety) agglutinating Rh-positive cells stronger at lower temperatures than at body temperature is obviously suitable for experiments of the type just described. Other Rh antisera obtained from Rh-negative mothers who had given birth to erythroblastotic

[3]The inhibition of agglutination experiments described here serve for the identification of the Rh substances proper and should not be confused with the "blocking antibody" recently described by Wiener (19).

children were also studied regarding their usefulness in demonstrating the occurrence of Rh substances in amniotic fluids.

In the following experiment two such sera were compared, one an anti-Rh′ serum (70 per cent variety), and the second, a different anti-Rh_0' serum (87 per cent variety, DiY, obtained from Dr. Diamond). The experiment itself was carried out as follows:—

Decreasing amounts of the same amniotic fluids used in Experiment 1, namely, one from an Rh-positive baby, and one from an Rh-negative baby, volume 0.05 cc., were mixed with, (I) 1:4 diluted anti-Rh′ serum (70 per cent variety); (II) 1:2 diluted anti-Rh_0' serum (DiY, 87 per cent variety). After incubation for 2 hours in the ice box (4° C.), 0.05 cc. of a 3 per cent suspension of Rh_1 cells belonging to blood group O was added. The tubes were shaken well and kept for 1 hour in the water bath at 37° C. They were then spun down in a centrifuge at medium speed for 1 minute and read macroscopically for agglutination. The results are given in Table II.

TABLE II

Agglutination of Rh_1 Cells by Anti-Rh Sera after Treatment of the Latter with Amniotic Fluids

Liquor amnii supernatants	I Anti-Rh′ serum (70 per cent)		II Anti-Rh_0' Serum (DiY, 87 per cent)	
	Amniotic fluid from Rh-positive baby	Amniotic fluid from Rh-negative baby	Amniotic fluid from Rh-positive baby	Amniotic fluid from Rh-negative baby
(1) Undiluted	−	+++	+	++
(2) 1:3	±	+++	+	+++
(3) 1:9	+	+++	++	+++
(4) 1:27	++	+++	+++	+++
(5) 0	+++	+++	+++	+++

Agglutination of Rh_1 cells is inhibited to a certain extent at least by the Rh-positive amniotic fluid. The specificity of the inhibition is proved by the failure of the Rh-negative fluid to inhibit the agglutination of the Rh_1 cells under the same conditions. However, the anti-Rh serum used in the first part of the experiment (anti-Rh′) seems to be better suited for the type of experiments described than the second serum (anti-Rh_0', DiY). In the latter case, the degree of inhibition is rather limited and would not be sufficient to draw any definite conclusions. It can easily be understood therefore, that investigators may fail to recognize the presence of Rh substances in amniotic fluid if such an anti-Rh serum is used. According to our experience, there is a considerable individual difference as to the usefulness of various anti-Rh sera for the demonstration of the Rh substances in amniotic fluid.

The importance of quantitative considerations in regard to the demonstration of Rh substances in amniotic fluid, is illustrated by the following experiment:—

Decreasing amounts of (A) an Rh-positive amniotic fluid, (B) an Rh-negative amniotic fluid (the same fluids as used in experiments 1 and 2), in the volume of 0.05 cc., were mixed

with 0.05 cc. of the anti-Rh serum Kru diluted, (I) 1:4; (II) 1:8; and (III) 1:16. The mixtures were incubated for 2 hours in the ice box and kept for 10 minutes at room temperature whereupon 0.05 cc. of a 3 per cent suspension of Rh_1 cells belonging to the blood group O was

TABLE III

Agglutination of Rh_1 Cells by Different Dilutions of Anti-Rh Serum after Treatment of the Latter with Amniotic Fluids

Liquor amnii supernatants	A Amniotic fluid from an Rh-positive baby	B Amniotic fluid from an Rh-negative baby
Part I		
Agglutination of Rh_1 Cells by 1:4 Diltued Anti-Rh Serum		
(1) Undiluted	++	+++
(2) 1:2	+++	++++
(3) 1:4	+++	++++
(4) 1:8	+++	++++
(5) 1:16	+++	++++
(6) 1:32	+++	++++
(7) 0	++++	++++
Part II		
Agglutination of Rh_1 Cells by 1:8 Diluted Anti-Rh Serum		
(1) Undiluted	±	+++
(2) 1:2	±	+++
(3) 1:4	+	+++
(4) 1:8	+	+++
(5) 1:16	++	+++
(6) 1:32	++	+++
(7) 0	+++	+++
Part III		
Agglutination of Rh_1 Cells by 1:16 Diluted Anti-Rh Serum		
(1) Undiluted	−	+++
(2) 1:2	−	+++
(3) 1:4	±	+++
(4) 1:8	±	+++
(5) 1:16	+	+++
(6) 1:32	++	+++
(7) 0	+++	+++

added. The tubes were shaken thoroughly, kept for 1 hour at room temperature, and spun down. The resulting agglutination is shown in Table III.

This experiment points to the importance of the Rh antiserum dilution used. The serum in a dilution of 1:4 barely allows the recognition of the presence of

the Rh substance in the Rh-positive amniotic fluid. In contrast, the same antiserum when diluted 1:16 is definitely inhibited by this amniotic fluid. The specificity of the inhibition is demonstrated by the failure of the Rh-negative amniotic fluid to show a similar degree of inhibitory potency. We, therefore, chose for our experiments antiserum dilutions producing a 3+ agglutination in the control tube rather than a 4+ agglutination which is frequently too strong to give clear cut results. On the other hand, definite agglutination has to be achieved in order to avoid mistaken interpretations of experimental findings. For this reason preliminary titrations with mixtures of decreasing amounts of the Rh antisera and the Rh-positive cells to be used in the main experiment were set up in order to find the optimal conditions.

The examination of amniotic fluids for the presence or absence of Rh substance is complicated by the existence of different subtypes of the Rh factor. Consequently, all experiments were set up with both Rh_1 and Rh_2 cells as routine. In addition, Rh_0 cells were used whenever the circumstances seemed to warrant them. To our surprise, the majority of amniotic fluids examined contained Rh_2 as well as Rh_1 substances. However, there was a small number of amniotic fluids which seemed to contain either the Rh_1 or the Rh_2 factor alone as shown in the following experiment:—

Decreasing amounts of amniotic fluid, volume 0.05 cc., were mixed with 0.05 cc. of our anti-Rh serum (Kru, 87 per cent variety) and kept for 2 hours in the ice box. After standing for 10 minutes at room temperature, 0.05 cc. of a 3 per cent suspension of Rh-positive cells was added. The mixtures were allowed to remain for 1 hour at room temperature and then spun down. The experiment was carried out in three parts. In Part I, Rh_1 group O cells, in Part II, Rh_2 group B cells, and in Part III Rh_0 group O cells were added. The anti-Rh serum was neutralized by the addition of the isolated blood group specific substances A and B (Lilly) (20) and used in final dilutions ranging from 1:4 to 1:6. The respective dilution was determined by preliminary agglutination tests with the test cells employed. Ten amniotic fluids were examined in this experiment. The first eight originated from Rh-positive babies, the ninth and tenth from Rh-negative ones. The results are recorded in Table IV.

The following facts manifest themselves from the experiment shown in Table IV: (1) The first two amniotic fluids, (*a*) and (*b*), inhibit the agglutination of Rh_1 cells but not of Rh_2 cells. (2) Amniotic fluids (*c*) and (*d*) inhibit the agglutination of Rh_2 cells but there is no inhibition or very little inhibition of agglutination of Rh_1 cells. (3) Amniotic fluids (*e*) and (*f*) inhibit both Rh_1 and Rh_2 cells. (4) The Rh-negative fluids (*i*) and (*k*) fail to show any inhibitory substances.

None of the fluids inhibits the agglutination of Rh_0 cells to any considerable extent. It should be mentioned, however, that our series of amniotic fluids came from white persons only and it is known that the Rh_0 type occurs very rarely (2.5 per cent) in the white population (21). Besides, the antiserum used in the experiment may have been poor in Rh_0 antibodies.

TABLE IV

Agglutination of Rh-Positive Human Red Blood Cells by Anti-Rh Serum after Treatment of the Latter with Amniotic Fluids

Liquor amnii supernatants	Amniotic fluids from Rh+ babies								Amniotic fluids from Rh− babies	
	a (No. 91)	*b* (No. 144)	*c* (No. 81)	*d* (No. 161)	*e* (No. 128)	*f* (No. 140)	*g* (No. 135)	*h* (No. 169)	*i* (No. 60)	*k* (No. 115)
Part I										
Agglutination of Human Red Blood Cells of the Rh_1 Type by Anti-Rh Serum after Treatment of the Latter with Amniotic Fluids										
(1) Undiluted	−	−	++	++	−	−	++	++	++	++
(2) 1:2	−	−	++	++	−	−	++	+++	+++	+++
(3) 1:4	−	±	+++	++	±	−	+++	+++	+++	+++
(4) 1:8	±	+	+++	+++	+	±	+++	+++	+++	+++
(5) 1:16	+	++	+++	+++	++	++	+++	+++	+++	+++
(6) 1:32	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
(7) 0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Part II										
Agglutination of Human Red Blood Cells of the Rh_2 Type by Anti-Rh Serum after Treatment of the Latter with Amniotic Fluids										
(1) Undiluted	+++	++	−	−	−	−	++	++	+++	+++
(2) 1:2	+++	++	−	−	−	−	+++	++	+++	+++
(3) 1:4	+++	+++	−	−	−	±	+++	+++	+++	+++
(4) 1:8	+++	+++	±	+	±	+	+++	+++	+++	+++
(5) 1:16	+++	+++	++	+	+	++	+++	+++	+++	+++
(6) 1:32	x	+++	+++	+++	++	+++	+++	+++	+++	+++
(7) 0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Part III										
Agglutination of Human Red Blood Cells of the Rh_0 Type by Anti-Rh Serum after Treatment of the Latter with Amniotic Fluids										
(1) Undiluted	+++	+++	+	++	+	++	+++	+++	+++	+++
(2) 1:2	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
(3) 1:4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
(4) 1:8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
(5) 1:16	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
(6) 1:32	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
(7) 0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

x, tube missing.

It is interesting to note that two fluids, (*g*) and (*h*), do not contain any demonstrable Rh substances in spite of the fact that the respective babies were Rh-positive. Assuming that the Rh substances found in the amniotic fluid

are derived from the baby and not from the mother, we are dealing here with two cases of non-secretors.

The question arose, therefore, whether the Rh substances found in the amniotic fluid came from the baby or from the mother. As mentioned before, the blood group specific substances found in the amniotic fluid correspond to the baby's blood group and not to the mother's (7). In order to elucidate the origin of the Rh substances in the amniotic fluid, the relationship between

TABLE V

Occurrence of Rh Substances in Amniotic Fluid in Relationship to the Rh Type of Mother and Baby

Case No.	Mother's blood		Baby's blood		Rh substances in amniotic fluid
	Blood group	Rh type	Blood group	Rh type	
1	A	Rh^-	A	Rh^+	Positive
2	O	Rh^+	O	Rh^+	Positive
3	O	Rh^+	A	Rh^+	Positive
4	A	Rh^-	A	Rh^+	Positive
5	AB	Rh^-	AB	Rh^+	Negative
6	O	Rh^+	A	Rh^+	Negative
7	O	Rh^+	A	Rh^+	Positive
8	O	Rh^+	A	Rh^+	Positive
9	O	Rh^+	O	Rh^+	Positive
10	O	Rh^+	O	Rh^+	Positive
11	A	Rh^+	O	Rh^-	Negative
12	A	Rh^-	A	Rh^+	Positive
13	O	Rh^+	O	Rh^-	Negative
14	A	Rh^+	A	Rh^+	Positive
15	AB	Rh^+	AB	Rh^+	Negative
16	A	Rh^+	O	Rh^+	Positive
17	B	Rh^+	B	Rh^+	Positive
18	O	Rh^+	O	Rh^+	Positive
19	A	Rh^+	A	Rh^+	Positive
20	AB	Rh^-	A	Rh^+	Positive

the occurrence of Rh substances on the one hand and the Rh types of mother and baby on the other hand was examined. The findings in twenty more or less consecutive cases are recorded in Table V.

There are five instances (Nos. 1, 4, 5, 12, and 20) of Rh-negative mothers with Rh-positive babies. In four cases (Nos. 1, 4, 12, and 20) the amniotic fluids contained Rh substances. Two examples (Nos. 11 and 13) of Rh-positive mothers with Rh-negative babies are given. No Rh substances were found in the respective amniotic fluids. Consequently, the Rh content of the amniotic fluid is determined by the baby's Rh type and not that of the mother.

In two instances (Nos. 6 and 15) no Rh substances were present in the amniotic fluids in spite of the fact that both mother and baby were found to be

Rh-positive. Therefore, we are dealing here with Rh non-secretors, two other examples of which were already reported in the preceding Table IV. Case 5 can also be readily explained on the basis of non-secretion of Rh substance. In this case there was no Rh substance in the amniotic fluid though the baby was Rh-positive and the mother Rh-negative. This particular case is of especial interest to us because it was a clinical case of erythroblastosis and will be discussed later.

The determination of the Rh factor of the baby is complicated by the fact that placental blood cells, as well as red blood cells obtained from newborn babies, occasionally are not strongly agglutinated by anti-Rh serum. This, too, parallels the experience gained with the four major blood groups. However, in the case of the Rh factor, the complications arising from the weak

TABLE VI

Comparison between Secretion and Non-Secretion of Rh Substances and of Blood Group Specific Substances into Amniotic Fluid

Case No.	Rh type	Rh secretion	Blood group	Blood group secretion
122	Rh^-	Negative	A	Positive
115	Rh^-	Negative	A	Negative
6	Rh^+	Negative	A	Positive
112	Rh^+	Negative	A	Negative
15	Rh^+	Positive	A	Positive
27	Rh^+	Positive	A	Negative
97	Rh^+	Negative	B	Positive
28	Rh^+	Positive	B	Positive
16	Rh^+	Negative	AB	Negative
143	Rh^+	Negative	AB	Positive
106	Rh^+	Positive	AB	Positive

agglutinability of the newborn cells are even more prone to give rise to mistaken determinations. We had several instances of seemingly Rh-negative babies whose amniotic fluids contained Rh substances. In some of these cases, repeated examination of the baby's cells revealed small clumps under the microscope provided stronger concentrations of anti-Rh serum were used.

As mentioned before the secretion of the blood group specific substances A and B into the body fluids is a constitutional property, the gene of secretion being dominant over the gene of non-secretion. The question arose whether the secretion of the Rh substances into amniotic fluid was governed by the same gene responsible for the secretion of the blood group specific substances or by an entirely independent gene. Ten amniotic fluids have been selected at random. The occurrence of group specific substances and of Rh substances in these fluids is recorded in Table VI.

According to the data presented in Table VI there were four of the ten cases

which could be at variance with the explanation of a single controlling gene; the strongest evidence for this assumption is case 27 in which Rh substance and not the blood group specific substance was found in the secretion.

Results reported in this paper are based upon the examination of a series of 100 amniotic fluids. Actually many more were collected but had to be excluded

TABLE VII

Analysis of a Series of 100 Amniotic Fluids

I. Blood Groups of Newborn		
Group O	41	
Group A	43	
Group B	11	
Group AB	5	
II. Blood Group Specific Secretion into Amniotic Fluid		
1. Group A	43	
Secretors	34	79.1 per cent
Non-secretors	9	20.9 per cent
2. Group B	11	
Secretors	11	100.0 per cent
Non-secretors	0	0.0 per cent
3. Group AB	5	
Secretors	2	40.0 per cent
Non-secretors	3	60.0 per cent
III. Rh Types of Newborn		
Rh-positive	86	
Rh-negative	14	
IV. Rh Secretion into Amnoitic Fluid		
1. Rh-positive	86	
Secretors	71	82.6 per cent
Non-secretors	15	17.4 per cent
2. Rh-negative	14	

from our final analysis because of one of the following four reasons: (1) The baby's blood cells were not obtained. (2) Occasional amniotic fluids proved to be hemolytic—a phenomenon for which we have no explanation at the present time. (3) The amniotic fluid was contaminated with blood, thus making a definite diagnosis difficult. (4) In some instances the diagnosis of the Rh type was not convincingly clear either as far as the baby's blood cells,

or as far as the occurrence of Rh substances in the amniotic fluid were concerned. This occurred in about 10 per cent of the amniotic fluids studied.

However, there was no doubt in our minds as to the baby's Rh type and as to the occurrence of the Rh substances in the amniotic fluids in the 100 remaining cases. The analysis of these 100 cases is given in Table VII.

The distribution of the major blood groups as well as their division into secretors and non-secretors follows the expected percentages as recorded in the American white population. The percentage distribution of Rh-positive and Rh-negative bloods, too, agrees with the figures related to the American white population—86 per cent being Rh-positive and 14 per cent Rh-negative. Among the Rh-positive newborns a great majority, at least four out of five secrete Rh substances into the amniotic fluid. It is possible that the percentage of non-secretors reported in this paper, namely 17.4 per cent might be reduced somewhat further because the content of Rh substances in some instances might have been so slight that it could not be detected by our present methods. A similar situation is found regarding the secretion of the blood group-specific substances. The occurrence of small amounts of A and B substances makes it difficult sometimes to decide whether we are dealing with a secretor or a non-secretor. Concentration and isolation of the Rh substances from the amniotic fluid will materially clarify the problem of weak secretors.

DISCUSSION

The Rh subtypes of the placental blood cells could not be determined as routine. We had to be satisfied with the diagnosis of the Rh factor as such, employing for that purpose our Rh anti-serum (Kru) which belongs to the 87 per cent variety. However, small amounts of anti-Rh′ and anti-Rh″ sera were available and were used occasionally. The diagnosis of the subtypes of the Rh factor of the red blood cells of the newborn proved to be a somewhat difficult and doubtful procedure in some cases. It should be mentioned in this connection that the amniotic fluid of a 5 months old fetus contained Rh substances.

While the determinations of the subtypes of the Rh factor of the newborn's cells were not included in this study, the amniotic fluids themselves were tested against Rh_1 and Rh_2 cells on a routine basis and frequently against Rh_0 and Rh_1Rh_2 cells also. The occurrence of the Rh_0 factor in amniotic fluid will have to be studied later preferably with fluids from colored patients because of the reported higher incidence of the Rh_0 type in colored people (41.6 per cent) (22).

The interest of the medical profession in the Rh factor has been aroused by its relationship to the pathogenesis of erythroblastosis. According to the observations of Levine and his coworkers erythroblastosis of the newborn is caused by isoimmunization in which the Rh factor plays a predominant rôle.

At the present time it is difficult to understand why differences in the four major blood groups do not constitute a cause of erythroblastosis. This has been explained tentatively by the fact that the blood group specific substances occur in all tissues and organs in addition to the red blood cells while the Rh factor is supposedly limited to the red blood cells.

The investigations reported in this paper prove the occurrence of Rh substances in amniotic fluid. From the quantitative standpoint the amounts are considerably smaller than those of the A and B substances also present in amniotic fluid.

Is there any connection between erythroblastosis on the one hand and the state of secretion or non-secretion of the Rh substances into amniotic fluid on the other hand? Such a possibility has been mentioned previously at a time however, when there was nothing known about the presence or absence of Rh factors in body fluids.

There was one case of erythroblastosis among the 100 cases reported here, namely, case 5 (Table V), exhibiting the following features:—

This patient's first pregnancy resulted in a normal, living child born in August, 1943. In November, 1944, being about 8½ months pregnant, she consulted a physician because she had felt no life for about 2 weeks. At this time no fetal heart sounds were heard and there was x-ray evidence of fetal death. About 2 weeks later she had a still-birth of a macerated, male fetus. The blood cells of the fetus were Rh-positive. The amniotic fluid did not contain any Rh substances, suggesting the fact that we were dealing with an Rh-positive non-secretor. This amniotic fluid though was contaminated with meconium. The mother was Rh-negative and "blocking antibodies" of high titer were found in her serum. There were no Rh agglutinins demonstrable by the usual test tube incubation method.

We had the opportunity to examine two more cases of erythroblastosis in addition to the 100 cases analyzed in this paper. Brief summaries of these cases are as follows:—

The second case of erythroblastosis (No. 176) presented the following features:—

In 1938 the patient bore a normal, living child. Her second pregnancy in 1943 ended at term with the delivery of a living child who died 3 days after birth of unknown causes. In March, 1945, the patient's third pregnancy terminated in the delivery a markedly jaundiced baby who showed 50 per cent nucleated red blood cells in the cord smear. The baby died 4 days after birth with typical manifestations of erythroblastosis neonatorum. The mother was found to be Rh-negative, the baby to be Rh-positive. "Blocking antibodies" were demonstrated in the mother's serum. The amniotic fluid, which was slightly bloody, showed no Rh substances to be present.

The third case (No. 179) of erythroblastosis gave the following history:—

In 1939 the patient had a spontaneous miscarriage at 4 months. Her second pregnancy ended in October, 1943, in a macerated still-born baby. When she became pregnant the third time her blood was examined for the Rh factor. The patient was found to be Rh negative. Examination of her serum revealed the presence of "blocking antibodies" in high

titer. As a result of this information, the obstetrician decided upon a Caesarean section 4 weeks before term in an attempt to save the baby's life. The operation was performed in March, 1945, with the delivery of a live but icteric child. The baby became increasingly jaundiced and died 4 days after birth in spite of the premature delivery and at postmortem presented typical findings of icterus gravis and other manifestations of erythroblastosis neonatorum. The baby was Rh-positive. Examination of the amniotic fluid obtained at delivery showed that it was free from Rh substances. This amniotic fluid was clean and not contaminated with either blood or meconium.

Our three cases of erythroblastosis came from Rh-negative mothers who had been delivered of Rh-positive children, all three of whom proved to be non-secretors. We are fully aware of the fact that the number of cases is insufficient to draw any conclusions regarding the pathogenesis of erythroblastosis. However, the findings seem to be of sufficient interest to warrant further investigations in that direction.

It seems conceivable that difference in sub-types might also have to be considered in this connection. For instance, the absence of one part of the Rh system in spite of the presence of a different part might constitute a sufficient reason for the development of erythroblastosis. Similar considerations might also be entertained to explain erythroblastosis on the basis of the Hr factor.

SUMMARY

1. Rh substances are found in amniotic fluid. Not all anti-Rh sera seem to be suitable for the detection of Rh substances in amniotic fluid. Careful selection of Rh antisera, as well as quantitative considerations, determine success or failure of their demonstration.

2. The baby's Rh type and not the mother's determines the occurrence of Rh substances in amniotic fluid.

3. There are Rh secretors and Rh non-secretors. At least four out of five individuals are secretors.

4. The secretion of Rh substance into the amniotic fluid would seem to be entirely independent of the secretion of the blood group specific substances.

5. The majority of Rh-positive amniotic fluids seem to contain both Rh_1 and Rh_2 substances. However, in certain instances fluids belonging to the pure Rh_1 type or pure Rh_2 type were found.

6. Three cases of erythroblastosis were described. All three came from Rh-negative mothers with Rh-positive babies. The amniotic fluids of all three failed to reveal the presence of Rh substances.

BIBLIOGRAPHY

1. Witebsky, E., *Z. Immunitätsforsch.*, 1926–27, **49**, 1, 517.
2. Witebsky, E., and Okabe, K., *Z. Immunitätsforsch.*, 1927, **52**, 359.
3. Kritschewski, I., and Schwarzmann, L., *Klin. Woch.*, 1927, **6**, 2090.
4. Hirszfeld, L., Halber, W., and Laskowski, J., *Klin. Woch.*, 1929, **8**, 1563.

5. Schiff, F., Über die gruppenspezifischen Substanzen des menschlichen Korpers, Jena, Gustav Fischer, 1931.
6. Friedenreich, V., and Hartmann, G., *Z. Immunitätsforsch.*, 1938, **92,** 141.
7. Putkonen, T., *Acta Med. Fenn. "Duodecim,"* 1930, series A, **14,** No. 2.
8. Hartmann, G., Group antigens in human organs, Copenhagen, Ejnar, Munksgaard, 1941.
9. Schiff, F., and Sasaki, H., *Z. Immunitätsforsch.*, 1932, **77,** 129.
10. Boorman, K., and Dodd, B., *J. Path. and Bact.*, 1943, **55,** 329.
11. Levine, P., and Katzin, E., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48,** 126.
12. Wiener, A. S., and Forer, S., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47,** 215.
13. Oettingen, K., and Witebsky, E., *Münch. med. Woch.*, 1928, **75,** 385.
14. Witebsky, E., and Reich, H., *Klin. Woch.*, 1932, **11,** 1960.
15. Reich, H., *Z. Immunitätsforsch.*, 1933, **77,** 449.
16. Dockeray, G., and Sachs, H., *J. Immunol.*, 1944, **48,** 241.
17. Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13,** 2306.
18. Belkin, R. B., and Wiener, A. S., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56,** 214.
19. Wiener, A. S., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56,** 173.
20. Witebsky, E., Klendshoj, N., and Swanson, P., *J. Am. Med. Assn.*, 1941, **116,** 2654.
21. Wiener, A. S., *Science*, 1944, **99,** 532.
22. Wiener, A. S., Belkin, R. B., and Sonn, E. B., *Am. J. Phys. Anthropol.*, 1944, **2,** 187.