# γA-BLOOD GROUP ANTIBODIES

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It is clear that anti-A and anti-B are sometimes γA-globulin (1–3) but evidence about other blood group antibodies is conflicting. The purpose of the present work was first to find out more about the serological characteristics of γA–anti-A and anti-B and second to search for γA-isoantibodies of other blood specificities. In view of the fact that γA-globulin is present in relatively large amounts in saliva and colostrum (4, 5), these secretions, as well as serum, were examined.

## *Methods*

*Donors of Anti-A and Anti-B.—*

*Selected potent antisera:* Five sera were selected; 4 were from group O women who had given birth to infants severely affected with hemolytic disease; 2 of the women (Bev. and Bak.) had each given birth to three infants affected with severe hemolytic disease due to A incompatibility; the 3rd (Byr.) had given birth to one infant with moderately severe hemolytic disease due to A incompatibility; and the 4th (Vel.) had given birth to one infant with hemolytic disease due to A incompatibility followed by one with hemolytic disease due to B incompatibility. The 5th serum was from a male group O subject who had been given two subcutaneous injections, each of 2 mg of group B substance, at an interval of 7 days and had been bled 10 days after the second injection.

*Anti-A and anti-B in colostrum and serum:* Samples of colostrum and serum were collected from 17 "random" mothers (8 group A, 6 group O, and 3 group B), between 3 days before and 1 day after delivery; the method of treating the colostrum is described below. Blood samples were taken from the same women by venepuncture within 2 days of delivery; the serum was separated from clotted blood as soon as possible and stored at −50°C if not tested immediately.

*Anti-A and anti-B in saliva and serum:* Samples of saliva and serum were obtained from 8 group O, 9 group A, and 7 group B normal subjects and examined for the presence of γA-anti-A or anti-B as described below.

*Donors of Anti-Rh.—*

*Anti-D:* Sera were regarded as containing anti-D when they reacted with D-positive red cells and not with D-negative red cells; no attempt was made to distinguish between anti-D and anti-CD etc.

From 25 women immunized to Rh(D) by pregnancy, serum alone was tested; 11 of the women had had at least one very severely affected infant (stillbirth, or live birth with cord hemoglobin concentration less than 9 g/100 ml).

One woman (Avg.) proved to be of special interest and for this reason further details of her history are given. She was born in 1909. Between 1934 and 1948 she gave birth to four

infants with Rh hemolytic disease including two stillbirths. Some time between 1945 and 1949 she was diagnosed as having hereditary spherocytosis and during this period was transfused with 28 units of blood. It is possible that some of the units transfused in 1945 may have been Rh positive. Splenectomy was carried out in 1949. She later developed hemochromatosis and between January 1961 and May 1962 approximately 35 liters of her blood were removed by venesection. During the subsequent 18 months the total amount of blood taken was less than 3 liters. In the middle of the course of venesection the patient agreed to receive some small injections of Rh-positive red cells in the hope of increasing the titer of Rh antibody in her serum. Further details are given below in Results.

From 5 additional women immunized by pregnancy to Rh(D), colostrum as well as serum was tested and in two of these cases saliva, obtained 7 days after delivery, was also tested.

Serum from 2 male subjects immunized by blood transfusion was examined; 1 patient had been transfused with Rh(D)-positive blood 7 yr previously and was examined 1 month after he had received a second transfusion of D-positive blood. The other patient had received transfusions of D-positive blood 3 yr previously and probably also 16 and 24 yr previously.

*Anti-c:* Two women with anti-c in their serum were examined; 1 woman had been immunized by pregnancy alone and the other by blood transfusion followed by pregnancy which resulted in a macerated stillbirth.

*Sera Containing Antibodies Other Than Those of the ABO and Rh Systems.*—Three sera containing Lewis antibodies were tested; 1 (containing anti-$Le^b$ and anti-$Le^a$) was from a subject (Cri.) who had received transfusions of Le(a+) plasma, injections of purified $Le^a$ and $Le^b$ substances and transfusions of Le(b+) red cells (6); the 2nd (containing anti-$Le^a$ only) was from a subject (Bur.) who had received transfusions of Le(a+) blood as well as injections of Le(a+) red cells (7); the 3rd, (Spe) kindly provided by Miss Carolyn Giles, contained mainly anti-$Le^a$ with a trace of anti-$Le^b$ and came from a woman who had never been transfused or been pregnant.

Three sera containing anti-K, 3 containing anti-$Fy^a$, and 3 containing anti-$Jk^a$ were also tested; all the subjects had been immunized by incompatible blood transfusions.

*Collection and Treatment of Colostrum and Saliva.*—Colostrum was collected by simple expression of the breast, between 3 days before and 1 day after delivery; the sample was centrifuged and the fatty layer removed; the centrifuged colostrum was then adjusted to pH 4.6 with acetic acid and the precipitate (casein) removed by centrifugation; the supernatant was adjusted to pH 7.4 with a solution of sodium hydroxide and stored at −20°C. (5).

In collecting saliva, the subject was asked to provide at least 2 ml in a clean beaker. The sample was centrifuged and any deposit discarded. The supernatant was stored at −20°C for not more than 3 days before being tested. $\frac{1}{10}$ volume of 5% NaCl was added to each sample to ensure that it would not be so hypotonic as to lyse red cells.

*Sera Used as a Source of Complement.*—Fresh group O serum containing low titer anti-A and anti-B was absorbed at 0°C three times with group A or group B red cells, previously washed three times with buffered saline.

Guinea pig serum, separated as soon as possible from freshly drawn blood, was absorbed once, at 0°C with human red cells.

*Human Group A and B Substances.*—Purified group A and B substances, prepared from pseudomucinous ovarian cyst fluids, was kindly provided by Professor W. T. J. Morgan. A solution containing 2 mg per 1 ml was used in the inhibition tests.

*DEAE-Cellulose Chromatography.*—Step-wise elution with phosphate buffers was used throughout. Two methods of fractionating the serum were used: In "method A" the first buffer was 0.0175 M, pH 6.5 and the following buffers were: 0.04; 0.06; 0.08; 0.12; 0.14; 0.16; and 0.2 M, at pH 8.1. In "method B" only three buffers were used: 0.0175 M, pH 6.5; 0.08 M, pH 8.1; and 0.2 M pH 8.1. The proteins eluted in each fraction were concentrated by pressure

dialysis and dialyzed against "buffered saline", (0.85% sodium chloride buffered with phosphate to pH 7.4).

Colostrum was fractionated following the method suggested by Tomasi et al. (5); thus three phosphate buffers were used: (*a*) 0.01 M, pH 7.5; (*b*) 0.1 M, pH 6.4; and (*c*) 0.3 M, pH 4.7. A single protein peak was obtained with eluants a and c but a double peak with eluant b, so that in all, four protein peaks were collected. The proteins present in each fraction were concentrated as in the method used for the serum fractions.

*Treatment with 2-Mercaptoethanol (2-ME).*—1 volume of serum, colostrum or fraction, was mixed with an equal volume of 0.1 M 2-ME in phosphate buffer pH 7.4 and left at 37°C for 2 hr. Iodoacetamide in phosphate buffer was then added to a final molarity 0.05. The mixture was left at 0°C for 15 min and then dialyzed against buffered saline at 4°C. As a control, 1 volume of serum, or colostrum or fraction, was treated with buffer alone in the same way.

*Heating at 56°C for Various Periods of Time.*—Aliquots of colostrum diluted with an equal volume of buffered saline, and the 0.017, 0.08, and 0.2 M fractions prepared from Bak. serum, were heated at 56°C for 1, 2, and 3 hr.

*Anti-A and Anti-B Agglutinins and Hemolysins.*—In testing anti-A and anti-B agglutinins, serial doubling dilutions of serum, colostrum, or saliva were incubated for 1½ hr with a suspension of appropriate red cells in saline. In testing serum and colostrum 1 volume was mixed with 1 volume of a 2% red cell suspension but in testing saliva, because the agglutinin titers were low, 4 volumes of saliva (or of a dilution of saliva) were mixed with 1 volume of red cell suspension.

In testing the hemolytic activity of colostrum, and of fractions of colostrum or serum, fresh complement was supplied. 4 volumes of each of a series of doubling dilutions of the antibody-containing samples were incubated at 37°C for 10 min with 2 volumes of complement and 1 volume of a 10% suspension of group A or B red cells. The tubes were spun and the degree of hemolysis scored. This method is referred to as the "one-stage" method.

In the "two-stage" method red cells were first sensitized with antibody, washed, and then incubated at 37°C with complement. Thus 4 volumes of colostrum, of serum (previously heated at 56°C for 20 min) or of fractions of colostrum or serum were incubated at 37°C for 90 min with 1 volume of a 10% suspension of appropriate red cells. The sensitized red cells were then washed three times with ice-cold saline and resuspended in saline to give a 10% suspension. 2 volumes of complement were added to each tube and the mixture incubated at 37°C for 30 min.

*Enhancement of Agglutination in Serum.*—Serial dilutions of serum, colostrum, or fractions were prepared using as a diluent group O serum, heated at 56°C for 15 min and then absorbed three times with group A and B red cells, and finally diluted with an equal volume of buffered saline.

*Inhibition of Anti-A Agglutinin with A Substance.*—The anti-A agglutinin present in colostrum (Roo.) and in the 0.0175, 0.08, and 0.2 M fractions of Bak. serum was tested before and after inhibition with various dilutions of A substance. The procedure used was similar to that described previously by Polley et al. (8).

*Indirect Antiglobulin Tests and Estimation of γG-, γA-, and γM-Globulins.—Anti-γA:* Four different sera were used. The first, kindly supplied by Dr. J. F. Soothill, had been made in a rabbit, by injecting urine from a patient with γA-myelomatosis. The second, kindly supplied by Professor S. Cohen, had been made in a rabbit by injecting serum from a patient with γA-myelomatosis; the remaining two sera were purchased from Hoechst Pharmaceutical Company, Frankfurt, Germany, and had been made by injecting γA-globulin from normal human serum into a rabbit and a goat respectively.

The samples of anti-γA were made specific by adding γG-globulin; after the treatment the immune sera gave only one line of precipitation when tested with immunoelectrophoresis

against human serum or a γA-globulin preparation kindly provided by Dr. S. Cohen; no line of precipitation was observed when the immune sera were tested against cord serum.

The immune sera did not agglutinate Rh-positive red cells sensitized with an incomplete anti-D eluted in the 0.0175 M fraction. As described in Results the specificity of the anti-γA-globulin sera was confirmed by showing that the reaction against Rh-positive red cells sensitized with an incomplete anti-D γA-antibody was not inhibited by cord serum.

*Anti-γG:* Sera obtained by injecting highly purified human γG-globulin into sheep and rabbits were used. The antisera contained 3 to 5 mg anti-γG and contained no demonstrable antibodies against other human serum proteins (9).

*Anti-γM:* Two sera obtained by injecting human pathological γM-globulin into rabbit were used; the immune sera were made specific by treatment with γG-globulin (10).

*Anti-$\beta_{1E}$ and anti-$\beta_{1C}$ (anti-C'):* Two sera were used: an anti-$\beta_{1E}$-globulin serum (M59) described by Polley et al. (11) and an anti-$\beta_{1E}$- + anti-$\beta_{1C}$-globulin serum (M 65), prepared as follows. Rabbit red cells were first sensitized with EDTA-treated horse serum, then washed and incubated at 37°C for 30 min with human fresh serum which had been previously absorbed with rabbit red cells and treated with zymosan (1 ml serum/3 mg zymosan type A) at 37°C. Several injections of red cells treated in this way were given intravenously to a rabbit.

For convenience the anti-$\beta_{1E}$- and anti-$\beta_{1E}$ + anti-$\beta_{1C}$-sera are referred to collectively as anti-C' globulin sera.

*Indirect Antiglobulin Tests.*—The anti-γG- and anti-γM-globulin tests were made as previously described (9), and a similar method was used with anti-γA (12); the dilutions of anti-γA-globulin serum used ranged from 1:50 to 1:200 and were those found optimal in testing Rh-positive red cells sensitized with an incomplete anti-D γA-antibody (see Results).

The anti-C' globulin tests were carried out using a two-stage method (13); thus 1 volume of a 20% suspension of red cells was incubated at 37°C for 90 min with 4 volumes of EDTA-treated serum (1 ml serum/4 mg EDTA) containing the incomplete antibody and then washed three times with buffered saline; the sensitized red cells were then incubated at 37°C for 20 min with 2 volumes of human complement. After being washed, the red cells were tested with a suitable anti-C' globulin serum.

*Estimation of the Degree of Agglutination.*—In the present paper the results of titrating antibodies are expressed either as an "end point" using the reciprocal of the last dilution of serum to show agglutination or as a "score" (14). In comparative titration the score method was found to be the more useful way of describing the pattern of an antibody in serum or fractions (14, 15).

*Estimation of γG, γM, and γA.*—The amounts of these three immunoglobulins in serum, colostrum, saliva, and in fractions obtained by DEAE-cellulose chromatography were estimated by the inhibition of antiglobulin serum method as previously described (16, 10).

γA was estimated by inhibiting the reaction between anti-γA and red cells sensitized with a particular anti-Rh serum (Avg.) which, as described below, was partly γA and partly γG (12).

*Elution of Antibody from Red Cells.*—The method of Rubin (17), slightly modified (18), was used in an attempt to demonstrate that anti-A eluates, prepared from A red cells incubated with colostrum, contained predominantly γA-globulin. In order to avoid interference from proteins absorbed nonspecifically onto the red cells and subsequently eluted, antibody was first eluted into 10% albumin; red cells were then sensitized with this eluate and a second elution performed. The concentration of γA- and γG-globulin in the second eluate was then estimated. An eluate prepared in the same way from group O red cells provided a control.

## RESULTS

*γA-Globulin in Cord Serum, Colostrum, Saliva, and in the Fractions Obtained from DEAE-Cellulose.*—In the present work estimations of γG-, γA-, and

γM-globulin in cord serum are expressed as percentages of the amounts present in the corresponding maternal serum; those in colostrum and saliva are expressed as percentages of the amounts present in the serum of a normal adult donor (Adi.) and those in the fractions obtained from DEAE-cellulose are expressed as percentages of the amount of γG-, γA-, and γM-globulins present in the original sample of serum or colostrum.

Table I shows the results. In the eight cord sera the concentration of γA-globulin was always less than 0.1% of that in the corresponding maternal serum. In the four samples of colostrum the concentration of γA-globulin ranged from 50 to 100% and in four salivas from 10 to 20% of the amount in normal serum.

The amount of γG-globulin in the four samples of colostrum was less than 1% of the amount in normal serum; only traces of γG-globulin were present

TABLE I

*Amount of γG, γM, and γA in Cord Serum, Colostrum, and Saliva, Estimated by Inhibiting Specific Antiglobulin Reactions*

| No. tested | Samples | Amounts present in normal adult sera* | | |
|---|---|---|---|---|
| | | γG | γA | γM |
| | | % | % | % |
| 8 | Cord serum | 120‡ | 0.1 | 5–20‡ |
| 4 | Colostrum | 0.2–0.6 | 50–100 | 5–10 |
| 4 | Saliva | 0.1 | 10–20 | 1–3 |

* Cord serum results expressed as percentages of concentrations in relevant maternal serum.

‡ From Polley et al. (9).

in the salivas tested. Similarly, only small amounts of γM-globulin were found in colostrum and saliva.

The results of estimating γA-globulin in the DEAE fractions obtained from serum using method A showed that about 40 to 50% of γA-globulin was recovered in the 0.08 M fraction, 20% in the 0.06 M fraction and 2% in the 0.04 M fraction (see Fig. 1). Using method B, 50 to 80% of γA-globulin was recovered in the 0.08 M fraction (see Table IV).

As mentioned in Methods four protein peaks were observed when colostrum was fractionated by DEAE-cellulose chromatography, since the 0.1 M buffer consistently resolved the colostrum protein into two peaks. The four fractions collected will be referred to as 0.01 M, 0.1 M(a), 0.1 M(b), and 0.3 M.

The results of estimating γA-globulin in these fractions showed that about 50% of γA-globulin was recovered in the 0.1 M(a) fraction and 20% in the 0.1 M(b) fraction; γG-globulin was recovered in the 0.01 M fraction and less than 1% of the γG-globulin present in whole colostrum was present in the other three fractions (see Table III).

*Frequency of Anti-A and Anti-B Antibodies in Colostrum.*—In all but one of the samples of colostrum tested, all expected isoagglutinins were present; the exceptional sample (lacking anti-B) was from a group A mother who had given birth to a premature infant at 31 wk following induction of labor.

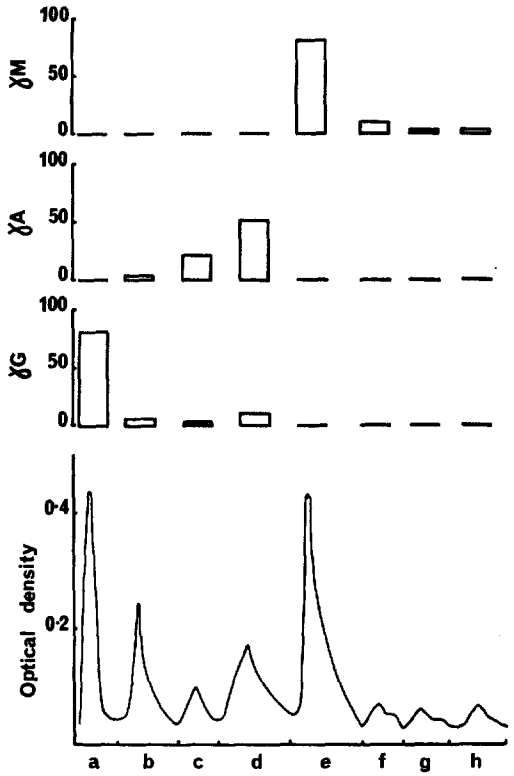


FIG. 1. Amounts of γM-, γA-, and γG-globulin, as percentages of the amounts present in whole serum, in fractions separated on DEAE-cellulose, using the following phosphate buffers as eluants: (*a*), 0.0175 M; (*b*), 0.04 M; (*c*), 0.06 M; (*d*), 0.08 M; (*e*), 0.12 M; (*f*), 0.14 M; (*g*), 0.16 M; and (*h*), 0.2 M.

The lower part of the figure shows the pattern of total protein eluted in the various fractions.

In 10 out of 16 cases in which isoagglutinins were present in colostrum the titer was higher than in serum from the same subject; and in the remaining 6 cases the titer was either the same or slightly lower in colostrum. The titers in colostrum, expressed as mean scores were as follows: in 8 group O mothers: anti-A, 62.5, anti-B, 48.0; in 3 group B mothers: anti-A 57.3; in 8 group A mothers: anti-B, 43.1.

*Frequency of Anti-A and Anti-B Antibodies in Saliva.*—The anti-A agglutinin

was present in 7 out of 8 salivas from group O donors and in 6 out of 7 salivas from group B donors. The anti-B agglutinin was present in 5 out of 8 salivas from group O donors and in 3 out of 9 salivas from group A donors.

Mean scores were as follows: group O donors: anti-A, 15.0, anti-B, 7.7; group A donors: anti-B, 6.2; group B donors: anti-A 10.1.

*Anti-A and Anti-B Antibodies in Fractions Eluted from DEAE-Cellulose.—*

*Selected potent group O sera:* Table II shows the results of testing the 0.08 M fractions which were found to contain the bulk of γA-globulin. In 2 (Bev. and Byr.) out of the 5 sera, no anti-A or anti-B could be detected in the 0.08 M fraction but in the remaining 3 the fraction agglutinated red cells to a titer of 16 to 32.

Anti-γG did not enhance the agglutination of red cells sensitized with fractions obtained from serum Vel. and serum Win.; since the indirect antiglobulin titer of γG–anti-A and anti-B is usually five dilution steps higher than the agglutination titer in saline (7) it was evident that these fractions contained no more than a trace of γG–anti-B. The fact that the antibody was mainly γA was indicated by distinct enhancement (two dilution steps) on adding anti-γA. The anti-A in the 0.08 M fraction from the serum Bak. was slightly enhanced by anti-γG and enhanced to a greater extent by anti-γA; it thus appeared that γA–anti-A was the predominant antibody although some γG–anti-A was probably also present.

TABLE II

*Results of Testing the 0.08 M Fractions from DEAE-Cellulose from Five Selected Sera Containing Potent Anti-A or Anti-B*

| | Indirect antiglobulin titer | | Saline control* |
|---|---|---|---|
| | Anti-γA | Anti-γG | |
| Bak. (anti-A) | 256 | 128 | 64 |
| Bev. " | 0 | 0 | 0 |
| Byr. " | 0 | 0 | 0 |
| Vel. " | 64 | 16 | 16 |
| Win.‡ (anti-B) | 128 | 32 | 32 |

* As a control each sample of sensitized red cells was tested with saline as well as with the specific antiglobulin sera.

‡ The first 4 sera were fractionated using "method A"; the 5th serum (Win.) was fractionated using "Method B".

Four samples of colostrum, three from group A donors (Law., Fee., and Whi.) and one from a group B donor (Roo.) were fractionated.

It was found that anti-A and anti-B agglutinins present in colostrum were recovered both in the 0.1 M(a) and 0.1 M(b) fractions, which together contain the bulk of γA-globulin. No anti-A or anti-B was found in the 0.01 M fraction;

in two out of the four samples tested, low titer agglutinins were found in the 0.3 M fraction (Table III).

In two of the group A sera fractionated, the anti-B agglutinin was found only in the 0.2 M fraction.

*Serological Properties of γA-Anti-A and Anti-B.*—The serological properties

TABLE III

*Results of Testing Anti-A and Anti-B Agglutinins in DEAE-Cellulose Fractions Prepared from Colostrum*

| | Content* of | | Anti-A or anti-B agglutinins‡ in: | | | |
|---|---|---|---|---|---|---|
| | γG-globulin | γA-globulin | Law. anti-B | Fee. anti-B | Whi. anti-B | Roo. anti-A |
| Whole colostrum | 100 | 100 | 67 | 121 | 38 | 77 |
| Fraction 0.01 M | 50 | 2 | 0 | 0 | 0 | 0 |
| Fraction 0.1 M(a) | 1 | 50 | 57 | 73 | 22 | 38 |
| Fraction 0.1 M(b) | 1 | 20 | 5 | 0 | 0 | 25 |
| Fraction 0.3 M | 1 | 2 | 8 | 0 | 0 | 0 |

* The content of γG- and γA-globulin present in the fractions is expressed as a percentage of the amount present in whole colostrum.

‡ The figures represent "scores" (see text).

TABLE IV

*Results of Testing Anti-B DEAE-Cellulose Fractions Prepared from Serum "Win."*

| | Content* of | | | Agglutination‡ | Hemolysis‡ |
|---|---|---|---|---|---|
| | γG | γA | γM | | |
| Whole serum | 100 | 100 | 100 | 152 | 51 |
| Fraction 0.0175 M | 75 | 2 | 1 | 37 | 23 |
| Fraction 0.08 M | 5 | 80 | 61 | 38 | 0 |
| Fraction 0.2 M | 2 | 2 | 100 | 131 | 39 |

* Expressed as a percentage of the content in whole serum.

‡ Results expressed as "scores".

of γA-isoagglutinins were studied either by testing samples of colostrum in which the isoagglutinins had been shown to be present only in association with γA-globulin or by testing fractions prepared from selected sera.

*Complement-Binding Activity of γA-Anti-A and Anti-B.*—Four samples of colostrum and fractions prepared from serum Win. were tested for hemolytic activity. It was found that the four samples of colostrum did not hemolyze group A or B red cells either when the red cells were incubated with colostrum in the presence of human or guinea pig complement ("one-stage" method) or

were first sensitized with EDTA-treated colostrum and then exposed to human complement ("two-stage" method). It was also found that the 0.08 M fraction prepared from Win. serum did not hemolyze group B red cells although the agglutinin titer of this fraction was very similar to that in the 0.0175 M fraction which did hemolyze group B red cells (Table IV).

Further tests were carried out to see whether γA–anti-A and anti-B were capable of binding complement components using the two-stage test (see Methods) in which cells are first incubated with antibody then with complement and finally tested with C′ globulin serum.

Addition of anti-C′ globulin serum never increased the titer of fractions con-

TABLE V

*Effect of Using Saline and Serum as Diluents in Titrating Anti-B Present in Serum or Colostrum*

| | DEAE-cellulose fraction | Diluent | Dilutions (reciprocals) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 2 | 8 | 32 | 128 | 512 | 2000 |
| Serum Win. | 0.0175 M | Saline | +++ | ++ | + | − | − | − |
| | | Serum | +++ | +++ | +++ | ++ | + | − |
| | 0.08 M | Saline | ++ | (+) | − | − | − | − |
| | | Serum | +++ | ++ | + | (+) | − | − |
| | 0.2 M | Saline | +++ | +++ | ++ | + | (+) | − |
| | | Serum | +++ | +++ | ++ | + | (+) | − |
| Colostrum Rei. | Untreated | Saline | +++ | ++ | + | (+) | − | − |
| | | Serum | +++ | +++ | ++ | + | (+) | − |

taining predominantly γA–anti-A and anti-B but regularly increased the agglutination produced by γG– or γM–anti-A and anti-B.

When whole colostrum was used as a source of anti-A or anti-B antibodies it was observed that two samples out of five sensitized red cells to anti-C′ globulin serum. One sample of colostrum (Tho.) giving a definitely positive result and one (Hol.) giving a negative result were fractionated on DEAE-cellulose. In both cases the 0.1 M(a) and 0.1 M(b) fractions, containing the bulk of γA-globulin, failed to sensitize red cells to agglutination by anti-C′ globulin serum. The 0.3 M fraction from sample Tho. gave a positive result indicating that the positive result given by whole colostrum was due to an antibody eluted with a buffer of higher molarity. Incidentally, the antibody eluted in this fraction was found to be completely inhibited by treatment with 2-mercaptoethanol at neutral pH.

*Enhancement of Agglutination by Using Serum Instead of Saline as a Medium.*

—The anti-A and anti-B agglutinins present in two samples of colostrum and in the 0.0175, 0.08, and 0.2 M fractions prepared from Win. serum were tested.

It was found that the titer of the antibody present in colostrum, in the 0.0175 M and in the 0.08 M fractions, but not that present in the 0.2 M fraction, was enhanced by using serum instead of saline as a diluent. The results of testing one sample of colostrum and the fractions obtained from serum using DEAE-cellulose are shown in Table V.

*Effect of A Substance on γA-Anti-A.*—The anti-A agglutinin present in one

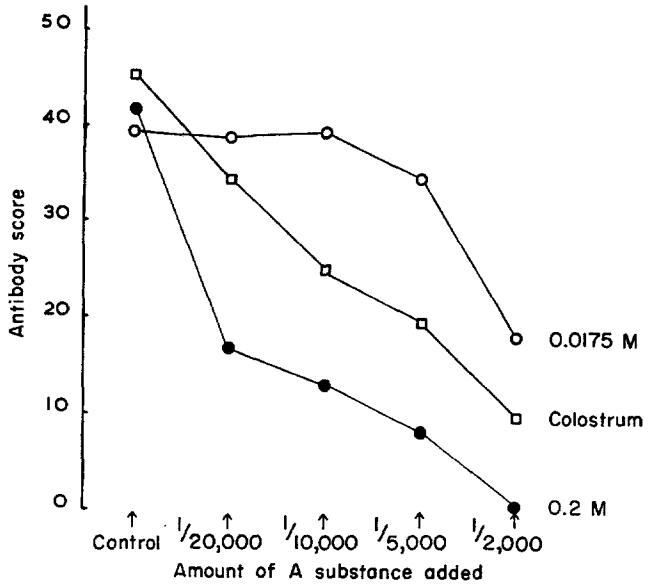


FIG. 2. Effect of varying amounts of purified human A substance (given as dilutions of a solution containing 2 mg/ml) on γG–, γA–, and γM–anti-A agglutinins.

0.0175 M and 0.2 M DEAE fractions, containing predominantly γG– and γM–anti-A respectively; colostrum predominantly γA–anti-A; see text.

colostrum (Roy.) and in the 0.0175 and 0.2 M fractions prepared from serum Bak. was tested before and after the addition of various dilutions of A substance. It was found that γA–anti-A was less easily neutralized by A substance than was γM–anti-A, but it was more easily neutralized than was γG–anti-A; see Fig. 2.

*Effect of Heating γA-Anti-A at 56°C for Various Periods.*—The results of testing anti-A agglutinin present in colostrum and in the 0.0175, 0.08, and 0.2 M fractions prepared from Bak. serum before and after heating at 56°C for 1, 2, and 3 hr are shown in Fig. 3. The anti-A agglutinin titer of the fractions containing the bulk of γG- and γA-globulins and of colostrum was unaffected by

heating at 56°C for 3 hr whereas the titer of the 0.2 M fraction (containing γM–anti-A) fell progressively.

*Effect of Treating γA–Anti-A and Anti-B with 2-Mercaptoethanol at Neutral pH.*—Three samples of colostrum and the 0.08 M fractions prepared from Win. and Bak. sera were tested before and after treatment with 2-ME; as a control the fractions containing the bulk of γG– and γM– anti-A or anti-B were also tested.

The agglutinin present in the colostrum and in the 0.08 M fraction was par-

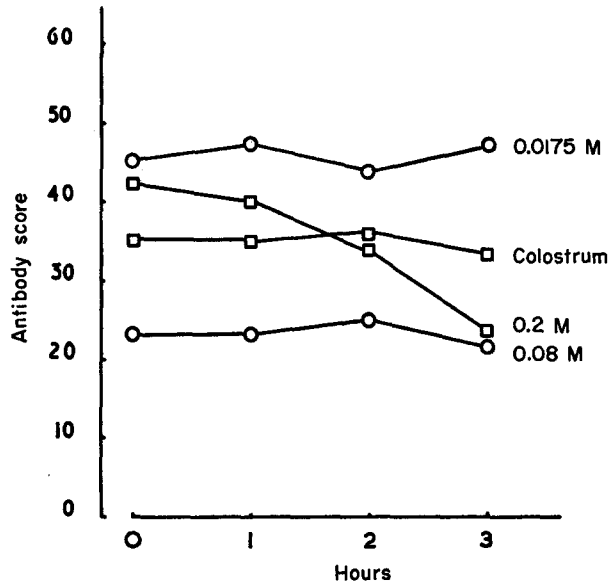


FIG. 3. Effect of heating at 56°C for periods of up to 3 hr on agglutinins in colostrum (γA–anti-A) and in DEAE fractions containing predominantly γG (0.0175 M), γA (0.08 M), and γM (0.2 M).

tially inhibited by the treatment whereas, as expected, the antibody present in the 0.0175 M fraction was not inhibited and that present in the 0.2 M fraction was inhibited. The results of testing three samples of colostrum and the 0.08 M fraction prepared from serum Win. are shown in Table VI.

*Tests on Eluates Prepared from Antibodies in Colostrum.*—As described in Methods an anti-A eluate was prepared from colostrum, using two successive elutions, the first being made into 10% albumin. The amount of γA-globulin in the second eluate was estimated by the inhibition of antiglobulin serum method using as a control an eluate made in the same way but from group O red cells. The sample of colostrum had an anti-A titer of 1000 and the second eluate a titer of 32. The second eluate was estimated to contain 12 μg per ml

γA-globulin but no detectable γG-globulin. The control eluate from group O red cells contained less than 0.1 μg per ml γA-globulin.

*Sera Containing Anti-Rh.*—All of the 32 sera containing anti-D which were tested were "incomplete" and all sensitized red cells to agglutination by anti-γG-globulin. All the sera were tested for their ability to sensitize red cells to agglutination by anti-γA-globulin serum, in each case using the four different anti-γA-sera described in Methods.

Of 10 sera with γG-anti-Rh titers of 256 to 2000, 1 (Avg.) sensitized red cells to agglutination by anti-γA; of 10 with γG-anti-Rh titers of 32 to 128, 2 (Gar. and Hol.) sensitized red cells to agglutination by anti-γA; and of 10 sera with γG-anti-Rh titers of 4 to 16 and 2 further sera, not titrated, none sensitized red cells to agglutination by anti-γA.

TABLE VI

*Effect of Treatment with 2-Mercaptoethanol on Anti-A and Anti-B Antibodies Present in Colostrum and in the 0.08 M Fraction Prepared from Serum "Win."*

| | ABO group | Anti-A* | | Anti-B* | |
|---|---|---|---|---|---|
| | | Control | 2-ME | Control | 2-ME |
| Colostrum Roo | B | 51 | 40 | ... | ... |
| Colostrum McD | O | 66 | 36 | 60 | 29 |
| Colostrum Thu | B | 98 | 40 | ... | ... |
| Serum "Win." 0.08 M fraction | O | ... | ... | 42 | 25 |

* The antibody titer is expressed as a "score". The antibody was incubated with 2-ME at neutral pH as described in Methods and as a control was incubated simply with buffer.
... , Not tested.

Of the 3 sera which sensitized red cells to agglutination by anti-γA, 2 (Gar. and Hol.) came from women immunized by pregnancy alone and 1 from a woman (Avg.) immunized originally by pregnancy but subsequently reimmunized with deliberate injections of Rh-positive red cells.

The subject, Gar., had had three pregnancies, the last of which resulted in the birth of an infant with moderately severe hemolytic disease; the indirect antiglobulin titer of this serum with anti-γG was 128 and with anti-γA, 4.

The subject, Hol., had had five pregnancies. The second and fifth infants were severely affected with hemolytic disease and the third and fourth moderately affected; the indirect antiglobulin titer of the serum with anti-γG was 64 and with anti-γA, 2.

As described earlier, numerous blood samples were available from the subject Avg. as she was undergoing regular venesection. When testing a sample taken from this patient in January 1964, it was realized that part of the antibody was γA-globulin; fortunately many earlier samples had been kept and it was thus

possible to trace the development of the antibody; the results are illustrated in Fig. 4. As Fig. 4 shows, no antibody could be detected in the samples taken after the first two injections of Rh-positive cells but γA-anti-D was readily detectable in the sample taken 5 wk after the third injection. After a further 3 wk the antibody was only just detectable and 6 wk after this, was no longer detectable. Following the fourth injection of Rh-positive red cells, the antibody was detectable again by the end of 9 days at the same titer as following the third injection, but this time the titer was maintained so that, about 3 months

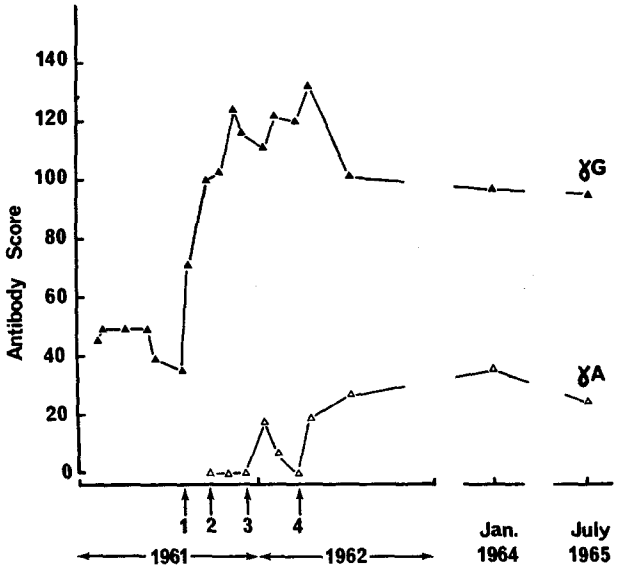


FIG. 4. Estimates of γG- and γA-anti-Rh in a patient (Avg.) immunized before 1950 by pregnancy and possibly also by transfusion. 1, 2, 3, and 4 indicate times at which intravenous injections of 1 ml of Rh-positive red cells were given.

after the third injection, it was still 16. After a further 6 months the titer had rather surprisingly risen to 64 although after 18 more months it was back again to 16. Thus 3 yr after the last Rh-positive stimulus the γA-antibody was still readily detectable.

Samples of serum from Gar., Hol., and Avg. were fractionated on DEAE-cellulose and it was confirmed that the 0.08 M fraction contained the bulk of the γA-antibody.

It was noted that in all three cases the undiluted 0.08 M fraction sensitized red cells more strongly to agglutination by anti-γA-globulin serum than did undiluted whole serum although the end point of the titer of γA-antibody was the same in whole serum as in the fraction.

Of the remaining 29 sera containing anti-D, 12 were fractionated on DEAE-cellulose but in no case did the 0.08 M fraction, containing the bulk of the γA-

globulin, sensitize red cells to agglutination by anti-γA-globulin serum. The sera fractionated included 10 out of 14 which had titers of 128 or more.

The two samples of serum containing incomplete anti-c had titers of 2000 and 128 respectively when tested by the indirect antiglobulin method using anti-γG but neither serum sensitized red cells to agglutination by anti-γA; both sera were fractionated on DEAE-cellulose and in both cases the 0.08 M fraction failed to sensitize red cells to agglutination by anti-γA.

*Tests on Saliva and Colostrum from Patients with Anti-D in their Serum.*—Saliva from Avg. whose serum at the time contained γA–anti-D with a titer of 16 and γG–anti-D with a titer of 2000 contained no detectable anti-D although it contained anti-B agglutinin with a titer of 1 in 16.

Colostrum was obtained from 5 women whose serum contained γG–anti-D with titers ranging from 8 to 64; the serum of 1 woman (Hol.) contained γA–anti-D. In no case was anti-D detected in the colostrum although in every case anti-A, anti-B, or both agglutinins were present. Saliva obtained from 2 of these women contained anti-A and anti-B but no anti-D.

*Sera Containing other Blood Group Antibodies.*—Serum from the donor Cri. with an anti-$Le^a$ titer of 128 and an anti-$Le^b$ titer of 32 contained only γM-antibody, all activity being recovered in the 0.2 M fraction.

Serum from Bur., with an anti-$Le^a$ titer of 128, contained predominantly γM-globulin as judged by the fact that it sensitized red cells to agglutination by anti-γM (10) but not by anti–γA- or γG-globulin serum; when the serum was fractionated on DEAE-cellulose most of the activity was recovered in the 0.2 M fraction although some was recovered in the 0.08 M fraction; this latter fraction did not sensitize red cells to agglutination by anti-γA (or by anti-γG) and the presence of anti-$Le^a$ could be deduced only by showing that the fraction would bring about complement binding to Le(a+) but not Le(a−) red cells.

Serum from donor Spe. (anti-$Le^a$ titer 32) was selected because, unlike any other Lewis antibodies investigated in this laboratory, it sensitized red cells to agglutination by anti–γG-globulin sera. It did not sensitize red cells to agglutination by anti–γA-globulin.

## DISCUSSION

The finding of an example of γA–anti-Rh(D) made it possible to apply the inhibition of antiglobulin serum method to estimating γA in colostrum, saliva, serum, and serum fractions. The specificity of the method is shown by the finding (as expected from previous work) that cord serum contained less than 1% of the amount of γA-globulin present in adult serum. The fact that colostrum was found to contain 50 to 100% and saliva 20% of the amount of γA in normal serum agrees well with previously published results (4).

Anti-A and anti-B in colostrum were found either entirely or mainly in the DEAE fractions containing the bulk of the γA-globulin. Evidence that the

antibodies themselves are γA depends on two findings: first that the agglutination produced by the antibodies was enhanced by anti-γA but not by anti-γG or anti-γM; second that anti-A eluted from red cells incubated with anti-A–containing colostrum was found to be entirely γA-globulin.

The finding that anti-A and anti-B titers are often higher in colostrum than in serum from the same subject whereas the concentration of γA is somewhat higher in serum, and that γA–anti-A may be present in colostrum but absent from the corresponding serum, emphasises that the γA in colostrum is not just a "filtrate" of serum. For this reason it cannot be assumed that the characteristics of γA-antibodies in colostrum will necessarily be the same as those of the corresponding γA-antibodies in the serum although in the present work no discrepancies were observed.

The present observation that γA–anti-A and anti-B are not hemolytic agrees with previous work (2, 3) as does the observation that the antibodies are partially inactivated by 2-mercaptoethanol (2, 3) and that in their relative ease of neutralization by A substance and the degree to which agglutination is enhanced by a medium of serum rather than saline, they are intermediate in behavior between γM– and γG–anti-A and anti-B (2).

The present finding that the agglutination produced by γA–anti-A and anti-B is enhanced by anti-γA agrees with that of Ishizaka et al. (3), although Rawson and Abelson (2) found no enhancement.

The present observation that only 3 out of 32 anti-Rh sera sensitized red cells to agglutination by an anti–γA-globulin serum is clearly at variance with that of Prager and Bearden (19) who found that 15 out of 15 Rh antibodies sensitized red cells to agglutination by an anti–γA-globulin serum. To explain the discrepancy one can only suggest either that the sera used in the present work gave false negative results although this seems a little unlikely since 4 different anti–γA-sera were used and some clear-cut positive results were observed; or that the serum used by Prager and Bearden cross-reacted with γG-globulin.

The present findings seem also to be at variance with those of Dodd and Wilkinson (20) who found that many Rh antibodies were partly γA. The technique used by these authors was to fractionate the anti–Rh-containing serum on DEAE-cellulose and to show that the serological activity of the fraction rich in γA was inhibited by the addition of anti–γA-globulin serum. The addition of anti–γG-globulin serum to the fraction also inhibited the reaction suggesting the possibility that the inhibition produced by anti-γA was due, at least partly, to a cross-reaction with γG-globulin. One sample of anti-Rh found by Dodd and Wilkinson to be partly γA was kindly made available to us by the authors but did not in our hands sensitize red cells to any of the examples of anti–γA-globulin serum which we used. It is of course possible that the method used by these authors was capable of detecting examples of γA–anti-Rh not detectable by the method used in the present work.

Studies in the present work on the patient Avg. who formed γA–anti-Rh after repeated stimulation provided new information about the ease with which γA-antibody is formed in relation to γG and on the persistence of the antibody in the serum. Thus an injection of Rh-positive cells which was sufficient to boost the titer of preexisting γG–anti-Rh to a high level failed to bring about the formation of detectable γA–anti-Rh although after a further booster injection some γA–anti-Rh became detectable. During the following few weeks the γA–anti-Rh fell to an undetectable level while the γG–anti-Rh remained at the same high level. Following a third booster injection the γA–anti-Rh reappeared, and after a fourth injection it reached a steady level at which it remained for 3 yr without further stimulation.

## SUMMARY

The serological characteristics of γA–anti-A and anti-B were studied using, as a source, either colostrum, or fractions relatively rich in γA obtained from selected potent antisera. γA–anti-A and anti-B were never hemolytic nor did they sensitize red cells to agglutination by anticomplement globulin sera. γA–anti-A, like γG–anti-A and unlike γM–anti-A was unaffected by heating at 56°C for 3 hr. On the other hand in the following three characteristics the behavior of γA fell between that of γG– or γM–anti-A: sensitivity to inactivation by 2-mercaptoethanol, ease of neutralization by A substance and degree of enhancement of agglutination in a medium of serum rather than saline. The agglutination produced by γA–anti-A was regularly enhanced by addition of anti–γA-globulin serum.

In searching for γA-blood group antibodies of other specificities the following sera were tested: anti-D (32 examples); anti-c (2 examples); anti-$Le^a$ or -$Le^b$ (3 examples); anti-K (3 examples); anti-$Fy^a$ (3 examples), and anti-$Jk^a$ (3 examples). Only 3 sera, all containing anti-D, sensitized red cells to agglutination by anti-γA. There were no discrepancies between results obtained with four different anti–γA-globulin sera. Approximately half the sera were fractionated on DEAE-cellulose, and the fractions rich in γA tested for their ability to sensitize red cells to agglutination by anti-γA; no additional examples of γA-antibodies were detected.

One of the three examples of γA–anti-D appeared in the serum of a woman during the course of deliberate reimmunization. γA–anti-D appeared only after three intravenous injections of red cells although the γG–anti-D titer rose considerably after a single injection. 3 yr after a fourth injection of Rh-positive cells γA–anti-D, as well as γG–anti-D, was still present in the serum.

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