

NATURE OF THE NORMAL INCOMPLETE COLD ANTIBODY IN HUMAN SERUM

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MATTEO ADINOLFI, M.D. (NAPLES, ITALY)

**Medical Research Council's Experimental Haematology Research
Unit, Wright-Fleming Institute of Microbiology, St. Mary's
Hospital Medical School, London**

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Abstract

It was hoped that the application of techniques found useful in distinguishing between different antibody proteins could be applied to clarify conflicting data which exist on some of the serological properties of the normal incomplete cold antibody.

Serum from normal adult donors and cord serum were therefore fractionated using precipitation methods, zone electrophoresis, DEAE-cellulose chromatography and gel filtration on Sephadex G 200. It was found that the antibody was absent in the fractions containing the bulk of γ G and γ M globulins, suggesting that the antibody was not associated with these globulins. The observation that the normal incomplete cold antibody was present in the serum of patients with severe hypogammaglobulinaemia, whilst confirming that the antibody was not γ G or γ M globulin, showed that it was not associated with γ A globulin.

It was confirmed that the antibody did not sensitize red cells to anti- γ G and anti- γ M globulin sera and it was observed that it did not react with anti- γ A globulin serum. The antibody was found to be inhibited by treatment with 2-mercapto-ethanol.

In studying the serological properties of the normal

incomplete antibody it was observed that this antibody differed from classical blood group antibodies associated with γ G, γ A and γ M globulin in that it was destroyed by heating at 56° for 1 hour and inhibited after incubation of serum at pH 4.6.

In the course of these investigations striking similarities were noticed between the properties of the normal incomplete cold antibody and those of some properdin-like factors present in normal human serum. Thus, for example, the antibody was found to be absorbed by zymosan, dextran, potato starch. In view of these similarities further investigations were carried out to see whether the n.i.c. antibody resembled the properdin-like factor responsible for the haemolysis of PNH red cells.

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SECTION I

Preface

The introduction in recent years of techniques for distinguishing or actually separating different plasma proteins has made it possible to show that antibodies in man and most species of animals are associated with a group of structurally related proteins which are referred to as "immunoglobulins".

The type of protein with which a particular antibody is associated assumes great importance in the neonatal period since in the human newborn infant there is a delay in the production of immunoglobulins and almost all the immunoglobulin present in cord serum is derived from the mother by placental transfer. With regard to blood group antibodies it has been repeatedly observed that the only antibodies present in cord serum are immunoglobulins of maternal origin.

These observations might suggest that the "normal incomplete cold" antibody present in cord serum is an immunoglobulin of maternal origin, but this would be surprising since the antibody is "naturally occurring" and "naturally occurring" antibodies are usually immunoglobulins which do not cross the human placenta. Furthermore, in the course of a systematic investigation, planned to classify human blood group antibodies, it was observed that the "normal incomplete cold" antibody did not have the characteristics of those immunoglobulins which are transferred from the mother to the newborn infant.

It was hoped that these conflicting data could be clarified by studying the serological characteristics of the antibody and, in particular, by isolating it, using some techniques found valuable in studying the nature of other blood group antibodies.

SECTION II

Historical introduction

Human red cells left in contact with normal human serum at 0° for 2 hours and then washed with warm saline in order to elute the cold agglutinin give a positive reaction when tested with a suitable antiglobulin serum. Dacie (1950), who described this reaction, termed the antibody responsible "normally occurring incomplete cold antibody" or "incomplete 'cold' antibody". Following a suggestion of Stratton and Renton the antibody is often referred to as "incomplete anti-H" (Stratton & Renton, 1958; page 126). The terminology "normal incomplete cold antibody" was introduced by Mollison (Mollison, 1956; page 253) and it is the one which will be used in this thesis, together with the abbreviation "n.i.c. antibody".

Evidence that fresh serum is necessary in the absorption of the antibody onto red cells was produced by Dacie (1950); thus he found that red cells suspended in serum previously heated at 56° for 5 to 30 minutes subsequently failed to give a positive reaction and the addition of a small amount of absorbed fresh human serum to the heat inactivated serum restored in part its antibody activity. Heparin (0.5 mg./1 ml sodium citrate (6 mg./1 ml.) or Wintrobe's oxalate (2 mg./1 ml.) added to fresh serum were found to inhibit the sensitization of the red cells.

Dacie (1950) also noticed that there were "qualitative" differences between the n.i.c. antibody and the incomplete

anti-Rh antibodies, for anti-human-globulin sera were not equally effective in revealing sensitization by the two kinds of antibodies; thus some anti-globulin sera highly potent in revealing sensitization by incomplete anti-D antibodies, gave weak reactions with red cells sensitized with the n.i.c. antibody and vice versa.

Later Dacie (1951) showed that antiglobulin serum to which γ globulin had been added would still react with red cells sensitized with the n.i.c. antibody whereas it was inactive against red cells sensitized with incomplete anti-Rh antibodies. Similarly Crawford & Mollison (1951) produced evidence that antiglobulin serum absorbed with red cells sensitized with incomplete anti-D antibodies would still react with red cells sensitized with the n.i.c. antibody, and that antiglobulin serum absorbed with red cells sensitized with n.i.c. antibody would still react with red cells sensitized with incomplete anti-D antibodies. These results were confirmed by Renton (1952).

In the course of the following years the role of complement in the antiglobulin test was fully investigated (Cutbush et al., 1955; Mollison & Cutbush, 1955; Dacie et al., 1957; Vaughan & Waller, 1957; Gandini, 1959; Jenkins et al., 1960; Stratton, 1960; Polley & Mollison, 1961; Harboe et al., 1963), and it is now clear that the positive antiglobulin reaction

against red cells sensitized with the n.i.c. antibody is not due to the detection of the antibody but to the reaction between the anti- β_1 (complement) globulin serum and complement components which are bound onto the red cells.

With regard to the antiglobulin test the n.i.c. antibody differs from other complement-binding blood group incomplete antibodies since attempts at sensitizing red cells with the antibody in the absence of complement have always been unsuccessful (Dacie, 1950; Polley & Mollison, 1961) and it has been shown that the sensitized red cells do not react with anti- ϕ -globulin sera (Dacie, 1951; Crawford & Mollison, 1951; Polley & Mollison, 1961).

The n.i.c. antibody has a specificity closely related to, or identical with, anti-H; thus, it reacts far more strongly with O cells than A_1B cells, the reactions are inhibited by the saliva of "secretors" but not by the saliva of "non-secretors" (Crawford et al., 1953) and finally the antibody does not react with "Bombay" red cells (Mollison, 1961).

It has been shown that the antibody is present in cord serum; (Crawford & Mollison in Mollison, 1956).

SECTION III

Methods and materials

a) Methods and materials used in detecting the n.i.c. antibody

1) Methods

The n.i.c. antibody sensitizes human red cells only when complement is present and can be detected only through the complement which is absorbed onto the red cells.

When fresh serum is used, the antibody is detected by mixing 10-20 volumes of serum with one volume of a 50 per cent suspension of red cells and leaving the mixture at 0° for 2 hours; the sensitized red cells are then washed in warm saline, in order to elute the cold agglutinins, and tested with a suitable anti- β_1 (complement) globulin serum.

In titrating the antibody present in fresh serum or in testing stored serum, serum heated at 56° and fractions of serum containing the antibody, fresh complement must be supplied. In these circumstances fresh human serum, previously absorbed at 0° (and therefore free of n.i.c. antibody) can be used (Dacie, 1950). The value of using horse complement and immuno-conglutinin (I-K) serum was shown by Mollison & Polley (1961) who observed that when human red cells were sensitized with complement-binding blood group antibodies and horse complement the strongest reaction was obtained using rabbit I-K serum.

Under appropriate conditions the n.i.c. antibody can also be detected through its haemolytic activity. Thus Dacie^{et al.} (1957) observed that red cells of patients with paroxysmal nocturnal haemoglobinuria (PNH red cells) were haemolysed by the antibody present in fresh serum; later Mollison & Thomas (1959) showed that if normal red cells were used and rabbit complement was supplied lysis occurred after incubation at 0° and then at 37°.

In the present work human and horse sera were used as sources of complement; red cells sensitized with the antibody and human complement were tested using an anti- β_1 (complement) globulin serum; red cells sensitized with the antibody and horse serum were tested using an I-K serum.

If not otherwise mentioned, the following ratio between serum, complement and red cells was used: 10 volumes serum, one volume complement and one volume of a 50 per cent suspension of red cells.

Titration of the n.i.c. antibody, and all other antibodies studied, was carried out by making serial, doubling dilutions of serum or fractions in buffered saline, as described by Mollison (1956); titrations were scored either as "end point" (Forester, 1897) or with the method suggested by Race & Sanger (1958); thus the highest dilution yielding detectable agglutination was considered the "end point" and the sum of the series of scores of each dilution constituted the "score".

11) Materials

Human serum containing the n.i.c. antibody. Human serum was obtained from defibrinated blood; if not used immediately the serum was partitioned into 1-2 ml aliquots and stored at -50° ; in the experiments in which the serological properties of the n.i.c. antibody were studied, serum from A_1B and A_1 donors was used.

Red cells. Normal human red cells were usually obtained from samples of blood stored in acid-citrate-dextrose solution (ACD) for not more than three weeks.

Complement. Fresh human serum was absorbed three times at 0° for a total of three hours with packed group O red cells; horse serum was separated within a few hours from freshly drawn blood and absorbed three or four times at 0° with group O red cells.

The absorbed human and horse sera were partitioned into 1-2 ml. aliquots and stored at -50° if not used immediately.

Anti- β_1 (complement) serum. Two anti- β_1 (complement) sera were used in the present work. One was kindly supplied by Dr. M.J. Polley; this serum "Pimm", contained antibodies against β_{1C} and β_{1E} (complement) globulins. As an anti- β_1 (complement) globulin, "Pimm" serum was used diluted 1 in 12.

The other serum was produced following a method suggested by Prof. P.L. Mollison: 1 ml. of a 20 per cent suspension of rabbit red cells was incubated at 37° for $1\frac{1}{2}$ hours with 1 ml.

of EDTA-treated horse serum; the red cells were then washed three times with buffered saline and mixed with 1 ml. of fresh human serum previously absorbed with zymosan (3 mg./1 ml.) at 37° for 1 hour; the mixture was incubated at 37° for 20 minutes; after being washed three times with buffered saline the sensitized red cells were re-suspended in saline and injected intravenously. The rabbit was injected on days 0, 2 and 5; after three weeks, the rabbit was re-injected in the same way with similar preparations on day 0, 3 and 5; 10 days later one large sample was collected.

The immune serum (M65) was heated at 56° for 30 minutes and absorbed three times with human red cells. To determine the titre of the anti- β_1 (complement) globulin present in the immune serum, serial doubling dilutions of the serum were prepared and one drop of each dilution was mixed on an opal tile with one drop of a 10 per cent suspension of red cells sensitized with a complement-binding antibody (anti-Le^a) and complement (see page 23).

The antibody was also tested against red cells sensitized with the n.i.c. antibody in presence of human complement.

The results are summarised in Table I; in this table the results of testing the antibody for anti- γ G and anti- γ M globulin (the techniques are described on page 23) are also reported.

Since the immune serum was found to agglutinate Rh+ red cells sensitized with an incomplete anti-D(γ G), the anti- γ G globulin present in the serum was inhibited with a

T A B L E I

REACTIONS OF THE ANTI- β_1 (COMPLEMENT) GLOBULIN SERUM FROM RABBIT
M65 WITH VARIOUS BLOOD GROUP ANTIBODIES

Red cells sensitized with: (1)	Dilutions of anti- β_1 (complement) serum (as reciprocals)								
	2	4	8	16	32	64	128	256	512
i) anti-Le ^a and human complement	++	+++	+++	+++	+++	++	+	(+)	-
ii) N.i.c.	++	+++	+++	+++	++	+	(+)	-	-
iii) anti-D (γ G)	+++	+++	++	+	(+)	-	-	-	-
iv) anti-Le ^a (γ M)	-	-	-	-	-	-	-	-	-

(1) The anti- β_1 (complement) globulin serum was tested against: i) Le(a+) red cells first sensitized with an EDTA-treated anti-Le^a and then coated with human complement; ii) red cells sensitized with the n.i.c. in the presence of human complement; iii) red cells sensitized with an incomplete anti-D (γ G) antibody; and iv) with an EDTA-treated anti-Le^a (γ M).

preparation of "pure" γ G globulin obtained by DEAE-cellulose chromatography; thus 10 volumes of serum were mixed with 1 volume of a γ G globulin solution containing about 2000 mg of γ G globulin per 100 ml. After the inhibition, the immune serum was shown to react only with red cells coated with human complement, that is to say it agglutinated red cells sensitized with the n.i.c. antibody, or with the anti-Le^a antibody, and complement whilst it did not agglutinate Rh+ red cells sensitized with the incomplete anti-D(γ G) antibody.

The absorbed immune serum was tested by immunoelectrophoresis against normal serum; the pattern of the reaction was compared with that obtained using an immune anti- $\beta_{1C}-\beta_{1A}$ serum kindly supplied by Dr. E. Rochna and Dr. M.J. Polley. As shown in Figure 1 the immune serum M65 gave two lines of precipitation of which one could be identified as $\beta_{1C}-\beta_{1A}$ globulins.

Immune-conglutinin (I-K) serum. Two sera were used; one, kindly supplied by Dr. M.J. Polley, had been prepared by repeated injections of a killed suspension of Salmonella pullorum using the method of "auto-stimulation" described by Coombs & Coombs (1953). The other was prepared in the same way, as follows: three injections of a killed suspension of Salmonella pullorum were given intravenously^{into a rabbit} on day 0, 3 and 7; after 10 days one large sample of blood was collected; the immune serum (M22) was heated at 56° for 30 minutes and absorbed three times with human red cells.



Fig. 1. Immuno-electrophoretic analysis of anti- β_1 (complement) globulin serum.

A) The circular well was filled with fresh serum; after electrophoresis the longitudinal trough on the top was filled with rabbit serum anti- β_1 (complement) globulin (M65); the lower trough with a specific anti- β_{1C} - β_{1A} globulin serum.

B) As shown in the diagram, M65 gave 2 lines of precipitation; one, biphasic, was similar to that obtained when using anti- β_{1C} - β_{1A} globulin serum; the other line was tentatively identified as β_{1E} globulin.

The serum was then tested against Le(a+) red cells sensitized with a complement binding antibody, anti-Le^a, and complement, before and after being treated with fresh human serum, as the human serum would inhibit the anti- β_1 (complement) antibodies without interfering with the immuno-conglutin activity.

The immune serum was also tested against red cells sensitized with human serum heated at 56° for 15 minutes (as a source of n.i.c. antibody) and horse complement. The results showed that the immune serum contained an immuno-conglutinin and that the optimal dilution, against red cells sensitized with n.i.c. antibody and horse complement, was 1:20.

b) Other methods and materials used in the course of the investigation on the nature of the n.i.c. antibody

Antiglobulin tests. Anti- γ G, anti- γ A and anti- γ M globulin tests were performed by incubating four volumes of antibody containing serum (or fraction) with one volume of a 20 per cent suspension of red cells containing the specific antigen at 37° for 1½ hours; the red cells were then washed three times with buffered saline and resuspended to give approximately a 10 per cent suspension. One drop of a suitable dilution of the antiglobulin serum was placed on an opal tile and to it one drop of the sensitized red cells was added. Each mixture was stirred with one applicator stick and the opal tile rocked gently for 10 minutes, after which the results were scored.

The anti- β_1 (complement) globulin test was performed using red cells coated with a complement-binding antibody and complement, as follows: one volume of a 20 per cent suspension of Le(a+) red cells was incubated at 37° for 90 minutes with four volumes of EDTA-treated serum (~~1~~ 1 ml serum/4 mg EDTA) containing an incomplete anti-Le^a (γ M). The sensitized red cells were then washed three times with buffered saline; all the saline was discarded before adding two volumes of fresh serum; the mixture was then incubated at 37° for 20 minutes; the red cells were washed three times with buffered saline and resuspended to give a 10 per cent suspension. One drop of a suitable dilution of anti- β_1 (complement) serum was mixed with one drop of the sensitized red cells placed on an opal tile. The result of the agglutination was scored after rocking gently for 10 minutes.

In the present work, in testing the anti-globulin sera and in estimating the concentration of γ G, γ A and γ M globulin in serum and in fractions, the following incomplete blood group antibodies were used:

1) an incomplete anti-D(γ G); this antibody was eluted in the 0.02M fraction from DEAE-cellulose chromatography, was not inhibited by treatment with 2-mercaptoethanol (2-me) and sensitized red cells only to anti- γ G globulin serum.

2) an incomplete anti-D(γ A) antibody, kindly supplied by Prof. P.L. Mollison and Dr. M. Polley; the serum

(Avga) contained a mixture of anti-D(γ G) and anti-D(γ A); the anti-D(γ G) was eluted in the 0.02M fraction and was not inhibited by treatment with 2-me; the anti-D(γ A) was eluted in the 0.06M fraction, was partially inhibited by treatment with 2-me and sensitized red cells only to an anti- γ A globulin serum (Prof. P.L. Mollison and Dr. M.J. Polley, personal communication).

3) an incomplete anti-Le^a(γ M), which was eluted in the 0.2M fraction, was inhibited by treatment with 2-me and sensitized red cells only to anti- γ M globulin serum.

The criteria used in the classification of the three blood group antibodies as γ G, γ A and γ M^{globulin}, are those described by Adinolfi et al. (1962).

Anti- γ G globulin serum. The serum "Pimm (see page) was used as its optimal dilution 1 in 32.

Anti- γ A globulin serum. One serum was kindly provided by Dr. Soothill. The other two sera were purchased from Hoechst Pharm. Ltd., London. The sera were shown to be specific and therefore to react only against γ A globulin either using agar gel precipitation methods or using red cells sensitized with γ G, γ A and γ M incomplete antibodies.

Anti- γ M globulin serum. Three sera were used; they were obtained by injecting into rabbits preparations of pathological γ M globulin. One serum was kindly provided by Dr. M.J. Polley; the other two were produced as follows: one volume (0.5 ml.) of a 2 per cent pathological γ M globulin was mixed with an equal

volume of Freund's complete adjuvant and injected intramuscularly into a rabbit in four different sites. After one month, 0.5 ml. of alum-precipitated antigen was injected intramuscularly at days 0, 4, 7 and 10. The alum-precipitated antigen was prepared using a minor modification of the method described by Proom (1943); 0.3 ml. of a 2 per cent solution of γ M globulin was mixed with 0.4 ml. of 10 per cent solution of aluminium potassium sulphate; the pH of the mixture was adjusted to 6.5 with 0.2M NaOH; the precipitate obtained was then washed three times with sterile 0.9 per cent NaCl solution and finally resuspended in 0.5 ml. of saline for injection.

Ten days after the last injection a large sample of blood was collected.

The immune serum was heated at 56° for 20 minutes and absorbed three times with packed human red cells.

Tested against red cells sensitized with γ G, γ A and γ M incomplete antibodies (see page 23), the immune sera (M58, M71) were found to contain anti- γ G and anti- γ M globulin antibodies. The immune sera were then made specific by adding cord serum diluted 1 in 4, so all antibodies, but the anti- γ M, would be inhibited.

After the inhibition it was found that the immune sera reacted only against red cells sensitized with incomplete γ M antibodies and gave only one line of precipitation when tested against normal serum or a partially purified γ M globulin preparation (Figure 2).

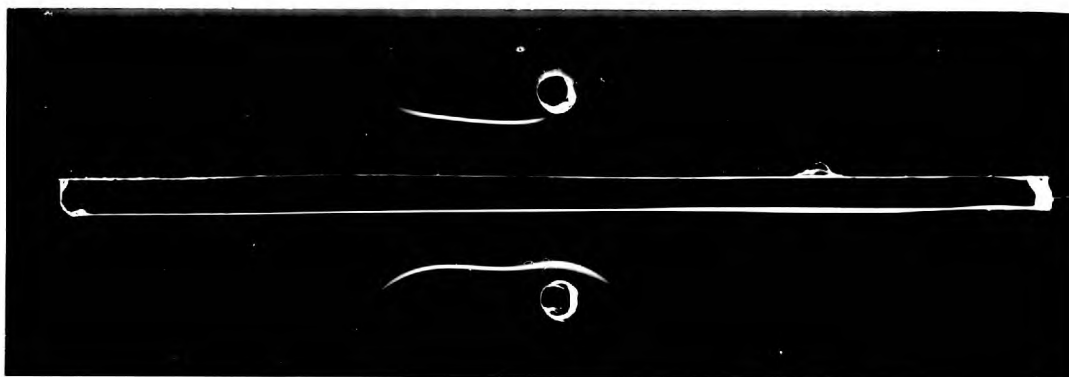


Fig. 2. Immunoelectrophoretic analysis of rabbit serum anti-human γ M-globulin. The immune serum (M58) was tested against normal serum (central well on the top) and against a preparation of pathological γ M-globulin (central well on the bottom).

Estimation of γ G and γ M globulins by the inhibition of antiglobulin sera

The method for the assay of γ G-globulin was that described in detail by Mollison (1956) and Kekwick et al. (1961). This method was based upon the inhibition of the reaction between an anti- γ G globulin serum and Rh sensitized red cells (Wadner et al., 1949; Wadner, 1955; Grubb, 1956).

Red cells were weakly sensitized with an anti-D(γ G) incomplete antibody; after being washed three times with buffered saline the sensitized red cells were resuspended to a 10 per cent suspension. An anti- γ G-globulin serum was used at a dilution which, when mixed with an equal volume of saline, would give only a moderately strong agglutination of the red cells.

In assaying the γ G-globulin content of serum (or fraction) a wide range of dilutions was made (e.g. from 1 in 100 to 1 in 100,000); one volume of each dilution was mixed with one volume of the diluted anti- γ G-globulin serum; after 10 minutes a drop of each mixture was placed on an opal tile and mixed with one drop of the sensitized red cells suspension. The highest dilution of serum giving definite inhibition of agglutination was taken as ^{the}end point.

When reading the end point a series of comparisons was made, either using as ^astandard, normal human serum or, in testing fractions, ~~using~~ the original serum. By taking the values of

γ G content in the normal serum or original serum as 100 per cent, results were expressed as "percentage of normal".

The method for assay of γ M-globulin was that described by Polley et al., 1962; Le(a+) red cells were sensitized with an EDTA-treated anti-Le^a(γ M) antibody at 37° for 90 minutes; the red cells were then washed three times with buffered saline and resuspended to give a 10 per cent suspension. The anti- γ M globulin serum was used at a dilution which, when mixed with an equal volume of saline, would give only a moderate strong agglutination of the sensitised red cells.

In assaying the γ M globulin content of serum or fraction, a wide range of dilution was made (usually from 1 in 10 to 1 in 500).

Inhibition of the anti- γ M globulin serum was then performed as described for inhibiting anti- γ G globulin serum.

γ A globulin was assayed using red cells sensitized with an anti-D(γ A) globulin serum. The method, similar to that described in assaying the concentration of γ M globulin, was suggested by Prof. P.L. Mollison and Dr. M.J. Polley, who have also provided the anti-D(γ A) antibody.

Bacterial antibody test

The indirect haemagglutination method was used (Neter, 1956). A suspension of bacteria in buffered saline (10 mg

wet-weight bacteria/1 ml saline) was heated in a water bath for one hour at 100°; the supernatant was then recovered by centrifugation.

One volume of the supernatant was mixed with one volume of a 10 per cent suspension of group O red cells in buffered saline and incubated at 37° for 20 minutes. The "modified" red cells were then washed three times with warm saline and resuspended in saline to make a 5 per cent suspension. The bacterial agglutinin in human serum was tested by making serial, doubling, dilutions of serum, or fractions, and adding to each tube an equal volume of the "modified" red cell suspension; after one hour at room temperature the deposited red cells were examined microscopically and the degree of agglutination scored.

(Paroxysmal nocturnal haemoglobinuria)

Serological test with PNH¹ red cells ("Ham's test" or "acid-serum test")

Method. 0.5 ml. of serum (or fraction) was acidified to pH 6.8 by adding 0.05 ml. of 0.2 N HCl; after mixing, the acidified serum (or fraction) was placed in a water-bath at 37° and a large drop of a 50 per cent suspension of PNH red cells was added. After incubation for 30 minutes the tubes were centrifuged and the degree of haemolysis scored.

As controls, additional tubes of unacidified and acidified serum were set up and tested with PNH and normal red cells.

In many experiments the method was made^{more}/sensitive by

adding one large drop of guinea pig complement to the serum (Dacie, 1949); the guinea pig complement was always supplied in testing serum fractions.

PNH red cells. The cells, obtained from two donors, were stored in ACD at 4° for not more than 3 weeks. They were washed three times with buffered saline before being used.

Human fresh serum. Only fresh serum from compatible donors was used.

Guinea pig complement. Samples of blood were collected by heart puncture; the serum, separated as soon as possible, was absorbed twice with packed human red cells at 0°.

Titration of serum complement

Doubling dilutions of serum were made in buffered saline to give dilutions ranging from undiluted serum to 1:64; volumes of 0.3 ml were used. To each tube of the serum dilutions, and to further tubes containing 0.3 ml of buffered saline and 0.3 ml of distilled water, respectively, 0.1 ml of a 10 per cent suspension of sheep red cells, previously sensitized with horse anti-sheep red cell antibody, was added. After incubation at 37° for 30 minutes, the tubes were centrifuged and the degree of haemolysis scored.

The sheep red cells were sensitized as follows: 1 volume of a 20 per cent suspension of red cells, previously washed with buffered saline, was mixed with 4 volumes of horse anti-sheep red cell antibody, diluted 1:100; the mixture was left

at 37° for 1 hour; the red cells were then washed three times with buffered saline.

Immunoelectrophoresis

The technique was that described by Scheidegger (1955); Ionagar II (Oxoid) and veronal buffer pH 8.6, 0.05M were used.

SECTION IV

The n.i.c. antibody in maternal serum
and in serum of newborn infants

The n.i.c. antibody is present in a high percentage of cord sera; thus Crawford and Mollison (in Mollison, 1956) observed the antibody in 29 out of 33 cord samples tested. Later, Polley et al. (1962) found that the titre of the antibody in cord serum was similar to that in the relative maternal serum.

Since it is believed that the only antibodies present in cord serum are those which have been derived from the mother by placental transfer and since the only immunoglobulin transferred is γ G-globulin (for review see: Hemmings & Brambell, 1961; de Muralt, 1962; Good & Papermaster, 1964) the inference would be that the antibody present in cord serum is γ G globulin, but, as mentioned previously, this would be surprising since "naturally occurring" antibodies are, at least usually, γ M globulin (see Mollison et al., 1962).

These conflicting observations, and therefore the problem whether the n.i.c. antibody present in cord serum is derived from the mother or is produced by the newborn infant, were initially investigated using only "biological" criteria, as follows:

a) by comparing the titre of the antibody in a large group of maternal and relative cord sera, and

b) by testing the antibody in the serum of a group of human infants from a few weeks to 5-6 months of age.

It was hoped that some information could be gained from these investigations since it is known that: (a) when antibodies are passed from the mother to the foetus there is a close relation between the titres in maternal and corresponding cord samples (Perkins et al., 1958; Evans & Smith, 1963) and (b) the passively acquired antibodies are slowly catabolized by the foetus and their concentration falls exponentially with a half-life variously estimated from 18 to as much as 42 days (Barr et al., 1949; Wiener, 1951; Dixon et al., 1952; Perkins et al., 1958; Lepow et al., 1961; Pagano et al., 1961).

Thus if it could be shown that there was no relation between the titres of the n.i.c. antibody in maternal and cord sera and that the antibody did not disappear from the circulation of the newborn infant as do γ G-antibodies, the inference would be that the n.i.c. antibody present in cord serum is produced before birth.

a) The n.i.c. antibody in maternal and cord sera

Twenty-three pairs of maternal and cord sera were tested; the cord blood was obtained from the umbilical vein with a syringe, carefully avoiding squeezing the cord; the maternal samples were taken by venepuncture within two days of delivery.

The sera were tested by incubating at 0° for 2 hours 10 volumes of each of a series of doubling dilutions of serum with 2 volumes of horse complement and 1 volume of a 50 per

T A B L E 2

N.I.C. ANTIBODY IN MATERNAL AND CORD SERA

	No. tested	No. with n.i.c.	Antibody mean score*
Mother	23	19 (82.6%)	31.7
Cord	23	20 (86.9%)	26.5

* The mean scores were calculated only from the sera containing the antibody.

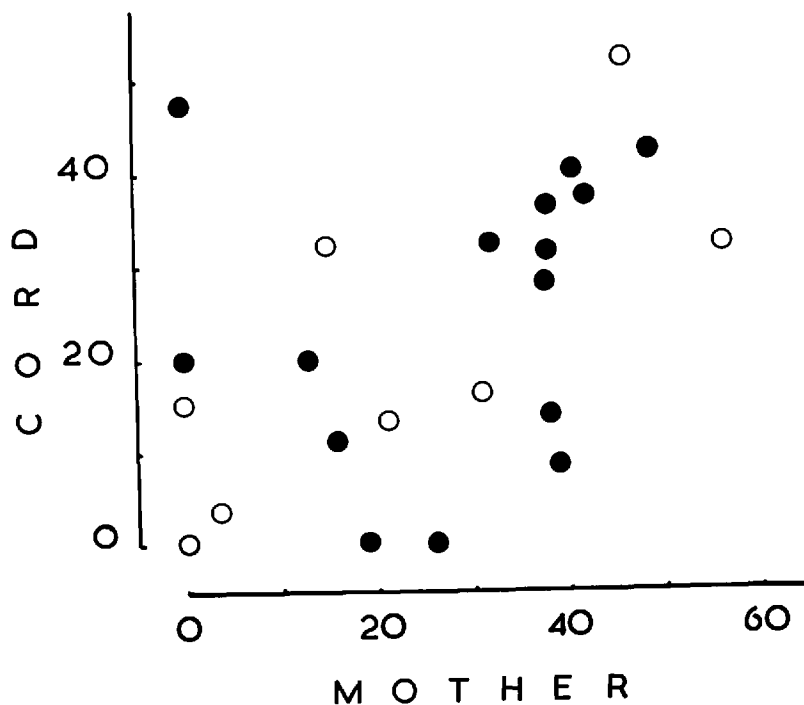


Fig. 3. N.i.c. antibody in 23 maternal sera and corresponding cord sera; results are expressed as scores.
 ● = mother and newborn infant of same ABO blood group.
 ○ = mother and newborn infant of different ABO blood group.

cent suspension of red cells; the sensitized red cells were then tested using an I.K. serum (see page 20).

The n.i.c. antibody was present in 19 out of 23 maternal sera (82.6 per cent) and in 20 out of 23 cord sera (86.9 per cent). The mean score of the antibody, calculated only for those samples in which antibody was present, was 31.7 for the 19 maternal samples and 26.5 for the 20 cord sera (Table 2). Figure 3 shows the score of the antibody in each maternal sample compared with that in the relevant cord serum. In 12 out of the 17 pairs of maternal and cord sera in which the antibody was present in both samples, the antibody had the same score or the difference did not exceed 10 units; in one case the antibody had a definitely higher score in the maternal sample; in four cases the antibody had a higher score in the cord serum.

Table 3 gives the distributions of the n.i.c. antibody in 23 pairs of sera in relation to the four possible combinations in which it may occur. The observed frequency for each of the four possible combinations was compared with the expected frequency calculated on the assumption that the presence of the antibody in the mother's serum did not influence its presence in the infant's serum. In three cases, ~~as expected~~, the antibody was present in the cord serum and absent from the corresponding maternal serum.

TABLE 3

N.I.C. ANTIBODY IN 23 PAIRS OF MATERNAL AND CORD SERA

	mother	cord	observed	expected *
i)	present	present	17	16.6
ii)	present	absent	2	2.5
iii)	absent	present	3	3.4
iv)	absent	absent	1	0.5

* The expected frequency was calculated assuming that the presence of the antibody in cord serum was independent of its presence in the corresponding maternal sample.

b) The n.i.c. antibody in normal human infants.

Eleven infants, from three to fifteen weeks old, were bled by heel prick, at least 1 ml. of blood being collected; six of these infants were born prematurely, but were otherwise normal. One of the infants was bled three times within a period of six weeks.

Due to the limited amount of serum available, the samples obtained by heel-prick from the eleven newborn infants were tested using the following dilutions: 1 in 2, 1 in 5, 1 in 10, 1 in 20 and 1 in 40. As controls, normal adult and cord sera were used at the same dilutions.

Seven normal infants, born at term, were bled by venepuncture three times at intervals of about five weeks. These infants were from seven to twenty-six weeks old and they were receiving a normal course of vaccination with oral poliomyelitis vaccine (Sabin) and with a combined diphtheria, tetanus and pertussis vaccine, following the schedule and the dosage described by Butler et al. (1962).

The antibody was present in the serum of all the eleven newborn infants whose blood was collected by heel-prick (Fig. 4). The score of the antibody ranged from 3 to 45 score units and was similar to that of the adult and cord sera used as controls. The score of the antibody in one infant bled three times within six weeks showed no significant change.

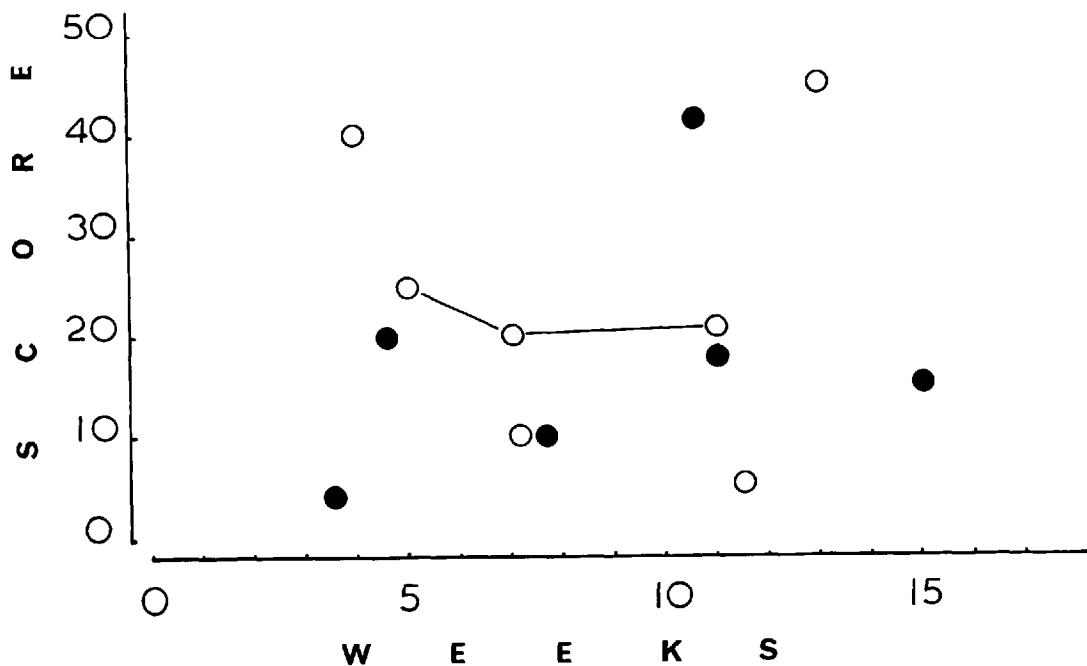


Fig. 4. N.i.c. antibody in 11 newborn infants bled by heel prick. ● = premature infants. The three observations joined by a line were made on the same infant.

The antibody was present in the serum of six of the seven infants bled three times with intervals of about five weeks between bleedings (Table 4 and Fig. 5). The antibody had a constant score in two infants; in another three infants the score of the second and third samples was a little higher than that in the first sample; in the remaining case the antibody rose progressively from 22 to 43 "score" units between the first and third samples.

The presence of anti-A, anti-B and anti-I agglutinins in the serum of the infants bled periodically at about five week intervals was also investigated.

In testing these antibodies, serial, doubling dilutions of the serum were made in buffered saline and to each was added an equal volume of a 2 per cent suspension of red cells. Anti-A and anti-B antibodies were tested leaving the mixtures at room temperature for 2 hours. Anti-I agglutinin was tested using group O, I, P₂ red cells or, as a control, group O, I₂, P₂ red cells, and leaving the mixtures at 0° for 2 hours.

The deposited red cells were examined microscopically and the degree of agglutination scored.

In all infants there was a "null" period during which no

T A B L E 4

ANTI-A, ANTI-B, ANTI-I AND N.I.C. ANTIBODIES IN 7 INFANTS TESTED
ON SEVERAL OCCASIONS IN THE FIRST WEEKS OF LIFE

No.	Age in weeks	ABO group	ANTIBODY SCORE*			
			anti-A	anti-B	anti-I	n.i.c.
1	9	A	...	0	10	29
	15		...	0	10	40
	19		...	0	10	44
	21		...	75	...	...
2	8	O	0	0	0	5
	14		0	0	0	13
	18		0	0	5	13
	20		18	0	7	15
3	14	O	0	0	0	13
	19		0	5	0	15
	24		12	7	0	15
	29		22	17	...	...
4	9	O	0	0	12	18
	14		0	0	12	35
	19		5	0	12	37
5	9	A	...	0	17	50
	14		...	0	25	50
	20		...	0	25	50
6	8	B	0	...	0	0
	13		0	...	0	0
	18		0	...	0	0
7	13	O	0	0	20	22
	20		21	0	28	39
	26		21	13	30	43

... Not tested

* See text

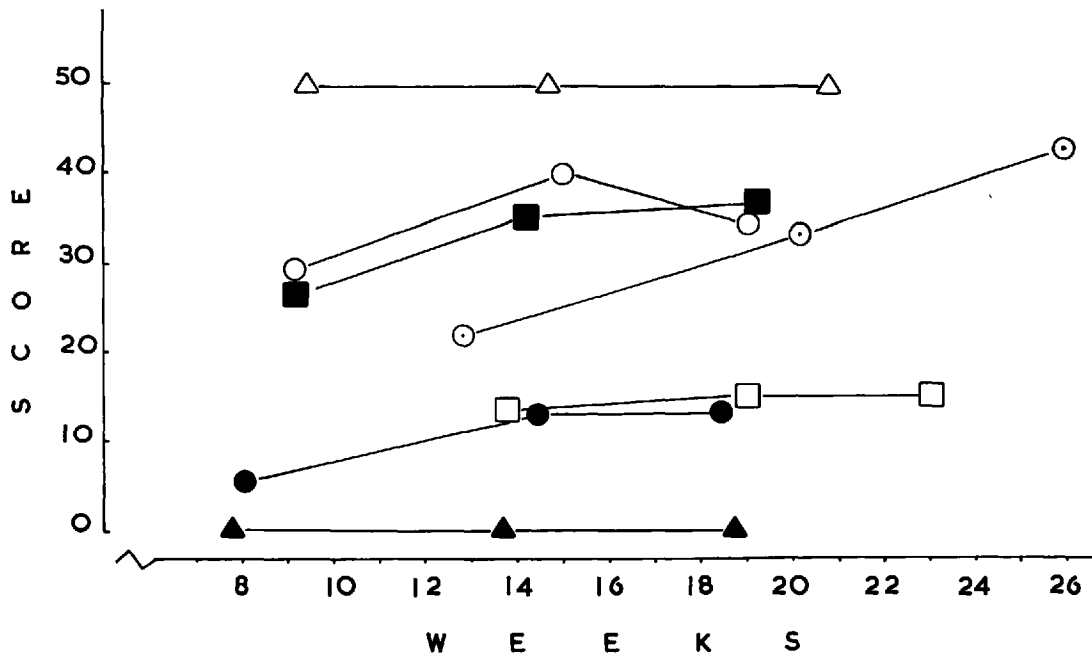


Fig. 5. N.i.c. antibody in seven newborn infants bled by venepuncture at intervals of about five weeks.

anti-A or anti-B were produced; the antibodies were first detectable when the infants were about 20 weeks old (Table 4).

In the seven normal infants bled periodically the anti-I antibody was regularly present in four (see Table 4); in two of them (nos. 1 and 4 in Table 4) the antibody had a constant titre during the whole period of observation; in the other two (nos. 5 and 7) the score increased by about 10 units between the first and the second blood samples taken 5 weeks apart. In two other infants out of the seven, the antibody was absent throughout the period of observation (nos. 3 and 6); in the last case (no. 2) the antibody was absent when the infant was 8 and 14 weeks old but was present when the infant was retested at the age of 18 and 20 weeks.

DISCUSSION

In the normal human newborn infant there is a delay in the production of immunoglobulins, in the development of lymphoid tissue and in the formation of plasma cells.

It is not until the 4th - 6th week of life that the human infant begins to produce its own γ G-globulin; thus Zak and Good (1959), studying two normal infants born to mothers with hypogammaglobulinaemia, found that the γ G-globulin concentration in the infant's sera, which was negligible at time of birth, started to increase between 35 and 42 days after birth in one case, and between 20 and 28 days in the other.

The synthesis of γ M-globulin occurs already, at a slow rate, in foetuses (Van Furth, 1964) but the concentration of this immunoglobulin in cord serum is only about one-tenth to one-twentieth of that in normal "adult" serum (Franklin and Kunkel, 1958; Derington and Soothill, 1961; Polley et al., 1962; West et al., 1962); the rate of the production of γ M-globulin increases rapidly during the first weeks of life and adult serum levels may be reached at the age of 9 months (West et al., 1962).

The production of γ A-globulin does not start until the 4th week of life; the rate of synthesis increases slowly and

steadily so that the average adult serum level is finally reached at puberty (West et al., 1962).

Although the newborn infant does not produce its own γ G-globulin in the first weeks of life, it nevertheless acquires γ G-globulin, and therefore antibodies, from the mother in utero. Consequently it may be postulated that the n.i.c. antibody present in cord serum, as anti-A and anti-B blood group antibodies, is a γ G-globulin derived from the mother. But the results of testing the n.i.c. antibody in a group of cord and corresponding maternal sera points to a different conclusion, namely that the antibody present in newborn infants is not of maternal origin and is produced by the newborn infants. Thus it was found that the titre of the antibody in cord serum was apparently unrelated to the titre of the corresponding maternal sample and that the frequency of the antibody in pairs of maternal and cord sera was similar to the expected frequency calculated on the assumption that the presence of the antibody in the mother's serum did not influence its presence in the infant's serum.

Evidence that the n.i.c. antibody was produced by the newborn infant was also obtained by testing the antibody in several infants from a few weeks to 5-6 months of life; thus, the n.i.c. antibody was present in almost all newborn infants and its titre remained approximately constant with time. If

the antibody had been of maternal origin and therefore a γ G-globulin, a fall in the titre after birth, and eventually a temporary disappearance of the antibody from the circulation would be expected.

The result of testing the newborn infants' sera for the presence of three other "naturally-occurring" blood group antibodies showed more clearly the difference in behaviour of the n.i.c. antibody. In fact in all the newborn infants bled periodically there was a period during which no anti-A or anti-B agglutinins were detectable; the antibodies were not produced by the infants until about 22 weeks of age. On the other hand, the anti-I cold agglutinin did not disappear from the circulation of the newborn infants as do antibodies which cross the human placenta. This cold agglutinin has been shown to be present in a high percentage of cord sera (Crawford and Mollison, in Mollison, 1956) and has been shown to be associated with γ M-globulin and evidently produced by the newborn infant before birth (Adinolfi, 1965).

Recent observations have shown that the human newborn infant does not pass through a period of complete inability to produce immunoglobulins and therefore antibodies (Smith, 1960; Smith & Eitzman, 1964). In fact it has been possible to immunize very young infants against a number of bacterial antigens and, according to Smith (1960), the antibodies formed

by such infants in response to the stimulation with H antigen of Salmonella typhi consisted, at least initially, of γ M-globulin.

Recently Fink et al. (1962) observed that premature infants produced γ M and little or no γ G globulins after stimulation with typhoid-paratyphoid antigens; typhoid O agglutinins were found only in the macroglobulin fraction and no change to γ G-globulin occurred with time and secondary stimulation. When bacteriophages were used for immunization by Uhr et al. (1963) it was observed that newborn premature infants produced antibodies as promptly as did older children and that antibodies formed after the stimulation were initially γ M globulin.

It has also been demonstrated that the newborn rabbit, thought to be immunologically incompetent prior to the third week of life, forms antibodies against certain antigens during this period even while rendered tolerant to others (Shaul, 1962).

Evidence has also been produced that antibodies may be formed before birth. In fact it has been shown that stimulation of lambs in utero by a variety of antigens induces an active antibody response (Silverstein et al., 1963); and that the foetal calf in utero, and the opossum embryo in the

pouch, form antibodies after stimulation (Kalmutz, 1962; La Via et al., 1963).

It is not surprising therefore that the human newborn infant produces blood group antibodies very early in life, evidently before birth. As mentioned previously, the anti-I agglutinin present in cord serum has been shown to be γ M globulin (Adinolfi, 1965); the question arises whether ~~the~~ the n.i.c. antibody produced by the newborn infant, is associated with the same class of immunoglobulin.

To investigate this possibility, attempts were made at isolating the antibody using several techniques of plasma protein fractionation and methods of dissociation of antigen-antibody complexes.

SECTION V

Attempts at isolating the n.i.c. antibody

The procedures used for isolating the n.i.c. antibody have been divided into two groups: a) non-specific methods which involve the fractionation of the antibody-protein on the basis of physicochemical properties common to both antibody and non-antibody proteins; and b) specific methods in which advantage is taken of the specific combination of the antibody with the appropriate antigen.

a) Non-specific methods

1) Precipitation methods. These are crude procedures which exploit the difference in solubility of various serum proteins.

Two methods were used:

1. Precipitation at low ionic strength and acid pH, and
2. Salting-out-fractionation with ammonium sulphate.

In the present work precipitation at low ionic strength and acid pH was carried out by dialysing normal serum containing the n.i.c. antibody against a phosphate buffer 0.02M, pH 5.4; 2ml. of serum was dialysed against 500 ml. of buffer at 0° for 16 hours; the supernatant and the precipitate were separated by centrifugation at 3,000 rev./min. for 20 minutes. The precipitate, washed three times with cold buffer, was then dissolved into 2 ml. of buffered saline. Supernatant and

precipitate were dialysed against 500 ml. of buffered saline for 4 hours before being used.

Three normal "adult" sera were treated as described; the n.i.c. antibody, tested using horse complement and an I.K. serum (see page 15), was always detected in the precipitates.

One further experiment was carried out as follows: a mixture of n.i.c. antibody and incomplete anti-D antibody was prepared by adding 9 volumes of fresh normal serum (group A₁) to 1 volume of "pure" γ G, incomplete anti-D antibody obtained from DEAE-cellulose chromatography using a phosphate buffer 0.02M pH 8.1 (see page 65). The titre (expressed as "end point") of the n.i.c. antibody in the mixture was 1:64; that of the incomplete anti-D was 1:128.

The mixture was dialysed against phosphate buffer 0.02M, pH 5.4, as previously described. The result of testing the supernatant and the precipitate showed that the n.i.c. antibody was present only in the precipitate, whereas almost all the anti-D antibody was found in the supernatant (Table 5).

In fractionating the n.i.c. antibody with the salting out method, ammonium sulphate was used.

A step-wise precipitation of serum proteins was obtained as follows: a mixture of n.i.c. antibody and incomplete anti-D antibody was prepared as previously described. To one volume

T A B L E 5

THE N.I.C. AND INCOMPLETE ANTI-D ANTIBODIES IN THE SUPERNATANT AND IN
THE PRECIPITATE AFTER DIALYSIS AGAINST PHOSPHATE BUFFER 0.02M pH 5.4

	Dilutions (as reciprocals)*								
	1	2	4	8	16	32	64	128	256
N.i.c. antibody: original serum	+++	+++	+++	++	++	+	(+)	-	-
supernatant	-	-	-	-	-	-	-	-	-
precipitate	+++	+++	++	++	+	(+)	-	-	-
Anti-D antibody: original serum	+++	+++	+++	+++	+++	++	++	+	-
supernatant	+++	+++	+++	+++	++	++	+	(+)	-
precipitate	++	++	+	(+)	-	-	-	-	-

* In testing the n.i.c. antibody horse complement was added to each dilution; the sensitized red cells were tested with an I-K. serum. The incomplete anti-D antibody was tested with an anti- γ G globulin serum. For details see: "serological methods", page 22.

of the mixture was added 0.54 volumes of 4.1M $(\text{NH}_4)_2 \text{SO}_4$ bringing the molarity of ammonium sulphate in the mixture to 1.43 (final concentration of ammonium sulphate: 35 per cent)*. After leaving the mixture at 25° for 20 minutes the precipitate was removed by centrifugation and resuspended in 1 ml. of buffered saline. To the supernatant more ammonium sulphate 4.1M was added bringing the final molarity in the mixture to 1.49 (concentration: 37.5 per cent), the precipitate was removed by centrifugation and the procedure was repeated successively bringing the ammonium sulphate molarity in the mixture to: 1.64, 1.86 and 2.05 (corresponding to the concentrations: 40, 45 and 50 per cent).

All precipitates (made up to the original volume) and the last supernatant (which was diluted 1 in 2, after adding

*The amount of saturated ammonium sulphate ($(\text{NH}_4)_2 \text{SO}_4$ 4.1M solution) added to the serum to obtain the desired concentration of ammonium sulphate in the mixture was calculated as follows: $X = V(C_2 - C_1) / (100 - C_2)$, where X is the amount of ammonium sulphate solution to be added, V is the volume of ^{solution} serum, C_2 the desired concentration and C_1 the existing concentration in the mixture.

TABLE 6

THE N.I.C. AND INCOMPLETE ANTI-D(γG) ANTIBODIES IN FRACTIONS OBTAINED AFTER
PRECIPITATION WITH AMMONIUM SULPHATE

	Ammonium sulphate ⁽¹⁾		Antibody titre(end point) ⁽²⁾		Content(%) of γ_M ⁽³⁾
	Molarity	Concentration	N.i.c.	anti-D	
Original serum			64	128	100
1st precipitate	1.43	35	4	16	0
2nd precipitate	1.49	37.5	4	64	0
3rd precipitate	1.64	40	8	16	10
4th precipitate	1.86	45	16	4	30
5th precipitate	2.05	50	2	4	30
final supernatant			0	2	20

(1) Molarity and concentration to which precipitation was obtained

(2) N.i.c. antibody tested using horse complement and I-K. serum; anti-D(γG) antibody tested using antiglobulin (anti- γG) test

(3) The content of γ_M globulin is expressed as percentage of the amount present in the original serum.

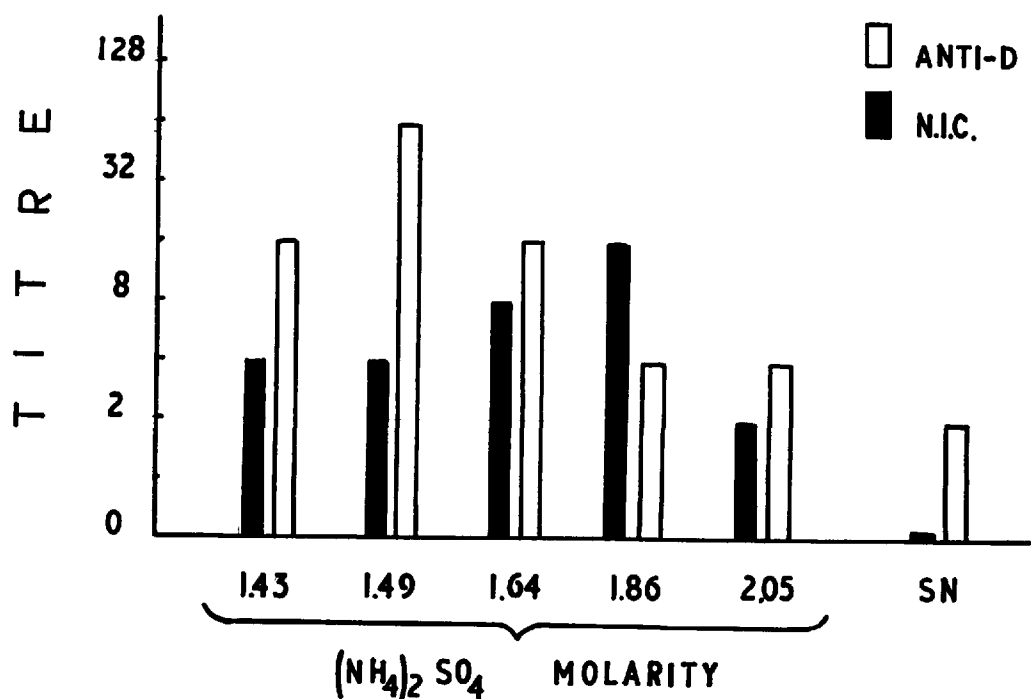


Fig. 6. N.i.c. antibody and anti-D (γ G) antibody in the precipitates obtained by treating serum with $(\text{NH}_4)_2\text{SO}_4$ at concentrations increasing from 1.43 to 2.05 M. The molarity at which each precipitate was obtained is indicated on the abscissa. SN : supernatant after precipitation with 2.05 M ammonium sulphate. Titre expressed as "end point" (reciprocals).

the ammonium sulphate solution) were dialysed against 5 changes of buffered saline at 0° for a total of 36 hours.

The incomplete anti-D antibody was detected by the indirect antiglobulin test; the n.i.c. antibody was tested using horse complement and an I.K. serum.

The higher titre of the n.i.c. antibody was observed in the precipitate obtained by increasing the molarity of $(\text{NH}_4)_2 \text{SO}_4$ in the serum to 1.86, whilst the optimum of precipitation of the incomplete anti-D antibody was obtained with a final molarity 1.64 (Table 6 and Fig. 5).

The concentration of γ M globulin in each fraction, estimated using the inhibition of antiglobulin method (see page 27), is expressed in Table 6 as a percentage of the amount present in the original mixture. It appears that γ M globulin is precipitated with ^ahigher concentration of ammonium sulphate; thus about 30 per cent of γ M globulin is precipitated in both the 1.86 and 2.05M fractions.

11) Zone electrophoresis. Fractionation of large volumes of serum on a preparative scale can be achieved by zone electrophoresis using different supporting materials, e.g. powdered cellulose, granular starch, polyvinylchloride

particles (for reviews see: Cann, 1959; Kunkel and Trautman, 1959; Longworth, 1959).

Since zone electrophoresis on potato starch has been found useful in separating blood group antibodies, attempts at isolating the n.i.c. antibody were initially made using this material but it was soon found that the n.i.c. antibody could not be recovered in any fraction after the electrophoretic migration. Further investigations, which will be described later (see page 131) showed that the antibody was "absorbed" by potato starch.

Consequently vinyl chloride/vinyl acetate copolymer particles (Geon 425, Geon British Ltd.) were tried as supporting material following a procedure described by Müller-Eberhard and Kunkel (1956).

The polyvinyl blocks (38 x 10 x 1 cm.) were prepared by pouring a barely-liquid mixture of Geon 425 and barbiturate buffer 0.1M, pH 8.6 into a mould lined with waxed paraffin paper on a glass plate.

The connections between the block and the buffer in the tanks were obtained using wicks made with filter paper Whatman No. 3.

2 ml. of serum was introduced directly into a narrow slit made across the 1 cm. deep block; the block was then allowed to equilibrate at +4° for 1 hour. The electrophoretic

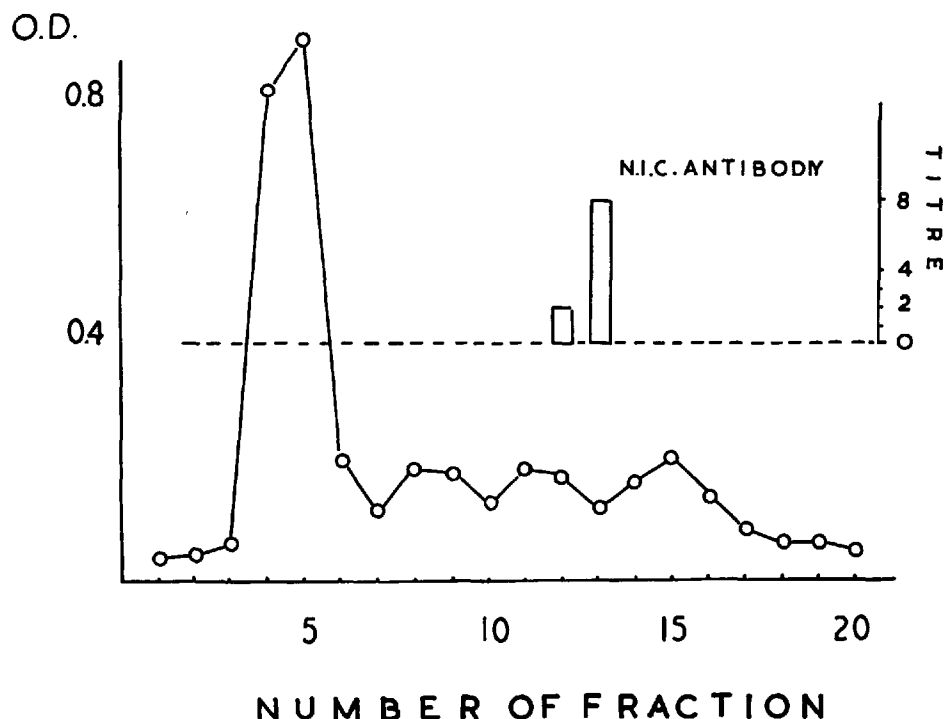


Fig. 7. The zone electrophoresis of 2 ml of fresh normal human serum (adult donor) was carried out on polyvivyl block (Geon 425 - Geon British Ltd.) using a barbiturate buffer 0.1M, pH 8.6; the protein content in each segment (1 cm) was estimated by determining the optical density of the eluates at 284 m μ in an Unicam spectrophotometer.

The n.i.c. antibody was recovered in fractions no. 12 and 13.

separation was carried out at $+4^{\circ}$ using a constant current of 4-6 volt per cm; in this condition an adequate separation was obtained in 18 hours.

After migration, the block was divided into serial segments (1 cm) which were transferred to test-tubes and mixed well with 5 ml. of buffered saline. The mixture was then filtered and the protein content of each eluate was estimated by determining the optical density at 280 m μ . in an Unicam DU spectrophotometer. The eluates were concentrated by ultra-filtration to 2 ml. and dialysed against buffered saline at $+4^{\circ}$ for 2 hours.

Three normal "adult" sera were fractionated with zone electrophoresis. The result of one fractionation is shown in Fig. 7; after migration the serum proteins were found to be distributed in 20 segments; each fraction was concentrated to the original volume and tested for the presence of the n.i.c. antibody using horse complement and an I-K serum. The antibody was detected in the fractions nos. 12 and 13 corresponding to the valley between β and γ globulin peaks.

Fraction nos. 11 to 15 and the original serum were tested with immuno-electrophoresis using a horse immune serum anti-human serum proteins. As shown in Fig. 8 fraction nos. 14 and 15, which were free of the n.i.c. antibody, contained the bulk of γ G globulins; the "active" fractions nos. 12 and

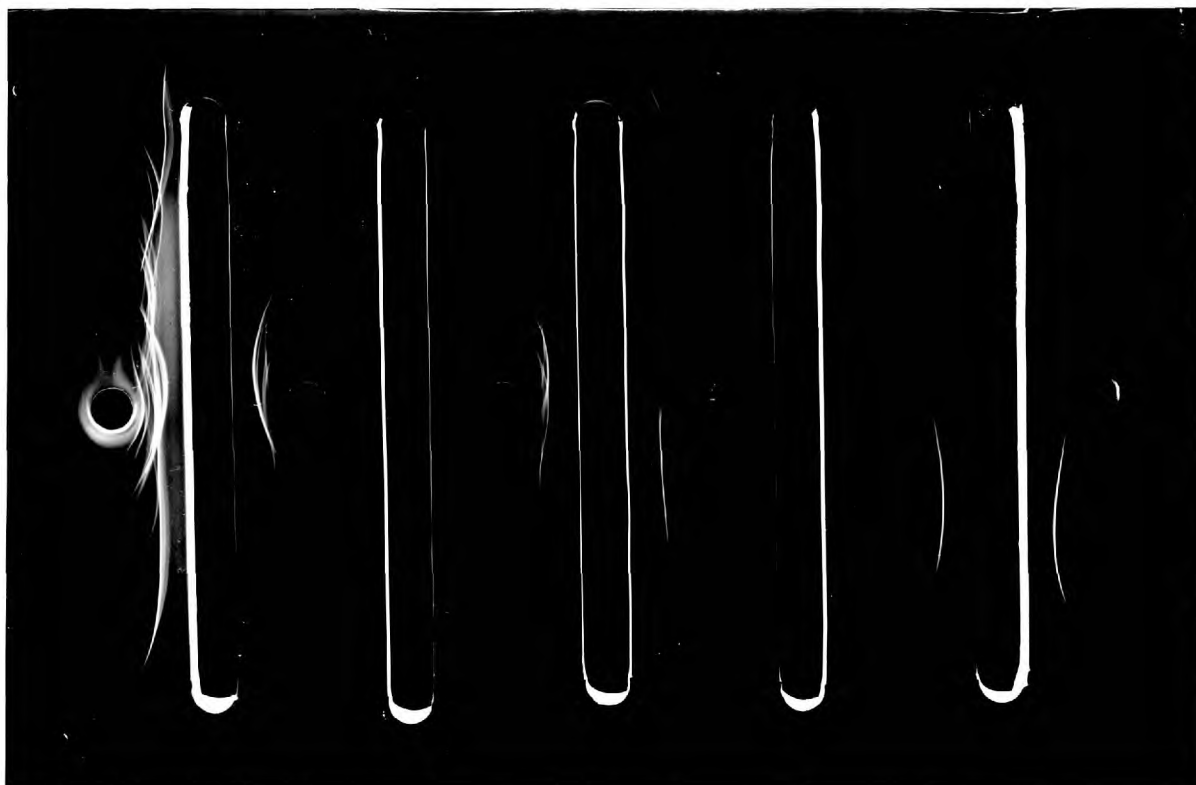


Fig. 8. Immunelectrophoretic analysis of fractions obtained by separating fresh serum with zone electrophoresis (see Fig. 7); whole serum (n.s.) and fractions nos. 11, 12, 13, 14 and 15 in the central wells; after migration the longitudinal troughs were filled with anti-human serum (horse immune serum).

13 contained trace of γ G globulin as well as A and β -globulins.

Similar results were obtained when the other two sera were fractionated; that is to say the n.i.c. antibody was detected in fractions corresponding to the valley between γ and β peaks.

A further fractionation was carried out using a mixture of n.i.c. and incomplete anti-D (γ G) antibodies made as described on page 52 ; since this mixture was made using group A₁ sera, it also contained an anti-B agglutinin, which was shown to be mainly γ M globulin; in fact after treatment with 2-mercaptoethanol (see page 100) its titre fell from 1:128 to 1:16.

After the electrophoretic migration, 22 fractions were collected; each fraction was tested for the incomplete anti-D(γ G) antibody, anti-B (γ M and γ G) agglutinin and the n.i.c. antibody.

The n.i.c. antibody was present mainly in fractions nos. 14 and 15; the incomplete anti-D (γ G) antibody was detected in 7 fractions (from fraction no. 13 to fraction no. 19) but the bulk of the antibody was found in fraction no. 17. The anti-B (γ M and γ G) agglutinin was present mainly in fractions nos. 15 and 16 (Table 7 and Fig. 9).

T A B L E 7

ANTI-B, ANTI-D AND N.I.C. ANTIBODY IN THE FRACTIONS
OBTAINED WITH ZONE ELECTROPHORESIS⁽¹⁾

			anti-B	anti-D	N.i.c.	Content of	
						γG globulin	γM globulin (2)
Whole serum			42	51	32	100	100
fraction no. 13			0	3	0	51	51
"	"	14	3	5	5	5	5
"	"	15	21	11	12	5	30-50
"	"	16	15	15	3	20	20
"	"	17	9	32	0	30	5
"	"	18	0	15	0	20	<1
"	"	19	0	5	0	5	<1

(1) After migration 22 fractions were collected (see Fig. 9); in the present table only the results of testing fractions nos. 13 to 19 are shown. The titre of the antibody is expressed as score.

(2) The concentration of the immunoglobulins is expressed as percentage of the amount present in whole serum.

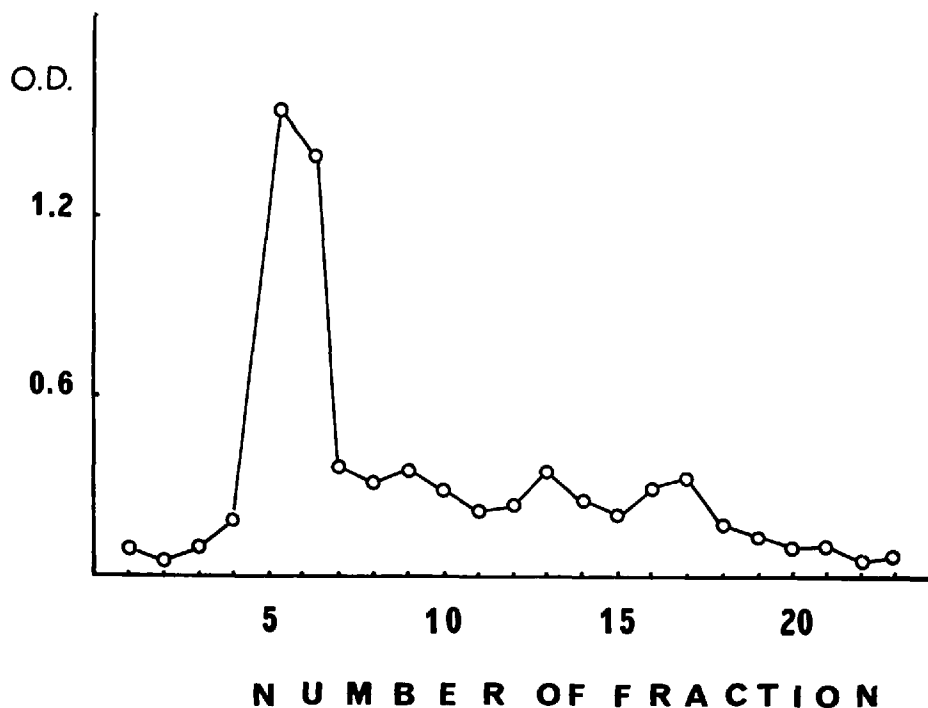


Fig. 9. Zone electrophoresis of 2 ml. of serum containing the n.i.c. antibody, an incomplete anti-D (γ G) antibody and an anti-B agglutinin. The electrophoresis was carried out as described in Fig. 7.

Estimates of γ G and γ M globulins, expressed as percentages of the amounts present in the original mixture, were obtained using the inhibition of antiglobulin methods; as shown in Table 7 the bulk of γ M globulin was recovered in fraction no. 15, whereas the bulk of γ G globulin was recovered in fraction no. 17.

111) Chromatography on DEAE-cellulose. Ion-exchange chromatography has proved a most useful technique for separating serum proteins (Peterson & Sober, 1960). Satisfactory fractionations have been achieved with ion-exchange derivatives such as diethylamino-ethyl-cellulose, triethylamino-ethyl-cellulose and carboxy-methyl-cellulose referred to as DEAE-, TEAE- and CM-cellulose (for reviews see: Peterson and Sober, 1956; Sober and Peterson, 1958; Fahey and Horbett, 1959; Peterson and Sober, 1960; Sober, Hertley, Carroll & Peterson, 1965)

DEAE-cellulose (Whatman DE 50 or Serva) was used in the present work; the material was washed with 1M NaOH, then with large volumes of distilled water and finally equilibrated with phosphate buffer 0.02M, pH 8.1.

The cellulose was packed in glass columns of between 1.2 to 1.6 cm. in diameter and 20 cm. in length; 2 gr. of DEAE-cellulose were used to prepare column for 0.5-1 ml. of serum.

Serum was dialysed overnight at 0° against phosphate buffer 0.02M pH 8.1, before being added to the DEAE-cellulose column.

The DEAE-cellulose column was mounted above a fraction collector and the eluate was automatically collected in fractions of 5 ml.

Serum proteins were eluted as follows:

Method A. This was a minor modification of the technique described by Levy and Bober (1960); only two buffers were used, the first 0.02M, pH 8.1 and the second 0.2M, pH 8.1; in most cases the second buffer contained in addition 1M NaCl. About 100 ml. of the first buffer was followed by a similar volume of the second buffer.

The effluent fractions of each tube were examined in a Unicam spectrophotometer at 280 mμ; the effluent of each fraction (0.02M and 0.2M) containing significant amount of protein, was pooled; the pool was concentrated by ultrafiltration and then dialysed against 500 ml. of buffered saline.

Method B. Elution was carried out using seven buffers pH 8.1 of increasing molarity: 0.02; 0.04; 0.06; 0.08; 0.12; 0.16 and 0.2M. Selected portions of the effluent were collected, concentrated and dialysed as described in "Method A".

T A B L E 8

THE N.I.C. ANTIBODY IN 0.02M AND 0.2M FRACTIONS OBTAINED FROM DEAE-CELLULOSE CHROMATOGRAPHY⁽¹⁾

No. of sample ⁽¹⁾	ABO Group	Antibody titre (expressed as score)			Content of ⁽²⁾			
		whole serum	fract. 0.02M	fract. 0.2M	G globulin		M globulin	
					0.02M	0.2M	0.02M	0.2M
1	O	39	0	32	80	10	0	100
2	O	22	0	19	60	5	0	15
3	O	31	0	34	150	10	0	150
4	A ₁	36	0	24	...	...	...	...
5	A ₁	42	0	26	100	10	0	100
6	A ₁	50	0	41	...	...	...	...
7	A ₁	25	0	30	100	5	0	150
8	A ₁ B	36	0	20	...	...	...	...
9	A ₁ B	42	0	35	...	...	...	...
10	O	29	0	19	80	10	0	100
11	A	40	0	31	...	...	...	...
12	O	26	0	17	...	...	...	...

(1) Sera nos. 1 to 9 were obtained from "adult donors"; sera nos. 10, 11 and 12 were cold sera.

(2) The concentration is expressed as a percentage of amount in whole serum.

All the chromatographic fractionations were carried out at $+4^{\circ}$.

The concentration γ G and γ M globulins in the fractions was estimated using the inhibition of antiglobulin methods (see page 27).

Nine "adult" sera and 3 cord sera were fractionated using only two buffers (Method A).

The titre of the n.i.c. antibody in the original serum and in the 0.02 and 0.2M fractions was determined by making a series of doubling dilutions of serum or fraction; to each dilution horse complement was added and the sensitised red cells were tested within I.K. serum. The results are shown in Table 8; in this table the titre of the antibody is expressed as ^d"score" (Race & Sanger, 1956) and the dilution of the fraction is taken into account (Adinolfi et al., 1962).
(see page).

The n.i.c. antibody was always recovered in the 0.2M fraction. Estimates of γ M globulin in the 0.2M fraction obtained from 5 "adult" sera, showed that this fraction contained from 80 to 150 per cent of γ M globulin present in the original sample; this fraction was contaminated with about 10 per cent γ G globulin.

The 0.02M fraction contained most of γ G globulin and was completely free of γ M globulin.

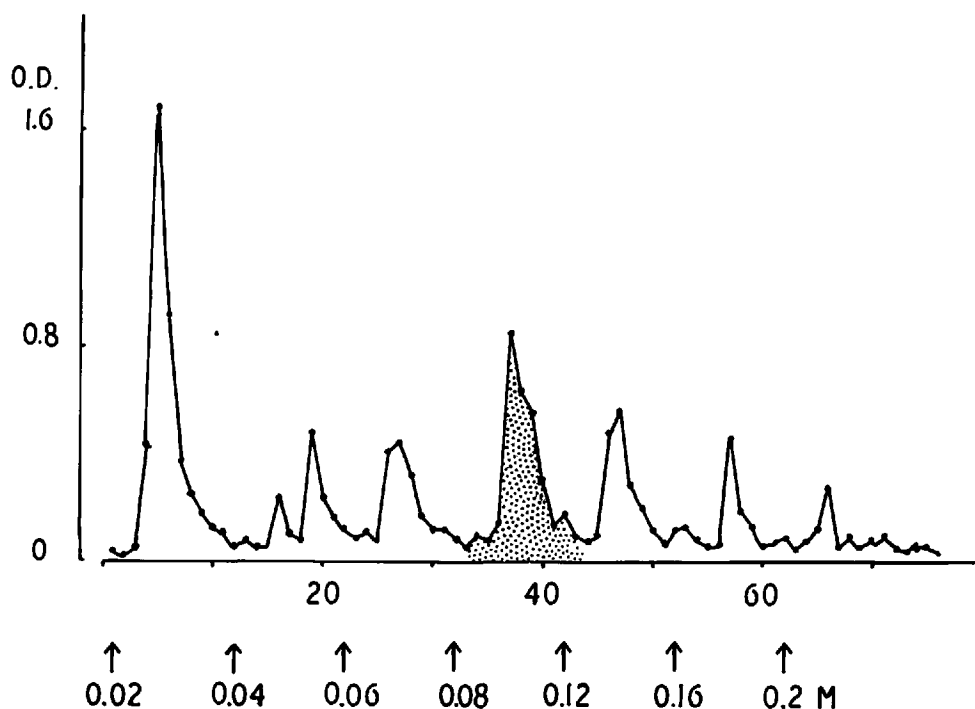


Fig. 10. Chromatography on DEAE-cellulose. Fractionation of human serum containing the n.i.c. antibody. Elution was carried out using a series of buffers of increasing molarity as indicated at the bottom of the diagram. The serum was separated into 7 fractions corresponding to the 7 major protein "peaks" of the chromatogram. The shadowed area indicates the fraction which was found to contain the n.i.c. antibody.

Two "adult" sera (nos. 1 and 4 in Table 8) and one cord serum (no. 10 in Table 8) were fractionated on DEAE-cellulose using "Method B".

Each serum was separated into 7 fractions corresponding to the 7 major "peaks" of the chromatogram; after concentration and dialysis, the titre of the n.i.c. antibody was determined using horse complement and an I.K. serum; the concentration of γ G and γ H globulins was estimated in the fractions obtained from serum no. 1 and the cord serum.

The n.i.c. antibody was recovered in the fractions eluted using phosphate buffer 0.08M; this fraction contained less than 5 per cent γ G globulin and less than 1 per cent M globulin present in normal "adult" standard serum.

Fig. 10 and Table 9 show the result of fractionating serum no. 1; the n.i.c. antibody was eluted in fraction 0.08M. The anti-A antibody present in this serum was eluted partially in the 0.16M fraction (γ M anti-A).

Serum no. 1 was also separated using a combined fractionation with zone electrophoresis and DEAE-cellulose chromatography. Thus 2 ml of serum were fractionated with zone electrophoresis on Geon 425 as previously described; after the migration the "active" fraction containing the n.i.c. antibody, eluted from 2 segments in the valley from γ and

TABLE 9

N.I.C. ANTIBODY AND ANTI-A AGGLUTININ IN THE FRACTIONS OBTAINED
WITH DEAE-CELLULOSE CHROMATOGRAPHY⁽¹⁾

	N.i.c. ⁽²⁾	anti-A ⁽²⁾	Content of ⁽³⁾	
			γ G globulin	γ M globulin
Whole serum	28	45	100	100
fraction 0.02 M	0	15	75	0
fraction 0.04 M	0	0	<2	0
fraction 0.06 M	0	0	<2	0
fraction 0.08 M	19	0	<5	0
fraction 0.12 M	5	0	<1	0
fraction 0.16 M	0	26	<1	>50 <100
fraction 0.2 M	0	5	<1	10

(1) Normal "adult" serum was fractionated as described in Fig. 10.

(2) The titre of the antibodies is expressed as "score".

(3) The concentration of the immunoglobulins is expressed as percentage of the amount present in whole serum.

β peaks, was dialysed against a phosphate buffer 0.02M, pH 8.1 and fractionated with DEAE-cellulose chromatography, using 7 phosphate buffers (Method B).

The n.i.c. antibody was eluted in the 0.08M fraction; the concentration of γ G and γ M globulins was estimated using the inhibition of antiglobulin method and it was found that the 0.08M fraction contained less than 1 per cent γ G globulin and less than 2 per cent γ M globulin present in the original serum.

When this fraction was tested with immunoelectrophoresis using a horse immune serum anti-human serum proteins, only one line of precipitation was observed.

In order to see if the protein detected with immunoelectrophoresis was related to the n.i.c. antibody, the fraction containing the antibody was absorbed at 0° with group O red cells in presence of fresh horse complement and then re-tested with immunoelectrophoresis. If the protein present in the fraction was related to the n.i.c. antibody it should have been partially or totally adsorbed, following the absorption of the antibody.

The investigation was carried out as follows: 2 aliquots (0.5 ml) of the fraction were mixed with group O red cells (0.2 ml of packed red cells); fresh horse serum, as a source of complement, was added to one sample; to the other sample a similar amount of horse serum, previously heated at 56°,

was added. The two mixtures were left at 0° for 2 hours and the supernatants recovered by centrifugation. Both supernatants were tested for the presence of the n.i.c. antibody and re-tested with immuno-electrophoresis. It was found that the supernatant absorbed with group O red cells in the presence of complement, was free of the antibody, whilst the protein detected with immuno-electrophoresis was not absorbed.

Gel filtration

Cross-linked dextran can be used to obtain an efficient separation of proteins on the basis of differences in molecular size. The gel consists of a three dimensional network of polysaccharide chains and the degree of cross-linking determines the porosity of the network. Molecules of larger size, such as 19 S γ - and α -globulins are essentially excluded from entering the gel; with smaller molecules the diffusion through the gel is restricted according to the size of the molecule and porosity of the gel. Using cross-linked dextran Sephadex G 200 (AB Pharmacia, Sweden) Flodin and Killander (1962) have shown that human serum separates into three major peaks corresponding to proteins with coefficients of sedimentation 19 S, 7 S and 3.5 S.

In separating the n.i.c. antibody the method described by Killander and Högman (1963) was used; a glass column approximately 80 x 2 cm was packed by gravity flow with Sephadex G 200 previously washed 5 times with an 0.05 M

potassium phosphate buffer, pH 7.3 containing 0.4M NaCl. Sodium azide 0.02%, was added in the buffer to prevent bacterial growth.

3 ml of fresh serum (donor group A₁) mixed with "pure" anti-D γ G (see page) was added to the column. The gel filtration was carried out at 4°; the elution flow rate varied between 10 and 15 ml for 1 hour; the effluent was collected in fractions of 5 ml and the protein content in each tube was recorded as UV transmission at 284 m μ .

The protein curve obtained showed the typical three peaks appearance (Fig. 11); the proteins present in each tube were concentrated by ultrafiltration and dialysed against buffered saline.

The n.i.c., anti-B and anti-D antibodies were tested using the techniques previously described; γ G and γ M globulins in each fraction were estimated (see page).

The anti-B agglutinin was detected in the fractions corresponding to the first and second peaks; the incomplete anti-D in the fractions corresponding to the second peak (Fig. 11). The n.i.c. antibody could not be detected in any fraction using human or horse serum as source of complement. As described in Section VIII it was later observed that dextran inhibited the n.i.c. antibody.

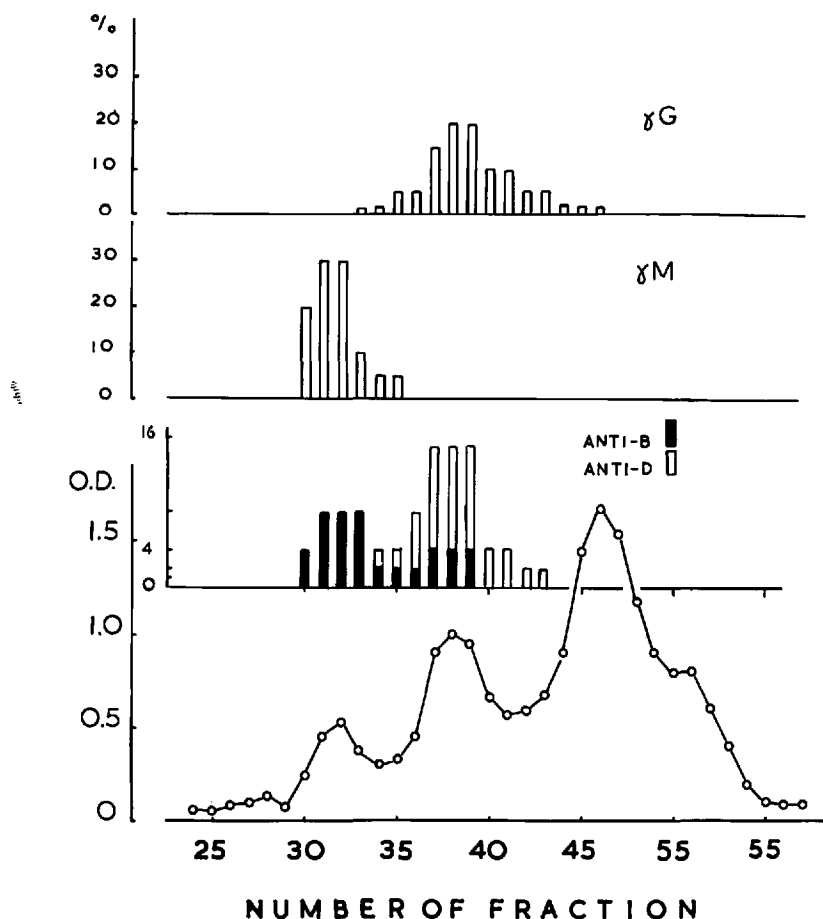


Fig. 11. Fractionation of 3 ml of human serum by gel filtration on Sephadex G 200. Elution was carried out using 0.05 M phosphate buffer pH 7.3 containing 0.4 M Na Cl. The protein content of the effluent was determined by absorption at 284 μ m.

The γG and γM globulin concentration in each fraction was estimated and is expressed as percentage of the concentration of the relevant protein in normal serum. The anti-D and anti-B titre of each fraction are shown in the lower part of the diagram.

b) Specific methods

These methods exploit the property that the antibodies have of combining specifically with antigens and of dissociating under special conditions. These methods involve the following steps: (1) formation of insoluble antibody-antigen complexes, (2) isolation of these complexes from all the other serum proteins, (3) dissociation of the complexes under mild conditions, and (4) separation of the antibodies from the antigens.

The dissociation of antibody-antigen complexes can be effected by methods which are not specific (e.g. changes of temperature, pH or salt concentration) or by means which may involve either displacement of the antigen from the complex by simple haptens, or the degradation of the antigen molecules within the complex, leaving the antibody molecules intact (for review see: Sehon, 1963). In the present work attempts at isolating the n.i.c. antibody were carried out using only methods involving changes of temperature, pH or salt concentration.

- 1) Attempts at dissociating the n.i.c. antibody from cellular antigens by heat.

Since the reactions between blood group antibodies and red cells are exothermic (Boyd, 1956; Wurmser & Filitti-Wurmser, 1957) the dissociation of blood group antibodies from

cellular antigens (red cells or red cell stroma) is usually obtained by incubating the complexes at a temperature higher than that used for promoting the formation of the complexes. Thus, for example, Landsteiner & Miller (1925) obtained anti-A and anti-B agglutinins by absorbing the antibodies onto red cells at room temperature and then eluting the agglutinins from the sensitized red cells at 56°. Very pure preparations of cold agglutinin were obtained by Gordon (1953) by absorbing the antibody onto red cell stroma at 0° and eluting it from the washed stroma-antibody complexes, at 37°.

In eluting the n.i.c. antibody from sensitized red cells a minor modification of the method described by Landsteiner & Miller (1925) was used. Thus 5 ml. of fresh serum was mixed with 1 volume of packed, previously washed group O red cells; the mixture was left at 0° for two hours; then the red cells were washed three times with cold buffered saline and re-suspended in 1 volume of saline; elution was carried out either at 56° for 5 minutes or at 37° for 20 minutes.

Both the eluates, tested using fresh horse serum as a source of complement and an I.K. serum, were found to be apparently free of n.i.c. antibody.

The n.i.c. antibody could only be detected in the eluate obtained from red cells sensitized at 0° when elution was carried out into serum instead of buffered saline as follows:

5 volumes of serum was mixed with 1 volume of group O packed red cells; the mixture was left 0° for 2 hours and then centrifuged; 4 volumes of the supernatant (absorbed serum) were removed and kept for testing and the remaining mixture of 1 volume of red cells and 1 volume of serum incubated at 37° for 20 minutes; the mixture was then centrifuged and the supernatant (eluate) removed for testing.

As shown in Table 10 this eluate contained the n.i.c. antibody.

A further attempt at isolating the n.i.c. antibody was made using a method which involved elution from red cell stroma (Weiner, 1957); human red cells were sensitized with the n.i.c. antibody at 0° , washed and packed tightly; the red cells were then lysed by freezing and thawing. Ten volumes of 50 per cent ethyl alcohol (pre-cooled at -6°) were added to the stroma and the mixture was left at -20° for about 1 hour. The supernatant was removed by centrifugation at 3000 rev./min. for 5 minutes, at 0° , and the sediment, washed once at 0° with distilled water, was then re-suspended in buffered saline and left at 37° for 30 minutes. The supernatant (eluate) was separated by centrifugation at 37° .

It was found that this method also failed to produce an eluate containing the n.i.c. antibody.

T A B L E 10

NORMAL INCOMPLETE COLD ANTIBODY ELUTED FROM
SENSITISED RED CELLS INTO SERUM AT 37°(*)

	Dilution of serum					
	1	2	4	8	16	32
Untreated serum	+++	+++	++	++	+	-
Absorbed serum	-	-	-	-	-	-
Elate	++	+	+	-	-	-

*Details of the elution procedure are described in "Methods" (p. 76)
Horse complement was added to each dilution of human serum;
the sensitised red cells were tested using an immuno-
comglutinin serum.

- 11) Attempts at dissociating the n.i.c. antibody from red cell stroma at acid pH.

The dissociation of blood group antibodies from cellular antigens can be brought about by changes of pH (Hughes-Jones et al., 1964). The presence of a charge on the antibody combining site in juxtaposition to an opposite charge on the antigenic determinant has been demonstrated (Singer et al., 1955; Grossberg & Freneman, 1960) and, therefore it would appear that the forces underlying antibody-antigen interaction are, at least in part, electrostatic (Sohn, 1962; 1964). Consequently, dissociation of such complexes can be obtained by changes of pH.

Antibody-antigen complexes are stable within the pH range of 4.5-11; dissociation gradually increases outside this range and it is complete at a pH of about 2.4 or 12.3 (Kleinschmidt and Boyer, 1952; Singer and Campbell, 1954, 1955; Hughes-Jones et al., 1964).

In the present work, a minor modification of the method described by Hughes-Jones et al. (1964) was made; 5 ml. of fresh serum was mixed with 100 mg. of freeze-dried, haemoglobin-free, red cell stroma; after keeping the mixture at 0° for two hours, the red cell stroma was washed three times with cold buffered saline and the elution performed at 37°

using a saline solution (0.17 M NaCl) buffered to pH 3.5.

As ^acontrol, an incomplete anti-D (γ G) antibody was used; whilst this antibody was recovered in the eluate with a titre which was about $\frac{1}{2}$ of that present in the original serum, the n.i.c. antibody could not be detected, even when fresh complement was supplied.

111) Dissociation with strong salt solution.

The observation by Heidelberger et al. (1936) that the same amount of pneumococcal polysaccharide precipitated less antibody nitrogen in 1.79 M NaCl than in 0.15 M NaCl₁ provided the basis for the development of methods for the purification of anticarbohydrate antibodies by dissociation of specific precipitates, or agglutinates with strong salt solutions (Heidelberger et al., 1936). By ultracentrifugal analysis at each step in the process of purification, it was shown by Kabat (1939) that the salt dissociation method did not produce any alteration in the size of the antibody molecules.

The salt dissociation method is probably applicable to many carbohydrate-anticarbohydrate systems (Kabat, 1961).

Since the n.i.c. antibody was found to react with some polysaccharides (see page 131) it was hoped that the application of this technique could be used to dissociate the n.i.c.

antibody from dextran-n.i.c. complexes.

Investigations, which will be described later in detail, had shown that the n.i.c. antibody was completely absorbed, or its titre was markedly reduced by mixing fresh serum with cross-linked dextran (Sephadex G200) and leaving the mixture at 0° for 1 hour.

In eluting the antibody from dextran (Sephadex) - n.i.c. complexes, the following procedure was used: ten volumes of serum was mixed with four volumes of previously washed Sephadex G200, and the mixtures were left at 0° or 37° for one hour; the Sephadex was then washed five times with buffered saline. In dissociating the complexes, a 15 per cent NaCl solution was used; thus the washed Sephadex, resuspended in five volumes of the salt solution, was left at 37° for one hour; the supernatant was recovered and the procedure of extraction repeated. The supernatants were then pooled, concentrated by ultrafiltration and dialysed against buffered saline.

The results of these investigations are summarised in Table 11; the n.i.c. was easily absorbed by dextran (Sephadex) at 0° and largely eluted from the complexes formed at 0°. The antibody was only partially absorbed at 37°.

The eluates, tested with immunoelectrophoresis, was found to be contaminated by several serum proteins; at least 3 lines

T A B L E 11

NORMAL INCOMPLETE COLD ANTIBODY ABSORBED
WITH AND ELUTED FROM DEXTRAN *

	Dilution of serum						
	1	2	4	8	16	32	64
untreated serum	+++	+++	+++	++	++	+	-
serum absorbed with dextran at 0°	-	-	-	-	-	-	-
eluate from dextran- n.i.c. antibody complex formed at 0°	+++	+++	++	+	-	-	-
serum absorbed with dextran at 37°	+++	+++	++	++	+	-	-
eluate from dextran- n.i.c. complex formed at 37°	+	±	-	-	-	-	-

*Normal serum containing the n.i.c. antibody was mixed with dextran (Sephadex G200) and the mixture left at 0° or 37° for 2 hours; the antibody was eluted from dextran-n.i.c. complex using a 15 per cent NaCl solution at 37°. For details see "Methods" pag. 82.

Horse complement was added to each dilution of human serum and an immunoconglutinin serum was used in testing the sensitized red cells.

of precipitation were noticed, corresponding to: one γ G globulin line, one β -globulin line and albumin. (see Fig. 13).

Discussion

Antibodies in man and most species of animal studied to date are associated with a group of structurally related proteins which were referred to as "gamma-globulins" (Tiselius, 1937) or "immunoglobulins" (Heremans, 1960).

The major fraction of these proteins is γ G-globulin which makes up approximately 80 per cent of the total immunoglobulins; the remainder consists of γ M and γ A globulins.

The γ G globulin has a molecular weight of approximately 160,000 and a sedimentation coefficient of approximately 7S (Svedberg units). On electrophoresis γ G globulin migrates as a continuous spectrum of heterogeneous proteins extending from the slowest immunoglobulin region to the α_2 -globulin region (William and Grabar, 1955; Wallenius et al., 1957).

The γ M-globulin has a molecular weight of approximately 1 million and an S rate of 19 (Deutsch et al., 1946; Müller-Eberhard et al., 1956); on electrophoresis it migrates from the fast γ G-globulin extending into the β -globulin region (Wallenius et al., 1957).

The γ M globulin is composed of six protein-units, approximately the size of γ G globulin, linked through disulphide bonds; Deutsch & Morton (1957) and Glenchur et al. (1958) have shown that γ M globulin can be dissociated into sub-units by treatment with sulphydryl compounds, e.g. 2-mercaptoethanol.

The γ A globulin, initially detected by William & Grabar (1955) is a heterogeneous group of proteins migrating in the fast γ G globulins and β -globulin regions. Most of the γ A globulin sediments as $\bar{v}$ 7S proteins in the ultracentrifuge, but larger components have been described with sedimentation coefficient 9, 11 and 13S (Heremans et al., 1959) and 18S (Tomasi et al., 1964).

In addition to these three classes of immunoglobulins, normal serum, and especially urine, contains a protein antigenically related to immunoglobulins (Webb et al., 1958; Franklin, 1959; Beggård, 1961) which according to some observers also carries antibody activity (Lerner et al., 1962). These micro- γ -globulins are heterogeneous on electrophoresis (Beggård, 1961) and have a molecular weight of between 10,000 (Webb et al., 1958) and 30,000 (Franklin et al., 1959).

Whereas the possible significance in biological phenomena of these micro- γ -globulins remains to be established, the association between the three major types of immuno-

globulins and bacterial and blood group antibodies has been repeatedly demonstrated.

It was hoped that the application of some methods which have been found useful in investigating the relation of many blood group antibodies to the different classes of immunoglobulin, might help in studying the nature of the n.i.c. antibody.

The first suggestion that the n.i.c. antibody was not associated with γ G globulin was obtained using a precipitation technique at low ionic strength and acid pH.

Certain of the globulins of plasma are relatively insoluble at low ionic strength or in absence of salt; a more effective precipitation at reduced ionic strength may be carried out in combination with isoelectric precipitation. Dialysis against a buffer of desired composition is the most common method used in approaching a given pH and ionic strength, while avoiding denaturation of the isolated protein.

In the present work normal serum containing the n.i.c. antibody was dialysed against a phosphate buffer 0.02M, pH 5.4. Under these conditions albumin and α -globulin - which have isoelectric points somewhat below 5 - almost all β -globulins, and γ G globulin are still soluble, whereas γ M globulin (see : Pennell, 1962), about 20% γ A globulin (Hermans, 1960),

12
 β -lipoproteins (Pennell, 1962) properdin (Pillemer et al., 1954) and two complement components - C'_1 and C'_2 - (see Mayer, in Vahat and Hey, 1961) are precipitated.

In the present work it was observed that the n.i.c. antibody was completely precipitated after dialysis against a buffer 0.02M, pH 5.4 whereas the incomplete anti-D antibody (γ G-globulin), which was used as a control, was recovered almost only in the supernatant, suggesting that the n.i.c. antibody was not associated with γ G-globulin.

The result of the serum protein fractionation with ammonium sulphate pointed to a similar conclusion; in fact the n.i.c. antibody was present mainly in the precipitate obtained with 1.86M ammonium sulphate, whereas the anti-D (γ G-globulin) antibody was present mainly in the precipitate obtained with 1.49M ammonium sulphate; as shown in Table 5 γ M-globulin was precipitated only at a high concentration of $(NH_4)_2 SO_4$.

Recently Olesen et al. (1964), using labelled γ G and γ M globulins, have confirmed that γ G globulin is precipitated at a lower concentration of ammonium sulphate than γ M globulin; these findings are in good agreement with the present work, as regards the precipitation of immunoglobulins (Fig. 12).

A further piece of evidence that the n.i.c. antibody was not associated with γ G globulin was obtained using zone

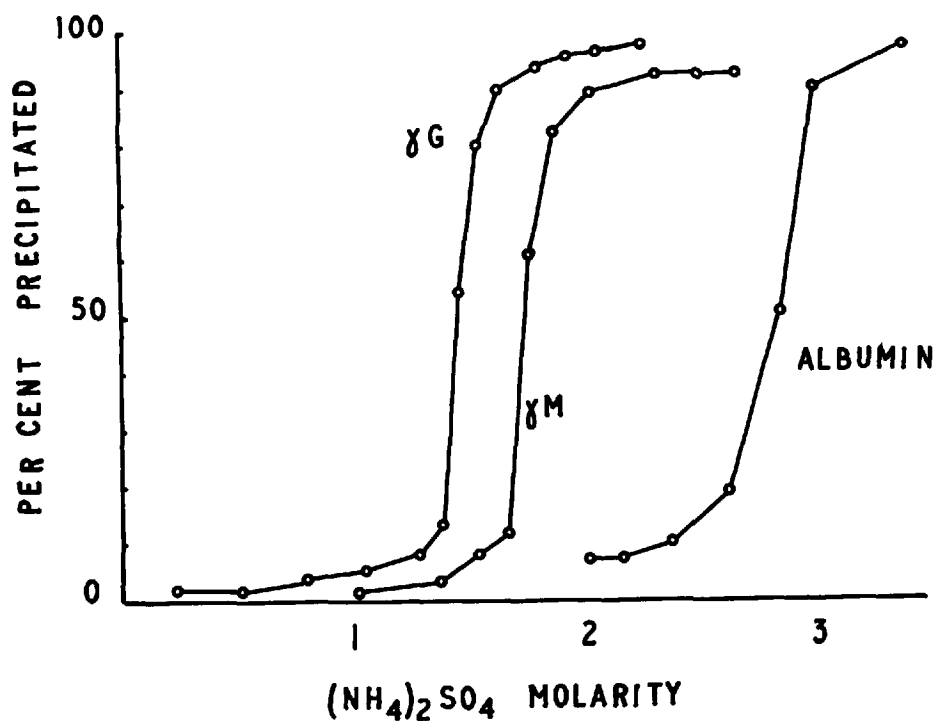


Fig. 12. Precipitation by ammonium sulphate at pH approximately 6.8. Percentage of albumin γG and γM globulin labelled with ^{125}I or ^{131}I , precipitated at various molarities. Modified from Olesen et al., 1964.

electrophoresis; the fractions which contained almost no protein apart from slow migrating γ G globulin, were found to be free of the n.i.c. antibody; the antibody was recovered in a narrow zone in the valley between the β and γ globulin peaks.

However the most compelling evidence that the n.i.c. antibody was not γ G globulin was obtained by fractionating serum containing the antibody with DEAE-cellulose chromatography.

Cellulose ion exchangers, notably DEAE-cellulose, have been shown to be of great value in the fractionation of serum proteins, especially in the separation of γ G globulin from γ M-globulin. Thus, if normal serum is fractionated by DEAE-cellulose chromatography using phosphate buffers pH 8 - 8.2 of increasing molarity (from 0.02 to 0.2M), about 90 per cent of γ G-globulin is recovered in the early fractions whereas γ M-globulin is eluted only in the later fractions (Fahey & Horbett, 1959); γ A-globulin is recovered between the γ G and γ M globulin peaks (Fahey, 1962). A small amount of γ G globulin is, however, eluted throughout the chromatogram (Fahey, 1962).

When the elution from^a DEAE-cellulose column is carried out using only two phosphate buffers, the first 0.02M and the second 0.2M, about 90 per cent of γ G globulin, present in the original serum, is recovered in the 0.02M fraction, which is free of γ M-globulin (Levy & Sober, 1960; Adinolfi et al., 1962).

The 0.2M fraction contains as much γ M globulin as is present in the original serum and between 5 and 10 per cent γ G-globulin (Levy & Sober, 1960; Adinolfi et al., 1962).

Therefore in fractionating serum with DEAE-cellulose chromatography using only two buffers, about 90 per cent of the antibody composed solely of γ G-globulin is expected in the 0.02M fraction whereas any antibody composed solely of γ M-globulin is expected only in the 0.2M fraction.

In the present investigations the n.i.c. antibody was never recovered from DEAE-cellulose columns in the 0.02M buffer either using a series of buffers of increasing molarity (method B) or only two buffers (method A). Using the latter procedure the antibody was recovered in the 0.2M fraction. When the elution was carried out with the 7 buffers the antibody was found in the 0.08M fractions. A similar result was obtained when fractionation of the antibody with zone electrophoresis was followed by DEAE-cellulose chromatography; the antibody was finally recovered in the 0.08M fraction which contained less than 1 per cent of the γ G and γ M globulins present in the original serum.

This latter finding was the first, in the series of results obtained using non-specific methods of isolating antibodies, which suggested that the n.i.c. antibody was

neither γ G globulin nor γ M globulin. Nevertheless as traces of both immunoglobulins were present in the "active" fraction, these observations were considered only contributory, rather than conclusive and it was decided that further investigations ought to be carried out, using procedures which exploit the dissociation of antigen-antibody complexes. These experiments were designed to answer two questions: a) ~~to see~~ whether it was possible to identify the antibody in the eluate as γ G, γ A or γ M globulin, using specific immune sera and agar gel precipitation techniques; and b) ~~to see~~ whether an eluate containing the antibody could be used as an antigen in the attempt at producing an immune serum specifically reacting against the n.i.c. antibody.

The procedure of classifying blood group antibodies by testing the eluates with agar precipitation using specific anti- γ G and anti- γ M globulin sera has not always been successful. Often, in fact, the eluates were contaminated by several serum proteins or were found to contain immunoglobulins, although they lacked antibody activity (Petoom, 1963). This method was only found useful in identifying the cold agglutinin present in patients with acquired haemolytic anaemia or cold antibody type (Mehotra, 1950). Petroom (1963) claimed to have identified an incomplete anti-D, an anti-Fy^a and an anti-Kell (the specificity of the latter antibody is doubtful since sometimes it is referred to as "anti-Kell", at other

times as an "anti-Kidd") as γ G-globulin, using this method.

Although the limitations of the technique were fully realised, it was hoped that some advantage could be gained by applying it to the study of the nature of the n.i.c. antibody.

Unfortunately the attempts at eluting the antibody from sensitised red cells or red cell stroma, using standard methods, were unsuccessful; the antibody could not be detected in the eluates obtained by heating at 56° or 37° or by using the dissociation at acid pH method.

Investigations which will be described in detail later (see page 126) showed that the failure at eluting the n.i.c. antibody at acid pH was due to the instability of the antibody at pH below 5.1.

The failure at eluting the n.i.c. antibody by heating sensitised red cells at 37° or 56° is more difficult to explain and might be tentatively ascribed to an interference of complement absorbed with the antibody onto the red cells, as Polley and Mollison (1961) have shown that some complement-binding antibodies are difficult to elute when absorbed in the presence of complement.

The n.i.c. antibody could be eluted from sensitised red

cells only when the elution procedure was carried out into serum instead of saline; whether this successful elution was due to a protective action of the medium is a matter of speculation.

Using a technique successful in isolating pneumococcal carbohydrate antibodies (Heidelberger et al., 1936) the n.i.c. could be dissociated from complexes formed with dextran (Sephadex), at 0°, but the eluate was found to be contaminated by several serum proteins. Incidentally, according to Kabat (1961) this method of dissociation with strong salt solution is applicable only to carbohydrate-anticarbohydrate systems and cannot be used for protein-antiprotein systems.

In conclusion, the failure in isolating the n.i.c. antibody using specific methods, even if disappointing, is not altogether surprising, as no studies have been reported so far of successful elution of the antibody from sensitized red cells. Since all other blood group antibodies can be eluted to some extent from sensitized red cells, the failure with the n.i.c. antibody must be accepted as further evidence that it differs from classical antibodies.

SECTION VI

Reaction of the n.i.c. with anti- χ G, anti- χ A and anti- χ M
globulin sera: effect of treatment with 2-mercaptoethanol

Two further methods were used to discover whether the n.i.c. antibody was γ G, γ A or γ M globulin; first, tests with specific anti- γ -globulin sera and second, treatment with 2-mercaptoethanol (2-me).

1) The reaction of the n.i.c. antibody with specific anti- γ -globulin sera

a) Classification of incomplete blood group antibodies as γ G, γ A and γ M globulin using anti- γ -globulin sera

The use of immune sera specifically reacting with γ G or γ M globulin has been shown to be a useful tool in classifying blood group antibodies (Polley et al., 1962; Mollison et al., 1962) since it has been demonstrated that in the antiglobulin test γ G and γ M globulins do not cross-react to any appreciable extent; thus red cells sensitized with incomplete anti-Rh (γ G) antibodies are agglutinated by anti- γ G globulin serum but not by anti- γ M globulin serum, and red cells sensitized with anti-Le^a (γ M) antibodies are agglutinated by anti- γ M globulin serum but not by anti- γ G globulin serum (Polley et al., 1962).

When thirty selected blood group antibodies (excluding anti-A and anti-B) were classified as γ G or γ M globulin (or mixtures of both) good agreement was found between the results

obtained using anti- γ G and anti- γ M globulin sera and the findings obtained using either DEAE-cellulose chromatography or treatment with 2-mercaptoethanol (Adinolfi et al., 1962)

With regard to the identification of blood group antibodies as γ A globulin, using anti- γ A-globulin serum, only a few studies have so far been reported.

In 1962 Rockey and Kunkel demonstrated that, in addition to γ G and γ M antibodies, anti-A and anti-B associated with immunoglobulin with a sedimentation rate $9_s - 15_s$ were present in the serum of individuals stimulated with specific blood group substances. These antibodies were eluted by DEAE-cellulose chromatography between the bulk of γ G and γ M globulin and were shown to be inhibited by treatment with 2-mercaptoethanol.

Evidence that these agglutinins were antigenically related to γ A globulin was produced one year later; thus Kunkel and Rockey (1963) using Ouchterlony plate analysis showed that anti-A antibodies, dissociated from complexes with A substance, reacted with anti- γ A globulin serum.

The presence of anti-A and anti-B γ A globulin in saliva, colostrum and ascitic fluid, other than in normal serum, has been demonstrated by Tomasi et al. (1964).

At present the association of other blood group antibodies with γ A globulins is still uncertain.

Recently Prager and Bearden (1964) claimed that anti- A globulin serum agglutinated the red cells sensitised with all 15 incomplete anti-D antibodies studied, whereas only 4 out of 21 other incomplete antibodies sensitised red cells to the anti- γ A globulin serum.

These observations lead to the question whether γ A antibodies are produced only against certain antigens.

According to Prager and Bearden (1964) the explanation lies elsewhere; thus they have observed that anti- γ A globulin serum may be diluted 100 times without altering the pattern of a weak agglutination, whereas, ordinarily, a several fold dilution of an anti- γ G globulin serum, giving weak agglutination leads to the disappearance of the agglutination altogether; this would indicate that the weak reaction reflected a weak binding of anti- γ A globulin serum to the γ A antibody and not the lack of the antibody on the red cells; accordingly Prager and Bearden claimed that almost all sera contained γ A antibodies, but the binding force of anti- γ A globulin sera was not strong enough to produce agglutination of red cells sensitised with some of these antibodies.

Such consideration is rather speculative at this time and does not seem to explain why the binding force of anti- γ A globulin serum is stronger with incomplete anti-D γ A antibodies and weaker with other incomplete γ A antibodies, e.g. anti-C or anti-K. Furthermore the findings of Prager and

Bearden are not in agreement with the data of Mollison et al. (1965) who have observed that only two incomplete anti-D, out of 30 sera tested, sensitised red cells to anti- γ A globulin serum (Prof. P.L. Mollison, personal communication).

b) The reaction of the n.i.c. antibody with anti- γ G, anti- γ A and anti- γ M globulin sera.

Evidence that the n.i.c. antibody does not sensitise red cells to agglutination by anti- γ -globulin sera was first produced by Dacie (1950) who noticed that antiglobulin sera, highly potent in revealing sensitisation by incomplete anti-D antibodies, gave a weak reaction with red cells sensitised with the n.i.c. antibody. This observation and those made later by Dacie (1951), Crawford and Mollison (1951) and Renton (1952) (described in detail in Section II) left little doubt that antiglobulin sera reacting with incomplete anti-D antibodies, were not active against red cells sensitised with the n.i.c. antibody. Since it is now known that incomplete anti-D antibodies are always associated with γ G globulin, these earlier observations imply that the n.i.c. antibody does not sensitise red cells to anti- γ G globulin serum.

The failure of anti- γ M globulin serum to agglutinate red cells sensitised with n.i.c. antibody was demonstrated by Polley et al. in 1962.

In the present work 3 anti- γ A and 3 anti- γ M globulin

sera were tested against red cells sensitised with the n.i.c. antibody; in view of the observations previously quoted, no attempt was made of using anti- γ G globulin serum.

Two anti- γ A globulin sera were obtained commercially⁽¹⁾; the other one was kindly supplied by Dr. J.F. Soothill; all three anti- γ M globulin sera were obtained by injecting pathological γ M globulin into rabbit. The immune sera were made specific by appropriate absorptions; details are reported on page 24.

In testing the immune sera against the n.i.c. antibody, group O red cells were sensitised with fresh A₁B serum (1 vol. 50% red cells suspension/10 vol. serum) at 0° for 2 hours. The immune sera were tested by making serial, doubling dilutions of each serum in buffered saline; one drop of each dilution was mixed, on an opal tile, with one drop of a 20 per cent suspension of sensitised red cells; the degree of agglutination was scored after 10 minutes.

As control, the immune sera were tested against appropriate red cells sensitised with an incomplete anti-D γ G, an anti-D γ A and an anti-Le^a γ M antibody.

The results showed that the three anti- γ A, as well as the three anti- γ M globulin sera, did not react with red cells sensitised with the n.i.c. antibody. The sera were shown to be specific since the anti- γ A globulin sera reacted only against red cells sensitised with anti-D γ A, whereas the anti- γ M globulin sera reacted only against red cells

(1) Hoechst Pharmaceuticals Ltd., London.

sensitised with the anti-Le^a γ M antibody.

ii) Treatment of serum containing the n.i.c. antibody with 2-mercaptoethanol

Deutsch and Morton (1957) observed that the addition of sulphydryl compounds, such as 2-mercaptoethanol or cysteine, resulted in the conversion of pathological γ M globulin and γ M globulin present in normal serum into molecular entities having a sedimentation coefficient near that of γ G globulin. The following procedure was used by Deutsch and Morton (1957): one per cent solution of the protein to be studied was treated with 0.1M 2-mercaptoethanol (2-me) in potassium phosphate buffer (ionic strength 0.2; pH 7.4) over a period of 24 to 48 hours at room temperature. Following this, the sulphydryl compound was removed by dialysis for 48 to 72 hours against the phosphate buffer or against solutions of the buffer containing 0.02M iodoacetate or iodoacetamide.

By ultracentrifugal analysis Deutsch and Morton (1957) showed that the removal of 2-me by dialysis resulted in the re-aggregation of the sub-units, whilst the presence of sulphydryl blocking agents prevented such re-association. Such results indicated that the depolymerization noted was the result of the breaking of disulphide bonds between the γ M globulin sub-units.

Treatment with 2-me was soon used successfully in distinguishing γ G from γ M antibodies; Fudenberg and Kunkel (1957), studying the physical properties of the red cell

antibodies present in patients with acquired haemolytic anaemia, observed that treatment of serum with 2-me inactivated "cold" agglutinins whereas it was ineffective against "warm" incomplete antibodies of anti-Rh type.

The effect of sulphhydryl compounds on the serological activity of blood group antibodies was investigated by Grubb and Swahn (1958); they found that anti-A and anti-B agglutinins in group O sera and anti-Rh agglutinins were inactivated by treatment with 0.02M-0.1M 2-me, whilst incomplete anti-Rh antibodies were not affected. The results were the same whether the treated sera were dialysed against buffer or buffer containing iodoacetate.

Since rearrangement of γ M antibody subunits take place during the dialysis against buffer, it is surprising that the loss of antibody activity on reduction with 2-me, or other reducing reagents, can be used successfully as a test for identifying γ M antibodies. To explain these intriguing observations Chan and Deutsch (1960) advanced the hypothesis that when whole serum containing the antibody is treated with reducing agents and then dialysed against buffer, the reaggregation of the sub-units takes place at random with other non-antibody γ M globulin fragments, to produce, at best, molecules containing only one combining site.

A few years later this hypothesis was proved to be correct; it was observed that γ M globulin retained its antibody activity after reduction and reassociation, provided that the treated

material contained a high percentage of "active" molecules and, on reassociation, the chance for "active" fragments coming together were relatively great; it was also observed that the addition of "foreign" fragments to the "active" sub-units lowered the antibody activity of reassociated γ_M globulin (Tomasi and Kunkel, 1961; Schrohenloher et al., 1964).

γ_G antibodies are not "inactivated" by treatment with 0.02-0.12M 2-me (or other reducing agents) since, under mild conditions of reduction from 3 to 5 interchain disulphide bonds are split (for review see Nisonoff, 1964) but the reduced γ_G globulin is not dissociated in sub-units unless it is subsequently treated with denaturing solvents (Edelman and Poulick, 1961) or dialysed against acid buffers (Palmer and Nisonoff, 1963).

At the present time the effect of reducing agents on γ_A globulin has not been fully investigated. It has been shown that γ_A globulin can occur in normal serum as a whole series of polymers from 7S to 17S (Vaerman et al., 1964) and that γ_A globulin is present in saliva and colostrum largely in the form of high polymers, the major component of which has a sedimentation coefficient of 11S (Tomasi et al., 1964).

According to Deutsch (1962) and Vaerman et al. (1964) treatment with 2-me dissociates the 10.5-11S γ_A globulin, present in normal serum, to 7S sub-units. However Tomasi et al. (1964) have observed that 11S γ_A globulins present in saliva and colostrum were not dissociated by 0.1M 2-me at neutral pH.

In the present work the effect of 2-me on the n.i.c. antibody was investigated using the following method: 1 volume of undiluted serum was mixed with an equal volume of 0.1M 2-me in phosphate buffer pH 7.4 and left at 37° for 2 hours; the mixture was then dialysed overnight against buffered saline at 0°. In a few experiments dialysis was carried out using buffered saline containing 0.02M iodoacetamide.

As controls, 1 volume of undiluted serum was treated with buffer alone, instead of 2-me.

Six "adult" sera and two cord sera containing the n.i.c. antibody were treated as described. As controls, one incomplete anti-D (Y G) and one anti-P (Y M) agglutinin were used.

It was found that the n.i.c. antibody was always inactivated after treatment with 2-me and dialysis against buffered saline with or without 0.02M iodoacetamide. Treatment with 2-me did not affect the anti-D antibody whereas the anti-P agglutinin was inactivated.

DISCUSSION

When antisera against Y G and Y M globulins are used in the antiglobulin sensitisation test, red cells sensitised with most incomplete "naturally occurring" blood group antibodies are agglutinated by anti-Y M globulin sera, whilst red cells sensitised with "immune" incomplete antibodies are usually

agglutinated by anti- γ G globulin sera (Polley et al., 1962).

Two major exceptions have been observed namely that red cells sensitised with anti-Jk^a (γ G) antibodies are only agglutinated by some anti- γ G globulin sera and that neither anti- γ G nor anti- γ M globulin sera agglutinate red cells sensitised with the n.i.c. antibody (Polley et al., 1962).

The present work, whilst confirming that anti- γ M globulin serum fails to agglutinate red cells sensitised with the n.i.c. antibody, shows^{also} that the antibody does not ~~even~~ react with anti- γ A globulin serum.

These findings might suggest either that the n.i.c. antibody is not a member of the immunoglobulin family at all, or that the antibody is an immunoglobulin but is qualitatively different from all other blood group antibodies.

If the second hypothesis is correct, that is to say the n.i.c. antibody, resembling in some way anti-Jk^a, is a qualitatively different immunoglobulin and sensitised red cells only to some specific anti- γ -globulin sera, it cannot be γ G globulin since it is inactivated by treatment with 2-me.

On the other hand treatment with 2-me does not exclude the possibility that the n.i.c. antibody is not a member of the family of the immunoglobulins since other serum proteins may be reduced and dissociated by treatment with sulphydril compounds.

It was at this stage of the investigation that it was thought to be worth while to test a few samples of serum of patients with severe hypogammaglobulinaemia to see whether they contained normal level of n.i.c. antibody.

SECTION VII

The n.i.c. antibody in the serum of patients with severe
hypogammaglobulinaemia

Hypogammaglobulinaemia is a rare syndrome characterized by a marked deficiency of circulating immunoglobulins and therefore of antibodies. Several variants of the syndrome have been observed; the more frequent primary hypoglobulinaemia is due to an hereditary error of metabolism occurring exclusively in males and transmitted as a sex-linked trait; this form is characterized by repeated bacterial infections beginning in infancy or early childhood (Bruton et al., 1952; Janeway et al., 1953). Primary hypoglobulinaemia also occurs as an acquired disease in individuals of either sex in whom the onset of the repeated severe infections may start at any time from the childhood to old age. Finally the syndrome may occur transiently in some infantsⁱⁿ whom, after the first two or three months of life, the level of the immunoglobulins in serum is very low for a period of several months and this is associated with recurrent infections.

Secondary acquired hypogammaglobulinaemia have been observed in patients with multiple myeloma, follicular lymphoma and chronic lymphatic leukaemia and in patients with general hypoproteinaemia (nephrosis, severe burns) (for references see Gitlin et al., 1956; Gitlin et al., 1959).

In patients with primary hypogammaglobulinaemia there is a deficiency of γ G-globulin as well as of γ A and γ M globulin (Gitlin et al., 1956; Giedion and Scheidegger, 1957). Anti-A and anti-B agglutinins are usually absent, or present in a very low titre (Gitlin et al., 1959; Soothill and Squire,

1963). The level of properdin remains normal in serum of patients with severe hypogammaglobulinaemia according to Barandum and Isliker (1959).

In the present investigation serum from 14 patients with primary hypogammaglobulinaemia was kindly supplied by the Medical Research Council's Working Party; six of these sera were tested only a few weeks after being collected and had been stored at -50° ; the other nine sera had been stored at -20° for many years; estimates of the concentration of γG , γA and γM globulins were kindly supplied by Dr. J.F. Soothill. All sera were tested for anti-A, anti-B and anti-I agglutinins. Some sera were also tested for the presence of bacterial antibodies. The n.i.c. antibody was tested using horse complement and an I-K serum.

The results are reported in Table 12; the n.i.c. antibody was found in all but one serum (no. 13). Due to the limited amount of serum available the antibody could be titrated only in one serum stored at -20 for 2 years (no. 7) and in six sera stored at -50° for a few weeks after being collected. The titre of the n.i.c. antibody in these sera was similar to that of normal sera used as controls.

Anti-A, anti-B and anti-I antibodies were absent from all sera; sera no. 3 and no. 4 were found to be free of bacterial agglutinins against E. coli C3, E. coli A55, Salmonella

enteritis variety Danisz, Staphylococcus aureus 80 and
Pneumococcus type XIV.

DISCUSSION

The demonstration that the n.i.c. antibody was present in 13 out of 14 sera of patients with severe hypogammaglobulinaemia strongly suggests that the antibody differs from other blood group and bacterial antibodies.

It may be suggested that the traces of the immunoglobulins present in these sera were specifically associated with the n.i.c. antibody, but it is difficult to conceive that in patients with hypogammaglobulinaemia, the synthesis of all antibody proteins, except those with n.i.c. specificity, should be inhibited.

Among the most challenging clinical problems presented by patients with severe hypogammaglobulinaemia is their capacity to react normally to many virus infections; for example, it has been repeatedly observed that the clinical course of episodes of rubeola, rubella, varicella poliomyelitis, influenza and infections with Echo viruses is not demonstrably different from that occurring in immunologically normal individuals and that repeated exposures to rubeola, varicella and influenza reveal an effective immunity even through extensive immunological investigations fail to detect specific antibodies (for references see Godd, 1959). On the basis of these

T A B L E 12

N.I.C. IN SERUM OF PATIENTS WITH HYPOGAMMAGLOBULINAEMIA SYNDROME(*)

No.	Initials	globulins ⁽⁺⁾			A B O blood group	Anti-A	Anti-B	Anti-I	N.i.c. ^(**)
		γ G	γ A	γ M					
1	H.H.	6	2	62	A	...	nil	nil	32
2	D.H.	17.5	3	100	A	...	nil	nil	16
3	S.S.	12.8	0.5	17	O	nil	nil	nil	64
4	T.W.	25	0.2	0	B	nil	...	nil	64
5	A.A.	300	...	...	O	nil	nil	nil	32
6	A.W.	20	8	6	B	nil	...	nil	16
7	C.W.	140	3	6	A	...	nil	nil	16
8	J.A.	40	2	6	O	nil	nil	nil	++
9	J.A.	2.5	0	25	O	nil	nil	nil	+
10	A.G.	25	0	300	AB	...	...	nil	++
11	M.B.	80	...	...	A	...	nil	nil	++
12	J.C.	30	2	200	A	...	nil	nil	+
13	B.W.	40	4	18	O	nil	nil	nil	-
14	E.H.	200	0	0	A	...	nil	nil	+++

- (*) The first six sera were stored at -50° for only a few weeks; the others were stored at -20° for periods between two and six years.
- (†) The concentration of the γ G globulin is expressed as mg./100 ml. of serum; the concentrations of the γ A and γ M globulins are expressed as percentages of the amount of standard normal sera.
- (**) The n.i.c. antibody was titrated in seven samples (titres expressed as reciprocals). The degree of agglutination of the other seven sera was compared with that of a normal serum which gave a +++ agglutination.

observations, Good (1959) suggested that "a specific immunologic mechanism, which is not dependent on the production of immunoglobulin or circulating antibody, is intact in patients with hypogammaglobulinaemia and results in their recovery from, and subsequent specific protection against certain virus disease".

Whether this immunological mechanism is in some way related to the properdin system has never been established; as mentioned previously, properdin is present in normal amounts in the serum of patients with severe hypogammaglobulinaemia; it is interesting that, in these patients, low levels of properdin have been observed in the course of severe bacteremias, apparently as a consequence of an increased consumption (Barandum and Isliker, 1956).

SECTION VIII

Serological characteristics of
the n.i.c. antibody

The experiments referred to in the previous sections support the view that the n.i.c. antibody differs from other blood group antibodies, in that it is not associated with γ G, γ A and γ M globulins. In starting the investigations which will be described in this chapter, it was hoped that these differences could be confirmed by comparing the serological properties of the n.i.c. antibody with those of some γ G, γ A and γ M blood group antibodies.

Furthermore, since a considerable body of evidence indicated that in some ways the n.i.c. antibody resembles properdin - for instance in being present in cord serum (Koch et al., 1957), and in the serum of patients with hypogammaglobulinaemia (Barandum and Isliker, 1956) - the serological characteristics of the n.i.c. antibody were compared with those of a properdin-like factor present in normal serum. In the present work, the property of normal serum requiring the properdin system was that which is involved in the haemolysis of the red cells of patients with paroxysmal nocturnal haemoglobinuria (PNH).

Before describing the methods used and the results obtained, the current concepts on the nature of the properdin system and on the property of normal serum involved in the haemolysis of PNH red cells, will be briefly described.

The properdin system

a) General characteristics

The concept of the properdin system emerged entirely from the studies on the mechanism of the inactivation of the third component (C'_3) of complement. It had been known since 1900 that yeast cells could inactivate complement (van Dungern, 1900) and since 1914, that this inactivation involved a component of complement which was subsequently designated C'_3 (Coca, 1914). Yeast powder (Whitehead, 1925) and zymosan (Pillemer and Ecker, 1941) were later shown to be effective substitutions for the whole yeast cells. The extension of these researches on the mechanism of inactivation of C'_3 with zymosan led directly to the isolation of properdin. It had already been shown that zymosan did not appreciably inactivate C'_3 in normal human serum at 17°C; Pillemer et al. (1954) showed that, under these conditions, zymosan removed from serum an active factor which formed a stable complex with the zymosan; that this new factor was required for the inactivation of C'_3 by zymosan at 37°C and that the material eluted from the properdin-zymosan complex formed at 17°C, when added to the supernatant of a serum absorbed with zymosan at 17°C, restored the ability of fresh serum to inactivate C'_3 at 37°C.

Pillemer and his collaborators termed this factor "properdin" (from perdere - to destroy) and showed that, in conjunction with complement and Mg^{++} , properdin participated

in the destruction of bacteria (Pillemer et al., 1954; Wardlaw and Pillemer, 1956), in the neutralization of viruses (Wedgwood et al., 1956) and in the lysis of PNH red cells (Hinz et al., 1956).

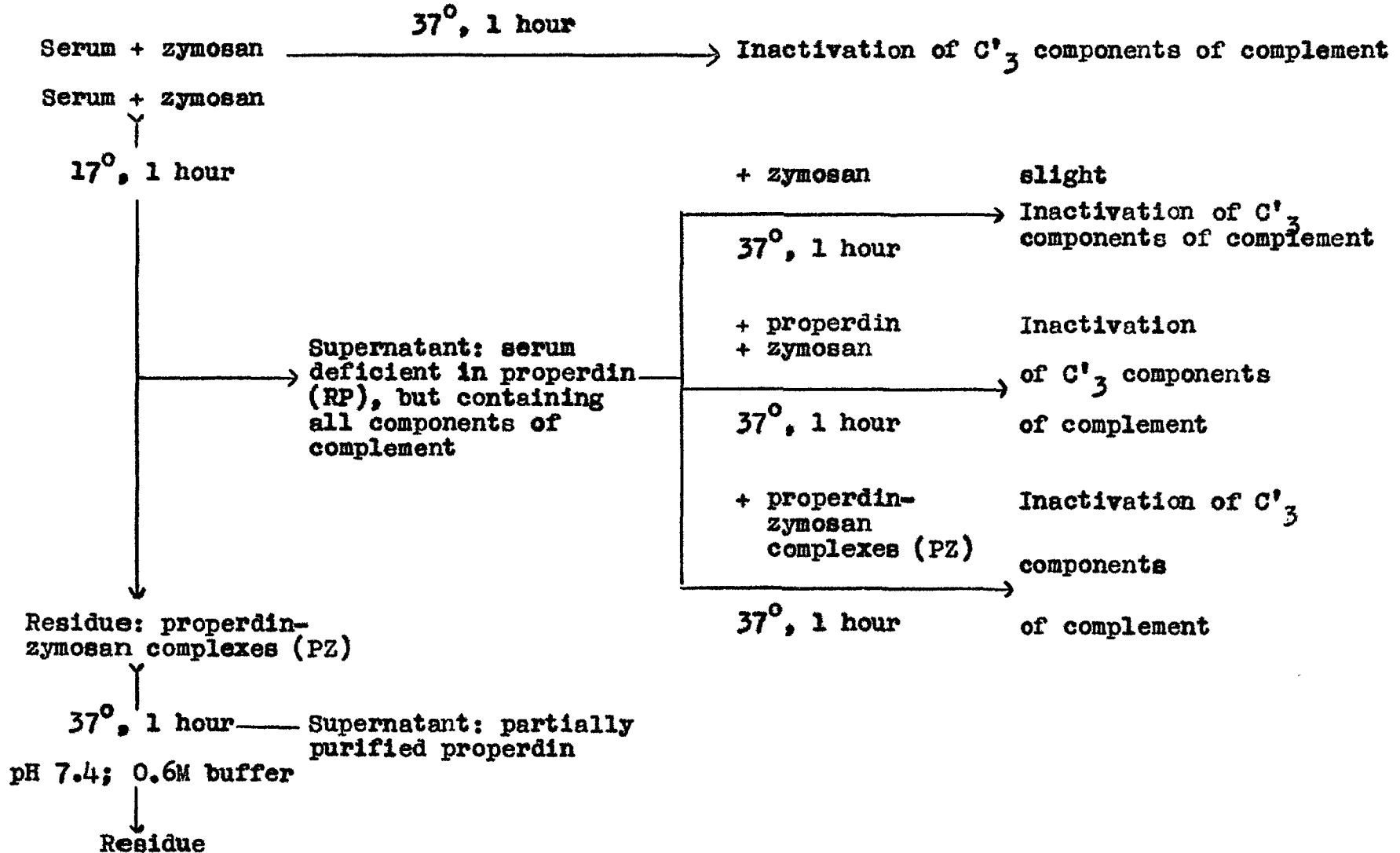
Properdin present/in normal human serum was found to be slowly inactivated by heating at 50° and completely destroyed after 30 minutes at 56° (Pillemer et al., 1954).

According to Pillemer et al. (1954) properdin is stable between pH 4.8 and 8.4, at ionic strength 0.15M; it is not destroyed by treatment with hydrazine and trypsin and it is not removed by adding γ globulin aggregates to fresh serum.

Properdin can be isolated by dissociating properdin-zymosan complexes, formed at 17°, using a buffer pH 7.4, 0.6 M at 37° (Pillemer et al., 1954) (see Table 13). Using free electrophoresis Lepow et al. (1959) showed that two components were present in these preparations; the first component, which formed at least 90 per cent of the total protein, had an electrophoretic mobility similar to fibrinogen; the other 10 per cent migrated as α_2 globulin.

Using potato starch zone electrophoresis, properdin present in normal human serum or in serum fortified with partially purified properdin, was recovered in a fraction at the leading edge of the γ -globulin peak (Lepow et al., 1959). A considerable amount (about 90 per cent) of properdin activity was lost during this procedure, suggesting inactivation or irreversible binding of properdin to potato starch.

TABLE XIII



Preparations of partially purified human properdin were found to be heterogeneous when studied with an analytical ultracentrifuge; Lepow et al. (1959) observed that at least four components were present in these preparations, corresponding to coefficients of sedimentation 6, 9, 12 and 18S. According to Lepow et al. (1959) at least 60 per cent of the total protein present in the properdin preparations was 6S and 9S components, but properdin activity, while distributed in all fractions, was associated primarily with the 18S component.

Recently, Rothstein (1965), using a new method for the partial purification of properdin, has found that properdin, prepared without absorption with zymosan, has a coefficient of sedimentation 10 ± 1 S. The discrepancy between this value and that observed by Lepow et al. (1959) was attributed by Rothstein to the presence of soluble zymosan in the preparations of properdin obtained from zymosan, using the method described by Pillemer et al. (1954).

According to Isliker (1958) properdin activity is inhibited by the treatment of normal serum with cysteine.

b) The problem of the specificity of properdin

According to Pillemer and his collaborators properdin was a new serum component involved in non-specific immunity. The hypothesis that properdin was distinct from antibodies and related to non-specific immunity received support from the observations that properdin was absorbed by zymosan and many

Gram-negative bacteria (Pillemer et al., 1954); that it was found in normal concentration in serum of patients with severe hypogammaglobulinaemia (Barandum and Isliker, 1956) and that it did not react with specific anti- γ G and anti- γ M globulin sera (Hinz et al., 1960).

The non-specificity of properdin was initially challenged by Nelson (1958) who observed that normal serum and preparations of partially purified human properdin contained antibodies which agglutinated zymosan at 0°C in presence of EDTA; he suggested, therefore, that properdin was an antibody to zymosan and that the properdin-zymosan complex (PZ) was a mixture of antigen, antibody and complement in the state EAC₁₄₂. The cross reactivity of properdin with zymosan, various polysaccharides, bacteria and viruses was explained on the assumption that closely related polysaccharides were widely distributed in nature.

In view of Nelson's findings, Pillemer and his collaborators (1959) showed that: a) there was no connection between the titre of zymosan agglutinin and properdin in the preparations of partially purified properdin; b) properdin activity could be destroyed by heating at 56° without affecting the agglutinin activity; and c) the agglutinin could be absorbed from purified properdin with zymosan without seriously affecting properdin activity.

Between 1958 and 1961, several papers appeared dealing with the specificity of human properdin; it was found that the inactivation of phage, which was shown to involve properdin

(Barlow et al., 1958) was not entirely nonspecific (Cowan, 1958) and required an antibody (Toussaint and Mushel, 1959), and that the bactericidal activity, attributed to the properdin system, showed serological specificity (Osawa and Mushel, 1959).

A major paradox appeared to exist. Investigation with serum depleted of properdin and properdin preparations suggested that properdin was a discrete protein functioning in a non-specific manner; the observation that the adsorption of serum with large amount of bacterial cell wall stroma at 0° inhibited bactericidal activity against the homologous, but not the heterologous organisms, suggested that properdin exhibited specific activity.

These conflicting data were made still more complicated by the demonstration, in normal human sera, of a new properdin-like factor which was absorbed by zymosan at 0° and was shown to be required for the inactivation of C₃ by zymosan (Blum and Lepow, 1959; Blum, 1964).

A tentative hypothesis which may, in part, reconcile some of the conflicting points of view has been advanced by Wedgwood (1964). This hypothesis suggests: a) that properdin is a single substance, distinct from antibody, with a variety of biological activities which are only partially specific; b) that the interaction of properdin and the other factors of the properdin system, in the various biological activities

which have been attributed to it, is dependent upon prior interaction with a specific antibody and c) that properdin, under these circumstances, can be conceived as having a relationship to the properdin system comparable with that of a component of complement to the antibody-antigen complement system.

There is insufficient data available to decide whether or not this hypothesis can be accepted; it appears that antibody-like substances are not required for all the activities of the properdin system; thus the possible role of specific antibodies in the inactivation of viruses with normal serum has not yet been directly shown (Wedgwood, 1960 and 1964) and, as described in detail later, Hinz et al. (1960) have not been able to show that an antibody is required for the haemolysis of PNH red cells.

c) Role of the properdin system in the lysis of PNH red cells.

Red cells of patients with paroxysmal nocturnal haemoglobinuria are abnormal in that they lyse readily in apparently normal plasma in vivo and in normal serum in vitro. The identification of the plasma components responsible for the haemolysis has a long and complicated history stretching back to the extensive studies of Ham and Dingle (1939), primarily concerned with the role of complement. Later the problem was investigated intensively by Crosby and Dameshek who concluded that clotting factors, in particular Ac-globulin and thrombin,

were involved (Crosby and Damesheck, 1950; Crosby, 1953). Crosby (1953) postulated that four plasma factors were required, two being heat-stable and two heat-labile; two of these factors were supposed to be inhibitors and two haemolytic; calcium and magnesium were required also.

This complex situation was partially resolved when it was demonstrated that at 17° zymosan absorbed a factor which is involved in the lysis of PNH red cells and that a properdin-like factor, complement and magnesium were necessary in lysing the abnormal red cells (Pillemer et al., 1954; Hinz et al., 1956).

The role of properdin in the haemolysis of PNH red cells was later confirmed by Hinz et al. (1960), who showed that the haemolytic activity of normal human serum was inhibited by adding to it an anti-properdin serum reacting specifically with human properdin and that the re-addition of partially purified properdin to the inhibited serum restored its lytic activity.

Properties of the n.i.c. antibody

The investigations which are described in this section were planned to study the serological relation between the n.i.c. antibody and "classical" γ G, γ A and γ M globulin blood group antibodies and to clarify the relationship between the n.i.c. antibody and the properdin-like factor involved in the lysis of PNH red cells.

1) Effect of heating the n.i.c. antibody at 56° and 63° for various periods of time

Two series of investigations were carried out; the first (a) to study the effect of heating the n.i.c. antibody and two other blood group antibodies selected for being γG and γM globulins; the second (b) designed to investigate the effect of heating at 56° the n.i.c. antibody and the anti-PNH red cell factor present in normal serum.

- (a) Activity of n.i.c., incomplete anti-D (γG) and anti-P₁ (γM) antibodies after heating at 56° and 63°.

One sample of serum (group A₁B) containing the n.i.c. antibody, one serum containing an incomplete anti-D and one containing an anti-P₁ agglutinin were used.

Aliquots of serum, previously diluted with equal volumes of buffered saline, were heated at 56° and 63° for 30 minutes, 1, 2 and 3 hours. The n.i.c. antibody was detected using horse serum as a source of complement and an I.K. serum; the anti-P₁ by agglutination in saline at 12° and the anti-D by the indirect anti-globulin test.

The results are shown in Fig. 13. Heating at 56° for 30 minutes produced a marked fall in the titre of the n.i.c. antibody; and heating for three hours destroyed it completely. By contrast heating at 56° for three hours did not alter the titre of the incomplete anti-D (γG), and the titre of the

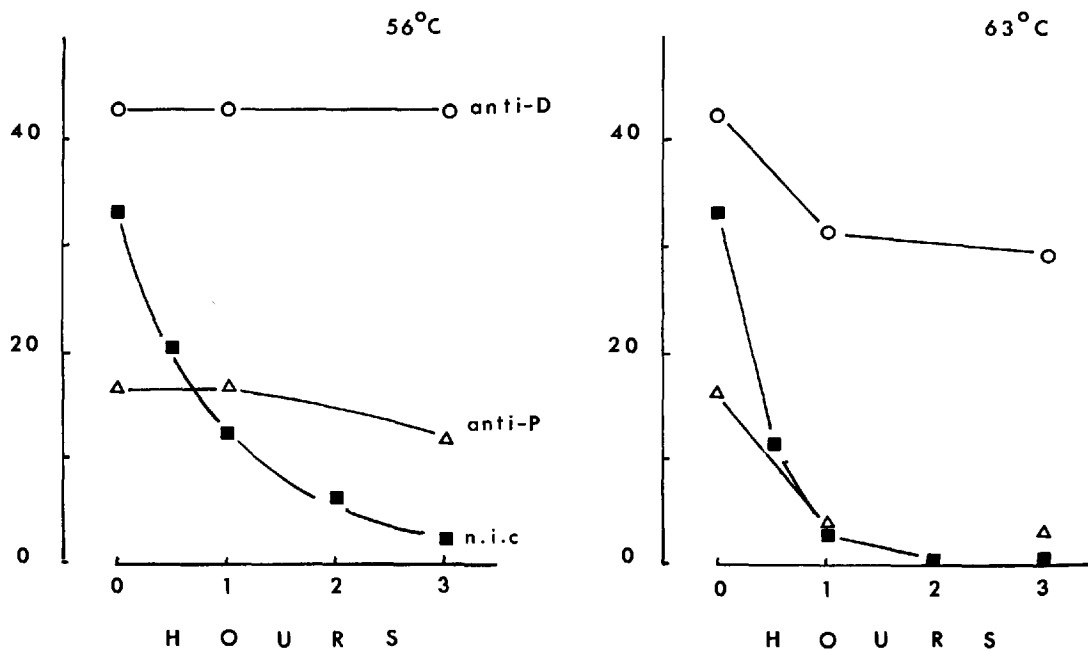


Fig. 13. Effect of heating anti-D, anti-P₁ and n.i.c. antibodies at 56° and 63°; titre are expressed as scores.

anti-P₁ (γ M) agglutinin was only slightly reduced. When the experiment was repeated at 63°, both n.i.c. and anti-P₁ antibodies were destroyed after one hour; after three hours at 63° the titre (expressed as score) of the anti-D antibody fell from 43 to 31 units.

(b) Effect of heat (56°) on the n.i.c. antibody and the anti-PNH red cell factor.

Aliquots of serum from a group A₁B donor, containing both the n.i.c. antibody and the anti-PNH factor were heated at 56° for 2, 5 and 20 minutes.

The treated samples and, as control, the untreated serum were tested for:

1) N.i.c. antibody activity before and after adding human complement; the sensitized red cells were tested using an anti- β ₁ (complement) serum (see page 16).

2) Anti-PNH red cell activity before and after adding guinea pig complement using the acid test (see page 29).

3) Complement activity using sheep red cells presensitized with anti-sheep red cell antibodies (see page 30).

It was observed that serum heated for 2 minutes showed reduced n.i.c. and complement activity; the haemolytic activity against PNH red cells was completely eliminated. The addition of complement (human and guinea pig complement respectively) to the samples heated for 2 minutes restored the n.i.c. antibody

activity whereas PNH red cells were only slightly haemolysed.

The samples heated at 56° for 5 and 20 minutes no longer sensitized human red cells at 0° or haemolysed PNH and sheep cells. Although the n.i.c. activity was restored by adding human complement to the samples, the anti-PNH red cell activity could not be restored by adding guinea pig complement.

ii) Effect of "incubating serum containing the n.i.c. antibody at acid or alkaline pH"

The effect of incubating the n.i.c. antibody at various pHs was compared with the effect of similar treatment on an incomplete anti-D (γ G) antibody, an anti-B agglutinin (mostly γ M) and the anti-PNH red cell factor present in normal serum. Complement activity of normal serum was also tested before and after incubation at various pH.

Successively a further investigation was carried out in which anti-A γ G, γ A and γ M antibodies were used.

a) Aliquots of 2 ml. of serum (group A₁) containing the n.i.c. antibody and the anti-PNH red cell factor were treated with NaOH 0.1M and HCl 0.2M to adjust the pH to 4.6, 5.2, 9.1 and 10.2; a further sample was not treated and its pH was 7.5. The samples were left at 37° for 1 hour and then the pH was adjusted to 6.8. The n.i.c. antibody activity was tested using horse complement and an I.K. serum; the anti-PNH red cell factor was tested using the acid test and

T A B L E X I V

ANTIBODY AND COMPLEMENT ACTIVITIES AFTER INCUBATION AT VARIOUS pHs
FOR 1 HOUR, AT 37°

pH of medium	N.i.c.	Antibody activity ⁽¹⁾			Complement activity ⁽²⁾
		anti-PNH	anti-D	anti-B	
4.6	-	-	84	48	++
5.2	++	-	...	...	+++
7.5	+++	++	78	47	+++
9.2	++	++	...	...	+++
10.2	++	-	80	48	-

- (1) The n.i.c. antibody was tested using horse serum and an I-K serum; the anti-PNH red cells factor using guinea pig complement and the acidified test; the anti-D (γG) antibody was tested by the indirect antiglobulin test and the anti-B antibody by agglutination in saline.
- (2) Complement activity was tested using sheep red cells previously sensitized with anti-sheep antibody (for details see the text).

guinea pig complement.

The other controls used were: the anti-B agglutinin present in the same A₁ serum and an incomplete anti-D (6G) antibody. The anti-B agglutinin was found to be partially inhibited by treatment with 2-me; thus its titre fell from 1:64 to 1:4. The incomplete anti-D antibody was tested using the indirect antiglobulin method; the anti-B by agglutination in saline.

As shown in Table 14 the n.i.c. antibody was completely inactivated by treatment at pH 4.6 while the incomplete anti-D and the anti-B agglutinin were not affected by similar treatment. The anti-PNH red cell factor was destroyed at pH 5.2 (and below) and at pH 10.2. Complement activity, tested using sheep red cells sensitized with anti-sheep red cells antibody, was completely inhibited by treatment at pH 10.1 and partially inhibited at pH 4.6.

b) Since, in the previously described investigation, 6A antibodies were not used as controls, a further experiment was carried out as follows:

a high titre anti-B agglutinin present in the serum of a group O subject previously injected with B substance was fractionated with DEAE-cellulose chromatography using 3 phosphate buffers, the first 0.0175M pH 6.5, the second 0.08M pH 8.1M and the third 0.2M pH 8.1. The titre of the anti-B agglutinin in each fraction and the relative concentration of

γ G, γ A and γ M globulins are shown in Table 15. Group B red cells were sensitized with each fraction and tested with anti- γ G and anti- γ A globulin sera; thus serial, doubling dilutions of each fraction were made in buffered saline; to 4 volumes of each dilution 1 volume of a 20 per cent red cell suspension was added and the mixtures incubated at 37° for 1½ hours; the sensitized red cells were then washed and tested against optimal dilution of anti- γ G and anti- γ A globulin sera; a normal rabbit serum, previously absorbed with human red cells, was used as control. It was observed that the titre of the anti-B antibody present in the 0.0175M fraction was enhanced only by the anti- γ G globulin serum, whereas the antibody present in the 0.08M fraction was enhanced only by the anti- γ A globulin serum. From these findings and the data on the concentration of the immunoglobulins in each fraction it was assumed that the 0.0175M fraction contained anti-B γ G antibody; the 0.08M fraction mainly anti-B γ A and the 0.2M fraction anti-B γ M antibody.

In studying the effect of the treatment at acid pH, the pH of 1 ml of each fraction and 1 ml of whole serum was adjusted at 4.6 (the pH which was found to destroy the n.i.c. antibody) and the samples were then incubated at 37° for 1 hour; as controls, equal amounts of serum and fractions were adjusted to pH 7.4 and incubated at 37° for 1 hour.

T A B L E X V

ANTI-B ANTIBODY AND γG , γA , γM GLOBULINS IN FRACTIONS
OBTAINED WITH DEAE-CELLULOSE CHROMATOGRAPHY⁽¹⁾

	anti-B ⁽²⁾ agglutinin	Concentration ⁽²⁾ of		
		γG	γA	γM
Whole serum	8000	100	100	100
Fraction 0.0175M	128	75	< 1	< 2
Fraction 0.08M	1.024	< 5	50	< 1
Fraction 0.2M	4.096	< 5	10	100

(1) A serum containing an anti-B antibody was fractionated with DEAE-cellulose chromatography using three buffers (see page 128).

(2) The titre of the anti-B antibody is expressed as "end point". the concentration of γG , γA and γM globulin is expressed as percentage of the amount present in whole serum.

After having re-adjusted the pH to 7.4, the titre of the anti-B agglutinin was determined and found to be unchanged in all samples.

iii) Treatment of serum containing the n.i.c. antibody with zymosan

Aliquots of 1 ml of serum containing the n.i.c. antibody and the anti-PNH red cell factor were mixed with 3 mg of zymosan type A (Fleishman Lab. U.S.A.); the mixtures were left for 1 hour at 0°, 17° and 37°; zymosan was maintained in suspension by frequent mixing. The supernatants were recovered by centrifugation at 15,000 rev. per min. at the same temperature used for the absorption stage.

The n.i.c. antibody was tested using horse complement and an I-K serum; the anti-PNH activity was tested using guinea pig complement and the acid test.

It was found that the n.i.c. antibody was absorbed by zymosan at 0° as well as at 17° and 37° whereas the anti-PNH red cell factor was absorbed by zymosan only at 17° or higher temperature.

iv) Treatment with potato starch, dextran and some simple sugars

(a) Potato starch

Aliquots of 2 ml. of serum were mixed with 0.3 g. (wet weight) of potato starch previously washed three times with buffered saline.

Two batches of potato starch were used, one was obtained from B.D.H., U.K.; the other from Griffin and George, U.K.

The mixtures were incubated for 1 hour at 0° or 37°; potato starch was maintained in suspension by frequent mixing; the supernatants were recovered by centrifugation at 15,000 rev./min at the same temperature used for the absorption stage.

The supernatants were found to be completely free of n.i.c. antibody after the absorption with both batches of potato starch.

(b) Dextran and simple sugars

The n.i.c. antibody and the anti-PNH red cell factor were tested after mixing 1 ml. of serum containing the antibody with the following amounts of soluble dextran and simple sugars:

- 60 mg. dextran (Dextraven; Bergers Lab. Ltd., U.K.)
- 100 mg. inulin (from Dahlia, from Difco Lab., U.S.A.)
- 100 mg. D-glucose (B.D.H., U.K.)
- 100 mg. L-fucose (B.D.H., U.K.)

In these experiments two aliquots of 1 ml. of serum containing the n.i.c. antibody and the anti-PNH factor (samples A and B) were mixed with each substance; after incubation at room temperature for 20 minutes, the B series was dialysed at 0° for two hours against two changes of buffered saline; the A series was used undialysed.

The n.i.c. activity was tested using human complement

and an anti- β_1 (complement) serum (see page 16); the anti-PNH red cell factor was tested using the acid test and guinea pig complement.

Complement activity in each sample was tested using pre-sensitized sheep red cells (see page 30).

It was found that the n.i.c. antibody was completely inhibited by treatment with dextran, inulin, glucose and fucose. The anti-PNH factor was inhibited only by dextran. After treatment with dextran the complement activity was slightly reduced; glucose, inulin and fucose were ineffective at the dosage used in the present investigations.

The property of soluble dextran (Sephadex G200) of absorbing the n.i.c. antibody and the anti-PNH red cell factor was studied as follows: aliquots of 5 ml of serum (A_1B donor) containing the n.i.c. antibody and the anti-PNH red cell factor, were mixed with 2 ml of Sephadex G200 previously washed with buffered saline (see page 82); one sample was left at 0° , the other sample at 37° for 1 hour. The supernatants were then recovered by centrifugation and tested. Since it was observed that the complement activity of the supernatant absorbed at 37° was markedly reduced, horse and guinea pig complement were supplied in testing the n.i.c. antibody and the anti-PNH red factor.

The results showed that whilst the n.i.c. antibody was completely absorbed at 0° and only partially absorbed at 37° ,

the anti-PNH red cell factor was slightly absorbed at 0° and completely absorbed at 37°.

(c) Inhibition of the n.i.c. antibody in vivo, using soluble dextran

Fifty ml of a 6 per cent (w/v) solution of dextran in 0.9 per cent sodium chloride solution (Deaxtraven, Bergers Lab. Ltd., U.K.) were injected intravenously into a normal subject. Samples of blood were taken three days before the injection, 10 minutes before and 10 minutes after the injection; further samples were collected one hour, three hours and one, two, five and eight days after the injection. The serum was separated as soon as possible and stored at -50°. The samples were all tested at the same time using the same source of complement and an I-K.

The samples were also tested against sheep erythrocytes previously sensitized with anti-sheep red cell antibodies (see page 30) and the degree of haemolysis scored.

As shown in Fig. 14, within 10 minutes after the injection of the dextran solution, the score of the n.i.c. antibody fell from 30 to 3 units; the pre-injection score was not regained for more than 2 days. The injection of dextran did not alter appreciably the titre of complement.

v) Absorption with bacteria

E. coli C3, E. coli A55, Streptococcus aureus 80,

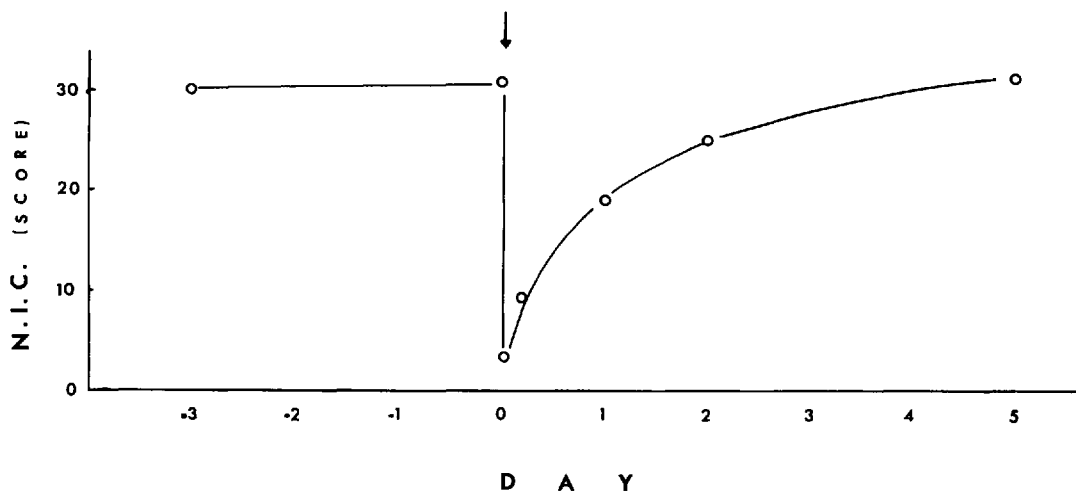


Fig. 14. Titre of n.i.c. antibody before and after an intravenous injection of 50 ml. of a 6% solution of dextran (Dextraven).

Salmonella enteritis variety Danysz and Pneumococcus type XIV were grown on nutrient agar, killed by heating at 60° for one hour and washed three times with distilled water. The bacteria were then weighed (wet weight), re-suspended in buffered saline to give approximately 10 mg. of bacteria in 1 ml. and were stored at 4° if not used immediately.

Aliquots of 1 ml of serum containing the n.i.c. antibody were absorbed with different amounts of bacteria (wet weight) at 0° for 30 minutes. The serum was then centrifuged at 0° at 15,000 rev./min. for 30 minutes and the precipitate discarded.

Three normal adult sera and two sera of patients with hypogammaglobulinaemia (nos. 1 and 3 in Table 12, page) were absorbed with bacteria; the n.i.c. antibody was completely removed after only one absorption with E. coli C3 whilst absorption with the other bacteria was ineffective.

The nutrient agar used in growing E. coli C3 did not inhibit the n.i.c. antibody.

A further investigation was carried out absorbing a normal fresh serum (A₁B donor) containing the n.i.c. antibody and the anti-PNH red cell factor with E. coli C3, Streptococcus aureus 80, Salmonella enteritis variety Danysz and Pneumococcus type XIV. The absorption was carried out as previously described. The results (Table 15) showed that whereas the n.i.c. antibody was absorbed only by E. coli C3, the anti-PNH

T A B L E XVI

RESULTS OF ABSORBING THE N.I.C. ANTIBODY AND THE ANTI-PNH
RED CELL FACTOR WITH BACTERIA⁽¹⁾

Bacteria	N.i.c. antibody	anti-PNH activity
E. coli C3	-	-
Streptococcus aureus 80	+++	-
Salmonella enteritis variety Danysz	+++	-
Pneumococcus type XIV	+++	-
Control: unabsorbed serum	+++	++

(1) For details of the absorption see text; the n.i.c. antibody was tested using horse complement and an I-K serum; the anti-PNH red cell factor was tested using the "acid test".

factor was absorbed by all bacteria used.

After having observed that E. coli C3 absorbed the n.i.c. antibody, the following investigations were carried out in order to study the serological relation between the antibody and the bacterial antigens:

(a) the titres of E. coli C3 agglutinins and the n.i.c. antibody were estimated and compared in 16 normal "adult" sera. The technique used in testing E. coli C3 agglutinin was that described on page 28.

As shown in Fig. 15 there was no relation between the titres of the n.i.c. antibody and the titres of the bacterial agglutinin.

(b) One normal serum and one serum from a patient with hypogammaglobulinaemia (no. 3, Table 12, page 110) were tested for the presence of the n.i.c. antibody and E. coli C3 agglutinin before and after:

- 1) being absorbed at 0° for 30 minutes with different strain of bacteria.
- 2) being absorbed with group O red cells at 0° for 2 hours.
- 3) being treated with H substance present in saliva of a "Secretor".
- 4) being heated at 56° for 30 minutes.

These treatments were carried out as previously described. The results (Table 17) showed that:

T A B L E 17

	Normal serum		Serum of a patient with hypogammaglobu- linaemia	
	n.i.c.	anti- <u>E. coli</u> C3	n.i.c.	anti- <u>E. coli</u> C3
Untreated serum	+++	+++	+++	+
Heated at 56° for 30 minutes	+	+++	...	...
Absorbed at 0° with human red cells	-	+++	-	-
Treated with H substance	-	+++	-	-
Absorbed with <u>E. coli</u> C3	-	-	-	-
Absorbed with <u>E. coli</u> A55	+++	+++	+++	-
Absorbed with <u>Staphilococcus aureus</u> 80	+++	+++	+++	-
Absorbed with <u>Salmonella enteritis</u>	+++	+++	+++	-
Absorbed with <u>Pneumococcus</u> type XIV	+++	+++	+++	-

- 1) only E. coli C3 absorbed both the n.i.c. antibody and E. coli C3 agglutinin; the other bacteria were ineffective in absorbing the bacteria agglutinin; the hypogammaglobulinaemia serum was free of E. coli C3 antibody whilst contained the n.i.c. antibody.
- 2) group O red cells absorbed only the n.i.c. antibody.
- 3) E. coli C3 agglutinin was not inhibited by H substance.
- 4) heating at 56° reduced the titre of the n.i.c. antibody whereas the E.coli C3 agglutinin was unaffected by a similar treatment.

Further data on the interaction between the n.i.c. antibody and E. coli C3 were obtained absorbing with E.coli C3 a human serum containing an anti-H agglutinin and a plant extract (*Ulex Europaeus*) containing an agglutinin with anti-H specificity.

The absorption was carried out mixing 0.5 ml. of serum or plant extract with 6 mg. (wet weight) of E. coli C3; as control, E. coli A55 was used. The mixtures were left at 0° for 30 minutes and the supernatants separated by centrifugation.

The anti-H activity was tested by adding one volume of a 5 per cent suspension of group O red cells to one volume of a series of doubling dilution of the serum or the plant extract. After 90 minutes at v.t. the deposited red cells were examined microscopically and the degree of agglutination scored.

It was found that neither the anti-H antibody present in normal serum or the anti-H agglutinin present in the plant were absorbed by E. coli C3.

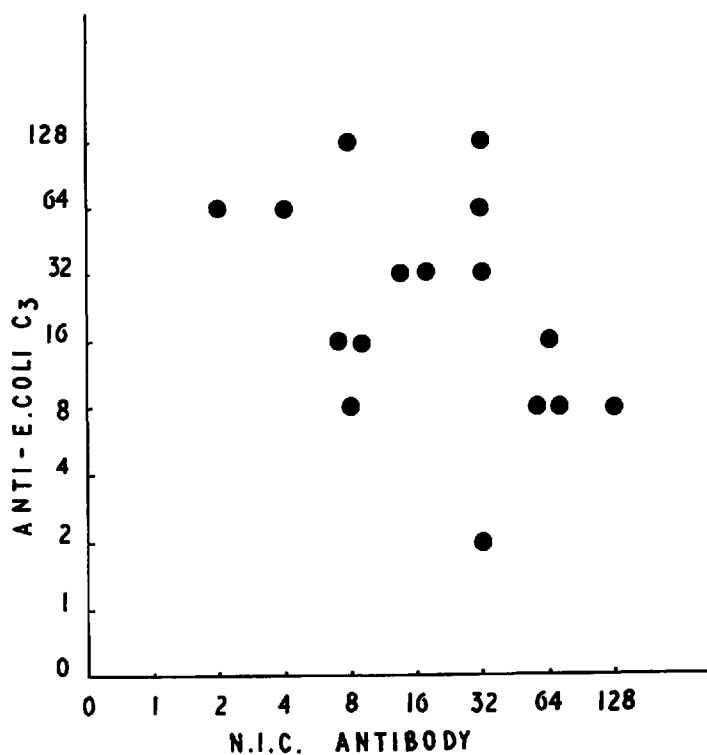


Fig. 15. N.i.c. antibody titre (expressed as ^{end point} score) plotted against Esch, coli C3 bacterial agglutinin titre in 16 normal human sera.

DISCUSSION

Variations in the resistance of antibodies to inactivation by heat have been known for many years (for reference see Landsteiner, 1945, page 141). One of the earlier and better documented differences in thermostability of bacterial antibodies is that relative to the agglutinins of flagellar antigens of Gram-negative bacilli as compared to the agglutinins of somatic antigens; Beyer and Reagh (1904) soon after it was recognized that flagellar and somatic antigens could be distinguished, reported that H agglutinins, produced in rabbit to hog cholera bacillus, withstood a degree of heating that inactivated the anti-O agglutinins. These results have been repeatedly continued (Orcutt, 1924; Jones, 1927) and more recently it has been shown that the relative heat stability of rabbit antibodies to various bacteria is directly related to the type of γ -globulin associated with the antibody; thus Pike and Schulze (1965) have found that rabbit γ_M antibodies against H or O antigens of *Salmonella typhosa*, *Leptospira grippotyphosa*, bovine and sheep red cells are ^{less} more resistant to heating than γ_G antibodies (Pike and Schulze, 1965).

The differences of thermostability of blood group antibodies were demonstrated, for the first time, by Coombs and Race (1945). These authors found that anti-Rh agglutinins were destroyed by heating at 65-75° for 5-10

minutes, whereas incomplete anti-Rh antibodies were resistant.

Boorman and Dodd (1946), while investigating the properties of immune anti-A sera which exhibited zoning, found that sera heated at 75° for 20 minutes failed to agglutinate group A red cells suspended in saline but did sensitize group A red cells to an anti- γ globulin serum.

The present investigations, while confirming that γ M globulin blood group antibodies were more thermolabile than γ G antibodies, showed that the n.i.c. antibody markedly differed in its thermostability from either the anti-D (γ G) or the anti-P (γ M) antibodies used as controls.

The n.i.c. antibody also differed from the anti-PNH red cell factor since the latter was more rapidly destroyed at 56° than the n.i.c. antibody.

The findings of treating serum containing antibodies at various pHs confirmed that the properties of the n.i.c. antibody were different from those of the other antibodies studied. Thus, whilst the titres of the incomplete anti-D (γ G) and anti-B (γ G, γ A and γ M) antibodies were not reduced by treatment at various pHs from 4.6 to 10.2, the n.i.c. antibody activity was inhibited by treatment at pH 4.6. It was also observed that, with regard to its stability at acid or alkaline pHs, the anti-PNH factor behaved differently

either from the n.i.c. antibody or the "classical" blood group antibodies studied, since it was inhibited at pH 4.6 as well as at pH 10.2.

The n.i.c. antibody differed from the anti-PNH red cell factor also in its reaction with zymosan; thus the antibody is absorbed at 0° as well as at 17° and 37°, whereas the anti-PNH red cell factor was absorbed only at 17° or higher temperature.

In the present work other polysaccharides were shown to absorb the n.i.c. antibody; some of these polysaccharides are well known for interfering with the property of normal serum requiring the properdin system; for example, dextran, which in the present work was shown to absorb the n.i.c. in vitro and in vivo, absorbs properdin (Pillemer et al., 1954) and the anti-PNH factor, either in vitro (Hinz et al., 1954) or in vivo (Stratton et al., 195).

However, once again, the differences between the properties of the n.i.c. antibody and those of the properdin-like anti-PNH red cell factor were clear, since whereas the former was best absorbed by insoluble dextran (Sephadex G200) at 0° the latter was best absorbed at 37°.

Finally, further evidence of the difference between the n.i.c. antibody and the anti-PNH red cell factor was obtained

by absorbing normal serum with different strain of bacteria. It was found that only E. coli C3 absorbed the n.i.c. antibody whereas the anti-PNH red cell factor was absorbed by all the bacteria tested.

In conclusion three principal points emerge from the results reported in this section:

(a) It appears that the n.i.c. antibody is definitely more sensitive to variations in temperature and pH than are γ G, γ A and γ M blood group antibodies.

(b) The n.i.c. antibody resembles properdin and the properdin-like factor involved in the haemolysis of PNH red cells since both are thermolabile, absorbed by zymosan, dextran, other polysaccharides and simple sugars.

(c) Nevertheless the n.i.c. antibody clearly differs from properdin and the anti-PNH red cell factor as, for instance, is less sensitive at variation of pH, is absorbed by zymosan and dextran at 0° and it is absorbed only by some bacteria.

At this stage of the investigation it was hoped that the production in rabbits of an antibody reacting against properdin and the n.i.c. antibody could be useful in studying the nature of the n.i.c. antibody and its relation with

properdin. The result of this study will be described in .
the next section.

SECTION IX

Attempt at producing an immune serum
specifically reacting with the n.i.c. antibody

An attempt at producing an antibody specifically reacting with properdin and the n.i.c. antibody was made by injecting into a rabbit the eluate obtained from zymosan-n.i.c. antibody complex. It was hoped that an eluate containing "pure" n.i.c. antibody could be used, but as mentioned in Section IV, all the attempts at eluting the antibody from sensitized red cells, using standard methods, were unsuccessful and the antibody eluted from dextran (Sephadex) - n.i.c. complex was found to be contaminated by several serum proteins. Furthermore, Chaplin and Cassell (1960) observed that rabbits injected with the eluate obtained from red cells sensitized with the n.i.c. antibody did not produce an immune-serum reacting specifically against the antibody.

Consequently, partially purified properdin preparations, eluted from zymosan had to be used. Similar preparations were found to be antigenic when injected into rabbits by Hinz et al. (1960).

The partially purified preparations of human properdin were prepared following the method described by Pillemer et al. (1954). Thus properdin-zymosan (and therefore n.i.c. - zymosan) complex was obtained by suspending zymosan in fresh serum (3 mg/1 ml) at 17° for 1 hour; the complex was then washed three times at 0° with buffered saline. Dissociation was achieved using an alkaline buffer (pH 7.4) 0.6M; the zymosan was separated by centrifugation and the supernatant

dialysed against phosphate buffer 0.02M, pH 5.8 for 24 hours; the precipitate was dissolved in buffered saline.

The primary course of immunisation consisted of three injections of properdin administered intramuscularly into an adult rabbit at intervals of one week. Each properdin preparation corresponded to the eluate obtained from 5 ml. of fresh serum absorbed with zymosan at 17° and it was mixed with an equal volume of Freund's adjuvant and 200,000 u.t. of penicillin. Three weeks after the last injection three doses of properdin mixed only with penicillin were administered intramuscularly at intervals of two days.

The rabbit was bled from a marginal ear vein before treatment, and one week after the last injection. The sera were heated at 56° for 30 minutes and absorbed three times with human red cells, previously washed with buffered saline.

The immune serum was tested by immunoelectrophoresis against normal serum; ~~as shown in Fig.~~ at least 4 lines of precipitation were observed; one line was biphasic and was tentatively identified as $\beta_{10}-\beta_{1A}$. To inhibit all the antibodies but those reacting against the n.i.c. antibody and properdin, one sample of the immune serum was absorbed with fresh serum free of the n.i.c. antibody and properdin. Serum lacking both factors was obtained by absorbing fresh human serum with zymosan (1 ml. serum/3 mg. zymosan) at 17° for 1 hour. Under these conditions, treatment with zymosan has been shown to remove completely the n.i.c. antibody

(see page 131) and properdin (Pillemer et al., 1954). In this paper serum free of n.i.c. antibody will be referred to as n.i.c.-free-serum. In absorbing the immune serum with n.i.c.-free-serum equal volumes of the reactants were mixed; the mixture was left at 37° for 1 hour and then at 0° overnight; the supernatant was recovered by centrifugation at 17,000 rev. per min. for 30 minutes.

As a control, one volume of the immune serum was absorbed with fresh normal serum.

When the immune serum absorbed with the n.i.c.-free-serum was tested by immunoelectrophoresis against normal serum only one line of precipitation was observed; the same line was noticed when the absorbed serum was tested against a human properdin preparation; the antibodies present in the immune serum were completely inhibited by treating the immune serum with whole normal serum (Fig. 16).

Successively the immune serum was tested for the presence of antibodies against α G, γ A and γ M globulins, using serological tests. The immune serum was also tested for the presence of antibodies against β_1 (complement) globulin, the n.i.c. antibody and the anti-PNH red cell factor present in normal serum. The immune serum was tested untreated, after being inhibited with n.i.c.-free-serum or after being inhibited with whole serum. Titration was carried out by making serial, doubling dilutions of the serum in buffered saline.

The tests were performed as follows:

a) Anti- γ G globulin test, Rh-positive red cells were sensitized at 37° for 1½ hours with an incomplete anti-D antibody which was shown to be a γ G globulin (see Adinolfi, Polley, Hunter and Hollison, 1962). After being washed with buffered saline, the sensitized red cells were tested with the immune serum on an opal tile.

b) Anti- γ A globulin test. Rh-positive red cells were sensitized, as previously described, using a serum containing an incomplete anti-D which had been shown to be partly γ A-globulin; thus this antibody sensitized Rh-positive red cells to an immune anti- γ A globulin serum and was partially eluted in the 0.08M fraction with DEAE-cellulose chromatography.

c) Anti- γ M globulin test. Le(a+b-) red cells sensitized with an EDTA-treated serum containing an anti-Le^a antibody which was shown to be a γ M globulin (see Adinolfi et al., 1962). The red cells were incubated at 37° for 1½ hours, washed with buffered saline and finally tested.

d) Anti- β_1 globulin (complement) test. The procedure described by Polley and Hollison (1961) was used (see page 23); thus Le(a+b-) red cells were incubated at 37° with a complement-binding antibody, anti-Le^a, in the presence of EDTA; the sensitized red cells were then washed with buffered saline and incubated again with fresh serum at 37° for 20 minutes; after being washed three times with

buffered saline, the sensitized red cells were tested.

e) Anti-n.i.c. antibody test. Group O red cells were sensitized at 0° with fresh human serum; the sensitized cells were then tested using the untreated immune serum, the immune serum absorbed with n.i.c.-free serum and the immune serum absorbed with whole serum.

The effect of the immune serum on the n.i.c. antibody was also investigated by mixing 0.5 ml. of fresh serum containing the antibody with 0.2 ml. of the immune serum which had been partially inhibited with a n.i.c.-free serum. After 1 hour at 37° one volume of a 50 per cent suspension of group O red cells and two volumes of complement (horse serum) were added; the mixture was incubated at 0° for 2 hours, then the red cells were washed with warm saline and tested with an I-K serum. As a control 0.2 ml. of buffered saline was used instead of the immune serum.

f) Anti-PNH factor test. 0.5 ml. of a compatible fresh serum and 0.5 ml. of the same serum acidified to pH 6.8 were mixed with one drop of a 50 per cent suspension of PNH red cells; the mixture was incubated at 37° for 20 minutes, centrifuged and the degree of haemolysis scored. The residual red cells were washed with buffered saline and tested on an opal tile with the immune serum previously inhibited with a n.i.c.-free serum.

The effect of the anti-human properdin serum on the haemolysis of PNH cells was also investigated following a

method described by Hinz et al. (1960): (a) 0.5 ml. of fresh serum, acidified to pH 6.8 was mixed with 0.2 ml. of the immune serum previously inhibited with a n.i.c.-free serum; (b) the mixture was left at 37° for 1 hour, then (c) one drop of a 50 per cent suspension of PNH red cells were added; (d) after 30 minutes at 37° the degree of haemolysis was scored. As a control, 0.5 ml. of fresh serum was mixed with saline instead of the immune serum.

Two further controls were carried out to discover whether the immune serum, previously inhibited with a n.i.c.-free serum was still reacting with complement components; thus (1) the previously described experiment was repeated, but guinea-pig complement was added at the stage "c"; (2) 0.5 ml. of serum containing anti-A haemolysin was mixed with 0.2 ml. of the partially inhibited immune serum; after 1 hour at 37°, a drop of a 20 per cent suspension of group A₁ red cells was added; the mixture was then incubated at 37° for 1 hour and the degree of haemolysis scored.

It was observed that the immune serum did not react with anti-D red cells sensitized with anti-D (γG and γA) or with anti-Le^a (γM) antibodies. Red cells sensitized with the anti-Le^a antibody and complement were agglutinated by the immune serum; the reaction was markedly and equally reduced when the immune serum was inhibited with fresh serum or a n.i.c.-free serum.

The red cells sensitized with the n.i.c. antibody were

T A B L E XVIII

REACTIONS OF ANTI-HUMAN PROPERDIN SERUM AGAINST RED CELLS SENSITIZED WITH VARIOUS ANTIBODIES AND PNH RED CELLS SENSITIZED WITH FRESH SERUM

Treatment of anti-human properdin serum	Titre of anti-human properdin serum against:				PNH red cells sensitized with fresh serum *
	anti- D(6G)	anti- Le ^a (6M)	anti-Le ^a + complement	n.i.c. + complement	
Untreated	0	0	64	16	0
Absorbed with human fresh serum	...	...	8	4	...
Absorbed with an RP-(17 ⁰) serum	...	...	8	4	0

* PNH red cells were sensitized with fresh serum untreated and with fresh serum acidified to pH 6.8 (see page).

T A B L E X I X

EFFECT OF THE ANTI-HUMAN-PROPERDIN SERUM ON (a) THE NORMAL INCOMPLETE COLD ANTIBODY,
(b) THE ANTI-PNH RED CELLS FACTOR, AND (c) COMPLEMENT COMPONENTS

(a) Serum containing the n.i.c. antibody

Serum	Anti-human properdin serum (*)	Saline	Horse complement	Group O ⁽⁺⁾ red cells	Test: Reaction with I-K serum
1) 0.5 ml.	0.2 ml.	...	2 drops	1 drop	---
ii) 0.5 ml.	...	0.2 ml.	2 drops	1 drop	+++

(b) Serum containing the anti-PNH red cells factor

Serum acidified to pH 6.8	Anti-human properdin serum (*)	Saline	Guinea pig complement	PNH red ⁽⁺⁾ cells	Test: haemolysis
1) 0.5 ml.	0.2 ml.	...	...	1 drop	---
ii) 0.5 ml.	...	0.2 ml.	...	1 drop	++
iii) 0.5 ml.	0.2 ml.	...	2 drops	1 drop	---
iv) 0.5 ml.	...	0.2 ml.	2 drops	1 drop	+++

(c) Fresh serum containing anti-A haemolysin

Serum	Anti-human properdin serum (*)	Saline	Group A ⁽⁺⁾ red cells	Test: haemolysis
1) 0.5 ml.	0.2 ml.	...	1 drop	++++
ii) 0.5 ml.	...	0.2 ml.	1 drop	++++

(*) The anti-human properdin serum was absorbed with an RP-(17) serum.

(+) A 50 per cent suspension of red cells was used in testing the n.i.c. antibodies and the anti-PNH factor; a 20 per cent suspension was used in testing the anti-A haemolysin.

agglutinated by the immune serum; the reaction was equally reduced when the immune serum was absorbed either with normal serum or a n.i.c.-free serum. PNH red cells "sensitized" with fresh serum or fresh serum acidified to pH 6.8 were not agglutinated by the immune serum previously inhibited with human serum free of properdin (n.i.c.-free serum).

The effect of the anti-human properdin serum on sera containing the n.i.c. antibody, the anti-PNH factor and the anti-A haemolysin are shown in Table 19. Treatment of sera containing the antibodies with the anti-human properdin serum, previously inhibited with a n.i.c.-free serum, resulted in the inhibition of the n.i.c. activity as well as in the inhibition of anti-PNH red cells activity. This inhibition was not due to an interference of the immune serum with complement components since the haemolytic activity of serum containing anti-A haemolysin was not affected.

DISCUSSION

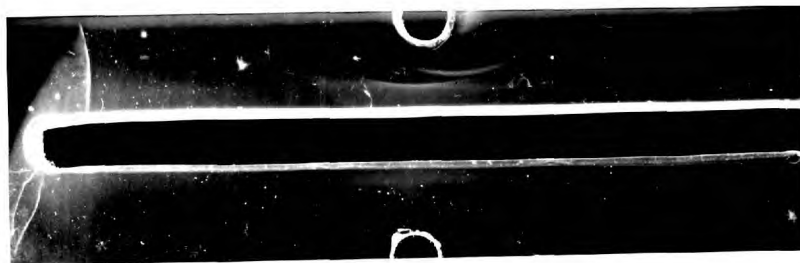
In view of the similarities between the n.i.c. antibody and properdin an attempt at producing an immune serum reacting against either the n.i.c. antibody or properdin was made by injecting a rabbit with human properdin preparation. Previous investigations have demonstrated that rabbits injected with human properdin produce antibodies to several components of human serum and that one of these antibodies specifically reacts with properdin (Hinz et al., 1960).

In the present work multiple antibodies were produced by a rabbit injected with human properdin preparation obtained from properdin zymosan complex. Before testing the immune serum for its activity against the n.i.c. antibody and properdin, all the antibodies except those reacting with these two factors were removed by absorbing the immune serum with fresh serum free of the n.i.c. antibody and properdin (n.i.c.-free serum). Following the absorption, only one line of precipitation was observed when the immune serum was tested with immunoelectrophoresis against normal serum or a properdin preparation, suggesting that an antibody to properdin had been produced.

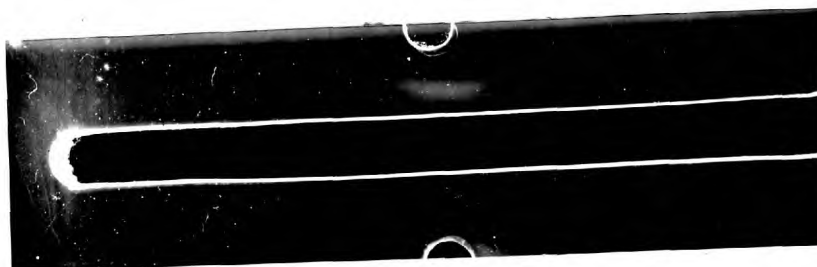
Since it has been demonstrated that the haemolysis of PNH red cells is due to a properdin-like factor present in normal serum (Pillemer et al., 1954; Hinz et al., 1956) the immune serum was tested to see whether it agglutinated "sensitized" PNH red cells or interfered with the reaction between the anti-PNH factor and PNH red cells.

It was found that, whereas the immune serum did not agglutinate "sensitized" red cells, it did inhibit the haemolytic activity of fresh serum for PNH red cells. These findings, which were similar to previous observations that anti-human properdin serum does not react with "sensitized" PNH red cells but inhibits their haemolysis when added to fresh serum (Hinz et al., 1960), confirmed that the immune serum contained an antibody which interfered with the property of normal serum

Properdin preparation



Normal serum



Properdin preparation

Fig. 16. Immunoelectrophoresis of the anti-human properdin serum (M65) against normal serum and a human properdin preparation. The immune serum was tested before and after absorption with an n.i.c.-free serum.

requiring the properdin system.

The immune serum was found to agglutinate red cells sensitized with the n.i.c. antibody but the reaction was presumably due to anti- β_1 (complement) antibodies and immunoconglutinin present in the immune serum; thus the reaction was inhibited to the same extent after treating the immune serum either with human serum free of n.i.c. antibody or with whole fresh serum. If the immune serum had contained an antibody specifically reacting with the n.i.c. antibody, a stronger reaction with the immune serum previously inhibited with n.i.c.-free serum would have been expected.

However, since the immunoconglutinin could mask the presence of an antibody reacting weakly but specifically against the n.i.c. antibody, fresh serum containing the n.i.c. antibody was treated with the immune serum which had been previously absorbed with n.i.c.-free serum. It was found that, under these conditions, small amount of the immune serum inhibited the n.i.c. activity. This inhibition, obtained with an immune serum free of anti- γG , anti- γA and anti- γM globulin confirmed that the n.i.c. antibody has characteristics which are different from those of blood group antibodies.

Section X

Conclusion

In man, the presence of antibodies in cord serum has come to be equated with their association with γ globulin of maternal origin; this concept, applied to the n.i.c. antibody, would associate this antibody with γ G globulin.

The finding that the titre of n.i.c. antibody in newborn serum is independent of the titre of the antibody in its mother's serum and that there is no fall in the titre of normal incomplete cold antibody during the first few months of life suggests conversely that this antibody is not a γ G globulin. More direct evidence is provided by the observation that the antibody does not sensitize red cells to an anti- γ G globulin serum, is not eluted from DEAE-cellulose in the fraction containing the bulk of the γ G globulin and, unlike γ G globulin, is inactivated by treatment with 2-mercaptoethanol.

Evidence that n.i.c. antibody is not associated with γ A or γ M globulin is not quite so conclusive, although the fact that the antibody is present in the serum of almost all patients with severe hypogammaglobulinaemia is very suggestive.

The failure to dissociate the n.i.c. antibody from antigen-antibody complexes using standard methods, prevented its isolation and identification as a specific serum protein. However the investigations undertaken to pursue this object were

useful, since they led to the observation that the antibody has properties which are different from those of classical blood group antibodies. It was noticed, for instance, that exposure to pH 4.6 or heating to 56° for one hour, which had no effect upon G, A or M blood group antibodies, inactivated the n.i.c. antibody. In these respects, as in its reaction with zymosan and other polysaccharides, the n.i.c. antibody resembles properdin and various 'factors' which have been associated with the properdin system.

The relation between the n.i.c. antibody, properdin and the properdin-like anti-PNH red cell factor has been already analysed in Section V; here only the similarities between the n.i.c. antibody, the heat labile factor responsible for the immune-adherence phenomenon (Turk, 1959) and the factor described by Evans and collaborators (1959, 1963) which haemolyses red cells of newborn rabbits with enteritis, will be discussed.

In 1959, Turk, using the immune-adherence of bacteria and polysaccharides to red cells, distinguished two types of activity of normal serum: a basic non-specific activity against all bacteria and polysaccharides tested, and a secondary, specific activity against individual antigens usually superimposed on the first to a varying degree. The non-specific activity was shown to be heat labile (being destroyed at 56°

for 20 minutes) and to be absorbed by a wide range of bacteria and a number of polysaccharides of different stereochemical structure. Furthermore, whereas the specific factor, heat stable, was found to be absent from the serum of newborn calves before suckling and to develop with age, the non-specific, properdin-like factor was observed in the serum of all newborn calves studied.

The serum factor concerned with the haemolysis of the red cells of weanling rabbits with enteritis has been described by Evans and collaborators in two reports (1959, 1963). Rabbits of about 5-8 weeks of age often develop a disease characterized by the association of enteritis and polyagglutinable red cells. ~~The red cells of these rabbits are susceptible to agglutinable red cells.~~ The red cells of these rabbits are susceptible to agglutination and haemolysis in normal rabbit serum; the agglutinating factor is heat stable, whereas the haemolytic factor is heat labile being destroyed at 56° for 30 minutes. The haemolytic factor, distinct from any complement components, is absorbed by zymosan, but it differs from properdin in that it is absorbed by zymosan at 0° as well as at 17° .

It is of further interest that Evans et al. (1963) have shown that the eluate derived from zymosan complex formed at 15° (complex obtained by mixing fresh rabbit serum with zymosan) is free of the haemolytic activity, although it appears to have properdin activity.

The similarities between these two "factors" and the anti-PNH red cell factor and the n.i.c. antibody are very remarkable. The question arises, therefore, whether the n.i.c. antibody may be referred to as an antibody.

It is worth mentioning that the attachment of polysaccharides and proteins other than Ig antibodies onto the red cell membrane has often been demonstrated; it has been shown, for instance, that human red cells may be specifically "coated" with bacteria polysaccharides (for reference see: Neter, 1956) or blood group polysaccharides (for references see: Sneath and Sneath, 1959). Furthermore, it has been observed that human red cells react specifically with plant extracts, although the agglutinating factor is not γ -globulin (for references see: Boyd, 1956).

Recently, using a human autologous system, the attachment of a complement component, namely C'_4 (β_{1E}) to the erythrocyte membrane has been demonstrated employing polyethylene glycol (Dalmasso, and Müller-Eberhard, 1964). Müller-Eberhard and Lepow (1965) have also shown that it is possible to induce the formation of erythrocyte complement complexes (EC'_4) in mixtures consisting of non-sensitized red cells (E), C'_4 (β_{1E}) and C'_1 esterase. Under these conditions the efficiency of C'_4 uptake by non-sensitized red cells is less than 1 per cent of that observed with EAC'_1 cells, but it increases considerably at higher concentrations of E.

It is of further interest that Yachnin (1964) has proposed

that the activation of complement may be effected in the fluid phase by reagents other than antigen-antibody complexes, probably mediated by an initial reaction between C'1_a and C'4.

The events underlying the reaction between the n.i.c. antibody, the red cell membrane and complement are still not fully understood and the available data do not permit one to distinguish between the possibility that the reaction of n.i.c. antibody with human red cells is similar to a "typical" antigen-antibody reaction or the possibility that the antibody is adsorbed onto the red cells with a reaction similar to that of the red cells which are coated with bacterial polysaccharides.

The most important differences, with regard to serological properties, between the n.i.c. antibody and classical blood group and bacterial antibodies, are that the n.i.c. antibody cannot be absorbed onto red cell membranes in the absence of complement and cannot be eluted from sensitized red cells using standard methods.

Nevertheless, on the evidence available it is not possible to rule out that the n.i.c. antibody reacts with red cells with a mechanism similar to that of classical antibodies and it is still appropriate to refer to it as an antibody.

ADDENDUM

The work described in this thesis was carried out at The Medical Research Council's Experimental Haematology Research Unit. The work was planned by myself following a suggestion made by Prof. P.L. Mollison, to whom I am also grateful for much advice and help through the course of the investigation and while writing the thesis.

The greater part of the laboratory work was carried out personally. For about six months I had the collaboration of Miss C. Daniels who carried out, under my control, the experiments on the fractionation of the n.i.c. antibody with zone electrophoresis and DEAE-cellulose chromatography. Miss M. Lindsay helped me in performing the investigation on the effect of the treatment of the n.i.c. antibody at acid or alkaline pHs. I would also like to thank Miss M. Lindsay for reading the thesis during its preparation and for her helpful suggestions.

As mentioned in Section VII, the samples of serum from patients with hypogammaglobulinaemia were kindly provided by Dr. J.F. Soothill, whom I would also like to thank for permission to quote estimates of γ G, γ A and γ M globulin concentration.

Thanks are due to Drs. P. Benson and H. Schrand for their

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I am particularly indebted to Dr. M.J. Polley for kindly supplying samples of immuno-conglutinin, anti- γ -globulin and anti- β_1 (complement) globulin sera.

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Eighteen papers published by me since 1958 are enclosed; the work described in the first 15 papers was carried out in collaboration with Dr. M. Siniscalco while I was an Assistant in the Genetics Department of the University of Naples (Italy) and with Prof. P.L. Mollison while I was a member of the Experimental Haematology Research Unit, London.

The work described in papers Nos. 1, 2, 3 and 4 was planned and carried out by myself. The work described in papers Nos. 5, 6, 7, 8 and 9 was planned by Dr. M. Siniscalco; I carried out all the serological and some of the haematological tests. The patient described in paper 10 was first observed by Dr. P. Barkhan and I collaborated with him in the investigations carried out on all the family.

The work described in papers Nos. 11, 12, 13, 14 and 15 was planned by Prof. P.L. Mollison. The method of DEAE-cellulose fractionation was worked out by myself and I also performed many serological tests. Dr. M.J. Polley carried out all the estimations of immunoglobulins; some of the studies with 2-mercapto-ethanol were carried out by D. Hunter.

In the remaining papers (Nos. 16, 17 and 18) I am responsible for the planning and for all the serological tests.

Larval and Adult Hæmoglobins of the Cyclostome *Petromyzon planeri*

SYSTEMATIC investigations of the electrophoretic behaviour of hæmoglobins of various vertebrates have so far been carried out mainly on the higher forms. We have started to investigate the electrophoretic behaviour of hæmoglobin of the cyclostome *Petromyzon planeri* in the adult or in the ammocoetes stage.

Paper electrophoresis did not permit the detection of any consistent differences between adult and larval blood, which by this technique showed the same electrophoretic pattern consisting of a single hæmoglobin band migrating toward the anode. On the other hand, starch-gel electrophoresis provided evidence for the presence of two distinct hæmoglobin bands with different mobility at each stage.

Adult and ammocoetes of *Petromyzon planeri* were collected from the River Sarno near Naples. Blood from 32 adults and 44 ammocoetes has been examined. Blood was collected, by decapitation, in Alsever solution. Erythrocytes were washed repeatedly, and then hæmolysed with distilled water.

Electrophoresis was performed with starch gel using the discontinuous buffer system described by Poulik¹. Fig. 1 shows the positions of all the hæmoglobin components in the adult and ammocoetes stages. Adult blood has two hæmoglobin bands (I and II). Hæmoglobin I migrates more rapidly and is present in much smaller amounts than II. Larval blood also has two distinct hæmoglobin bands (I' and II'), which are apparently present in the same concentrations. They migrate more rapidly than the adult ones.

Apart from the difference in the electrophoretic mobility between adults and larvæ, no other differences were detected among animals of the same stage and in particular no animals with only a single band were found in Nature.



Fig. 1

It is conceivable, therefore, that the double-band pattern obtained only by gel electrophoresis might be the result of some kind of degradation of the hæmoglobin molecule due to the supporting medium itself.

Further researches are in progress to clarify this aspect of the problem, bearing in mind what is known for mammalian hæmoglobins²⁻⁴.

Nevertheless we think that the results given above allow the conclusion that in these lower vertebrates the electrophoretic behaviour of hæmoglobin in the larval stage is different from that of the adult stage. Although this may be obviously interpreted as to be due to the presence of some foetal-like hæmoglobin in the larvæ, it must be noted that unlike the mammalian foetal hæmoglobin, here the larval hæmoglobin migrates faster than the adult one.

M. ADINOLFI
G. CHIEFFI

Institute of Genetics,
University of Naples.
June 21.

¹ Poulik, M., *Nature*, **180**, 1477 (1957).

² Chernoff, A. S., and Singer, K., *Blood*, **6**, 413 (1951).

³ Beaven, G., Hoch, H., and Holiday, E. R., *Biochem. J.*, **49**, 374 (1951).

⁴ Huisman, T. H. J., Visser, H. K. A., and van der Helm, H. J., *Nature*, **180**, 758 (1957).

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Hæmoglobin Pattern of the Cyclostome *Petromyzon planeri* during the course of Development

Two hæmoglobin components have been demonstrated by starch gel electrophoresis in both larval and adult forms of *Petromyzon planeri*¹. Although the components are most clearly separated by electrophoresis in starch gel, separation has also been obtained by starch block electrophoresis and on acetate cellulose paper. Furthermore, after elution of the individual bands it was found that each one then runs again as a single component in the starch gel system (Fig. 1). It seems therefore quite likely that the separation in the different hæmoglobin bands is due to differences in the net charge more than to differences in molecular size. Concerning the electrophoretic mobility of these hæmoglobins as compared to the human ones it must be noted that their isoelectric point, unlike that of other species of lampreys², seems to be in the region of neutrality.

All the larvæ and adults collected between April and May 1958 from the River Sarno (Naples) show only two such hæmoglobin components, but in the larval forms each of them has an appreciably greater mobility than the corresponding one in the adult forms. Furthermore the two components are present in about the same amount in the larvæ, while in the adults the fast band is always very faint as compared to the slow one. It has now been found that if the material is collected between January and March, several individuals are observed which possess three, or all the four components (Fig. 2). This is a period when metamorphosis is in progress and the specimens with three or four hæmoglobin components are much commoner among individuals showing obvious signs of metamorphosis.

In late April and May all individuals found in Nature are either of the adult or of the larval type, and no animals with more than two hæmoglobin components are found. Later on the sexually mature individuals die after spawning. Only ammocetes survive and they show the larval hæmoglobin pattern until the beginning of the following winter. It is conceivable that the changes in the pattern may be of some physiological importance, possibly connected with metamorphosis and sexual maturation.

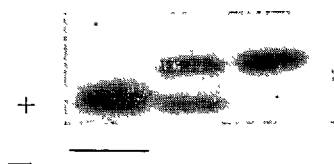


Fig. 1. Re-migration of the single hæmoglobin bands from larvæ of *Petromyzon planeri*. The slow and the fast components (on the sides) were split by electrophoresis on starch gel and then eluted and run again together with another sample of the original hæmolysate (in the middle). Starch gel electrophoresis at pH 8.8 (tris-borate system of Poulik, 2.5 V./cm. for 14 hr.)

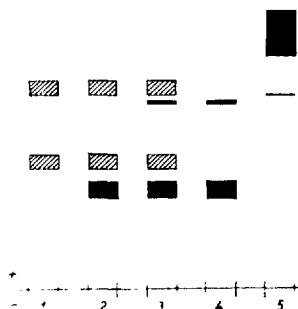


Fig. 2. Electrophoretic patterns in the lamprey *Petromyzon planeri* hæmoglobin. Size of the rectangles in the diagram is proportional to the intensity of the hæmoglobin bands after staining with benzidine: 1, typical larval pattern between May and December; 2, 3, patterns observed between January and March mostly in individuals showing obvious signs of metamorphosis; 4, typical adult pattern from May onwards; 5, human hæmoglobin pattern in the same experimental conditions. (Conditions of the experiments as described under Fig. 1.)

It should, however, be noted that apart from the gross morphological and physiological changes, which take place at metamorphosis, there is also a corresponding change in the ecology of the organisms: the adults swim freely in the river, the larvæ remain in the mud of the bottom. Thus at the present stage the significance of the difference in the character of larval and adult hæmoglobins remains still uncertain.

Finally it must be added that there was no obvious evidence of genetic polymorphism in the hæmoglobin pattern of either the 119 ammocætes or the 102 adults from the River Sarno so far investigated. The same adult type hæmoglobin pattern was observed in adult individuals of *Petromyzon fluviatilis*, a species closely related to *Petromyzon planeri*,

which lives in the sea during the early larval stage and comes up to fresh water to reach maturity.

Further investigations are now in progress to characterize the structure and the properties of each of the hæmoglobin components described above, bearing in mind what is already known of the hæmoglobins in lampreys^{2,3} and in other Cyclostomata⁴.

M. ADINOLFI

G. CHIEFFI

M. SINISCALCO

Institute of Genetics,
University of Naples.
July 6.

¹ Adinolfi, M., and Chieffi, G., *Nature*, **182**, 730 (1958).

² Roche, J., and Fontaine, M., *Ann. Inst. Oceanogr.*, **20**, 77 (1940)

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⁴ Manwell, C., *Biol. Bull.*, **115**, 227 (1958).

CHIEFFI G., SINISCALCO M. e ADINOLFI M. — *Modificazioni elettroforetiche dell'emoglobina durante lo sviluppo degli Ittiopsidi*. *Acta Embryol. Morphol. Exper.*, 3: 338-339, 1960.

Con il progredire delle ricerche si vanno scoprendo nel corso della ontogenesi accanto a modificazioni morfologiche, profonde variazioni di ordine biochimico non solo quantitative ma anche qualitative.

Gli esempi più conosciuti riguardano due proteine: il pigmento retinico e l'emoglobina.

Le prime ricerche sulle modificazioni dell'emoglobina durante lo sviluppo dei Mammiferi risalgono a molti anni fa (BISCHOFF, 1926; KRUGER, 1927; BARCROFT, 1936). Solo recentemente queste ricerche sono state estese alle altre classi di Vertebrati. Sono state infatti individuati due tipi di Hb: la Hb fetale o larvale e quella adulta.

Per alcuni Vertebrati inferiori esistevano chiare indicazioni di variazioni delle caratteristiche fisiologiche dell'Hb durante lo sviluppo. Al fine di giungere ad una chiara separazione dell'Hb adulta da quella larvale negli Ittiopsidi ci siamo serviti di tecniche elettroforetiche sia su carta sia in gel o salda d'amido secondo la tecnica di POULIK (1957).

Abbiamo preso in considerazione inizialmente i Ciclostomi, finora ignorati da questo punto di vista. Le ricerche sono state eseguite su *Lampetra planeri* (Fiume Sarno). Le modificazioni elettroforetiche dell'Hb sono state seguite prima, durante e dopo la metamorfosi. Sia il pattern larvale sia quello di adulti consiste di due componenti emoglobiniche con velocità di migrazione diversa (quelle larvali più veloci di quelle adulte). Durante la metamorfosi alcuni individui presentavano un pattern con tutte e quattro le emoglobine (due larvali e due adulte).

Anche in *Anguilla* (Fiume Garigliano) abbiamo osservato un pattern emoglobinico per le cieche diverso da quello degli adulti. L'Hb delle cieche presenta una sola banda, mentre quella degli adulti (anguille gialle) ne presenta due di cui una migrante verso l'anodo e l'altra verso il polo opposto.

Per gli Anfibi già si conoscevano modificazioni delle caratteristiche fisiologiche dell'Hb durante la metamorfosi (MCCUTCHEON, 1936; RIGGS, 1951): diversa affinità per l'O₂ e diverso effetto Bohr per l'Hb larvale e quella adulta di *Rana catesbiana*.

Noi siamo riusciti a separare in *Rana esculenta* (Sarno) mediante elet-

troforesi in gel d'amido due bande emoglobiniche larvali con velocità di migrazione maggiore delle due bande dell'adulto. Durante la metamorfosi critica accanto all'Hb larvale compare la banda più veloce dell'Hb adulta; solo 10 giorni dopo la metamorfosi scompare l'Hb larvale.

Nelle tre specie studiate non è stato osservato polimorfismo genetico propriamente detto.

Queste ricerche indicano che le modificazioni biochimiche dell'Hb durante lo sviluppo rappresentano un fenomeno esteso a tutte le classi di Vertebrati dai Ciclostomi in su. Inoltre il momento in cui l'Hb larvale è sostituita da quella di tipo adulto sembra coincidere con variazioni delle condizioni ambientali o con un particolare periodo della metamorfosi della specie osservata.

4

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ACCADEMIA NAZIONALE DEI LINCEI

Estratto dai *Rendiconti della Classe di Scienze fisiche, matematiche e naturali*

Serie VIII, vol. XXVIII, fasc. 2. — Febbraio 1960.

Biologia. — *Modificazioni del comportamento elettroforetico dell'emoglobina durante la metamorfosi di Rana esculenta* (*). Nota di GIOVANNI CHIEFFI, MARCELLO SINISCALCO e MATTEO ADINOLFI, presentata (**) dal Corrisp. G. MONTALENTI.

Sono note le profonde modificazioni, non solo morfologiche ma anche biochimiche, che intervengono nel corso della metamorfosi degli Anfibi. Due proteine sono state particolarmente studiate per quanto riguarda la loro evoluzione nello sviluppo degli Anfibi: il pigmento retinico (Wald, 1945, p. 41) [1] e l'emoglobina (McCutcheon, 1936; Riggs, 1951) [2-3].

In queste ultime ricerche è stato dimostrato che le emoglobine di tipo adulto e di tipo larvale di *Rana catesbiana* presentano caratteristiche differenti per quanto riguarda l'affinità per l'O₂ e l'effetto Bohr.

La discriminazione tra i due tipi di emoglobine è nettissima per quanto riguarda l'effetto Bohr: mentre l'Hb della Rana adulta ha un elevato effetto Bohr l'Hb delle larve non sembra risentire alcuna influenza per le variazioni del pH (Riggs, 1951) [3].

Le due Hb non sembrano invece differenziarsi per quanto riguarda il peso molecolare stimato dal Riggs [3] per mezzo della ultracentrifugazione intorno a 68.000. Egli ha comunque trovato nell'emolisato una piccola frazione di materiale eterogeneo (tra il 5 e il 10 %) che includeva due componenti di natura imprecisata con costanti di sedimentazione (S₂₀) rispettivamente di 7 e 2 unità.

Variazioni della morfologia delle emazie adulte e larvali sono state descritte da McCutcheon; questo autore inoltre, confrontando le curve di affinità per l'O₂ di campioni di sangue di animali a diversa epoca di sviluppo, ha identificato in un ristretto periodo della metamorfosi, durante il quale viene modificato il tipo di respirazione, il momento in cui l'Hb larvale è sostituita da quella di tipo adulto.

In queste nostre ricerche ci siamo proposti di studiare contemporaneamente sia il comportamento elettroforetico delle due Hb sia il loro succedersi nel corso della metamorfosi di *Rana esculenta*. Solo incidentalmente Dessauer e coll. (1957) [4] avevano osservato, in una nota preliminare sulle proprietà elettroforetiche di Hb di Anfibi e di Rettili, che l'Hb di girini di *Rana clamitans* ha una velocità di migrazione inferiore all'Hb dell'animale adulto.

Utilizzando l'elettroforesi su gel di amido, con buffer discontinuo (tris-borato, pH 8,8, 2,5 V/cm), secondo la tecnica di Poulik [5], abbiamo innanzitutto potuto dimostrare che esistono in *Rana esculenta* due distinte Hb di

(*) Lavoro eseguito nell'Istituto di Genetica dell'Università di Napoli, con il contributo parziale della Rockefeller Foundation.

(**) Nella seduta del 13 febbraio 1960.

tipo larvale e due Hb di tipo adulto. È stato inoltre possibile precisare il periodo in cui, nel corso della metamorfosi le Hb si succedono le une alle altre.

Sia gli adulti, sia le larve di *Rana esculenta* sono state raccolte nel bacino del fiume Sarno. Alcune larve sono state allevate in laboratorio ed alimentate con lattuga cotta e dopo la metamorfosi con drosophile. Il sangue di ciascun individuo è stato raccolto mediante decapitazione e reso incoagulabile con soluzione di Alsevers; le emazie, lavate per tre volte con soluzione di Ringer per Anfibi, sono state emolizzate con H₂O distillata.

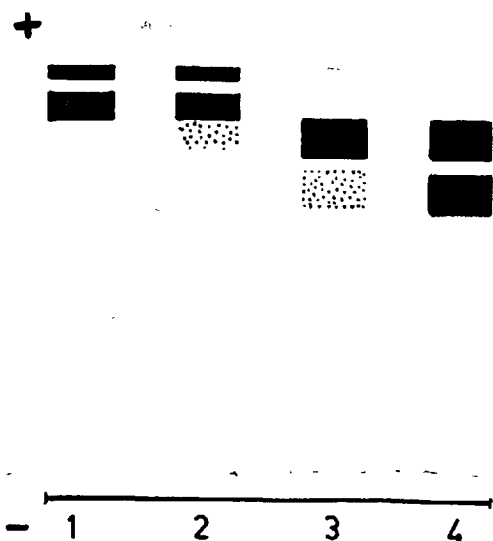


Fig. 1. - Elettroforesi in gel di amido con buffer discontinuo sec. Poulik di Hb di *Rana esculenta* a diversi stadi della metamorfosi (n. 1: stadio 27-32; n. 2 e 3 dallo stadio 33 a circa 10 giorni dalla metamorfosi; n. 4: rana adulta).

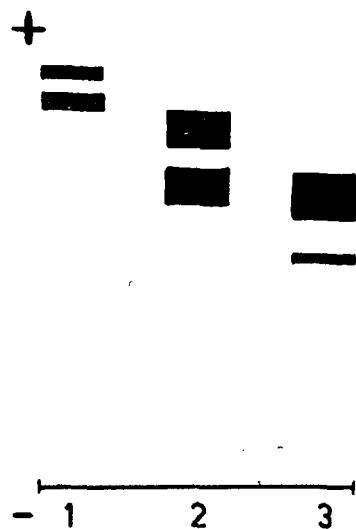


Fig. 2. - Elettroforesi in gel di amido con buffer discontinuo sec. Poulik di Hb larvale (1) ed adulta (2) di *Rana esculenta* ed Hb umana (3).

I tracciati elettroforetici su gel di amido hanno permesso di discriminare due bande emoglobiniche sia negli adulti sia nelle larve (fig. 1). Le due Hb larvali, in concentrazione minore quella più rapida e maggiore quella più lenta, presentano tutte e due una velocità di migrazione superiore alle due Hb di tipo adulto. Queste ultime apparentemente, dopo colorazione del tracciato con benzidina, sembrano essere presenti in proporzioni uguali. Nella fig. 2 viene messa a confronto la mobilità della Hb di Rana con quella umana.

Tracciati elettroforetici di tipo larvale sono stati osservati negli stadi dal 27 al 32⁽¹⁾; negli stadi successivi, dal 33 fino a circa 10 giorni dopo la

(1) Sono state usate per la seriazione degli stadi le tavole del WITSCHI (1956) [6]. Lo stadio 25 corrisponde alla metamorfosi iniziale (comparsa degli abbozzi degli arti posteriori), lo stadio 31 alla metamorfosi critica; lo stadio 33 alla fine della metamorfosi,

metamorfosi compaiono le Hb adulte per sostituire progressivamente quelle di tipo larvale. Queste ultime scompaiono del tutto intorno al 12°-15° giorno dopo la metamorfosi.

Non sono state osservate differenze tra le Hb di tipo adulto o larvale in animali di sesso diverso né è stato osservato nel materiale esaminato un polimorfismo genetico propriamente detto (40 animali adulti; 64 girini e 64 individui giovani).

Queste osservazioni confermano che tra i Vertebrati inferiori anche gli Anfibi presentano emoglobine con proprietà differenti a seconda della epoca del loro sviluppo.

Già nei Ciclostomi (*Petromyzon planeri*) (Adinolfi e Chieffi, 1958; Adinolfi, Chieffi e Siniscalco, 1959) [7-8] sono state differenziate Hb di tipo adulto e di tipo larvale.

Anche nei Pesci Selaci e Teleostei (Manwell, 1957, 1958) [9-10-11] sono state osservate Hb con caratteristiche differenti in animali allo stadio adulto e allo stadio embrionale.

Il momento in cui le Hb larvali e embrionali sono sostituite da quelle di tipo adulto sembra coincidere con variazioni delle condizioni ambientali o con un particolare periodo della metamorfosi della specie osservata.

Nel caso degli Anfibi, come abbiamo detto, questa fase di transizione coincide con l'epoca in cui alla respirazione di tipo bronchiale subentra quella di tipo polmonare. Non è possibile allo stato attuale interpretare il significato di questa singolare coincidenza ed attribuire ad emoglobine che hanno comportamento elettroforetico diverso, proprietà funzionali differenti in stretto rapporto con la metamorfosi o con il variare dell'*habitat*.

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- [10] C. MANWELL, « Science », 127, 592-593 (1958).
- [11] C. MANWELL, « Physiol. Zool. », 31, 93-100 (1958).

RENDICONTI - Febbraio 1960

Classe di Scienze fisiche, matematiche e naturali

Seduta del 13 febbraio 1960

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(Classe di Scienze fisiche, matematiche e naturali)
serie VIII, vol. XXVI, fasc. 6 – Giugno 1959

Genetica. — *Dati sulla genetica dei gruppi gamma-globulinici con particolare riguardo alla loro distribuzione in alcune popolazioni italiane.* Nota I ^(*) di MATTEO ADINOLFI, IDA BIANCO, CAROLYN GILES, BACHISIO LATTE, EZIO SILVESTRONI e MARCELLO SINISCALCO, presentata dal Corrisp. G. MONTALENTI ^(**).

Fino a non molti anni or sono l'unico esempio di polimorfismo genetico nell'Uomo, riconducibile ad uno schema mendeliano semplice, era rappresentato dai gruppi sanguigni che, da soli, individuano almeno nove loci autosomici.

Oggi il numero delle caratteristiche ereditarie per le quali la specie umana si rivela polimorfa nel senso di Ford (1940) ⁽¹⁾ è almeno raddoppiato. Per non parlare della talassemia o microcitemia e delle varianti emoglobiniche, basterà riferirsi alle recenti acquisizioni sul polimorfismo biochimico delle proteine del plasma umano.

Il plasma umano, come è noto, presenta una miscela profondamente eterogenea di proteine di classe e specie diverse. Questa complessità è stata specialmente messa in luce dai recenti progressi dei metodi di analisi delle miscele proteiche come l'elettroforesi zonale mono e bidimensionale in gel di amido (Smithies, 1955) ⁽²⁾, la immunoelettroforesi (Grabar, *et al.* 1953) ⁽³⁾, la tecnica del « fingerprinting » (Ingram, 1956) ⁽⁴⁾ ecc.

È così che dalle sei componenti proteiche della elettroforesi classica si è giunti ora a distinguere almeno 20 classi di proteine diverse.

Tra queste si sono imposte all'attenzione del genetista le varianti aptoglobiniche (Smithies, 1955; Harris *et al.* 1959) ^(5,5), le beta-globuline o transferrine (Smithies 1957; Harris *et al.* 1959) ^(6,5) e le gamma-globuline (Grubb e Laurell, 1956) ^(7,8) in quanto si è riusciti, per queste tre classi di proteine

(*) Ricerche eseguite con un contributo concesso dalla Rockefeller Foundation (New York) all'Istituto di Genetica dell'Università di Napoli. Gli autori ringraziano vivamente il dott. A. E. Mourant (Lister Institute-Londra) nel cui laboratorio sono state eseguite le determinazioni dei gruppi sanguigni ed il dott. H. Harris (Dept. of Biochemistry, London Hospital Medical College, Londra) nel cui laboratorio sono state eseguite le determinazioni delle aptoglobine.

(**) Nella seduta del 1° giugno 1959.

(1) E. B. FORD, *Polymorphism and taxonomy*, in « The new systematics » (Editor: J. S. Huxley), p. 493, Clarendon Press Oxford (1940).

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(6) O. SMITHIES, « Nature », 180, 1482 (1957).

(7) R. GRUBB, A. B. LAURELL, « Acta Path. Microbiol. Scand. », 39, 390 (1956).

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del plasma umano, a riconoscere l'esistenza di una variabilità discontinua intra-specie riconducibile ad un meccanismo mendeliano semplice.

In questa sede si riferirà sul polimorfismo delle gamma-globuline (*Gm*) specialmente per quanto riguarda la distribuzione dei tipi *Gm* in Italia e quegli aspetti di genetica formale di questo nuovo carattere monofattoriale dell'uomo a tutt'oggi non ancora o non sufficientemente studiati.

Contrariamente a quello che accade per le altre varianti proteiche del plasma umano, il polimorfismo delle gamma-globuline è dimostrabile per ora solo con metodo indiretto.

È noto che alcuni sieri di individui affetti da artrite reumatoide sono capaci di agglutinare emazie Rh positive sensibilizzate con anticorpi Rh incompleti (Grubb, 1956)⁽⁹⁾. Gli autori svedesi Grubb e Laurell (1956)⁽⁷⁾ mentre erano intenti ad indagare la natura di questo fenomeno si accorsero che il siero di circa il 60 % di individui di ambo i sessi in Europa settentrionale inibiva tale reazione. Essi riuscirono a stabilire che il « fattore inibente » faceva parte delle gamma-globuline e che rappresentava una caratteristica ereditaria trasmissibile secondo un meccanismo monofattoriale.

Quest'ipotesi è stata in seguito confermata da numerosi altri Autori (Moullec *et al.* 1956; Linnet - Jepsen, 1958; Lawler, 1958; Harboe e Lundevall, 1959)^(10,11,12,13) e pertanto si può affermare con sufficiente attendibilità che la presenza del fattore inibente o della « sostanza *Gm* » — come più semplicemente si usa chiamarlo — è controllata da un gene autosomico *Gm^a* dominante sul suo allelomorfo *Gm* in modo che:

sieri di individui dal genotipo <i>Gm^a/Gm^a</i>	} sono inibitori
sieri di individui dal genotipo <i>Gm^a/Gm</i>	
sieri di individui dal genotipo <i>Gm/Gm</i>	

Il fenotipo degli individui che posseggono il fattore inibente viene indicato con il simbolo *Gm* (*a* +); e rispettivamente quello degli individui che non lo posseggono con il simbolo *Gm* (*a* —).

Recentemente Harboe (1959)⁽¹⁴⁾ in Norvegia ha trovato un siero di paziente affetto da artrite reumatoide, capace di reagire con sieri di individui *Gm* (*a* —) e con i *Gm* (*a* +) eterozigoti. Se tale osservazione venisse confermata bisognerebbe concludere che in effetti esistono due tipi di sostanze *Gm* entrambe presenti nell'eterozigote e ciascuna da considerarsi come il prodotto specifico dei due allelomorfi.

Per concludere questa premessa bibliografica riferiremo che Harboe e Lundevall (1959)⁽¹³⁾ hanno pubblicato dati che lasciano prevedere l'esistenza

(9) R. GRUBB, « Acta Path. Microbiolog. Scandinav. », 39, 195 (1956).

(10) J. MOULLEC, R. KHERUMIAN, E. SUTTON, P. ESPAGNON, « Revue Hémat. », 11, 512 (1956).

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(14) M. HARBOE, « Nature », 183, 1468 (1959).

di un terzo allelomorfo al locus *Gm*, mentre Oudin (1956 *a*-1956 *b*)^(15,16) Dray e Young (1958)⁽¹⁷⁾ e Dubiski *et al.* (1959)⁽¹⁸⁾ hanno dimostrato per mezzo di una reazione di precipitazione, che un polimorfismo gamma-globulinico è presente anche nei conigli.

Altro dato fondamentale è quello di Moullec *et al.* (1956)⁽¹⁹⁾, - confermato poi da Brönnestam e Nilsson (1957)⁽²⁰⁾ e Linnet-Jepsen (1958)⁽²¹⁾ - che i neonati alla nascita hanno sempre il fenotipo *Gm* della madre, verso il secondo mese sono tutti *Gm* (*a* —), e solo verso la fine del 7°-8° mese sviluppano il fenotipo *Gm* definitivo.

L'influenza di alterazioni patologiche come la ipo ed iper gamma-globulinemia non è stata sufficientemente chiarita, per quanto sembrerebbe che la prima determini la scomparsa della attività inibente in individui geneticamente *Gm* positivi mentre la seconda non ne determina necessariamente la comparsa in individui geneticamente *Gm* negativi (Grubb e Laurell, 1956; Vaughan e Good, 1958; Moullec *et al.*, 1958; Grubb, 1959)^(7,20,21,8).

Tenendo presente infine le circostanze che hanno reso possibile la individuazione della sostanza *Gm* nel siero umano, vien fatto spontaneo di chiedersi quali siano i rapporti tra fenotipo *Gm* ed artrite reumatoide.

I dati di Grubb (1957)⁽²²⁾ e Podliachouck *et al.* (1958)⁽²³⁾ sembrano deporre per una completa indipendenza in quanto la frequenza di individui *Gm* (*a* +) tra i pazienti affetti da artrite reumatoide è praticamente la stessa che tra i normali.

È però significativo il fatto (vedi Tab. I) che finora i soli due collettivi che presentano quasi il 100 % di *Gm* (*a* +) sono gruppi di Africani ed Esquimesi cioè individui appartenenti a popolazioni presso le quali l'artrite reumatoide è praticamente sconosciuta. (Grubb, 1959)⁽⁸⁾.

Nella stessa tabella I si osserva che la frequenza dei *Gm* (*a* +) è sorprendentemente omogenea in tutte le popolazioni europee e nord-europee finora esaminate cioè (intorno al 60 % con unica eccezione per l'Italia che sembra presentare la più bassa frequenza di *Gm* (*a* +) finora osservata (Lawler, 1958)⁽¹²⁾,⁽²⁴⁾.

(15) J. OUDIN, «C. R. Acad. Sci.», 242, 2489 (1956 *a*).

(16) J. OUDIN, «C. R. Acad. Sci.», 242, 2606 (1956 *b*).

(17) S. DRAY, G. O. YOUNG, «J. Immunol.», 81, 142 (1958).

(18) S. DUBISKI, Z. DUBZIAK, S. SKALBA, «J. Immunol.», 2, 84 (1959).

(19) R. BRÖNNESTAM, S. B. NILSSON, «Vox. Sang.», 2, 316 (1957).

(20) J. H. VAUGHAN, R. A. GOOD, «Arthritis and Rheumatism», 1, 99 (1958).

(21) J. MOULLEC, J. M. FINE, C. HENRY, Dati presentati al «7° Congr. Int.^{le} della Trasfusione del Sangue», Roma (1958).

(22) R. GRUBB, «Vox Sang.», 2, 305 (1957).

(23) L. PODLIACHOUCK, F. JAQUELINE, A. EYQUEM, «Ann. Inst. Pasteur», 94, 590 (1958).

(24) Si tratta di materiale raccolto nel 1957 da Silvestroni e Bianco in Berra (Ferrara) durante una campagna di ricerche sulla microcitemia e in cui i gruppi *Gm* sono stati determinati dalla D.ssa S. D. Lawler del Galton Laboratory di Londra.

TABELLA I.
Distribuzione dei gruppi Gm in alcune popolazioni umane.

Località	Autori	n°	Gm (a+) %	Frequenze geniche		E. S. ±
				Gm ^a	Gm	
Francia. . . .	Moullec <i>et al.</i> ; 1956 Podliachouck <i>et al.</i> ; 1958	600	55.6	0.33	0.67	0.014
Danimarca . .	Linnet-Jepsen <i>et al.</i> ; 1958	1084	55.6	0.33	0.67	0.018
Inghilterra . .	Lawler; 1958	133	61.0	0.38	0.62	0.040
Italia	Lawler; 1958	125	40.2	0.22	0.78	0.050
Norvegia . . .	Harboe e Lundevall; 1959	320	60.6	0.38	0.62	0.024
Svezia	Grubb e Laurell; 1956	360	59.7	0.37	0.63	0.020
Africani A.O.F.	Moullec <i>et al.</i> ; 1958	300	100.—	1.00	—	0.029
Esquimesi . .	Grubb e Laurell; 1956	74	94.6	0.72	0.18	0.056

Poiché il materiale su cui si basano queste osservazioni proviene da un piccolo villaggio della provincia di Ferrara noto peraltro per presentare una elevata incidenza di talassemia o microcitemia, abbiamo ritenuto opportuno di raccogliere altri dati sia dalla stessa sia da altre regioni italiane.

I dati della presente ricerca, in buona parte costituiti da famiglie del villaggio di Cologna (Ferrara), in cui sono state determinate la microcitemia, se presente, le aptoglobine, i gruppi sanguigni, hanno inoltre reso possibile la ricerca di eventuali associazioni (*linkages*) del locus *Gm* con la maggior parte degli altri loci autosomici finora noti.

DATI SPERIMENTALI.

La tecnica usata per la determinazione dei gruppi *Gm* in questa ricerca è quella di agglutinazione-inibizione secondo la metodica originale descritta da Grubb (Grubb e Laurell, 1956) ⁽⁷⁾.

Sono state usate emazie di individui CDe/cDE sensibilizzate con siero anti-Rh incompleto diluito 1/200 (il titolo agglutinante di tale siero con la tecnica di Coombs era di circa 1/1000). La determinazione veniva eseguita con due sieri di individui affetti da artrite reumatoide (gentilmente inviatici dal Dr. A. E. Mourant e dal Dr. R. Grubb) entrambi usati al titolo di 1 : 30. Il potere inibente dei sieri in esame veniva saggiato alle diluizioni di 1 : 4, 1 : 8, 1 : 16, 1 : 32 in presenza degli opportuni controlli.

La tabella II riporta i dati sulla distribuzione dei fenotipi *Gm* in gruppi di individui non parenti tra loro e prelevati da quattro diverse aree della popolazione italiana.

TABELLA II.
Distribuzioni dei gruppi Gm in alcune località italiane.

Località	n° casi	Gm (a+)	Gm (a—)	Stima delle frequenze geniche		
				Gm ^a	Gm	E. S.
Cologna (Prov. Ferrara)	185	70 (0,378)	115 (0,622)	0,21	0,79	± 0,023
Atena (Prov. Salerno)	73	34 (0,465)	39 (0,535)	0,27	0,73	± 0,040
Galtelli (Prov. Nuoro)	102	32 (0,314)	70 (0,686)	0,17	0,83	± 0,028
Napoli	84	34 (0,405)	50 (0,595)	0,23	0,77	± 0,035
TOTALI . . .	444	170 (0,383)	274 (0,617)			

Eterogeneità nella frequenza dei gruppi Gm nei vari centri $\left\{ \begin{array}{l} \chi^2 = 4,25 \\ g.l. = 3 \\ P > 0,20 \end{array} \right.$

TABELLA III.
Sommario dei dati familiari (Cologna, Prov. Ferrara)

Tipo d'incrocio	n° Famiglie		Figli	
	osservato	atteso	Gm (a +)	Gm (a —)
Gm (a +) × Gm (a +)	8	12,0	16	4
Gm (a +) × Gm (a —)	48	39,9	65	71
Gm (a —) × Gm (a —)	29	21,1	—	73
TOTALI . . .	85		81	148

Stima delle frequenze geniche tra i genitori $\left\{ \begin{array}{l} Gm = 0,79 \pm 0,024 \\ Gm^a = 0,21 \end{array} \right.$

χ^2 per l'equilibrio secondo Hardy-Weinberg nella generazione parentale $\left\{ \begin{array}{l} \chi^2 = 3,48 \\ g.l. = 1 \\ P < 0,10 \\ > 0,05 \end{array} \right.$

Per quanto queste osservazioni siano ancora troppo limitate per consentire una generalizzazione, è da notare tuttavia che la bassa incidenza degli individui $Gm(a+)$ sembra essere una caratteristica comune a tutte le regioni studiate siano esse zone talassemiche (Prov. di Ferrara e di Nuoro) sia non talassemiche (Prov. di Napoli e Salerno). La più bassa incidenza è quella riscontrata in Sardegna, mentre la più elevata è in provincia di Salerno; comunque sembra opportuno concludere per il momento che la incidenza dei $Gm(a+)$ nelle popolazioni studiate è circa del 38 %, come si ricava cioè dal totale generale, e che le fluttuazioni intorno a questo valore, osservate nelle singole località, sono casuali.

TABELLA IV.

Analisi delle segregazioni familiari.

(i) Incrocio $Gm(a+) \times Gm(a-)$ n° figli per famiglia	n° famiglie con almeno un recessivo	n° figli recessivi			
		osservato	atteso	varianza	
1	1	1	1,00	0,00	$\chi^2 = 0,23$ $g.l. = 1$ $P < 0,70$ $> 0,50$
2	14	21	18,66	3,11	
3	13	22	22,28	6,37	
4	3	8	8,53	2,35	
5	4	12	10,32	4,33	
7	1	1	3,53	1,67	
9	1	6	4,51	2,21	
TOTALI	37	71	68,83	20,04	
(ii) Incrocio $Gm(a+) \times Gm(a+)$					
2	1	1	1,14	0,12	$\chi^2 = 0,01$ $g.l. = 1$ $P > 0,90$
3	1	2	1,30	0,26	
4	1	1	1,46	0,42	
TOTALI	3	4	3,90	0,80	

Un sommario generale dei dati familiari raccolti nella provincia di Ferrara è riportato nella tabella III.

Da questa tabella si può notare: 1) che la popolazione parentale è da considerarsi in equilibrio rispetto alle stime di frequenze geniche ottenute

dalla stessa (χ^2 per la deviazione dell'equilibrio = 3,48, per 1 grado di libertà $P < 10\%$, $> 5\%$); 2) che su 29 famiglie del tipo $Gm(a-)\times Gm(a-)$ con un totale di 73 figli non sono state osservate eccezioni nella segregazione attesa del 100% di figli $Gm(a-)$.

TABELLA V.

Numero atteso delle famiglie segreganti (per $Gm^a = 0,21$, $Gm = 0,79$).

(i) Incrocio $Gm(a+)\times Gm(a-)$ n° figli per famiglia	n° famiglie esaminate (n_c)	n° famiglie segreganti			
		osservato (m_c)	atteso	varianza	
1	5	1	2,20	1,23	$\chi^2 = 0,96$ $g.l. = 1$ $P > 0,30$
2	18	14	11,88	4,04	
3	16	13	12,32	2,83	
4	3	3	2,49	0,42	
5	4	4	3,41	0,51	
7	1	1	0,87	0,003	
9	1	1	0,88	0,002	
TOTALI . . .	48	37	34,05	9,04	
(ii) Incrocio $Gm(a+)\times Gm(a+)$	(N_c)	(M_c)			$\chi^2 = 0,004$
1	1	—	0,19	0,15	
2	3	1	1,02	0,67	
3	3	1	1,34	0,74	
4	1	1	0,53	0,25	
TOTALI . . .	8	3	3,08	1,71	

L'analisi delle segregazioni familiari è stata poi eseguita in modo completo servendosi del metodo proposto da C.A.B. Smith (1956)⁽²⁵⁾ ed i risultati riportati nelle tabelle IV, V, VI, sono in ottimo accordo con l'ipotesi monofattoriale di Grubb e Laurell.

Lo stesso materiale familiare studiato come si è detto anche per i gruppi sanguigni, la talassemia e le aptoglobine, è stato utilizzato per la ricerca di eventuali associazioni (*linkages*) del locus Gm con questi altri loci autosomici.

(25) C. A. B. SMITH, «Ann. Hum. Genet., Lond.», 20, 257, 1956.

Dopo aver controllato che nessuna associazione fenotipica si presentava nel materiale in oggetto tra il fenotipo *Gm* e i caratteri sopra menzionati, sono state selezionate le sole famiglie informative a genotipo dei genitori « certo » per la ricerca del linkage propriamente detto. Il metodo adoperato per questo scopo è quello delle statistiche $-u$ di Fisher (1935 *a, b*)^(26, 27) secondo le modificazioni suggerite da C.A.B. Smith (1953)⁽²⁸⁾.

TABELLA VI.

Riepilogo dell'analisi genetica.

	Tipo di incrocio	χ^2	g. l.
N° figli recessivi dato m_c	1) <i>Gm</i> (<i>a</i> +) $\times$ <i>Gm</i> (<i>a</i> —)	0,23	I
N° figli recessivi dato M_c	2) <i>Gm</i> (<i>a</i> +) $\times$ <i>Gm</i> (<i>a</i> +)	0,01	I
m_c dato n_c	1) <i>Gm</i> (<i>a</i> +) $\times$ <i>Gm</i> (<i>a</i> —)	0,96	I
M_c dato N_c	2) <i>Gm</i> (<i>a</i> +) $\times$ <i>Gm</i> (<i>a</i> +)	0,01	I
		1,21	4

m_c : numero di famiglie segreganti tra gli incroci di tipo (1) con un dato numero di figli

M_c : numero di famiglie segreganti tra gli incroci di tipo (2) con un dato numero di figli

$P > 0,80$

n_c : numero totale di famiglie del tipo (1) con un dato numero di figli

N_c : numero totale di famiglie del tipo (2) con un dato numero di figli

I risultati sono riportati nella tabella VII dove si leggono i punteggi $\Sigma(\lambda)$ delle misure dirette della deviazione dall'indipendenza e le rispettive varianze $\Sigma(v)$. Com'è noto valori di $\Sigma(\lambda)$ negativi sono contro l'ipotesi di linkage, valori positivi vanno considerati degni di nota quando superano almeno di 2 volte la radice quadrata della loro varianza. È chiaro dunque che nessuno dei computi eseguiti depone effettivamente in favore dell'ipotesi di linkage, sebbene il confronto *sistema Gm-gruppo sanguigno P*, richieda ulteriore considerazione per essere il rapporto: $\Sigma(\lambda)/\sqrt{\Sigma(v)} = \sim 2$.

In realtà il livello di significatività che si richiede nella ricerca di linkages per la specie umana deve essere molto più elevato per le note difficoltà che riguardano sia i limiti della sperimentazione genetica dell'uomo, sia l'efficienza dei metodi statistici proposti a tale scopo (vedi Siniscalco 1954)⁽²⁹⁾.

(26) R. A. FISHER, « Ann. Eugen. », 6 187 (1935 *a*).

(27) R. A. FISHER, « Ann. Eugen. », 6, 339 (1935 *b*).

(28) C. A. B. SMITH, « J. R. Statist. Sc. », B, 15, 153 (1953).

(29) M. SINISCALCO, « Atti 1° Convegno A.G.I. », Suppl. a « La Ric. Sci. », 25, 13 (1954).

TABELLA VII.

Risultati dei confronti per la ricerca di associazioni (linkages) tra gruppi Gm ed altri loci autosomici

Carattere a confronto		n° famiglie informative (*)	$\Sigma (\lambda)$	$\Sigma (v)$
Aptoglobine		16	— 3.055	63,00
Talassemia o microcitemia		10	— 5.778	20.000
Gruppo sanguigno:				
Sistema	ABO	11	— 7.000	28,75
»	MNS	12	— 1.556	69,28
»	P	10	+ 5.110	7,12
»	Rh	10	— 4.333	37,15
»	Lutheran	—	—	—
»	Kell	5	+ 0.333	10,11
»	Duffy	11	+ 7.778	46,37

(*) Solo famiglie a genotipo dei genitori « certo ».

Quanto sin qui riportato ci permette di concludere che:

1) la distribuzione dei gruppi *Gm* in Italia appare singolarmente diversa da quella trovata in altri paesi d'Europa e nord-Europa: v'è una relativa scarsità di individui *Gm* (*a* +) che sembra accentuata in aree di popolazione dov'è frequente la microcitemia (Provincia di Ferrara e Provincia di Nuoro);

2) il materiale familiare raccolto è in buon accordo con la ipotesi monofattoriale di Grubb e Laurell e dimostra che i vari tipi d'incrocio possibili si presentano nella generazione parentale con frequenze compatibili con quelle attese per l'equilibrio genico secondo Hardy-Weinberg;

3) non si sono riscontrate prove in favore di eventuali associazioni (*linkages*) del locus *Gm* con i loci dei gruppi sanguigni, della microcitemia e delle aptoglobine sebbene il confronto sistema *Gm-gruppo P* richieda ulteriore considerazione.

ACCADEMIA NAZIONALE DEI LINCEI

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Genetica. — *Dati sulla genetica dei gruppi gamma-globulinici nell'Uomo.* Nota II (*) di MATTEO ADINOLFI, ACHILLE MARONE, FRANCO OLIVELLI, PIETRO POLOSA e MARCELLO SINISCALCO, presentata (**) dal Corrisp. G. MONTALENTI.

Nella Nota precedente (Adinolfi et al. 1959) [1] è stato descritto il nuovo esempio di polimorfismo proteico geneticamente controllato, individuato nel 1956 da Grubb e Laurell [2] nelle frazioni gamma-globuliniche del plasma umano.

Queste varianti gamma-globuliniche sono state scoperte quasi contemporaneamente alle varianti aptoglobuliniche (Smithies, 1955) [3] ma a differenza di queste ultime classi di plasma-proteine esse sono individuali solo indirettamente per mezzo di una complessa tecnica immunologica, che si fonda essenzialmente su di una reazione specifica di agglutinazione-inibizione.

Era noto da qualche tempo che alcuni sieri di pazienti con artrite reumatoide sono capaci di agglutinare emazie umane Rh+ sensibilizzate con anticorpi incompleti anti-Rh. Nel 1956 Grubb e Laurell [2] si accorsero che questa reazione poteva essere inibita dal siero di circa il 60 % degli individui adulti di ambo i sessi della popolazione svedese.

Essi riuscirono inoltre a dimostrare che il «fattore inibente» è identificabile in una frazione gammaglobulinica e che esso rappresenta una caratteristica ereditaria trasmissibile secondo un meccanismo mendeliano semplice.

La Tabella I riassume le reazioni di agglutinazione-inibizione e la ipotesi genetica di Grubb con la classificazione dei genotipi e fenotipi gamma-globulinici.

Per concludere questa premessa bibliografica riferiremo che Harboe e Lundevall [4, 5] hanno individuato un siero tipo artrite reumatoide capace di reagire con i sieri di individui *Gm* (*a*—) e con i *Gm* (*a*+) eterozigoti ed hanno pubblicato dati che lasciano prevedere la esistenza di un terzo allelomorfo al locus *Gm*.

Altro dato fondamentale è che i neonati alla nascita hanno sempre un fenotipo *Gm* uguale a quello della madre, verso il secondo mese sono tutti *Gm* (*a*—) e solo verso la fine del settimo mese manifestano il fenotipo definitivo [7, 8, 9]. La sostanza *Gm* sarebbe inoltre assente nella secrezione lattea (Grubb, 1959; Lawler, 1960) [9, 10].

(*) Ricerche eseguite con un contributo della Rockefeller Foundation (New York) e del C.N.R. all'Istituto di Genetica della Università di Napoli e dirette dal prof. M. Siniscalco. I dati di cui alla presente Nota sono stati comunicati alla 5ª Riunione dell'Associazione Genetica Italiana, svoltasi in Napoli il 22-24 ottobre 1959.

(**) Nella seduta del 12 marzo 1960.

Come abbiamo già riportato in precedenti comunicazioni (Adinolfi et al., 1959; Siniscalco, 1959) [1, 11] la frequenza dei *Gm* (*a*+) è sorprendentemente omogenea in tutte le popolazioni europee finora esaminate e cioè intorno al 60% con unica eccezione per l'Italia che sembra presentare la più bassa frequenza di *Gm* (*a*+) con punte minime nelle provincie di Ferrara e Nuoro.

TABELLA I.

Reazione normale				Sistema <i>Gm</i>	
Siero tipo artrite reumatoide	+	Emazie sensibilizzate	= agglutinaz.	Fenotipo	Genotipo
<i>Azione del Siero umano</i>					
Siero tipo artrite reumatoide	+	Siero umano normale	+ Emazie sensibilizzate = agglutinaz. <i>inibita</i>	<i>Gm</i> (<i>a</i> +)	<i>Gm^a/Gm^a</i> <i>Gm^a/Gm</i>
Siero tipo artrite reumatoide	+	Siero umano normale	+ Emazie sensibilizzate = agglutinaz. <i>presente</i>	<i>Gm</i> (<i>a</i> —)	<i>Gm/Gm</i>

I dati relativi al materiale familiare da noi raccolto in Italia (Adinolfi et al., 1959, Tabelle III-IV-V-VI-VII [1]) sono in buon accordo con la ipotesi monofattoriale di Grubb e Laurell e dimostrano che i vari tipi di incrocio si presentano nella generazione parentale con frequenze compatibili con quelle attese per l'equilibrio secondo la legge di Hardy-Weinberg.

Il locus *Gm* sembra inoltre segregare indipendentemente, nelle famiglie studiate, dai loci per i gruppi sanguigni, la talassemia e le aptoglobine, sebene il confronto con il sistema P richieda ulteriori ricerche.

CONTRIBUTO SPERIMENTALE.

Ulteriori dati sulla distribuzione dei tipi Gm in Italia.

La raccolta dei dati popolazionistici dei tipi *Gm* in Italia è stata estesa a diverse provincie ed ulteriormente accresciuta in quelle già esaminate. Il quadro generale resta quello segnalato precedentemente (Tabella II) sebene i dati della provincia di Catania mostrino una distribuzione del sistema *Gm* pressoché identica a quella delle altre provincie europee.

Viene inoltre confermato il rilievo delle più basse frequenze del fenotipo (*Gm* (*a* +)) nelle popolazioni sarde.

GENOTIPO *Gm* E MALATTIE.

L'influenza delle alterazioni patologiche, come le ipo- e le iper-gammaglobulinemie sul sistema *Gm* non sono state sufficientemente chiarite per quanto sembri che la prima condizione possa determinare la scomparsa della

attività inibente del siero di individui $Gm(a+)$ mentre la seconda non determini necessariamente la comparsa del fattore inibente gamma-globulinico in individui $Gm(a-)$ (Grubb e Laurell, 1956; Podliachouk et al., 1958; Grubb, 1959) [2, 12, 9].

TABELLA II.

Distribuzione dei Gruppi Gm in alcune località italiane.

Località	N° indiv. esamin.	$Gm(a+)$	$Gm(a-)$	$Gm(a+)$ %	Frequenze geniche		
					Gm	Gm	e.s. $\pm$
VERCELLI	109	51	58	46,8	0,24	0,76	0,033
COLOGNA (Prov. Ferrara) .	185	70	115	37,8	0,21	0,79	0,023
NAPOLI	84	34	50	40,5	0,23	0,77	0,035
ATENA (Prov. Salerno) . .	73	34	39	46,5	0,27	0,73	0,040
CATANIA	190	122	68	64,2	0,41	0,59	0,029
GALTELLI (Prov. Nuoro) . .	102	32	70	31,4	0,17	0,83	0,028
CABRAS (Prov. Cagliari). . .	97	33	64	34 -	0,19	0,81	0,029

Abbiamo da qualche tempo iniziato la ricerca di eventuali correlazioni tra fenotipi Gm e malattie. Da un collettivo di dati raccolti da Silvestroni e Bianco [1] in provincia di Ferrara, si rileva che i fenotipi del sistema Gm sono distribuiti indipendentemente dalla talassemia (Tabella III).

TABELLA III.

Distribuzione dei gruppi Gm in Individui normali e talassemici nella Provincia di Ferrara (Cologna).

	$Gm(a+)$	$Gm(a-)$	
Normali	131 (124,3)	210 (216,7)	341
Talassemici	32 (38,7)	74 (67,3)	106
	163	284	447

$$\chi^2 = 2,40 \quad \text{g. l.} = 1 \quad P > 0,10$$

La frequenza del fenotipo $Gm(a+)$ in un gruppo di donne affette da neoplasie dell'utero e della mammella è invece significativamente più elevata che nella popolazione di controllo (Tabella IV). La frequenza del fenotipo $Gm(a+)$ si aggira infatti intorno al 64,8 nelle donne affette da neoplasia contro il 40,5 del controllo.

TABELLA IV.

Distribuzione dei gruppi Gm in individui normali (I) ed in donne affette da neoplasie (II).

	$Gm(a+)$	$Gm(a-)$	
Gruppo I	34 (43,3)	50 (40,7)	84
Gruppo II	46 (36,7)	25 (34,3)	71
	80	75	155

$$\chi^2 = 9,36 \quad \text{g. l.} = 1 \quad P < 0,01$$

Non siamo ancora in grado di precisare se gli individui $Gm(a+)$ presentano effettivamente un più elevato coefficiente di morbidità per i tumori oppure se lo stato morboso sia capace di modificare il fenotipo del sistema Gm . Indagini su base familiare sono in corso per chiarire questo interessante aspetto del problema.

Dati biochimici e immunologici relativi al sistema Gm.

Dai recenti contributi pubblicati da Grubb risulta che la sostanza Gm non viene inattivata con il calore (63°C) o dal trattamento con il periodato di K e con composti sulfidrilici (2 mercaptoetanol e etilmercaptano), mentre viene completamente distrutta dalla papaina. La natura chimica della sostanza Gm e il meccanismo immunologico che ne permette l'individuazione sono tuttora oscuri. Ciò è d'altronde comprensibile dal momento che ancora oggi solo in linea ipotetica le reazioni dei test diagnostici per l'artrite reumatoide sono interpretate come reazioni tra un antigene e un anticorpo e l'agglutinazione delle emazie Rh positive sensibilizzate con anticorpi incompleti ad opera di un siero tipo artrite reumatoide è ritenuto dal Grubb simile alla reazione di Coombs in mezzo albuminoide.

Se tale ipotesi fosse giusta ne deriverebbe per conseguenza la possibilità di identificare il fattore inibente in una sostanza a potere antigene capace di adsorbire gli anticorpi presenti in un siero tipo artrite reumatoide. In tal

caso dovremmo attenderci reazioni post-trasfusionali ogni qualvolta del plasma *Gm* ($a +$) viene trasfuso in individui con siero tipo artrite reumatoide. Ciò in effetti non si è mai verificato anche quando il siero del recettore era capace di agglutinare ad alto titolo, a 37°C , emazie Rh positive sensibilizzate (Grubb, 1958) [13]. Neppure la introduzione per via intradermica di siero *Gm* ($a +$) in questi stessi pazienti ha mai provocato reazioni locali a tipo allergico.

Poiché evidenti appaiono le correlazioni tra la metodica rivelatrice il polimorfismo del sistema *Gm* e le tecniche immunologiche recentemente introdotte nella pratica clinica per le diagnosi delle malattie reumatiche croniche, abbiamo ritenuto utile un confronto tra sistema *Gm* e fattori plasmatici proteici che inibiscono altri test diagnostici per l'artrite reumatoide.

In precedenza erano già state riferite osservazioni sulla inibizione delle reazioni diagnostiche per l'artrite reumatoide da parte dei sieri normali umani. Heller et al. 1954 [14] avevano infatti notato che undici sieri normali, su un totale di ventotto sieri umani da loro esaminati, inibivano l'agglutinazione positiva del test di Heller. Svartz e Schlossmann (1955), anche Ziff et al. 1956 [15, 16] avevano avuto modo di osservare fenomeni analoghi per il test di Waaler-Rose. La incostanza dei risultati acquisiti e la mancanza di una impostazione genetica non permise a questi Autori di conseguire risultati utili.

Nella presente indagine è stata ricercata in primo luogo una eventuale correlazione tra sostanza *Gm* e fattore inibente il test di Heller; questo test diagnostico si basa sulla proprietà che presentano alcuni sieri di pazienti affetti da artrite reumatoide di agglutinare emazie di pecora sensibilizzate con dosi non agglutinanti di emolisine di coniglio anti-globuli rossi di pecora.

Nel materiale da noi esaminato è stato possibile confermare la esistenza di sieri che inibiscono il test di Heller ma questo fattore inibente, che sembra anche essere legato ad una frazione gamma-globulinica, non è inattivato dal calore (63°C) e non è per nulla correlato con il fenotipo *Gm*.

Un analogo confronto è stato eseguito anche fra fenotipi *Gm* e fattore inibente del test diagnostico per l'artrite reumatoide descritta da Plotz 1957 [17]. Olivelli (1959) [18] aveva infatti osservato che circa il 60% dei sieri umani normali inibiscono l'agglutinazione del test al lattice-globulina per l'artrite reumatoide. Dalle nostre osservazioni preliminari risulta che anche questo fattore inibente è indipendente dal fenotipo *Gm*.

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DISTRIBUZIONE DEI LIVELLI EMATICI DELLA
GLUCOSO 6-FOSFATO-DEIDROGENASI (G6PD) IN SARDEGNA.
DATI FAMILIARI E POPOLAZIONISTICI ⁽¹⁾

M. ADINOLFI, L. BERNINI, U. CARCASSI, B. LATTE, A. MOTULSKJ
M. SINISCALCO

Istituto di Genetica, Università, Napoli

RIASSUNTO

Dati popolazionistici e familiari sulla distribuzione dei livelli ematici della G6PD (glucoso-6-fosfato-deidrogenasi dei globuli rossi) sono stati raccolti in Sardegna allo scopo di ottenere materiale opportuno per un'ulteriore indagine sulla genetica del favismo. E' stato osservato che, mentre l'attività di questo enzima è scarsa o del tutto assente nel sangue circolante di individui con anamnesi positiva per il favismo, il contrario non è sempre vero. La maggior parte degli individui enzimopenici (identificati con la tecnica di Motulskj) non avevano mai sofferto di favismo sebbene in molti di essi sia stato riscontrato qualche segno indiretto di emolisi (diminuita concentrazione delle aptoglobine) specialmente durante la stagione delle fave.

E' stato inoltre notato che in questo periodo dell'anno il difetto enzimatico non è rilevabile in parecchi individui enzimopenici con la semplice determinazione quantitativa dell'attività enzimatica del sangue circolante. Ciò è dovuto alla aumentata produzione di eritrociti giovani (sempre molto ricchi di enzima) che segue evidentemente la lieve ma continua crisi emolitica determinata in questi individui dai principi tossici delle fave.

Per la stessa ragione la talassemia costituisce un'altra causa d'errore nella diagnosi dell'enzimopenia perchè l'abbreviata durata di vita

⁽¹⁾ Ricerche eseguite con il contributo del Consiglio Nazionale delle Ricerche e della Rockefeller Foundation, New York.

media degli eritrociti in questa forma di anemia emolitica ereditaria conduce nuovamente alla comparsa in circolo di un'elevata percentuale di globuli rossi giovani ricchi di enzima.

Dopo aver preso le opportune misure sperimentali per evitare le suddette cause di errore, si è visto che la distribuzione dell'enzimopenia, tanto nelle famiglie quanto nelle popolazioni, risulta praticamente in buon accordo con l'ipotesi di Childs *et al.* che ci si trova di fronte ad un tipico esempio di polimorfismo genico controllato da una coppia di geni legati al sesso con dominanza incompleta.

Le stime di frequenza genica (ottenute dalle popolazioni maschili delle scuole elementari in diversi villaggi) dimostrano che la distribuzione del gene responsabile dell'enzimopenia è ineguale in Sardegna e si presenta, come il gene per la talassemia, con delle punte di frequenza massime nei villaggi di pianura un tempo fortemente malarici, e minime nei villaggi di alta collina e montagna. I villaggi della costa occidentale presentano inoltre frequenze più elevate che quelli della costa orientale pur rilevandosi in entrambe le aree delle distribuzioni di gruppi sanguigni che sono quelle tipiche delle popolazioni sarde.

In due villaggi della costa occidentale è stato anche possibile determinare la frequenza degli individui anomali, intermedi e normali tra le femmine trovandole in buon accordo con quelle attese per l'ipotesi del linkage al sesso e date le frequenze geniche osservate tra i maschi. Vengono infine discusse le possibili interazioni tra favismo, talassemia, aptoglobine e malaria al livello fenogenetico e popolazionistico.

ABSTRACT

Population and family data on the distribution of G6PD-deficiency have been collected in Sardinia with the purpose of obtaining suitable material for a further investigation on the genetics of Favism.

It was thus found that while the enzyme activity is always deficient or absent in all red cell populations from individuals with positive history of Favism, the reverse is not necessarily true.

The great majority of the G6PD-deficient people, identified with the screening method of Motulskj, did in fact never suffer of Favism though most of them were found to show indirect signs of haemolysis (decreased haptoglobin concentration), especially during the fava beans season.

It was noted furthermore that in this period of the year many of the enzyme deficient people do not show their defect because of the significant increase in the proportion of young erythrocytes (always very rich in enzyme activity) which presumably follows the haemolysis as a compensating factor.

For the same reason thalassemia turns out to be another perturbing factor in the detection of the G6PD-deficiency since the shortened red cell life span in this type of haemolytic anemia, results again into an abnormally high percentage of young erythrocytes in the blood stream.

When the appropriate measures to overcome the above described difficulties were taken, the distribution of the G6PD-deficiency, studied within families and at population level, was found to be quite consistent with the hypothesis of Childs et al. of a true genetic polymorphism controlled by a sex linked gene pair with incomplete dominance.

Estimates of gene frequencies (obtained by screening for the enzyme deficiency a consistent number of school boys in several villages) showed that the « abnormal gene » is unevenly distributed in Sardinia and follows the same pattern as the thalassaemic gene, i.e. it is very common in the lowland districts (once highly malarial) and quite rare on the mountains.

A significant difference in the gene frequency has also been observed between the villages of the East and West coast: the latter being the one with the highest incidence.

The population samples on which all these estimates were based had the typical « Sardinian » blood group distribution.

In two villages of the West coast it was also possible to estimate the frequencies of abnormal, intermediate and normal individuals among females: they were found to be in very good agreement with those expected under the sex-linkage hypothesis and for the gene-frequency observed in males.

The possible interactions between G6PD-deficiency, thalassemia, haptoglobin type and malaria are discussed.

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Genetica. — *Indagini genetiche sulla predisposizione al favismo.* —

I. *Il problema ed i metodi. Fluttuazioni stagionali dei livelli di glucoso-6-fosfato-deidrogenasi in Sardegna. Interazione con la talassemia al livello fisiologico* (*). Nota di MATTEO ADINOLFI, LUIGI BERNINI, UGO CARCASSI, BACHISIO LATTE, ARNO G. MOTULSKY, MARCELLO SINISCALCO, presentata (**) dal Corrisp. G. MONTALENTI.

È stato di recente dimostrato che diverse forme di anemie emolitiche di tipo tossico ad incerta classificazione fino a non molti anni or sono, hanno una patogenesi comune riconoscibile in un alterato metabolismo dei globuli rossi.

Il merito di aver proposto questa teoria unitaria spetta soprattutto ad Alving ed alla sua scuola [1] che dimostrarono, con una serie di lunghe ed esaurienti ricerche, che l'azione emolitica della primachina e di altri farmaci antimalarici, da loro osservata in oltre il 10% dei negri d'America, colpisce soltanto gli individui che sono enzimopenici per la glucoso-6-fosfato-deidrogenasi (G6PD) e di conseguenza hanno un basso livello ematico di glutazione ridotto.

Childs, Zinkham e coll. [2], studiando la distribuzione dei livelli di G6PD nell'ambito sia delle popolazioni sia dei gruppi familiari, si accorsero che questo nuovo esempio di polimorfismo enzimatico della specie umana era praticamente limitato, negli Stati Uniti, alla popolazione negra e si presentava con un tipico dimorfismo sessuale nel senso che tra i maschi era possibile distinguere solo individui normali o enzimopenici mentre le femmine si distribuivano in tre distinte classi: normali, intermedie e totalmente enzimopeniche. La frequenza della enzimopenia totale era inoltre tra i maschi sensibilmente più elevata che tra le femmine.

Queste osservazioni condussero gli Autori americani alla conclusione che dovevasi trattare di un polimorfismo genetico controllato da una coppia di geni legati al sesso con dominanza incompleta, per quanto le circostanze descritte (e specialmente la variabile espressione della intensità del fenomeno enzimopenico riscontrato nelle donne intermedie) non permettessero di escludere l'ipotesi alternativa di una coppia di geni autosomici con diversa penetranza nei due sessi (eredità limitata dal sesso).

(*) Ricerche eseguite, con contributi della Rockefeller Foundation (New York) e del Consiglio Nazionale delle Ricerche all'Istituto di Genetica della Università di Napoli, sotto la guida del prof. M. Siniscalco. I dati di cui alla presente Nota sono stati comunicati alla 5ª Riunione della Associazione Genetica Italiana, svoltasi in Napoli il 22-24 marzo 1959.

(**) Nella seduta del 14 maggio 1960.

Szeinberg e Sheba (1957) [3] in Israele, Sansone e Segni (1956) [4] in Italia avevano intanto dimostrato che la stessa anomalia metabolica descritta negli individui sensibili alla primachina era presente nei soggetti di razza bianca con anamnesi positiva per il favismo. Questa è una forma di anemia emolitica molto diffusa nelle regioni del Mediterraneo, che insorge dopo ingestione di fave fresche o inalazione del polline di fava e che si presenta negli individui colpiti con manifestazioni cliniche molto variabili, le quali vanno da un lieve malessere stagionale con astenia, pallore e cefalea fino all'ittero emoglobinurico grave (per una recente messa a punto degli aspetti clinici del problema cfr. Sansone e coll. 1959 [4]).

Zinkham, Lenhard e Childs (1958 [5]) conclusero che l'ipotesi del *linkage* al sesso era sostenibile anche per spiegare l'eredità della predisposizione al favismo rilevando tuttavia che l'intensità della enzimopenia nei soggetti di razza greca da loro osservati era molto più elevata di quanto fino allora descritto tra i negri sensibili alla primachina e che pertanto poteva trattarsi di un allelomorfo diverso.

Queste conclusioni sulla eziopatogenesi del favismo furono in seguito confermate ampiamente per quanto riguarda gli aspetti biochimici da Szeinberg e Sheba in Israele; Larizza e coll.; Sartori e coll. e Carcassi e coll. in Italia [6].

Mentre però i dati degli Autori israeliani concordavano abbastanza bene con l'ipotesi genetica del *linkage* al sesso, quelli degli Autori italiani, tutti raccolti in Sardegna dove il favismo è molto diffuso, presentavano frequenti eccezioni, tanto da sembrare in accordo piuttosto con l'ipotesi di una eredità di tipo recessivo autosomico (Sartori) [7].

Le cose stavano presso a poco a questo punto quando abbiamo cominciato la presente indagine della quale riferiremo i risultati più salienti in una serie di Note in questi « Rendiconti ».

IL PROBLEMA ED I METODI.

Sulla base di quanto è stato sommariamente riassunto ci siamo proposti nella presente indagine i seguenti obbiettivi fondamentali:

a) raccolta di dati popolazionistici e familiari in Sardegna sulla distribuzione della descritta anomalia biochimica e ulteriori indagini sulla genetica della predisposizione al favismo;

b) interazione al livello fisiologico e popolazionistico con la microcitemia o talassemia che, come è noto, è anch'essa molto diffusa nella popolazione sarda;

c) eventuale conferma diretta del meccanismo della eredità legata al sesso in famiglie di fabici segreganti anche per il gene del daltonismo.

Per tale programma era necessario raccogliere dalle popolazioni naturali dati molto numerosi ed accurati, in tempo relativamente breve e in circostanze non sempre idonee per fini indagini biochimiche.

Ciò fu reso possibile solo quando Motulsky [8] mise a punto un metodo rapido ed efficiente, particolarmente adatto a determinare l'attività glucoso-6-fosfato-deidrogenasica in tali circostanze.

Questo metodo è praticamente simile a quello elaborato per le deidrogenasi da Thunberg [9]. In esso, come è noto, si prende come misura della attività enzimatica il tempo di decolorazione, in ambiente anaerobico ed a 37°C., di una quantità ottimale di bleu di metilene aggiunta alla miscela di incubazione contenente l'enzima, il substrato ed un coenzima piridinico (DPN, TPN).

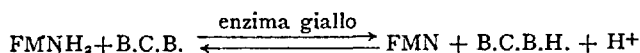
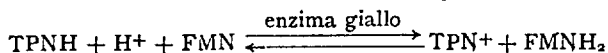
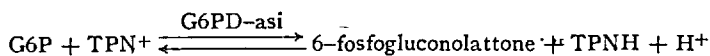
L'esperienza ha mostrato che, quando si tratta di misurare l'attività enzimatica nei maschi, questo metodo è altrettanto efficiente quanto quello spettrofotometrico (a patto che i valori dell'ematocrito non scendano al di sotto del 20%), mentre per le femmine non dà risultati attendibili a meno che non si ricorra ad opportune precauzioni, che lo rendono però troppo laborioso (tempo di decolorazione con letture molto ravvicinate ed espresso in funzione dell'ematocrito o della concentrazione dell'Hb).

Per un'esatta valutazione quantitativa dell'attività deidrogenasica nelle emazie dei soggetti in esame ci siamo serviti del metodo spettrofotometrico di Kornberg [10] modificato da Motulsky [8].

La Tabella I e la fig. 1 riassumono lo schema della reazione di Motulsky e la distribuzione dei tempi di decolorazione in gruppi di maschi normali e di enzimopenici.

TABELLA I.

Schema della reazione di Motulsky per la misura dell'attività G6PD.



B.C.B.	= bleu brillante di cresile	(colore bleu)
B.C.B.H	= » » » » ridotto	(leucoderivato incolore)
F.MN.	= flavin mononucleotide.	

L'enzimopenia a carico della G6PD non è l'unica alterazione biochimica dei globuli rossi degli individui sensibili alla primachina o predisposti al favismo. Diverse altre alterazioni sono state descritte (Tabella II), ma, per quanto ci risulta, l'enzimopenia sembra essere la più costante e deve considerarsi certamente primaria almeno rispetto alla diminuita concentrazione del glutatione ridotto ed alla sua instabilità (Tabella III).

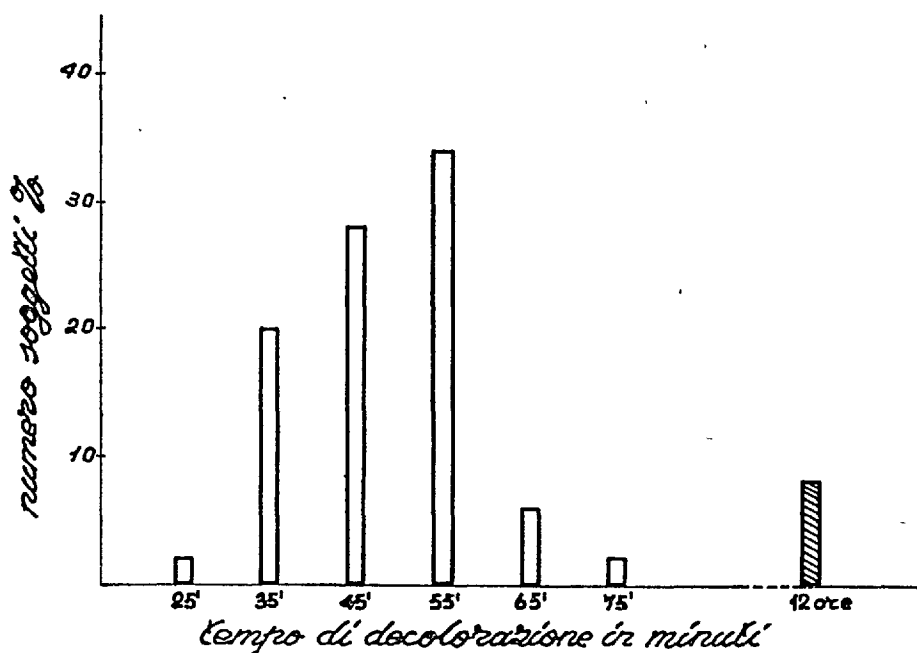


Fig. 1. - Reazione di Motulsky eseguita su 100 individui di sesso maschile.

TABELLA II.

Fondamentali alterazioni biochimiche degli eritrociti sensibili alla primachina.

- 1) Diminuzione del tasso di glutatione ridotto.
- 2) Instabilità del glutatione.
- 3) Riduzione dell'attività della G6PD
- 4) Riduzione dell'attività della glutatione-riduttasi.
- 5) Aumento dell'attività della aldolasi.
- 6) Diminuzione della attività della catalasi.

da CHILDS e ZINKHAM

TABELLA III.

1. $G-6-P + TPN^+ \xrightleftharpoons{G6PD} 6\text{-phosphogluconolactone} + TPNH + H^+$
2. $6\text{-phosphogluconolactone} \xrightarrow{\text{Lactonasi}} 6\text{-PG.}$
3. $6\text{-PG.} + TPN^+ \xrightleftharpoons{6\text{-PG.D.}} R-5\text{-P} + CO_2 + TPNH + H^+$
4. $GSSG + TPNH + H^+ \xrightarrow{GSSG \text{ rid.}} 2 \text{ GSH} + TPN^+$

da MARKS

È stato per questi motivi che abbiamo deciso di basare la nostra classificazione fenotipica su questa caratteristica dei globuli rossi e d'ora in poi indicheremo con i simboli:

Gd (+)	gli individui con attività della G6PD normale
Gd (—)	» » » » » G6PD molto bassa o assente
Gd (i)	» » » » » G6PD intermedia.

Per la raccolta dei dati si è proceduto nel modo seguente: in un certo numero di paesi di circa 3-4000 abitanti e dislocati ad altitudini variabili dal livello del mare ai 1000 metri, è stato esaminato un campione rappresentativo della popolazione scolastica maschile per determinare la frequenza dell'enzimopenia e della talassemia ⁽¹⁾.

Agli individui trovati positivi ed ai loro familiari veniva quindi eseguito un prelievo di sangue venoso per una più accurata valutazione quantitativa dell'attività enzimatica (determinata spettrofotometricamente su sangue conservato a — 20° C) e della talassemia (conta dei globuli rossi e curva di Price-Jones al contatore elettronico «Celloscope»; dosaggio della HbA₂ ed altri dati ematologici). Il plasma veniva conservato per la determinazione del fenotipo aptoglobinico ⁽²⁾ che, a parte l'interesse genetico, poteva essere un sistema rivelatore di un eventuale processo emolitico in atto.

Fluttuazioni stagionali dei livelli di G6PD.

I primi dati furono raccolti nelle provincie di Nuoro e di Cagliari nell'aprile del 1959. Si trattava di tre villaggi dislocati sulle alture del Gennargentu: Fonni, Desulo, Tonara, tra i 600 e i 1000 metri e di cinque villaggi marini: Galtelli, Orosei e Siniscola sulla costa orientale e Cabras e S. Giusta su quella occidentale.

I dati popolazionistici apparvero subito di notevole interesse perché mettevano in evidenza una notevole eterogeneità nella distribuzione della enzimopenia che poteva essere connessa con le già note differenze ecologiche e razziali che distinguono le popolazioni sarde.

I dati familiari per contro rivelarono frequenti eccezioni incompatibili con l'ipotesi del *linkage* al sesso come già era stato osservato dagli altri Autori che si sono occupati della eredità del favismo in Sardegna.

(1) I metodi adoperati per questo preliminare accertamento erano la prova di Motulsky per l'enzimopenia; la prova della resistenza degli eritrociti ad una soluzione clorosodica 0,375 % e l'esame morfologico del sangue per la talassemia. Queste prove richiedevano solo il prelievo di poche gocce di sangue al dito.

(2) Le aptoglobine, come è noto, costituiscono un tipico esempio di polimorfismo delle proteine del plasma umano controllato da una coppia di geni autosomici senza dominanza. Esse hanno la caratteristica proprietà di legare l'Hb libera nel plasma e pertanto scompaiono durante i processi emolitici (cfr. SINISCALCO e coll.; HARRIS e coll.) [11].

Appena però furono completate sul materiale raccolto, tutte le indagini diagnostiche sopra descritte, fu subito chiaro che tali eccezioni erano soltanto apparenti e dovute precisamente: 1° alla difficoltà di individuare i soggetti enzimopenici nel corso di un processo emolitico cronico; 2° alla interazione fisiologica tra talassemia e livello della G6PD negli individui colpiti da entrambe le sindromi.

Si mise infatti in evidenza che le aptoglobine del plasma erano presenti in quantità minima o erano del tutto scomparse nella maggior parte degli individui enzimopenici e che le « eccezioni » erano rappresentate sempre da individui privi del tutto o quasi di aptoglobine, oppure talassemici.

Queste circostanze e i dati pubblicati da Marks (12) sulle variazioni quantitative della G6PD in ragione inversa alla età delle emazie, ci fecero concludere che verosimilmente le eccezioni trovate potevano essere spiegate con la presenza nel sangue periferico di questi soggetti di una più elevata aliquota di globuli rossi giovani.

Questa evenienza è, come è noto, un dato di fatto per gli individui talassemici, per essere la durata di vita media dei loro globuli rossi inferiore alla norma e potrebbe verificarsi come fenomeno di compenso (rigenerazione midollare) negli individui enzimopenici che vanno incontro ad episodi emolitici cronici di una certa entità durante la stagione delle fave.

Di recente abbiamo avuto modo di provare la fondatezza di queste ipotesi, 1° ripetendo in epoche successive la determinazione dell'attività enzimatica e della concentrazione delle aptoglobine in un certo numero di individui aberranti; 2° verificando che la frequenza degli enzimopenici nell'autunno e nell'inverno è significativamente più elevata di quella osservata in primavera; 3° studiando la distribuzione dei livelli di G6PD in collettivi di talassemici e di normali.

Le figg. 2 e 3 e la Tabella IV riassumono i risultati di queste indagini.

La fig. 2 riporta le variazioni stagionali della frequenza dell'enzimopenia che risultavano evidenti in tutti i villaggi studiati ad eccezione dei soli centri a bassa frequenza, che sono quelli montani dove non viene praticata la coltura delle fave.

La fig. 3 descrive la distribuzione dei livelli di G6PD nel sangue totale di individui talassemici e normali dei due sessi. Appare qui evidente che gli individui talassemici cadono tra i plus-varianti in ciascuna classe dei fenotipi Gd.

Quest'ultimo fenomeno, che per la verità appare poco evidente per la classe degli individui Gd (—), probabilmente per via dello scarso numero di osservazioni e per la difficoltà di porre in evidenza modesti aumenti ad un così basso livello di attività enzimatica, può essere dimostrato anche più chiaramente confrontando in ciascun individuo l'attività enzimatica della popolazione eritrocitaria totale con quella della sottopopolazione di emazie giovani selezionate con il metodo della iper-resistenza alle soluzioni ipotoniche (Marks e coll. [13]).

In assenza di talassemia l'attività della G6PD, misurata nelle emazie iper-resistenti ad una soluzione clorosodica 0,37%, risulta infatti aumentata di circa il 100% rispetto ai valori della popolazione eritrocitaria totale negli individui Gd (+) e di oltre il 500% nei Gd (-). In presenza di talas-

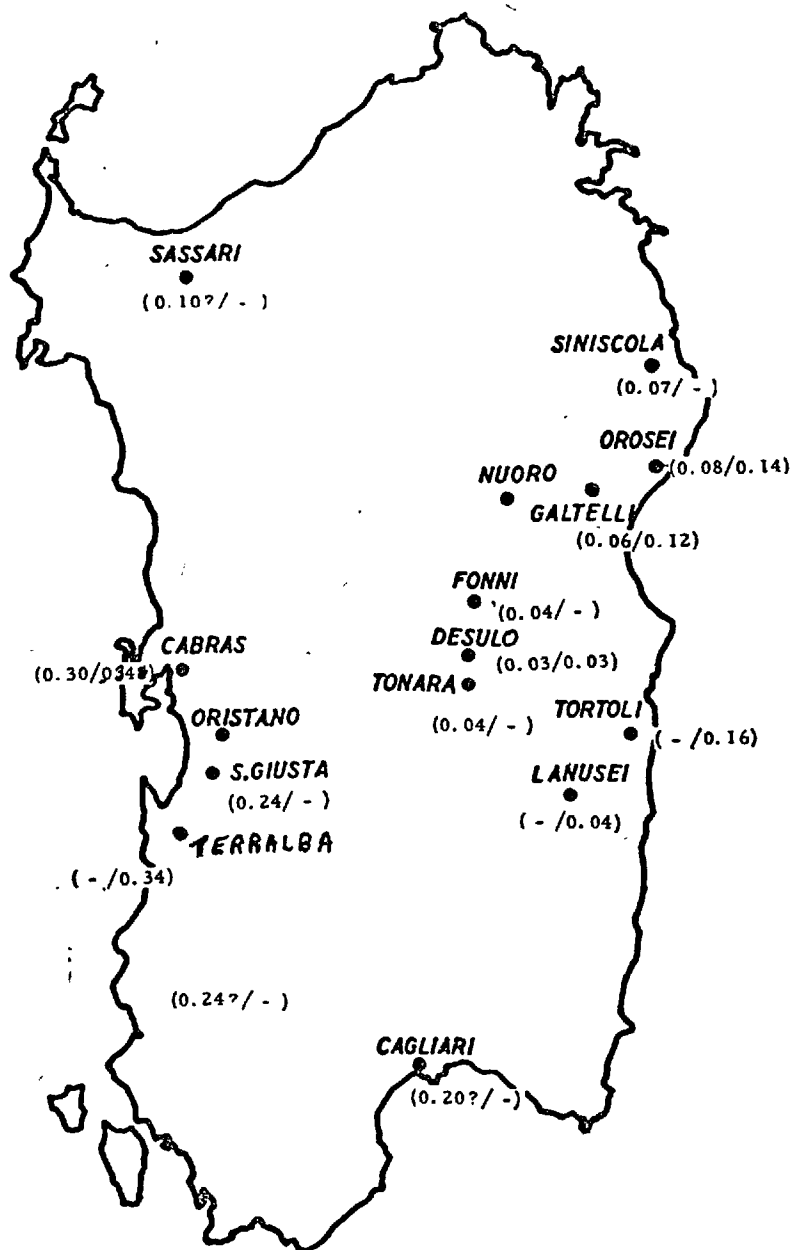


Fig. 2. - Variazioni stagionali della frequenza dell'enzimopenia in alcune località della Sardegna:

le due cifre si riferiscono ai rilevamenti fatti nei mesi di aprile e di ottobre.

INTERAZIONE TRA TALASSEMIA E LIVELLI G - 6 - P - D (103 ♂♂ e 159 ♀♀ dati familiari)

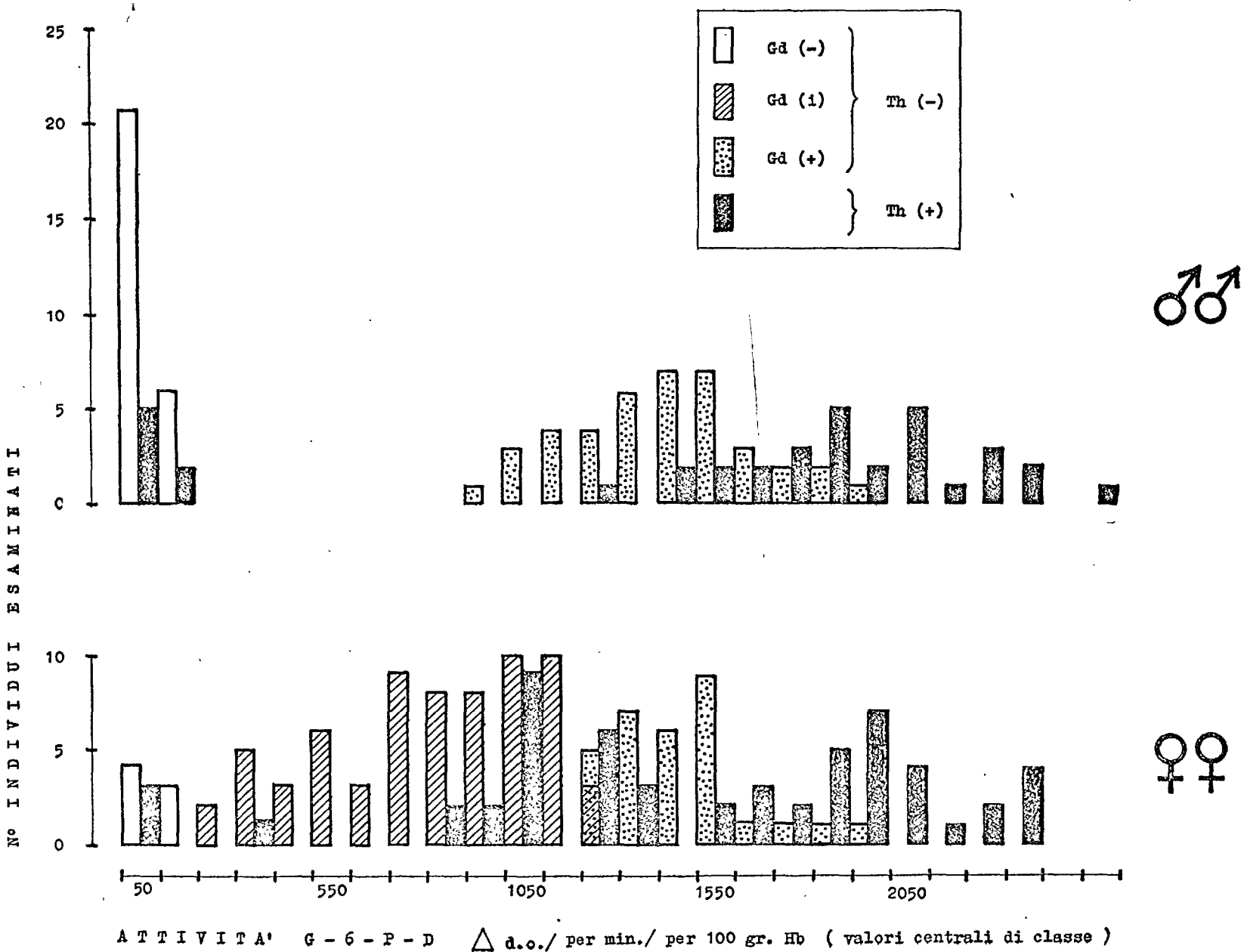
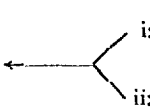
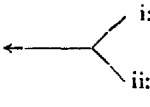
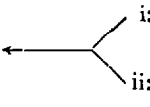


Fig. 3.

semia si hanno invece aumenti molto più modesti e precisamente del 30% per i Gd (+) e del 100% per i Gd (—) (fig. 4).

La Tabella IV riporta i valori medi dei livelli di G6PD calcolati separatamente per gli individui talassemici e non e in ciascuna classe di genotipi Gd. Sono anche riportate le soglie discriminanti tra i diversi fenotipi Gd rispettivamente per i talassemici e i non talassemici con la percentuale dell'errore di classificazione (cfr. Cavalli, 1954 [14]).

TABELLA IV.

Sesso	Fenotipo Gd	N° oss.	Medie livelli G-6-P-D	D. S.	E. S.	Limiti fiduciali medie (P=0,05)	Soglie discriminanti tra fenotipi Gd per i Th(—) e Th(+)	Errore di classificazione	
Femmine	Gd (—) i:	7	88	± 37	± 14	61-115		0,75%	
	ii:	5	99	± 124	± 55	0-207		ii: 460	0,17%
	Gd (i) i:	60	813	± 273	± 35	744-882		6,1 %	
	ii:	24	1263	± 277	± 56	1153-1373		ii: 1643	8,0 %
	Gd (+) i:	24	1496	± 170	± 35	1427-1565			
	ii:	18	1944	± 218	± 51	1844-2044			
	Maschi	Gd (—) i:	27	67	± 40	± 8	51-83		0,01%
		ii:	10	55	± 43	± 14	28-82		ii: 307
Gd (+) i:		52	1330	± 197	± 27	1277-1383			
ii:		30	1896	± 289	± 53	1792-2000			

i: non talassemici (Th—)

ii: talassemici (Th+)

Gd (—) : individui con enzimopenia totale o quasi

Gd (i) : " " " intermedia

Gd (+) : " " " livelli di enzima normali.

N. B. — Le differenze tra le medie dei talassemici e non talassemici entro ciascun gruppo Gd sono significative al livello dell'1% tranne che per i gruppi di individui Gd (—).

Per il calcolo delle soglie discriminanti e relativi errori di classificazione cfr.: CAVALLI, 1954 [14].

Allo stato attuale delle nostre conoscenze non è possibile interpretare il significato di questa interazione fisiologica tra enzimopenia e talassemia, né siamo in grado di dire se l'associazione delle due anomalie risulti vantaggiosa o meno all'individuo portatore per quanto riguarda sia l'espressività del gene talassemico sia l'entità del rischio di crisi emolitiche da ingestione

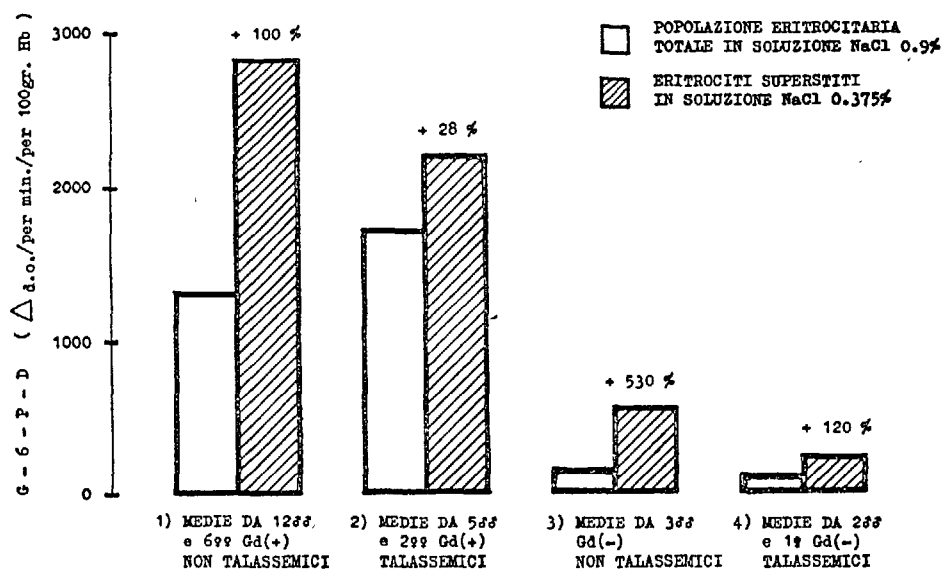


Fig. 4. - Valori medi dell'attività glucoso-6-fosfato-deidrogenasica presente in popolazioni eritrocitarie totali e in eritrociti iperesistenti.

di fave o di altri fattori tossici. Vale la pena tuttavia di sottolineare i dati riportati dalla Tabella V che sono indicativi a questo proposito pur non prestandosi ad una accurata valutazione statistica per la scarsità del numero di osservazioni. In tale tabella viene riportata l'incidenza del « favismo clinico » in 295 soggetti di ambo i sessi, di età superiore ai 50 anni, per i quali la classificazione dei fenotipi Gd e Th e la raccolta dei dati anamnestici è stata eseguita con particolare accuratezza; sembra dunque lecito poter affermare che: a) l'incidenza di episodi di favismo clinico tende in genere ad essere più elevata tra maschi e femmine Gd(—) rispetto alle femmine Gd(±); b) tra i Gd(—) sono più frequentemente colpite le femmine; c) in ciascuna classe di enzimopenici gli individui talassemici sembrano meno colpiti.

Da quanto fin qui esposto (usando i predetti criteri di orientamento per la diagnosi dei casi dubbi), possiamo pertanto concludere che, molto probabilmente, non vi è favismo senza enzimopenia e che pertanto la misura dell'attività della G6PD consente sempre di individuare la condizione di predisposizione al favismo.

Le osservazioni a cui si è accennato sulle variazioni stagionali del tasso di aptoglobine in soggetti con anamnesi negativa per il favismo clinico e tuttavia enzimopenici, permettono inoltre di confermare l'opinione già espressa da numerosi Autori (cfr. Marcolongo [15]) circa la esistenza di un « favismo minore » che forse si limita a lievi forme di anemie emolitiche a decorso stagionale per le quali non si osserva mai emoglobinuria perché la soglia di saturazione delle aptoglobine non viene superata.

TABELLA V.

Anamnesi relativa al favismo clinico.

		♂♂	♀♀	♂♂	♀♀	Totali
		Positiva		Negativa		
Gd (—)	N	13	4	37	5	59
	Th	2	1	18	5	26
Gd (i)	N	—	6	—	56	62
	Th	—	0	—	24	24
Gd (+)	N	—	—	52	24	76
	Th	—	—	30	18	48
		15	11	157	122	295

Tuttavia, considerata la modesta quota di enzimopenici che vanno incontro ad episodi acuti di favismo clinico, non si può fare a meno di pensare che probabilmente l'enzimopenia rappresenta la condizione necessaria ma non sufficiente per la insorgenza delle crisi itteroemoglobinuriche gravi.

Questi rapporti tra enzimopenia e favismo sono stati trattati da Carcassi in una recente monografia [15]).

È chiaro che per una corretta impostazione del problema genetico della predisposizione al favismo tre circostanze sono essenziali: 1° evitare la raccolta di dati durante la stagione della fioritura delle fave; 2° classificare il fenotipo Gd separatamente per gli individui talassemici e quelli normali ricorrendo eventualmente alla descritta prova della determinazione in doppio sul sangue totale e sulle emazie iper-resistenti; 3° non limitare la raccolta del materiale familiare ai soli casi più gravi selezionati attraverso i centri ospedalieri.

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ULTERIORI DATI SULLA GENETICA FORMALE E DI POPOLAZIONE DELL'ENZIMOPENIA G6PD (GLUCOSIO-6-FOSFATO DEIDROGENASI)

M. ADINOLFI, R.G. DAVIDSON, B. LATTE, P. MEERA KHAN, S. PIOMELLI
M. RATTAZZI, M. SINISCALCO

Istituto di Genetica, Facoltà di Scienze, Università, Roma

Ospedale Civile, Nuoro

Johns Hopkins Hospital, Dept. of Paediatrics, Baltimore, USA

RIASSUNTO

I dati raccolti in Sardegna negli anni scorsi ci hanno permesso di documentare definitivamente, sia attraverso l'analisi delle segregazioni familiari, sia con lo studio dei rapporti di linkage con altri geni legati al sesso, che l'enzimopenia G6PD è dovuta ad un gene dominante incompleto localizzato sul cromosoma X.

Anche a noi, tuttavia, come ad altri Autori, è capitato di osservare delle eccezioni a tale ipotesi, rappresentate prevalentemente dal reperto di madri e figlie di individui enzimopenici con livello enzimatico normale. Ciò prescindendo dalla talassemia e dai fenomeni emolitici occasionali che, com'è noto, si accompagnano sempre a livelli enzimatici più elevati. Recentemente è stata avanzata l'ipotesi che tali eccezioni, analogamente a quanto postulato da Mary Lyon nel topo, possano essere spiegate ammettendo si tratti di una variabilità somatica dovuta al fatto che solo uno dei due cromosomi X sarebbe attivo dopo le prime fasi dello sviluppo embrionale. Dai nostri dati risulta invece evidente che la variabilità dell'espressione fenotipica del gene G6PD non è innanzitutto limitata alle sole femmine eterozigoti e che verosimilmente le eccezioni risultanti debbono essere messe in rapporto con fenomeni di interazione genetica, per il momento non sempre chiaramente intelligibili.

Molte di queste eccezioni sono infatti raccolte in singole famiglie o alberi genealogici come nel caso di 4 sorelle delle quali due sono del tutto enzimopeniche con alcuni figli normali e due apparentemente normali con figli enzimopenici. Inoltre si è osservato che nella grande maggioranza dei casi i fratelli normali di maschi enzimopenici con madri apparentemente normali

hanno livelli di attività G6PD 2 o 3 deviazioni standard al disopra della media.

I dati popolazionistici hanno confermato ulteriormente la correlazione positiva tra pregressa morbidità malarica e frequenze geniche attuali. Anche la correlazione tra daltonismo di tipo Deutan e deficienza enzimatica ha ricevuto ulteriore convalida dagli ultimi dati, risultando pertanto avvalorata l'ipotesi della possibile selezione del gene per il daltonismo a causa dello stretto linkage con quello per l'enzimopenia G6PD.

Questi dati, accanto alla distribuzione non casuale dei genotipi in coupling e repulsion, ci fanno ritenere che la selezione abbia determinato l'accumulo di favorevoli combinazioni geniche (gene complexes) inibendo il crossingover.

I dati sul linkage e sullo screening delle anomalie eterocromosomiche in Sardegna, che sono presentati a parte nel corso di questa stessa Riunione, sono in buon accordo con questa ipotesi.

ABSTRACT

The segregation and linkage data collected on G6PD-deficiency in Sardinia by our group in the last 3 years, contributed the final proof of the hypothesis that this red cells abnormality is due to an incompletely dominant gene located on the X-chromosome.

However we have encountered some exceptions to the sex linkage hypothesis, as have many other investigators. These consist mainly in the occurrence of women who are normal in their enzyme activity in spite of being daughters or mothers of enzyme deficient males. These exceptions might be explained by the Mary Lyon hypothesis, based on observations in the mouse. According to the theory, somatic variation would be expected in females heterozygous for a sex-linked gene, where only one of the two X-chromosomes remains active after early differentiation.

From our data it appears however that the variable phenotypic expressions of the G6PD gene is not limited to heterozygous females alone and that the exceptions found could actually be better explained in terms of some genetical interaction for the time being not fully understood. Many of these exceptions tend in fact to be concentrated in the same pedigrees, as in the case of four sisters two of whom are totally enzyme deficient and have normal children. The two others have normal enzyme levels and are mothers of enzyme deficient males. Moreover, it has been observed that in the great majority of the cases the normal brothers of enzyme deficient males with apparent normal mothers show levels of G6PD activity 2 or 3 standard deviations above the mean.

The population data have confirmed the positive correlation between the past malarial morbidity and the gene frequencies for G6PD deficiency and thalassaemia in Sardinia. Also the positive correlation between the incidence of colourblindness of the Deutan type and of the G6PD deficiency in small Sardinian isolates has been strengthened by our recent data, lending further credit to the hypothesis that the gene for colourblindness might have been selected for by virtue of its close linkage with the gene for G6PD deficiency.

These data, together with the non-random distribution of coupling and repulsion genotypes, make possible the conclusion that selection might have favoured the accumulation of adaptive gene complexes through inhibition of crossing-over. The results of the linkage investigations and the data on some heterochromosomal aberrations found in Sardinia (and presented in another session of this meeting) seem to give further support to this hypothesis.

**OBSERVATIONS ON THE HIGH FOETAL HAEMOGLOBIN
GENE AND ITS INTERACTION WITH THE
THALASSAEMIA GENE**

BY
P. BARKHAN and M. ADINOLFI

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Observations on the high foetal haemoglobin gene and its interaction with the thalassaemia gene

P. BARKHAN AND M. ADINOLFI

From the M.R.C. Experimental Haematology Research Unit, Wright-Fleming Institute, and the Department of Haematology, St. Mary's Hospital Medical School, London

SYNOPSIS A family of mixed Indian-Portuguese ancestry is reported in which there is a hereditary persistence of foetal haemoglobin and β -chain thalassaemia.

The propositus, a 17-year-old boy, was found to have a mild haemolytic anaemia characterized by slight splenomegaly, microcytosis, numerous target cells, decreased osmotic fragility, a very high level of foetal haemoglobin (75%), and normal haemoglobin A_2 level.

Examination of 12 other members of the family showed the following: Three individuals (father, sister, and nephew) had high levels of foetal haemoglobin (25%) but without other clinical or haematological abnormalities. Two individuals (mother and sister) had the features of thalassaemia trait with increased haemoglobin A_2 and normal levels of foetal haemoglobin.

The condition in the propositus appears to be the result of heterozygosity for a gene which is responsible for the hereditary persistence of foetal haemoglobin (high F gene) combined with heterozygosity for a β -thalassaemia gene and provides further evidence for allelism of these genes.

The possible genetic basis for the high F state and β -chain thalassaemia is discussed.

Fifty to eighty per cent of the haemoglobin of newborn infants consists of foetal haemoglobin. This is gradually replaced by adult haemoglobin which constitutes the major fraction after the age of 3 months. By the end of the second year of life normal children possess less than 1% of alkali-resistant haemoglobin (White and Beaven, 1959).

It now appears that foetal haemoglobin may persist in some families in the absence of any clinical or other haematological abnormalities (Edington and Lehmann, 1955; Jacob and Raper, 1958; Went and MacIver, 1958; Herman and Conley, 1960; Kraus, Koch, and Burckett, 1961; Thompson, Mitchener, and Huisman, 1961). However, it remains uncertain whether this is the consequence of a primary defect in the genetic mechanism responsible for the switchover from foetal haemoglobin to the production of adult haemoglobin or whether it represents a compensatory response to some undetectable abnormality in the synthesis of adult haemoglobin. For convenience, the term 'high foetal' or 'F gene' (Went and MacIver, 1958) will be used in referring to this condition. The quoted studies suggest allelism of the F gene to the S and C haemoglobin

genes. Family II of Kraus *et al.* (1961) and the patients reported by Wheeler and Krevans (1961) suggest allelism also with the gene of classical thalassaemia; the possibility of an interaction of this kind was suggested by Oleson, Oleson, Livingstone, Cohen, Zuelzer, Robinson, and Neel (1959) in their study of atypical thalassaemia cases in Liberia.

In this paper we report the hereditary persistence of foetal haemoglobin in three generations of a family of mixed Indian and Portuguese descent; as far as we know this appears to be the first account of this condition in a non-Negro family. One of the members has a mild haemolytic anaemia which is believed to result from the simultaneous presence of a thalassaemia gene derived from his mother with an F haemoglobin gene derived from his father, and this lends further support for the possibility of allelism between the F gene and the classical thalassaemia gene.

MATERIALS AND METHODS

Haemoglobin, packed cell volume, red cell count, and red cell osmotic fragility were determined by standard methods (Dacie, 1956). Haemolysates were prepared and foetal haemoglobin (Singer, one-minute method) was determined as described by Lehmann and Ager (1961).

ELECTROPHORESIS OF HAEMOGLOBIN (a) On cellulose acetate paper (Oxoid) at room temperature using veronal buffer, pH 8.6, ionic strength 0.05 (duration of run approximately four hours); the strips were examined in the unstained state.

(b) On starch gel with a tris-borate discontinuous buffer system (Poulik, 1957); hydrolysed starch (Connaught Laboratories, Toronto) was used for making the gel.

(c) On a starch block at 4°C. for the determination of haemoglobin A₂, essentially as described by Kunkel, Ceppellini, Müller-Eberhard, and Wolf (1957); the Dutch potato starch (Mulder) supplied by Griffin and George, London, was found to be satisfactory for this purpose. The maize starch supplied by British Drug Houses, Poole, Dorset, was equally satisfactory.

Red cells containing foetal haemoglobin were demonstrated in peripheral blood films by the acid elution method of Kleihauer, Braun, and Betke (1957) as modified by Zipursky, Hull, White, and Israels (1959).

FAMILY STUDIES

THE PROPOSITUS This boy (Fig. 1, II.9) aged 17 years, an apprentice decorator, attended St. Mary's Hospital with the complaint of intermittent attacks of pain in the left hypochondrium which occurred, apparently spontaneously, at intervals of a few weeks. The pain was stabbing in character, remained localized to the left hypochondrium, and was sometimes associated with nausea. Apart from the pain he felt well. On physical examination he was of normal development for a boy of his age. The only abnormal physical finding was an enlarged spleen which was palpable about two finger-breadths below the left costal margin on inspiration; it was firm and somewhat tender.

The blood count was as follows: Haemoglobin 11.6 g./100 ml., red cells 5.8 m./c.mm., P.C.V. 36%, M.C.H. 20 µg., M.C.V. 66 c.µ., reticulocytes 5% (Table I). The film showed anisocytosis and numerous target cells. The leucocyte and platelet counts were within normal limits. Serum bilirubin was 0.5 mg./100 ml. and serum iron 102 µg./100 ml. Electrophoresis of the haemoglobin on cellulose-acetate paper showed a single component with a mobility between that of haemoglobin A and haemoglobin S. The pattern suggested the presence of an increased amount of foetal haemoglobin; this was confirmed by the alkali denaturation test which showed 75% alkali-resistant haemoglobin.

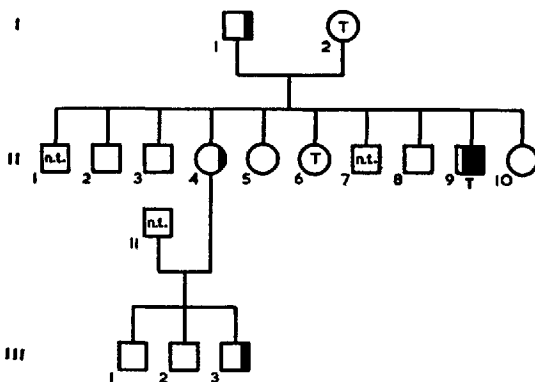


FIG. 1. The family tree. The shaded symbols indicate those individuals with high foetal haemoglobin, the degree of shading representing approximately the amount of foetal haemoglobin.

n.t. = not tested T = thalassaemia trait.

The haematological examination was repeated several times during the succeeding six months and showed a persistent mild anaemia with reticulocytosis, numerous target cells, increased foetal haemoglobin, and a normal serum iron concentration; the red cell osmotic fragility was markedly decreased (Fig. 2). The proportion of A₂ haemoglobin was within normal limits (Table I). Nucleated red cells were not seen in the peripheral blood films. At no time did he appear jaundiced. There was no evidence of blood loss.

Since the blood picture suggested a haemoglobinopathy, the other members of the family were examined.

THE FAMILY Thirteen members were available for study (Fig. 1). All were born in India, and came to England nine years ago. They look typically Indian. Both parents have Indian and Portuguese ancestry, the Portuguese element having been introduced from Goa a few generations earlier.

The parents were clinically well but had some abnormal blood findings (Table I). The father (I.1) had 23% alkali-resistant haemoglobin but no other abnormal haematological findings; the red cells appeared normocytic and normochromic and their osmotic fragility was within normal limits (Fig. 2). The film of the mother's

TABLE I

HAEMATOLOGICAL FINDINGS IN AFFECTED MEMBERS OF THE FAMILY

Pedigree	Sex	Age (yr.)	Hb (g./100 ml.)	P.C.V. (%)	Red Cells (m./c. mm.)	M.C.H.C. (%)	M.C.H. (µg.)	M.C.V. (c.µ.)	Reticulocytes (%)	Foetal Hb (%)	A ₂ Hb (%)	Target Cells	Osmotic Fragility	Serum Iron (µg./100 ml.)
I.1	M	59	15.5	48	5.4	32	28	94	1.5	25	1.3	Nil	Normal	76
I.2	F	54	13.6	44	5.7	31	24	77	2.0	<1	4.5	Some	Decreased	150
II.4	F	29	11.9	37	4.1	32	28	90	1.2	28	1.5	Nil	Normal	82
II.6	F	26	8.8	28	3.0	31	29	93	2.5	<1	4.5	Nil	Not done	Not done
II.9	M	17	11.2	36	5.7	31	20	63	5.8	75	2.2	Many	Decreased	102
III.3	M	12	11.8	35	4.1	33	29	85	2.0	25	1.6	Nil	Normal	100

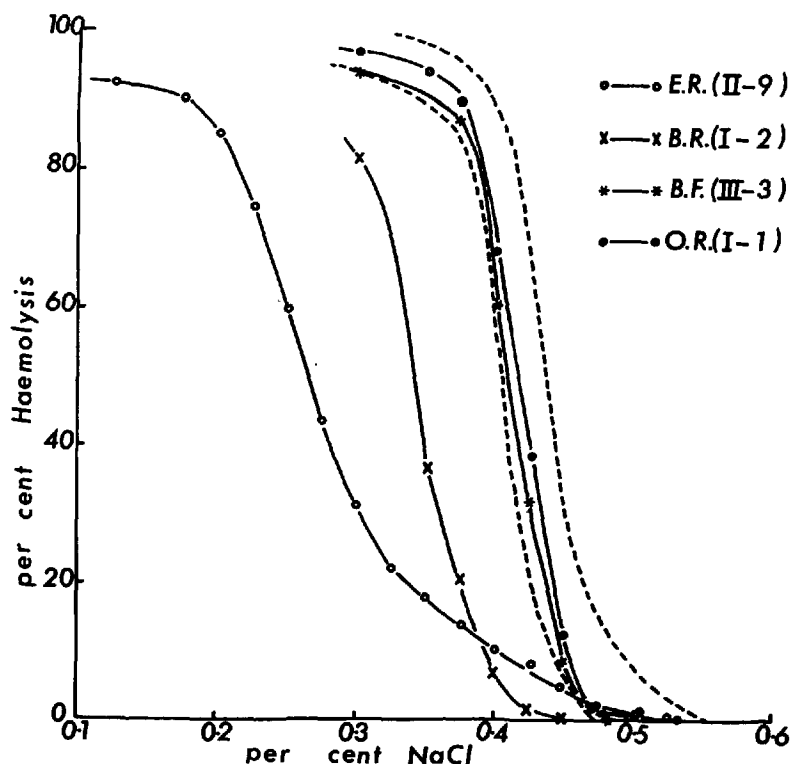


FIG. 2. Red cell osmotic fragility curves from I.1, I.2, II.9, and III.3. The area enclosed by the interrupted lines is the normal range.

(I.2) blood showed an increased number of target cells; the M.C.H.C. was only slightly reduced, but the M.C.H. was low; the osmotic fragility was decreased (Fig. 2); the serum iron and bilirubin concentrations were normal; the haemoglobin A_2 fraction was increased to 4.5% (Table I). The A_2 component was easily visible after electrophoresis of the mother's haemolysate on cellulose acetate paper.

An older sister (II.4) and one of her sons (III.3) were found to have increased amounts of foetal haemoglobin (25%) but their blood pictures appeared to be otherwise normal (Table I); the osmotic fragility of the blood of III.3 was within normal limits. Another sister (II.6) who was about 36 weeks pregnant at the time of examination had a moderate anaemia. Some target cells were present. Foetal haemoglobin was within normal limits. However, since she showed a haemoglobin A_2 value of 4.5% (Table I) which is definitely raised, she was presumed to have the thalassaemia trait. The blood films of the remaining members of the family appeared normal and their haematological values were within normal limits. Two brothers of the propositus were in India and were not studied. They were said to be well.

STARCH GEL AND STARCH BLOCK ELECTROPHORESIS PATTERNS The haemoglobin patterns, on starch gel, of the parents (I.1 and I.2), the propositus (II.9), his sister II.4, and his nephew (III.3) were as follows:—

The major haemoglobin band of the propositus moved somewhat more slowly than that of the other members of the family and appeared to have a similar mobility to that of cord blood but unlike cord blood it showed distinct A_2 and protein X components. Starch block electrophoresis of the haemolysates from II.9 and III.3 showed a distinct separation between the haemoglobin A and F components, the latter constituting in the case of II.9 the major component (Fig. 3).

CELLULAR DISTRIBUTION OF FOETAL HAEMOGLOBIN Examination of the treated films showed that all the red cells contained some foetal haemoglobin. In the case of the propositus there appeared to be a fairly uniform distribution (Fig. 4b); his sister (II.4) and his nephew (III.3) showed a similar distribution. The father's cells, however, varied in their foetal haemoglobin content; all appeared positive but there was a gradation from cells which stained relatively palely to those which were deeply stained (Fig. 4c). The red cells of the mother reacted like normal cells but many showed small inclusion bodies whose nature is unknown (Fig. 4d).

INTERPRETATION OF THE HAEMATOLOGICAL FINDINGS

THE PROPOSITUS The clinical and haematological findings are suggestive of thalassaemia minor but the

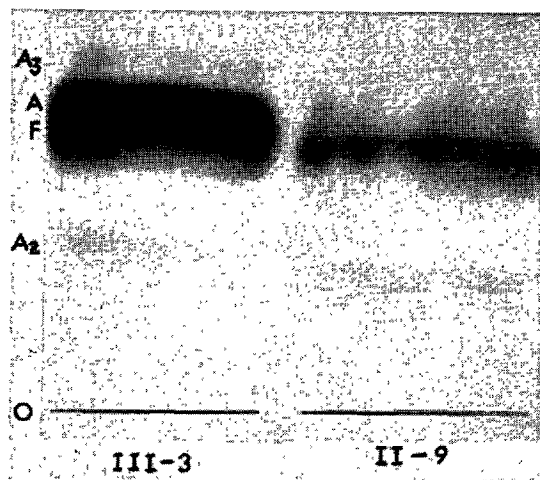


FIG. 3. Starch block electrophoretic patterns of haemolysates from III.3 and II.9 showing the separation of haemoglobins A, F, and A₂. O = origin.

very high percentage of foetal haemoglobin appears to exclude this diagnosis. Thus, Beaven, Ellis, and White (1961), in a study of blood samples from 131 cases of thalassaemia minor, found that the percentage of foetal haemoglobin never exceeded 8 and was within normal limits in two-thirds of the cases. Very high levels of foetal haemoglobin are found in thalassaemia major but the clinical and haematological findings considered together make it most unlikely that the *propositus* has thalassaemia major.

THE PARENTS Apart from the large amount of foetal haemoglobin, the father is haematologically normal. This picture conforms with those cases reported as having a hereditary persistence of foetal haemoglobin. The mother has the haematological features of classical thalassaemia trait.

The most likely interpretation of the findings in the *propositus* is that he has acquired a thalassaemia gene from his mother and a 'high foetal' haemoglobin gene from his father. He is therefore carrying two abnormal genes which have interacted to produce a mild haemolytic anaemia.

The other two members (II.4 and III.3) with high foetal haemoglobins presumably possess a 'high foetal' gene and a normal gene.

DISCUSSION

The hereditary persistence of foetal haemoglobin has been described only in Negro families, and this report is the first account of this condition in a non-

Negro family, and the third report of the apparent interaction with the gene of classical thalassaemia.

The persistent alkali-resistant haemoglobin appears to be identical with the foetal haemoglobin of cord blood. Bradley, Brawner, and Conley (1961) found it indistinguishable from foetal haemoglobin by electrophoresis in various buffers and by elution from chromatography columns. Like foetal haemoglobin it could be separated from adult haemoglobin by electrophoresis on agar in a citrate buffer at pH 6.0 where it showed the same mobility as the major component from cord blood. The kinetics of its denaturation by alkali and its absorption curve in the ultra-violet spectrum were similar to those of foetal haemoglobin. The amino-acid sequence of the globin moiety appears to be similar to that of foetal haemoglobin (Thompson *et al.*, 1961).

A further similarity with foetal haemoglobin is in the acid elution test; the red cells from the 'high foetal' individuals were found to behave like those from normal cord blood. It would, therefore, seem that all the red cells contain foetal haemoglobin in greater or lesser degree. This makes it seem unlikely that two different clones are involved, the one producing cells with only adult haemoglobin and the other producing cells with only foetal haemoglobin. The *propositus* has therefore inherited two genes, each of which presumably impairs or suppresses the formation of haemoglobin A and allows its replacement with foetal haemoglobin. It is interesting that he has approximately three times as much foetal haemoglobin as the AF individuals, a finding similar to that reported by Kraus *et al.* (1961) and Wheeler and Krevans (1961).

That the basis for thalassaemia may be a structurally abnormal but electrophoretically normal haemoglobin synthesized at a subnormal rate was suggested by Pauling (1955) and Itano (1957). Ingram and Stretton (1959) then proposed that thalassaemia is a mutation either of an α or of a β haemoglobin gene; they suggested that the gene of classical thalassaemia which characteristically is associated with a raised A₂ value was at the Hb β^A locus. On this hypothesis the mother of the *propositus* has a β -chain thalassaemia. Direct demonstration of a molecular alteration involving an amino-acid substitution is still awaited. It is possible, however, that the various thalassaemic genes only suppress the synthesis of α or β chains without leading to the formation of an abnormal haemoglobin. This block may operate at different points in the synthetic pathway depending on the particular gene involved and may secondarily affect the synthesis of γ chains (foetal haemoglobin) accordingly. It is generally assumed that there is a reduced rate of synthesis of

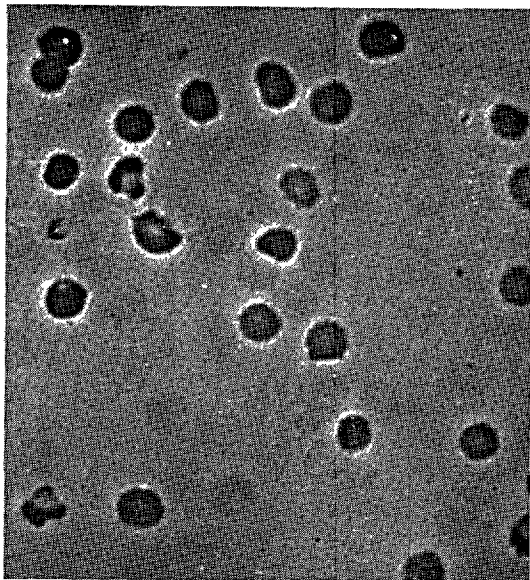


FIG. 4a

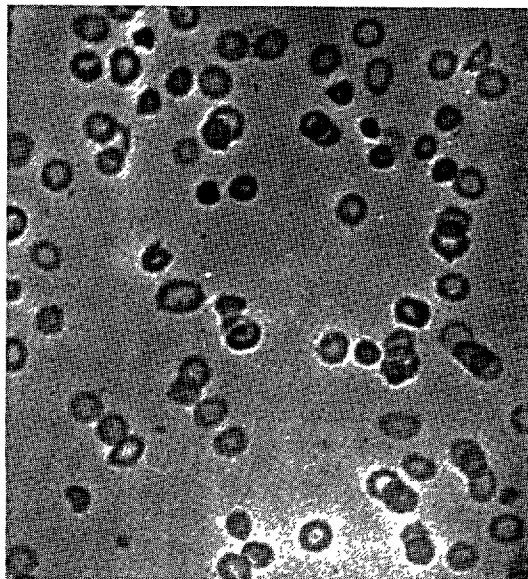


FIG. 4b

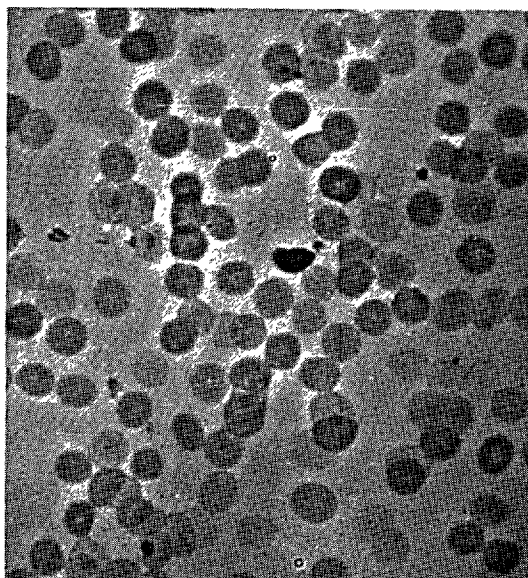


FIG. 4c

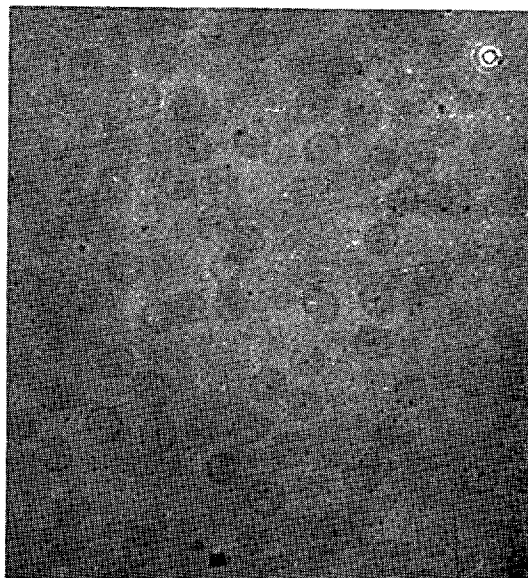


FIG. 4d

FIG. 4. *Appearance of red cells after acid elution (phase-contrast microscopy). Cells containing foetal haemoglobin stand out darkly. Normal adult cells appear as ghosts. (a) Mixture of cord and adult blood, (b) 11.9, (c) 1.1, (d) 1.2.*

adult haemoglobin in classical thalassaemia but it remains to be established whether this is due primarily to an abnormality of haem synthesis or an abnormality of globin synthesis, or of both, or whether a failure in one determines the abnormal production of the other.

On the basis of the hypothesis and haemoglobin symbols proposed by Ingram and Stretton (1959) we may write the genotype of the affected members of our family as follows: The father $\text{Hb}_{\alpha}^{\text{A}}/\text{Hb}_{\alpha}^{\text{A}}$, $\text{Hb}_{\beta}^{\text{A}}/\text{Hb}_{\beta}^{\text{x}}$; the mother $\text{Hb}_{\alpha}^{\text{A}}/\text{Hb}_{\alpha}^{\text{A}}$, $\text{Hb}_{\beta}^{\text{A}}/\text{Hb}_{\beta}^{\text{Th}}$; the propositus, $\text{Hb}_{\alpha}^{\text{A}}/\text{Hb}_{\alpha}^{\text{A}}$, $\text{Hb}_{\beta}^{\text{x}}/\text{Hb}_{\beta}^{\text{Th}}$, where x is the gene responsible for the persistence of foetal haemoglobin and Th the gene for classical thalassaemia. The genotypes of the other two members (II.4 and III.3) with just the 'high F' gene would be the same as that of the father. If these genotypes are correct it follows that the haemoglobin A of the propositus is abnormal since both genes for the $\text{Hb}_{\beta}^{\text{A}}$ locus are abnormal. The fact that his haemoglobin A component appears normal electrophoretically is still consistent with this interpretation since the molecular abnormality may be electrophoretically 'silent'. A detailed study of the amino-acid sequence of his haemoglobin A might give further helpful information.

The basic assumption of these interpretations is that the 'high F' gene and the thalassaemia gene are allelic but we have no conclusive proof of this since the propositus has not yet produced any children. A further assumption is that the persistence of foetal haemoglobin is a consequence of a genetic defect in the production of the β chains of A haemoglobin and not due to a primary activity of a specific 'high F' structural gene; this abnormality in some way prevents the switchover from γ chain (of F haemoglobin) production to normal β chain production. Wheeler and Krevans (1961) have reported that a child apparently homozygous for the high F gene possessed no detectable A or A_2 haemoglobin and they consider that this absence of both β and δ chains favours the concept of the high F gene as resulting from mutation at a controller locus. The findings in this child have led Neel (1961) to a similar concept. The terms 'controller', 'regulator', or 'operator' gene were suggested by Jacob and Monod (1961) and would appear to be similar to the 'tap' genes of Freese (1958). Ingram and Stretton (1959) considered the hypothesis of 'tap' gene mutations as an alternative to the 'substitution' hypothesis but felt the latter to be more tenable. As yet, there is no reported evidence for the substitution hypothesis despite what

must be, according to Neel (1961), a brisk search in several laboratories, and he feels that this lends indirect support to the 'tap' hypothesis. Motulsky (1961) has put forward somewhat similar views. He postulates that the high haemoglobin F condition represents a mutation of a switching gene which exerts reciprocal control over the formation of γ and β chains. During foetal life this gene normally suppresses β chain formation. After birth, the identical gene suppresses γ chain formation but allows β chain production. A mutation of the switching gene (high F gene) causes failure of the normal switchover from γ to β chains. He considered that the normal to low haemoglobin A_2 levels in the high F syndrome and the low haemoglobin A_2 levels in normal neonatal bloods suggests that the switching gene also controls the δ locus. This mutant switching gene and the β -thalassaemia gene are presumably linked or allelic to the normal β chain locus.

If the regulator or switchover gene concept is the correct one, then there is no need to postulate a β chain mutation as the basis of the high F syndrome. The genetic apparatus for producing normal β chains must then be presumed to be present but functionally suppressed by the mutant regulator gene and no abnormal haemoglobin would be expected. However, assuming the validity of this hypothesis, it is not clear why the interaction of the mutant switchover gene and β -thalassaemia gene should result in an even greater production of F haemoglobin since the heterozygous state of the latter, unlike the heterozygous state of the former, is usually not associated with increased levels of F haemoglobin. But, on the other hand, homozygous β thalassaemia (thalassaemia major) is usually associated with a greatly increased production of F haemoglobin which suggests a close relationship amongst these genes.

While the individuals with the 'high F' trait (I.1 and III.3) appear to have a normal red cell osmotic fragility, and the mother of the propositus has a moderately reduced osmotic fragility, the propositus has a marked reduction in osmotic fragility. It would seem that though the 'high F' gene by itself does not produce any abnormality in red cell osmotic fragility, its association with a β -thalassaemia gene enhances the osmotic resistance found with the thalassaemia gene alone. The explanation of these findings is unknown.

A further finding for which no adequate explanation can be given is the normal haemoglobin A_2 value in the propositus. Having acquired the gene of classical thalassaemia, which is characteristically associated with a high A_2 value, he might be expected to have had a raised A_2 component. However, many

patients with thalassaemia major (homozygous β -gene thalassaemia) also show normal A_2 values. Kraus *et al.* (1961) also observed a normal A_2 value in their high F-thalassaemia patient. They suggest that the A_2 value should be related only to the amount of A haemoglobin. If this is done then the A_2 percentage is markedly raised in these individuals.

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Evidence that 'Normal Incomplete Cold Antibody' is not a Gamma-globulin

DACIE¹ discovered that if normal human red cells were left at 0° C with their own fresh serum and afterwards washed in warm saline, they could be agglutinated by anti-human globulin serum, and he postulated that serum normally contained an incomplete antibody reacting at 0° C with human red cells. Later, Dacie, Crookston and Christenson² produced evidence that the positive anti-globulin reactions were not due to the direct detection of the antibody, but to a reaction with complement which was evidently bound to the cells at 0° C and was not removed by the subsequent washing.

Crawford, Cutbush and Mollison³ showed that normal incomplete cold antibody had anti-H specificity. Thus the strongest reactions were obtained by incubating group AB serum with group O red cells, and the reactions could be inhibited by the addition to the serum of H-containing saliva.

It has been shown that normal incomplete cold antibody is present in the serum of new-born infants^{4,5}. Since it is believed that the only antibodies present in cord serum are those which have been derived from the mother by placental transfer, and since the only γ -globulin transferred is 7S γ -globulin (for review, see ref. 6), these observations might suggest that normal incomplete cold antibody is a 7S γ -globulin, but this would be surprising since natural antibodies are, at least usually, 19S γ -globulin.

The problem was further investigated in the following way: samples of normal adult serum were fractionated by DEAE-cellulose chromatography⁷ using a modification described elsewhere⁸ and the fractions tested for the presence of normal incomplete cold antibody; in testing fractions, in order to overcome the difficulty of having to supply complement without normal incomplete cold antibody, horse serum was added as a source of complement to the fractions, and the cells were finally tested with immunocoagulatin serum to see if complement had been bound⁹. The normal incomplete cold antibody was recovered in the fractions eluted with 0.10-0.12 M phos-

Table 1

Initials	γ -Globulins (% normal)*			A B O blood group	Anti-A	Anti-B	Anti-I	Titre of normal incomplete cold anti- body (re- ciprocals)
	7S γ	γ_{1A}	γ_{1M}					
S.S.	0.8	0.5	17	O	nil	nil	nil	64
W.T.	2	0.2	1	B	nil	—	nil	64
C.V.†	11	3	6	A	—	nil	nil	16

* Concentration expressed as percentage of standard normal sera.

† This sample had been stored for almost three years; the other two had been stored for only a few weeks.

phate buffers and was not present in the 0.02 M fraction, in which the bulk of the 7S γ -globulin is eluted.

Because normal incomplete cold antibody is thus apparently not a 7S γ -globulin, and because it is present in cord serum, the possibility must be considered that normal incomplete cold antibody is not a member of the γ -globulin family at all. Two further pieces of evidence support this view. First, we have found that red cells sensitized with normal incomplete cold antibody are not agglutinated either by potent anti-7S γ -globulin or by anti-19S γ - (γ_{1M} -) globulin sera. Secondly, we have found that normal incomplete cold antibody is present in approximately normal amounts in the serum of patients with hypogammaglobulinæmia; thus it was present in samples from 10 out of 11 patients, even though some of these samples had been stored at -20° for some years. Table 1 shows three examples; in all three, concentrations of 7S γ -, γ_{1M} - and γ_{1A} -globulin were very low, and none of the samples contained iso-agglutinins or the common cold auto-agglutinin anti-I; but all contained normal amounts of normal incomplete cold antibody.

Normal incomplete cold antibody differs in various ways from classical antibodies; thus we have found that it is relatively heat-labile, being destroyed in 30 min at 63° C, that it is absorbed by zymosan (as previously noted by Dr. F. Stratton, personal communication), and that it cross-reacts with certain bacteria. In these ways, normal incomplete cold antibody resembles properdin, and incidentally properdin is present in cord serum¹⁰ and in the serum of patients with hypogammaglobulinæmia¹¹. However, we have found that normal incomplete cold antibody and properdin differ in many respects; for example, whereas normal incomplete cold antibody is best absorbed by red cells at 0° C and is absorbed by zymosan just as well at 0° C as at 37° C, properdin is absorbed by zymosan only at 17° C or higher; again, whereas a wide range of bacteria absorb properdin¹², only a few bacteria (for example, *E. coli* C₃) absorb normal incomplete cold antibody.

We thank the Medical Research Council's Working Party on Hypogammaglobulinæmia for samples of serum,

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M. ADINOLFI

CHRISTINE DANIELS

P. L. MOLLISON

Medical Research Council,
Experimental Haematology Research Unit,
Wright-Fleming Institute of Microbiology,
St. Mary's Hospital Medical School,
Paddington, London, W.2.

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**CLASSIFICATION OF BLOOD-GROUP ANTIBODIES AS β_2 M
OR GAMMA GLOBULIN**

BY
M. ADINOLFI, MARGARET J. POLLEY, DENISE A. HUNTER
and P. L. MOLLISON

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Classification of Blood-Group Antibodies as β_2 M or Gamma Globulin

M. ADINOLFI, MARGARET J. POLLEY, DENISE A. HUNTER AND P. L. MOLLISON

Medical Research Council's Experimental Haematology Research Unit, Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2

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Summary. Thirty selected blood-group antibodies (excluding anti-A and anti-B) have been classified as β_2 M (19S γ) globulin, γ (7S γ) globulin or mixtures, using the following three methods: fractionation on a DEAE-cellulose column; indirect anti-globulin tests, using specific anti- β_2 M-globulin and anti- γ -globulin sera; and treatment with 2-mercapto-ethanol. With only minor exceptions, results obtained with the three methods were in agreement.

Most blood-group antibodies within the Le, MNSs and P systems appear to be 'naturally occurring' and these were found to be β_2 M globulin. Blood-group antibodies within the Rh, K and Jk systems, which had arisen after an antigenic stimulus, were usually γ globulin but were occasionally β_2 M globulin.

Antibodies composed of β_2 M globulin usually behave as agglutinins but may behave as incomplete antibodies (e.g. some examples of anti-Jk^a); conversely, antibodies composed of γ globulin usually behave as incomplete antibodies but may behave as agglutinins (e.g. an example of anti-M).

The ability to bind complement seems to be related more to the blood-group specificity of the particular antibody than to its molecular size. For example, anti-Jk^a, when composed either of γ or β_2 M globulin, seems invariably to bind complement, whereas potent anti-M or anti-Rh, whether composed of γ or β_2 M globulin, do not bind complement.

INTRODUCTION

Several different terms are in use to describe the various proteins of which antibodies are composed. In the present paper the terminology of Grabar and Burtin (1960) has been followed. Thus the protein of which most antibodies are composed is described as γ globulin, rather than 7S γ globulin and the macroglobulin (19S γ globulin) of which some antibodies are composed is described as β_2 M globulin.

The use of an ion-exchange column to separate plasma proteins (Peterson and Sober, 1956; Sober, Gutter, Wyckoff and Peterson, 1956) has proved particularly valuable in separating γ globulin from β_2 M globulin. Thus, if a fraction containing γ and some β globulin is first separated from whole serum by an electrophoretic technique and is subsequently subfractionated by anion-exchange DEAE-cellulose chromatography using phosphate buffer pH 8 of increasing molarity (from 0.02 to 0.2M), a large part of γ globulin is recovered in the early fractions while the β_2 M globulin is present only in the later fractions (Fahey and Horbett, 1959). Whole serum can be fractionated in the same way to discover whether particular blood-group antibodies are associated with γ or

β_2 M globulin (Abelson and Rawson, 1959; Kochwa, Rosenfield, Tallal and Wasserman, 1961).

'Incomplete' blood-group antibodies can also be classified as γ globulin or β_2 M globulin by using the indirect antiglobulin test and employing specific anti- γ and anti- β_2 M globulin sera (Stratton, 1961; Polley, Mollison and Soothill, 1962).

A further useful aid to classification is to treat antibody-containing sera with a sulphhydryl compound, which results in the dissociation of macroglobulins and thus inactivates β_2 M globulin antibodies (Deutsch and Morton, 1957; Grubb and Swahn, 1958).

The object of the present work was to apply these three different methods to human sera containing various blood-group antibodies and thus to classify the antibodies as γ or β_2 M globulin and to relate these findings to the serological characteristics of the particular antibodies.

MATERIALS AND METHODS

DONORS. Thirty donors were selected to provide a variety of blood-group antibodies. For convenience the sera have been divided into two main classes: those containing blood-group antibodies belonging to the Le, MNSs and P systems, together with isolated examples of antibodies of the ABO and Ii systems. These various blood-group antibodies are considered together because in most cases they appear to be 'naturally occurring'; that is to say, they are found in subjects who have received no recognizable antigenic stimulus. The results of studying examples of anti-A and anti-B are not included in the present paper but will be reported separately. Antibodies belonging to the Rh, K and Jk systems have been considered together because they are usually found in subjects who have received a blood transfusion or have given birth to an infant affected with haemolytic disease of the newborn and have in one or other of these two ways received an appropriate antigenic stimulus. A single example of serum from a patient with syphilitic paroxysmal cold haemoglobinuria was also studied.

Antibodies within the Le, MNSs and P Systems (with Single Examples of Anti-O(H) and Anti-I)

Anti-Le^a. Nine examples of anti-Le^a were studied (cases 1-9). Of these, eight were found in subjects who had not previously been transfused; the remaining subject (case No. 9) was a man who within the previous few months had received at least two transfusions of Le(a+) blood and several test injections of Le(a+) red cells; other details about this patient were reported by Cutbush, Giblett and Mollison (1957). Of the eight subjects who had not previously been transfused, seven were women, all but one (case No. 4) of whom had had one or more pregnancies.

The male subject (case No. 8) who had not been transfused at the time when anti-Le^a was first detected in his serum, was given a test injection of Le(a+) red cells preceding a cardiac operation, during which he was transfused with blood from many Le(a+) donors. A sample of serum taken 2 weeks after the injection of Le(a+) red cells, at a time when the titre of anti-Le^a had risen from 4 to 64, was used in the present investigation.

All the examples of anti-Le^a agglutinated Le(a+) red cells in saline at 20°, although the reactions of some samples, particularly from case No. 9, were very weak. All the sera gave a positive indirect antiglobulin test with Le(a+) red cells at 37°.

Anti-M. Two examples of anti-M were tested; one came from a woman (case No. 10) who had not previously been transfused but 28-36 years previously had undergone four normal pregnancies. This patient's serum agglutinated red cells strongly at 20° but only

very weakly at 34° and not at all at 37°. The serum did not sensitize red cells to an anti-globulin serum. Other details of this case were published by Cutbush and Mollison (1958).

The other example of anti-M came from a woman (case No. 11) who had not been transfused but had had three pregnancies, the last of which resulted in the birth of twins affected with haemolytic disease of the newborn due to anti-M. Details of the case were published by Stone and Marsh (1959) who found that the patient's serum agglutinated M-positive cells just as strongly at 37° as at lower temperatures.

Anti-S. Of the two examples of anti-S, the first came from a woman (case No. 12) who had had seven previous pregnancies and had had blood transfusions on three occasions. The anti-S was found only after the third blood transfusion and was not present in the earlier samples of serum.

The second example of anti-S (case No. 13) came from a patient who had had a hysterectomy and who had been deliberately immunized with S-positive red cells. The serum agglutinated red cells suspended in saline.

Anti-s. The example of anti-s came from a patient described by O'Riordan and Cann (1959). The patient (case No. 14) had not been transfused and the antibody was discovered during her third pregnancy, which resulted in the birth of a normal infant. The patient's serum agglutinated red cells suspended in saline. The reactions were as strong at 37° as at 19°. The reactions were not significantly enhanced by using enzyme-treated cells, or by suspending the cells in albumin.

Anti-P₁. Of the two samples of anti-P₁, the first came from a woman (case No. 15) who had never been pregnant and had not been transfused. The other (case No. 16) came from a man who had not previously been transfused. Both sera agglutinated P₁-positive red cells suspended in saline at temperatures up to about 30°. At 37° serum from case No. 15 sensitized P₁-positive red cells to an antiglobulin serum. Serum from case No. 16 originally sensitized red cells to an antiglobulin serum but the sample available in the present work has been stored for some years and was virtually unreactive at 37° in all tests.

Anti-P + P₁. Of the two samples of anti-P + P₁ (anti-Tj^a), the first (case No. 17) came from a woman who was pregnant for the first time. She had never been transfused with red cells but 5 years previously she had received a transfusion of plasma, some of which presumably came from P₁-positive donors. This patient gave birth to an infant of genotype P₂p. The mother's serum agglutinated red cells suspended in saline and in the presence of complement produced brisk haemolysis.

The serum obtained from her infant agglutinated P₁-positive red cells suspended in saline but it did not agglutinate P-negative red cells suspended in saline. However, it did react weakly at 4° with P-negative red cells which had been enzyme-treated (personal communication from Drs. R. R. Race and Ruth Sanger).

The other example of anti-P + P₁ came from a woman (case No. 18) who had never been transfused and had not been pregnant. The characteristics of this antibody were very similar to those of case No. 17.

Anti-O(H). The only antibody of the ABO system investigated in the present work was an example of anti-O(H) found in a woman (case No. 19) who had had four normal pregnancies and had never been transfused. The serum agglutinated group O red cells strongly at temperatures up to about 30° and was not inhibited by H substance; at 37° the serum sensitized group O red cells to an antiglobulin serum; other details have been published previously (Mollison, 1959).

Anti-I. The single example of anti-I (case No. 20) came from the normal donor described by Jenkins, Marsh, Noades, Tippet, Sanger and Race (1960). The serum strongly agglutinated red cells at temperatures up to 30°, but in tests carried out strictly at 37° with cells and serum warmed to this temperature before being mixed together, the serum did not agglutinate cells but sensitized them to agglutination by an antiglobulin serum.

Antibodies within the Rh, K and Jk Systems (with a Single Example of Serum from a Patient with Syphilitic Cold Haemoglobinuria)

Anti-D. Three examples were tested; all came from women who had been immunized by pregnancy alone and had given birth to infants affected with haemolytic disease of the newborn. One of the sera (case No. 21) agglutinated cells suspended in saline and when diluted to a point at which it would no longer produce agglutination, it gave a positive indirect antiglobulin test. The other two sera (cases Nos. 22 and 23) failed to agglutinate Rh-positive cells suspended in saline but strongly sensitized them to agglutination by an antiglobulin serum. Serum from case No. 23 was selected because it was the only one, out of about twenty examples of incomplete anti-Rh, the activity of which was diminished by treatment with 2-mercapto-ethanol.

Anti-c. Of the two examples tested, one came from a woman (case No. 24) who had originally been immunized by transfusion and had subsequently given birth to infants affected with haemolytic disease of the newborn. The other example came from a woman (case No. 25) who had previously been pregnant and had also received many blood transfusions. Both examples of anti-c were 'incomplete'.

Anti-E. The single example tested came from a patient (case No. 26) with chronic lymphatic leukaemia who had received many blood transfusions. The serum agglutinated red cells suspended in saline and no incomplete antibody could be demonstrated.

Anti-Jk^a. One example came from case No. 26 referred to above and the other from a subject (case No. 27) who had received many injections of red cells.

Anti-K. Two examples were tested: one came from a woman (case No. 28) who had never been pregnant but had received a large number of blood transfusions and the other came from a woman (case No. 29) who had been pregnant with one K-positive infant after receiving a transfusion of K-positive blood.

Serum from a Patient with Syphilitic Cold Haemoglobinuria. Serum from a patient with syphilitic paroxysmal cold haemoglobinuria (case No. 30) was tested. When fresh serum from this patient was mixed with normal group O red cells at temperatures in the range 0° to 18°, antibody was bound as shown by the fact that the red cells, after washing, were agglutinated by an antiglobulin serum. When the mixtures were left at 18°, the red cells were only very weakly sensitized but at 0° the cells were very strongly sensitized and on subsequent incubation at 37°, haemolysis occurred. The method of titrating the serum is described below.

ION-EXCHANGE CHROMATOGRAPHY. The method used was a minor modification of that described by Levy and Sober (1960). The diethylaminoethyl-cellulose anion exchanger used was Whatman's powder DE 50.

The material was treated with N NaOH, then washed with a large volume of distilled water followed by 0.02 M NaH₂PO₄ solution. The column was then packed and equilibrated with 0.02 M phosphate buffer pH 8.1. About 2 g. of DEAE-cellulose was used to prepare a column for 0.5–1.0 ml. of serum.

The column was mounted above a fraction-collector and separate 5 ml. amounts were

collected in tubes. Only two buffers were used, the first at 0.02 M (pH 8.1) and the second at 0.2 M (pH 8.1). In most cases the second phosphate buffer contained in addition 1 M NaCl. About 75 ml. of the first buffer was used followed by a similar volume of the second. The effluent fractions of each tube were examined in a Unicam spectrophotometer at 280 m μ . From each fraction (0.02 M and 0.2 M) tubes containing significant amounts of protein were pooled and the pool was concentrated by dialysis against polyethylene glycol ('Carbowax') M.W. 20,000 for 36-48 hours at 4° and then dialysed against 1 per cent saline buffered to a pH of 7.4 for 24 hours at 4°.

In order to study the distribution of antibody in one particular serum in more detail, step-wise elution using buffers ranging from 0.02 M to 0.2 M was used. Selected portions of effluent were collected, concentrated with 'Carbowax' and then tested for their content of γ globulin and β_2 M globulin and of antibody.

Assay of γ and β_2 M Globulin in Fractions from the DEAE-Cellulose Column

The concentration of γ and β_2 M globulin in fractions obtained from the anion-exchange column was estimated in every case, using the inhibition of antiglobulin method. The γ globulin concentration was estimated by inhibiting the reaction between anti- γ -globulin and Rh-sensitized red cells, following the technique of Mollison (1956) (see also Kekwick, Vallet, Cutbush, Mollison, Thomas, Geil and Soothill, 1961); β_2 M globulin was estimated by inhibiting the reaction between anti- β_2 M-globulin and Lc^a-sensitized red cells, using the technique described by Polley *et al.* (1962).

SEROLOGICAL TESTS

Agglutination of Red Cells Suspended in Saline

Serial, doubling, dilutions of the serum were made in saline and to each was added an equal volume of a 2 per cent suspension of red cells in saline. In titrating Rh antibodies the mixtures were left at 37°, but in all other cases were left at room temperature (approx. 20°). After 2 hours the deposited red cells were examined microscopically. The degree of agglutination was scored, using the system recommended by Race and Sanger (1956).

Indirect Antiglobulin Tests

The two-stage antiglobulin test described by Polley and Mollison (1961) was used; that is 1 volume of a 20 per cent suspension of red cells was first incubated at 37° for 90 minutes with 4 volumes of EDTA-treated antibody-containing serum; the cells were then washed three times and incubated at 37° for 15 minutes with 2 volumes of fresh normal serum, as a source of complement. Finally the cells were washed and tested with specific anti- γ -, anti- β_2 M- and anti- β_1 - (complement) globulin sera. (The preparation of the anti- γ and anti- β_2 M sera used in the present work is described by Polley *et al.* (1962).)

The fact that the reactions were due respectively to anti- γ and anti- β_2 M was confirmed by showing that the reactions were abolished by adding to the diluted antiglobulin serum an equal volume of 0.01 per cent γ globulin or of 0.005 per cent β_2 M globulin. The fact that the reactions were due to anti- β_1 -(complement) globulin was confirmed by showing that the addition to the antiglobulin serum of a 1 in 40 dilution of serum from a patient with hypogammaglobulinaemia, inhibited the reactions but that the addition of γ and β_2 M globulin (as above) did not; further confirmation was obtained by incubating the red cells with EDTA-treated serum alone and showing that no reaction was then obtained with an anti- β_1 -globulin serum.

The score of each antibody-containing serum was determined by preparing a series of doubling dilutions of the serum and testing each one as above. The degree of agglutination, with the antiglobulin serum, of each dilution was scored and the sum of the scores was recorded as the antibody score.

When sera agglutinated red cells suspended in saline at 37° the indirect antiglobulin test could not be applied (see Tables 2 and 3).

When testing serum from the patient with syphilitic cold haemoglobinuria (case No. 30) by the indirect antiglobulin test, a two-stage test was not used because, although some antibody was adsorbed on red cells in the absence of complement, more was adsorbed when complement was present at the same time. Antibody-containing serum and fresh normal serum were absorbed with Zymosan (3 mg./ml.) to prevent haemolysis, and 4 volumes of both were mixed with 1 volume of a 20 per cent suspension of normal group O red cells and allowed to stand at 0° for 90 minutes. The mixtures were then transferred to a water bath at 37° and after 15 minutes' incubation the cells were washed and tested with antiglobulin sera.

Haemolysis

Four volumes of each of a series of doubling dilutions of the antibody-containing sera were incubated at 37° for 90 minutes with 4 volumes of fresh normal serum and 1 volume of a 5 per cent suspension of appropriate red cells. The tubes were then spun and examined for haemolysis and the results were scored, using an arbitrary scale.

When testing samples of serum from the patient with syphilitic cold haemoglobinuria, the mixture of the patient's serum, fresh normal serum and red cells was first allowed to stand at 0° for 90 minutes; then, without washing, the mixture was transferred to a water bath at 37° and incubated for 30 minutes before being examined for haemolysis.

Comparison of Results on Serum and on Fractions

In comparing results obtained on fractions, dilution had to be taken into account. The method of doing this was as follows: supposing the fraction was diluted 1 in 2, then in considering the score the reaction of the undiluted serum was excluded and the first reaction counted was that for the 1 in 2 dilution. This is illustrated in Table 1.

TABLE 1
METHOD OF CORRECTING THE 'SCORE' OBTAINED WITH WHOLE SERUM FOR COM-
PARISON WITH THE SCORE OBTAINED WITH A DILUTED FRACTION

	Dilutions of serum (reciprocals)					
	1	2	4	8	16	32
Observed reaction	+++	++	+	+	(+)	—
Score*	10	10	8	5	3	0

*See Racc and Sanger (1956, p. 246).

In this example the observed score is 36; if the reaction with undiluted serum is omitted, the 'corrected score' becomes 26.

TREATMENT WITH 2-MERCAPTO-ETHANOL. One volume of undiluted serum was mixed with an equal volume of 0.1 M 2-mercapto-ethanol in phosphate buffer pH 7.4 and left at 37° for 2 hours; the mixtures were then dialysed overnight against buffered saline at 4°. As a control, 1 volume of undiluted serum was treated with buffer alone in the same way and examined at the same time as the treated serum.

RESULTS

Amount of γ Globulin and β_2M Globulin in Fractions from the DEAE-Cellulose Column

Estimates of the γ globulin recovered in the 0.02 M fraction ranged from 80 to 130 per cent; a small amount of γ globulin could always be detected in the 0.2 M fraction but the amount never exceeded 10 per cent of the total. The recovery of β_2M globulin in the 0.2 M fraction ranged between 80 and 130 per cent; no β_2M globulin could be detected in the 0.02 M fraction.

Antibodies of the Le, MNSs and P Blood-Group Systems (with Single Examples of Anti-O(H) and Anti-I)

Results of examining antibodies belonging to these various blood-group systems are shown in Table 2. As will be seen, with only three exceptions, the antibodies appeared to be exclusively β_2M globulin. Thus, apart from these three exceptions, serological activity was found only in the 0.2 M fraction from the DEAE-cellulose column; in those cases in which a positive indirect antiglobulin test was obtained, the cells reacted with an anti- β_2M -globulin but not with an anti- γ -globulin serum and, finally, all these antibodies were inactivated by treatment with 2-mercapto-ethanol.

The three exceptions were examples of anti-Le^a (case No. 9), anti-M (case No. 11), and anti-P+P₁ (case No. 17):

The anti-Le^a from case No. 9 was apparently composed partly of γ globulin because some serological activity was found in the 0.02 M fraction from the DEAE-cellulose column. The 0.02 M fraction, in the presence of complement, sensitized Le(a+) red cells to agglutination by an anti- β_1 -globulin serum but this fraction, with or without complement, did not sensitize the red cells to agglutination by an anti- γ -globulin serum. These findings are referred to again in the Discussion.

The anti-P+P₁ from case No. 17 also appeared to be composed to a small extent of γ globulin; thus the 0.02 M fraction from the column agglutinated enzyme-treated P₁-positive red cells but not P₁-negative red cells (according to the terminology of Sanger (1955)).

The anti-M serum from case No. 11 was apparently composed exclusively of γ globulin, being present only in the 0.02 M fraction from the column, but treatment of the whole serum with 2-mercapto-ethanol slightly reduced the titre of the antibody.

Antibodies belonging to the Rh, K and Jk Systems (with a Single Example of the 'Donath-Landsteiner' Antibody)

Of the blood-group antibodies listed in Table 3, only two — the anti-D from case No. 21 and the anti-E from case No. 26 — were capable of agglutinating red cells suspended in saline. In both cases only the 0.2 M fraction eluted from a DEAE-cellulose column displayed agglutinating activity; moreover, in both cases the agglutinating activity of whole serum was abolished after treatment with 2-mercapto-ethanol. Evidently, then, these agglutinins were β_2M globulin. The 0.02 M fraction prepared from the serum of case No. 21 behaved as an incomplete antibody, demonstrating that this serum contained a mixture of γ globulin (incomplete) and β_2M globulin (agglutinating) antibody molecules.

Of the remaining incomplete antibodies, almost all appeared to be composed solely of γ globulin. Thus they were found wholly or predominantly in the 0.02 M fraction eluted from a DEAE-cellulose column, they sensitized red cells to an anti- γ -globulin serum, and

TABLE 2

TESTS ON SERA CONTAINING ANTIBODIES OF THE Lc, MNSs AND P SYSTEMS (WITH SINGLE EXAMPLES FROM THE ABO AND Ii SYSTEMS)

Case No.	Specificity of antibody	Test	Corrected score (see text)			Reactions with antiglobulin sera			Serological activity after SH treatment†
			Whole serum	0.02 M fraction*	0.2 M fraction*	Anti-γ	Anti-β ₁	Anti-β ₂ M	
1	Anti-Le ^a	Antiglob.	46	0	40	—	+	+	Nil
2	Anti-Le ^a	Antiglob.	36	0	30	—	+	+	Nil
3	Anti-Le ^a	Antiglob.	22	0	16	—	+	+	Nil
4	Anti-Le ^a	Antiglob.	10	0	7	—	+	+	...
5	Anti-Le ^a	Antiglob.	24	0	26	—	+	+	Nil
6	Anti-Le ^a	Antiglob.	10	0	10	—	+	+	Nil
7	Anti-Le ^a	Antiglob.	27	0	29	—	+	+	Nil
8	Anti-Le ^a	Antiglob.	41	0	28	—	+	+	...
9	Anti-Le ^a	Antiglob.	31	7	20	—	+	+	Nil
10	Anti-M	{ Agglut. (20°)	26	0	23	...	...	...	Nil
		{ Antiglob.	0	0	0	—	—	—	Nil
11	Anti-M	Agglut.	24	25	0	...	...	...	Diminished (16)
12	Anti-S	Agglut.	23	0	23	...	...	...	Nil
13	Anti-S	Agglut.	26	0	24	...	...	...	Nil
14	Anti-s	Agglut.	38	0	44	...	...	...	Nil
15	Anti-P ₁	{ Agglut. (20°)	31	0	34	...	...	...	Nil
		{ Antiglob.	37	0	39	—	+	+	Nil
16	Anti-P ₁	{ Agglut. (20°)	24	0	20	...	...	...	Nil
		{ Antiglob.	0	0	0	—	—	—	Nil
17	Anti-P + P ₁	{ Agglut.	53	0	58	...	...	...	Diminished (17)
		{ Haemol.	20	0	23	...	...	...	Diminished (4)
18	Anti-P + P ₁	{ Agglut.	40	0	44	...	...	...	Nil
		{ Haemol.	53	0	47	...	...	...	Nil
19	Anti-O(H)	{ Agglut. (20°)	15	0	12	...	...	...	Nil
		{ Antiglob.	18	0	18	—	+	+	Nil
20	Anti-I	{ Agglut. (20°)	36	0	22	...	...	...	Nil
		{ Antiglob.	26	0	18	—	+	+	Nil

Antiglob. = antiglobulin test after incubating red cells with antibody-containing serum and complement at 37°. When the antibody-containing serum agglutinated red cells at 20° but not at 37° the antiglobulin test could be carried out at 37° (cases 10, 15, 16, 19 and 20). When the antibody-containing serum agglutinated red cells at 37° the antiglobulin test could not be carried out on whole serum but those fractions which contained no agglutinating activity could be tested (for example the 0.02 M fraction in cases 12, 13, 14, 17 and 18 and the 0.2 M fraction in case 11). Negative antiglobulin tests were obtained in all these cases although the 0.02 M fraction from case 17 weakly agglutinated papain-treated red cells.

Agglut. = agglutination of saline-suspended red cells (at 37° unless otherwise specified).

Haemol. = haemolysis of red cells suspended in fresh serum at 37°.

... = not tested.

*Fractions obtained from a DEAE-cellulose column.

†Serological activity after treatment of the serum with a sulphhydryl compound (2-mercapto-ethanol). When the activity was diminished, but not abolished, the figure in parentheses refers to the 'score' of the treated serum.

TABLE 3

TESTS ON SERA CONTAINING ANTIBODIES OF THE Rh, K AND Jk SYSTEMS (ALSO SERUM FROM A PATIENT WITH SYPHILITIC COLD HAEMOGLOBINURIA)

Case No.	Specificity of antibody	Test	Corrected score (see text)			Reactions with antiglobulin sera			Serological activity after SH treatment†
			Whole serum	0.02 M fraction*	0.2 M fraction*	Anti-γ	Anti-β ₁	Anti-β ₂ M	
21	Anti-D	{ Agglut.	70	0	57	..	...	...	Nil
		{ Antiglob.	...	69	...	+	—	—	Unchanged
22	Anti-D	Antiglob.	101	121	56	+	—	—	Unchanged
23	Anti-D	Antiglob.	71	71	22	+	—	—	Diminished (53)
24	Anti-c	Antiglob.	105	87	46	+	—	—	Unchanged
25	Anti-c	Antiglob.	25	20	0	+	—	—	Unchanged
26	{ Anti-E	Agglut.	31	0	23	...	..	...	Nil
	{ Anti-Jk ^a	Antiglob.	59	49	0	+	+	—	Unchanged
27	Anti-Jk ^a	Antiglob.	49	0	44	—	+	+	..
28	Anti-K	Antiglob.	37	38	5	+	—	—	Unchanged
29	Anti-K	Antiglob.	18	16	8	+	+	+	Diminished (12)
30	Syphilitic cold haemoglobinuria	{ Haemol.	28	21	0	.	...	...	Unchanged
		{ Antiglob	26	10	0	+	+	—	Unchanged

Except in case No. 30 (see text), tests were carried out at 37°.

Antiglob = antiglobulin test, after incubation of cells with antibody-containing serum and complement. In cases 21 and 26 (anti-E) the antiglobulin test could not be carried out on whole serum or on the 0.2 M fraction because these produced agglutination at 37°. The 0.02 M fraction could be tested and the results obtained in case 21 are shown. The results in case 26 were negative and are not shown.

Agglut. = agglutination of saline-suspended red cells.

Haemol. = haemolysis of red cells in fresh serum.

... = not tested.

*Fractions obtained from a DEAE-cellulose column

†Serological activity after treatment of the serum with a sulphydryl compound (2-mercapto-ethanol) When the activity was diminished, but not abolished, the figure in parentheses refers to the 'score' of the treated serum.

they were not affected by treatment with 2-mercapto-ethanol. The only exceptions were one example of anti-Jk^a (case No. 27) which appeared to be entirely β_2 M globulin; one example of anti-K (case No. 29) which appeared to be partly β_2 M globulin and one example of anti-D (case No. 23) which behaved in an anomalous way.

The anti-D from case No. 23 behaved as an incomplete antibody but after treatment with 2-mercapto-ethanol the titre was reduced, suggesting that the antibody might be composed partly of β_2 M globulin; on the other hand red cells sensitized with the antibody were agglutinated only by anti- γ globulin and not by anti- β_2 M globulin and the reaction with anti- γ globulin was completely inhibited by the addition of a preparation (G₄) of γ globulin. On fractionating the serum on a DEAE-cellulose column, most of the antibody was found in the 0.02 M fraction. In order to study the distribution more completely fractionation was repeated with a much larger number of steps than usual. As Fig. 1 and Table 4 show, the activity was distributed between several fractions but none was found in the fraction containing the greatest amount of β_2 M globulin.

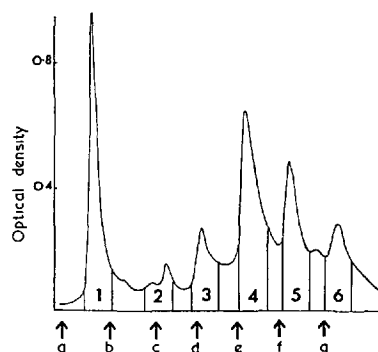


FIG. 1. Fractionation of serum from case No. 23. Elution was carried out from a DEAE-cellulose column using a series of phosphate buffers, pH 8, of increasing molarity, thus: a, 0.02 M; b, 0.04 M; c, 0.05 M; d, 0.06 M; e, 0.12 M; f, 0.16 M; g, 0.2 M. Successive 5 ml. samples were collected and the optical density of each determined. Samples were pooled as shown ('1', '2' etc.); serological tests on the samples are shown in Table 4.

TABLE 4
TESTS ON SEVERAL FRACTIONS OBTAINED FROM SERUM OF CASE 23

	Content of		Antibody 'score'		Reactions with antiglobulin serum†
	γ globulin (as a percentage of amount in whole serum)	β_2 M globulin	Before SH* treatment	After SH* treatment	
Whole serum	100	100	42	31	+
Fraction 1	40	<2	19	8	+
Fraction 2	4	<2	5	2	+
Fraction 3	6	<2	14	0	+
Fraction 4	<2	5	0	0	—
Fraction 5	<2	25	0	0	—
Fraction 6	<2	25	0	0	—

The fractions are those illustrated in Fig. 1.

*Treatment with a sulphhydryl compound (see text).

†The reactions were inhibited completely by adding to the antiglobulin serum an equal volume of 0.01 per cent γ globulin. Reactions with an anti- β_2 M-globulin serum were all negative.

DISCUSSION

AGREEMENT BETWEEN METHODS

Fahey and Horbett (1959) used continuous-gradient elution from a DEAE-cellulose column with phosphate buffer pH 8 from 0.02 M to 0.30 M and divided the protein-containing effluent into five consecutive fractions of equal volume. On analysis in the ultracentrifuge, γ globulin only was detected in the first four fractions and most of this (about 75 per cent) was found in the first fraction. The fifth fraction contained predominantly β_2 M globulin.

In the present work, elution was carried out with only two buffer solutions, the first of 0.02 M, pH 8.1 and the second of 0.2 M, pH 8.1. After concentrating each effluent fraction as described, the concentration of γ globulin in each fraction was determined by the inhibition of antiglobulin serum method. This method has been shown to be at least as sensitive as any other in estimating γ globulin; thus, it is possible to estimate concentrations considerably below 1 mg./100 ml. (Kekwick *et al.*, 1961). The method used here has a coefficient of variation of the order of ± 20 per cent.

The results confirm that the 0.02 M fraction eluted from the column contains most of the γ globulin present in the original serum. These findings agree broadly with the findings of Levy and Sober (1960) that 50–100 per cent γ globulin could be recovered from the column and with Fahey and Horbett's estimate of 90–100 per cent recovery.

No β_2 M globulin could be detected in the 0.02 M fraction; to be more precise, this fraction, when added undiluted to diluted anti- β_2 M-globulin serum, failed to inhibit the reaction with Le^a -sensitized red cells, indicating that the concentration of β_2 M globulin was less than 0.3 mg./100 ml. in a solution containing more than 500 mg./100 ml. of γ globulin.

The 0.2 M fraction appeared to contain about as much β_2 M globulin as was present in the original serum and between 3 and 10 per cent of the γ globulin.

Antibody composed solely of β_2 M globulin is expected to be found exclusively in the 0.2 M fraction. The finding of any blood-group antibody activity whatever in the 0.02 M fraction indicates that the antibody is composed at least partly of γ globulin.

Antibody composed solely of γ globulin would be expected to be present mainly in the 0.02 M fraction. Accordingly, the results observed in cases 22, 23 and 24 call for comment. In these cases the antibody appeared to be composed solely of γ globulin, as judged by the fact that the sera sensitized red cells only to an anti- γ -globulin serum and not to an anti- β_2 M-globulin serum. Nevertheless, instead of finding about one-tenth of the serological activity in the 0.2 M fraction obtained from the DEAE-cellulose column there appeared to be about one-third of the total, as judged by antibody scores. This is, in all probability, an artefact because the method of scoring overestimates the amount of antibody in relatively dilute samples.

It has been shown that in the antiglobulin test, γ globulin and β_2 M globulin do not cross-react to any appreciable extent. Thus, red cells sensitized with incomplete anti-Rh are agglutinated by anti- γ -globulin but not by anti- β_2 M-globulin sera and red cells sensitized with incomplete anti- Le^a are agglutinated by anti- β_2 M-globulin but not by anti- γ -globulin sera (Polley *et al.*, 1962). In the present work, none of the antibodies classified as β_2 M globulin on the fractionation results sensitized red cells to an anti- γ globulin and none of the antibodies classified as γ globulin on the fractionation results sensitized red cells to an anti- β_2 M-globulin serum.

There was one unexpected negative result. Thus the anti- Le^a from case No. 9, a small

amount of which was recovered in the 0.02 M fraction from the column, failed to sensitize red cells to an anti- γ -globulin serum. The fact that this particular example of anti-Le^a was composed partly of γ (7S γ) globulin was previously reported (case No. 8) by Kekwick and Mollison (1961) as a result of tests on ether-fractionated samples.

Results following treatment with 2-mercapto-ethanol were in general those expected on the assumption that such treatment dissociates β_2 M globulin but not γ globulin. Thus in all cases in which antibody appeared to be composed solely of β_2 M globulin, treatment with 2-mercapto-ethanol resulted in loss of all serological activity. On the other hand, in a few cases in which an antibody appeared to be composed partly or solely of γ globulin there was some loss of activity after treatment with 2-mercapto-ethanol.

The anti-Le^a from case No. 9 which, as described above, appeared to consist predominantly of β_2 M globulin but partly of γ globulin, was completely inactivated by treatment with 2-mercapto-ethanol, an observation which was repeatedly confirmed. The example of anti-M from case No. 11, which appeared to be composed solely of γ globulin, was partially inactivated by treatment with 2-mercapto-ethanol. Finally, the example of incomplete anti-D from case No. 23 which sensitized cells to an anti- γ -globulin serum but not to an anti- β_2 M-globulin serum was partially inactivated by treatment with 2-mercapto-ethanol. This last result calls for some discussion, particularly in view of the fact that Kekwick and Mollison (1961) also concluded that this particular example of anti-D (case S.T. in that paper) was composed partly of β_2 M globulin. As described in the Results, the antibody was subjected to a more detailed fractionation than that used for the remaining sera and it was found that all the antibody activity was in the fraction containing γ globulin and none in the fraction containing β_2 M globulin. There was some evidence that the antibody in fraction 3 was particularly susceptible to damage by 2-mercapto-ethanol (see Table 4).

It must be concluded that the anti-D in case No. 23 was composed of protein which was antigenically identical with normal γ globulin, as shown by the fact that all fractions from the serum sensitized red cells to agglutination by an anti- γ -globulin serum. The unusual susceptibility of this antibody to treatment with 2-mercapto-ethanol cannot at present be explained.

The conclusion of Kekwick and Mollison (1961) that this incomplete anti-D was composed to some extent of β_2 M globulin was based partly on the finding that the serological activity was reduced by 2-mercapto-ethanol and partly on the finding that a considerable part of the serological activity was recovered in the 'G 2/2' fraction obtained by the ether-fractionation process. It is now apparent that results with 2-mercapto-ethanol are not decisive and it has become apparent that separation of γ and β_2 M globulin using the ether-fractionation method is sometimes rather unsatisfactory (unpublished findings based on estimates made by the inhibition of antiglobulin serum method).

In conclusion, the idea that incomplete anti-Rh may sometimes be composed of β_2 M globulin is now seen to be unsupported by any really satisfactory findings.

Relation between Known Antigenic Stimuli and Molecular Size of Antibodies

In this paper antibodies have been divided into those belonging to blood-group systems in which the antibodies are usually 'naturally-occurring' and those in which the antibodies are usually 'immune'. Antibodies of the Le, MNSs and P systems are usually found in subjects who have not received a recognized antigenic stimulus. In the present work, eight out of nine examples of anti-Le^a were composed of β_2 M globulin; the ninth was apparently

composed partly of γ globulin but this subject had received several transfusions of Le(a+) blood before his serum was examined for anti-Le^a.

One example of anti-M, two examples of anti-S and one of anti-s were all β_2 M globulin; one example of anti-M was a γ globulin but this came from a woman who had been recently delivered of M-positive twins and she may have been immunized by pregnancy.

Two examples of anti-P₁ and one of anti-P + P₁ were composed solely of β_2 M globulin; one other example of anti-P + P₁ was composed partly of β_2 M globulin but this came from a woman who had been transfused with plasma which may well have contained P₁ substance; moreover, she had been pregnant with an infant of the genotype P₂p. The fact that some of this antibody was γ globulin had been expected from the fact that anti-P₁ had been found in the cord serum of her last infant.

A single example of anti-O(H) from a pregnant woman and one of anti-I from a normal donor described by Jenkins *et al.* (1960) were also found to be β_2 M globulin.

Observations on case No. 8 showed that an increase in the titre of anti-Le^a following an antigenic stimulus could be due solely to the production of β_2 M antibody. Similarly persons of group B when injected with human A substance, if they have an immune response, produce mainly β_2 M anti-A (unpublished observations; see also Rawson and Abelson, 1960).

Antibodies of the Rh, K and Jk systems are usually γ globulin but may be β_2 M globulin. For instance, out of fifteen examples of anti-Jk^a tested by Polley *et al.* (1962) twelve were γ globulin and three were β_2 M globulin.

In the Rh system, β_2 M-globulin antibodies behave as agglutinins; see case No. 21, and the anti-E in case No. 26; see also Campbell, Sturgeon and Vinograd (1955), Fudenberg, Kunkel and Franklin (1959) and Kekwick and Mollison (1961). However, in other blood-group systems β_2 M globulin antibodies may behave as incomplete antibodies; see the example of anti-Jk^a in case No. 27.

Complement-Binding by Different Blood-Group Antibodies

The ability of particular blood-group antibodies to bind complement seems to be related more to their blood-group specificity than to their molecular size. Thus in the ABO, Le, P and Jk systems all antibodies active at 37° bind complement. Some of these antibodies, for example antibodies of the Le system, are almost always β_2 M globulin but on the other hand most examples of anti-Jk^a are γ globulin.

Conversely, antibodies of the Rh, MNSs and Lu systems do not as a rule bind complement, the only exceptions being some examples of anti-S. Antibodies of the K and Fy systems bind complement in about 50 per cent of cases (unpublished observations).

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Serological Characteristics of Anti-A Related to Type of Antibody Protein (7 S γ or 19 S γ)

MARGARET J. POLLEY, M. ADINOLFI and P. L. MOLLISON

Medical Research Council's Experimental Haematology Research Unit,
Wright-Fleming Institute of Microbiology, St. Mary's Hospital Med. School, London

The introduction of techniques for distinguishing between or actually separating different antibody proteins has made it possible to relate the serological characteristics of particular antibodies to the type of protein of which they are composed. It was hoped that application of these techniques to a study of anti-A sera might clarify the interpretation of present tests and in particular help to show which characteristics should be regarded as "immune".

Materials and Methods

Sera from 104 random group 0, 12 random group A and 10 random group B, donors were tested. In a few selected cases further samples were obtained a few weeks after the initial donation to confirm or extend observations made on the original samples.

Five group 0 and 5 group B volunteers were tested before and after being injected with human group A substance. Group 0 subjects were selected so as to provide a variety of serological patterns (see Table I). Thus, before injection the serum of one subject (M. A.) failed to haemolyse A cells, using the standard test (see below). The serum of a second subject (P. R.) had anti-A haemolysin, but no 7 S γ anti-A. The serum of another subject (R. L.) had a little 7 S γ anti-A only, and the remaining two subjects (R. P. and D. B.) had more 7 S γ than 19 S γ haemolysin.

In preliminary experiments, 3 of the 5 group 0 subjects were given 2 subcutaneous injections, each of 0.1 mg of human group A substance at an interval of 3 days; 5 days and 11 days after the second injection the subjects were bled. As only 1 of the 3 subjects showed a definite immune response it was decided to inject the same 3 subjects and the remaining volunteers (2 more group 0 and 5 group B subjects) with a larger amount of antigen. Accordingly all 10 subjects were given

2 subcutaneous injections each of 1.5 mg of human group A substance at an interval of 1 week and were bled 10 days after the second injection. The human group A substance used for these injections and for certain inhibition tests *in vitro* (see below) was prepared by Professor W. T. J. MORCAN, F. R. S., from pseudo-mucinous ovarian cyst fluid.

Tests were also made on the serum of a group 0 woman (T. B.) who had given birth to 2 infants severely affected with haemolytic disease of the newborn due to anti-A, and on samples of serum obtained from the cord blood of 8 normal group 0 infants born to group 0 mothers.

Ion-Exchange Chromatography

The method used was a minor modification of that described by LEVY and SOBER [12]. In most cases only 2 phosphate buffers were used, 0.02 M and 0.2 M (both pH 8.1). In 5 cases (serum from 4 volunteers after stimulation with A substance, and from 1 mother - T. B. -, see above) elution was carried out successively with 0.02 M, 0.04 M, 0.06 M, 0.08 M, 0.1 M, 0.12 M, 0.14 M, 0.16 M, 0.18 M and 0.2 M phosphate buffers, pH 8.1. Seven fractions containing the protein "peaks" were concentrated by dialysis against "Carbowax", M. W. 20,000, and then dialysed against buffered saline. Further details have been published elsewhere [3]. The amount of 7 S γ and 19 S γ globulin in all fractions was assayed by the inhibition of anti-globulin serum method [9, 15].

Serological Tests

Red cells were obtained from normal donors of sub-group A₁ or group B. Red cells taken on the day of testing were used for haemolysis tests but for agglutination tests samples stored in ACD at 4° C for periods up to 2 weeks were used. The cells were in all cases washed 3 times in 0.17 M NaCl to which sodium phosphate ($\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$) in a final concentration of 0.01 M had been added to bring the pH to approximately 7. The cells were then resuspended in the same solution for testing.

Pig group 0 (0^p) and A ("strong" A^p) were kindly supplied by Mr. Lockyer of the Blood Transfusion Service, Bristol. The A^p cells were considered as having a "strong" A^p antigen when they were

completely haemolysed at room temperature by a serum with a haemolysin titre of at least 1 in 8 (at 37° C) against human group A red cells.

The pig cells were stored for periods of up to 1 week before being used. Following the method used by LOCKYER [13] the cells were washed and then resuspended in 1.5 per cent NaCl which was also buffered to approximately pH 7.

Serum which was to be tested against A^p cells was first absorbed twice with one fifth its volume of six-times washed group O pig cells (O^p) and then titrated against A^p cells, using O^p cells as a control.

Agglutination of red cells suspended in saline. Serial, doubling dilutions of the serum were made in saline and to each was added an equal volume of a 2 per cent suspension of red cells in saline. After 2 hours at room temperature, the deposited red cells were examined microscopically. The degree of agglutination was scored [16].

Haemolysis. Since the sera tested had been stored for a variable period and the fractions from DEAE-cellulose contained no active complement, it was necessary, as a routine, to add fresh serum as a source of complement. For this purpose group O serum containing low titre anti-A was absorbed at 0° C with group A red cells. The resulting absorbed serum was stored at -50° C for periods not exceeding 2 weeks before being used.

Four volumes of each of a series of doubling dilutions of the antibody-containing sera were incubated at 37° C for 90 minutes with 2 volumes of absorbed O serum and 1 volume of a 10 per cent suspension of group A red cells; thus the ratio of anti-A serum: cells in the first tube of the titration was 40:1. The tubes were then spun and examined for haemolysis and the results were scored, using an arbitrary scale.

When it was desired to make the test more sensitive 1 volume of a 5 per cent suspension of red cells was substituted for the 10 per cent suspension to give a ratio, serum:cells, of 80:1. In a few cases in which no haemolysis was obtained even with the 5 per cent suspension of cells, the following method was used:

Sensitive method for detecting haemolysis. The principle was to label group A red cells with ⁵¹Cr, to incubate a very dilute suspension of the labelled cells with anti-A serum and to estimate the release of ⁵¹Cr into the supernatant as an index of haemolysis. In these experiments 4 volumes of anti-A serum were added to 1 volume of absorbed group O serum (complement) and 1 volume of an approximately 2 per cent suspension of red cells so that the final ratio of anti-A serum to

red cells was approximately 200:1. (The concentration of red cells, as haemoglobin, in the final mixture was checked in a photoelectric colorimeter). As controls, labelled group A red cells were incubated (a) with group AB serum and (b) with group A serum.

Haemolysis of A^p Cells. The method was as described above to achieve a ratio of anti-A serum:cells of 40:1, the only difference being, first that the anti-A serum used as a source of complement was previously absorbed with O^p cells, and second that the test was carried out at room temperature.

Enhancement of agglutination in serum. To discover whether antibodies contained in particular fractions would agglutinate cells more strongly in a medium of serum than in saline, the following method was used: serial dilutions of the fraction were prepared using as a diluent group AB serum, mixed with an equal volume of saline. To each volume of the fraction, diluted in AB serum, was added an equal volume of a 2% suspension of A₁ cells in AB serum and the mixtures were left at room temperature for 2 hours before examining the deposit microscopically for agglutination.

The AB serum used in these tests was specially selected for its relatively low content of A substance; it was diluted with an equal volume of saline because undiluted serum tended to cause rouleaux formation.

Indirect antiglobulin tests. Tests with anti-7 S γ , anti-19 S γ and anti- β_1 (complement) globulin sera were made as described previously [15]. When A^p cells were being tested, the antiglobulin sera were first absorbed with A^p and O^p cells. In some cases the two-stage antiglobulin test, that is incubation first with antibody and second with complement was used [14].

Indirect antiglobulin tests after "partial neutralization". A range of dilutions of group A substance was first prepared. One volume of each dilution was then added to an equal volume of undiluted anti-A serum, or of a particular dilution of anti-A serum, or to a fraction obtained from a DEAE-cellulose column and the "partially neutralized" solution was then tested with group A red cells. In some cases preliminary tests were carried out to discover the least amount of A substance which would just prevent the anti-A from agglutinating red cells suspended in saline and the anti-A serum was then tested for its ability to sensitize group A red cells to an antiglobulin serum; this test is described as the "indirect antiglobulin test after partial neutralization".

Treatment of sera with 2-mercapto-ethanol. This was carried out as described previously [3].

Absorption by pig cells. The serum was first absorbed twice at 0° C with one fifth its volume of six-times washed O^p cells, and was then divided into 2 aliquots. The first was absorbed at 0° C with an equal volume of packed, six-times washed A^p cells; the second was absorbed with an equal volume of packed six-times washed O^p cells and is referred to in Table III as "unabsorbed".

Estimation of the amount of complement bound. One volume of a 50% suspension of A₁ cells was incubated for 1½ hours with the serum or fraction previously treated with EDTA. The cells were then washed 3 times and as much supernatant as possible removed. To the packed cells were added 5 volumes of a 1 in 10 dilution of fresh group O serum, previously absorbed at 0° C with A₁ cells. After 15 minutes incubation at 37° C the mixtures were centrifuged and the supernatant was separated and its complement titre determined, using sheep cells sensitized with bovine antibody. As a control the fresh absorbed group O serum was incubated with group O cells which had previously been incubated with the same preparation of anti-A.

Results

Random Donors

Frequency of haemolysins. Ninety-four of the 104 group O sera produced at least a trace of haemolysis of A₁ red cells using the "standard test" in which the ratio of serum:cells was 40:1. In 76 of the 104 cases the serum was titrated (in the remaining cases the amount of serum was insufficient) and in Fig. 1 the distribution of haemolysin titres is shown; 7 of the 76 samples gave no detectable haemolysis under these conditions and are scored as Nil.

Since the frequency (approximately 90%) of haemolytic anti-A sera was much higher than that estimated previously (18%) in this laboratory [5] using a method in which the ratio of serum: cells was about 20:1, some comparative tests were made. When the ratio of serum:cells was reduced from 40:1 to 20:1 the number of sera producing haemolysis fell from 90% to 58%.

In order to see whether even those group O sera which failed to haemolyse A cells in a ratio of 40:1 (serum:cells) would haemolyse red cells if the ratio were increased further, tests were carried out on

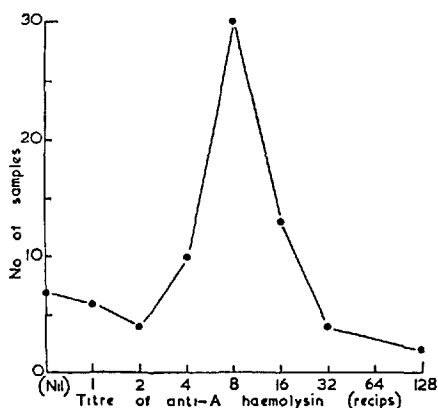


Fig. 1. Distribution of anti-A haemolysin titres in 76 random group O donors. Ratio of volume of serum to volume of cells in the tests, 40:1.

5 of the 10 sera which failed to haemolyse A cells using the standard test. At a ratio of 80:1, 3 of the 5 sera were haemolytic. The remaining 2 sera were tested at a ratio of serum:cells of approximately 200:1, using the ^{51}Cr -release method and both were found to be haemolytic; thus the amount of haemolysis given by these 2 sera was 15 and 32% respectively compared with less than 2% in negative controls. As a positive control, an anti-A serum with a haemolysin titre of 16 using the standard method was tested at a ratio of serum:cells of 200:1; the amount of lysis was 90%.

Using the standard ratio of serum:cells of 40:1, 12 random group A sera all haemolysed group B red cells and 10 random group B sera all haemolysed group A red cells.

Correlation between agglutinin titre and haemolysin titre. The results of testing 51 group O sera are shown in Fig. 2. Although agglutinin titres and haemolysin titres are evidently correlated, some sera with agglutinin titres of 16–32 were non-lytic under the conditions of the test.

Results of treating sera with 2-mercapto-ethanol. Seventy-six of the 104 random group O sera were tested before and after treatment with 2-mercapto-ethanol. Of 69 which haemolysed A cells before treatment only 14 still haemolysed cells after treatment. On the other hand 34 of the 76 still agglutinated group A red cells after treatment. The conclusion was reached that in group O sera most anti-A haemolysins are 19 S γ globulin but that many anti-A agglutinins are 7 S γ globulin.

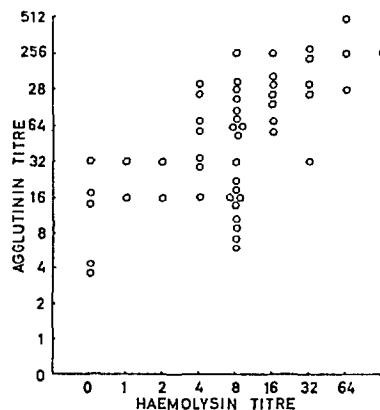


Fig. 2. Anti-A agglutinin titre plotted against anti-A haemolysin titre in 51 group O samples.

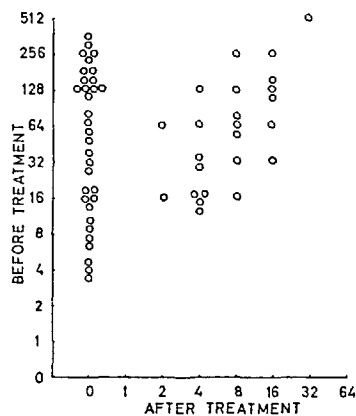


Fig. 3. Anti-A agglutinin titre of serum in 57 group O samples, before and after treatment with 2-mercapto-ethanol.

Fifty-seven of the 76 sera treated with 2-mercapto-ethanol were titrated before and after treatment and the results are shown in Fig. 3. All sera showed some reduction in titre after treatment suggesting that in every case, in the original serum, the titre of 19 S γ anti-A agglutinin exceeded that of 7 S γ anti-A agglutinin. Thirty-three of the 57 sera after treatment failed to agglutinate A₁ cells showing that the agglutinin in these cases was exclusively 19 S γ .

As Fig. 2 shows, all of the 5 sera which failed to haemolyse group A red cells in the standard test were of comparatively low agglutinin

TABLE I

Tests on Group O Sera; Relation between the Haemolysin Titre vs. Human A₁ Cells and the Ability to Agglutinate Pig Group A (A^p) Cells

Haemolysin titre (reciprocals) vs. human A ₁ cells	No. of samples	No. agglutinating A ^p cells
0-1	11	0
2-4	14	5
8-16	35	32
32-128	11	11
	71	48

titre; the serological activity of 3 of these 5 was completely abolished by treatment with 2-mercapto-ethanol suggesting that these sera contained only 19 S γ anti-A.

Sera from 8 normal newborn group O infants, born to group O mothers, were tested before and after treatment with 2-mercapto-ethanol. The anti-A agglutinin titre in the samples varied from 1 to 16 and was in all cases the same after treatment.

Agglutination of A^p cells. Of 71 group O sera tested 23 failed to agglutinate A^p cells; the haemolysin titre of these 23 sera against human A₁ cells was determined by the "standard" technique with the following results: 11 had a haemolysin titre of 0-1, 9 had a titre of 2-4, 2 had a titre of 8 and 1 a titre of 16 (see Table I). When sera which failed to agglutinate A^p cells were absorbed with A^p cells, their titre against human A₁ cells was not reduced; 2 examples are shown in Table III. Serum I. D. was typical of the majority in that it had a haemolysin titre of 4 or less (actually nil) against human A₁ cells and had a low anti-A agglutinin titre. Serum J. J. was exceptional in having a moderately potent anti-A haemolysin (titre 16; score 26) and agglutinin.

Of the samples of serum from normal newborn group O infants, referred to above, none agglutinated A^p cells.

Donors Stimulated with A Substance

Table II shows the effect of injection of A substance on the agglutinin and haemolysin scores of the anti-A in 5 group O and 5 group B subjects and the distribution of antibody between the 0.02 M and 0.2 M fractions.

Results following the injection of 0.1 mg A substance. As described in Methods, 3 of the group O subjects were first injected with 0.1 mg A substance; only 1 (M. A.) showed an appreciable response. This subject had been specially selected because his serum, before injection, failed to haemolyse A cells at a ratio of 40:1. After the 2 injections of 0.1 mg of A substance his serum became moderately haemolytic and the agglutinin titre rose slightly. In the other 2 subjects (P. R. and R. P.) there was no increase in haemolysin titre and little or no rise in agglutinin titre.

Results following injections of 1.5 mg A substance. In the 5 group O subjects, before injection, the agglutinating activity of the sera was found wholly in the 0.2 M fraction in 2 cases, mainly in the 0.2 M fraction in 2 further cases but equally in the 0.02 M and 0.2 M fractions in the fifth case; after injection of A substance, in all cases there was some increase in the agglutinating activity associated with both fractions although in some cases the amount in the 0.2 M fraction was distinctly greater.

TABLE II

Amount of 7S γ and 19S γ Anti-A in Serum of 5 Group O and 5 Group B Subjects, before and after the Injection of Purified Human Group A Substance

Subject Initials	Group	Agglutination (Scores)						Haemolysis (Scores)					
		Before			After			Before			After		
		Whole serum	Fractions 0.02 M 0.2 M		Whole serum	Fractions 0.02 M 0.2 M		Whole serum	Fractions 0.02 M 0.2 M		Whole serum	Fractions 0.02 M 0.2 M	
M. A.	O	45	0	31	137(71) ¹	79	121	0	0	0	90(18) ¹	32	72
P. R.	O	43	0	50	79(42)	23	69	20	0	20	53(23)	0	50
R. P.	O	50	24	46	82(56)	26	62	52	29	6	56(52)	29	6
R. L.	O	40	7	36	110	60	107	20	0	23	64	30	64
D. B.	O	52	43	43	129	50	128	30	25	6	104	42	97
S. C.	B	30	0	32	92	13	91	32	0	25	66	0	64
E. B.	B	47	0	39	85	13	71	18	0	13	49	0	43
A. S.	B	31	0	32	40	0	49	17	0	20	27	0	25
A. K.	B	41	0	40	48	0	47	20	0	15	33	0	27
O. L.	B	31	0	31	41	20	31	25	0	20	25	0	20

¹ The figures in brackets show the scores 10 days after the second of 2 injections of 0.1 mg A substance.

All other "After" scores refer to tests made 10 days after the second of 2 injections of 1.5 mg A substance.

In the 5 group B subjects there was no agglutinating or haemolytic activity associated with the 0.02 M fraction before injections of A substance. Following the injections, in 2 of the 5 subjects (S.C. and E.B.) there was a considerable increase of agglutinating and haemolytic activity associated with the 0.2 M fraction; 2 more subjects (A. S. and A. K.) showed smaller increases and in the fifth subject (O. L.) the only change was the development of agglutinin in the 0.02 M fraction.

In 3 of the group O subjects and in 1 of the group B subjects, serum taken after the second injection of A substance was fractionated using

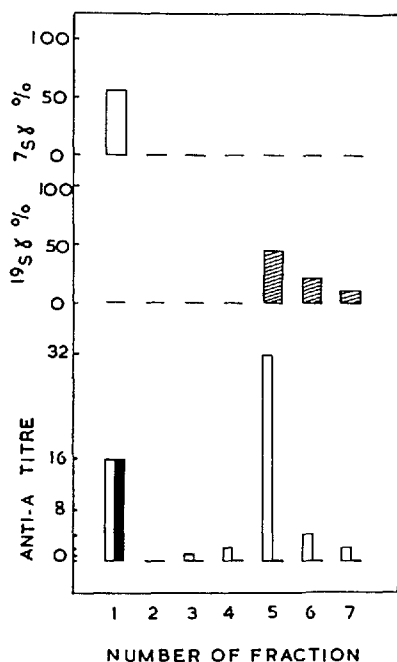


Fig. 4. Fractionation of serum from a group O subject (M. A.) previously injected with human A substance. The fractions 1-7 were obtained from DEAE-cellulose by eluting with phosphate buffers (pH 8.1) of the following molarities:

- 1 = 0.02 M:
- 2 = 0.04 M:
- 3 = 0.08 M:
- 4 = 0.10 M:
- 5 = 0.12-0.14 M:
- 6 = 0.14-0.16 M:
- 7 = 0.16 M-0.2 M:

The 7S γ and 19S γ concentration of each fraction was estimated and is expressed as a percentage of the concentration of the relevant protein in normal serum. Fractions 2-7 inclusive contained less than 1% of 7S γ and fractions 1-4 contained less than 2% of 19S γ . The anti-A titre of each fraction is shown in the lower part of the figure.

□ = anti-A titre of untreated fraction

■ = anti-A titre after treatment of 2-mercapto-ethanol

As the figure shows, the "peaks" of antibody activity corresponded to the peak concentrations of 7S γ and 19S γ , respectively.

10 solutions of increasing molarity, from 0.02 M to 0.2 M (see Methods). In 2 of the group O subjects (M. A. and D. B.) and the group B subject (A. S.) antibody activity was confined to the fractions containing the peaks of 7 S γ and 19 S γ globulin; see (Fig. 4). The serum from 1 of the group O subjects (R. P.) gave a further peak of antibody activity in fractions (0.08 M and 0.1 M) intermediate between those containing the peaks of 7 S γ and 19 S γ globulin.

Serum from subject T. B. was also fractionated using the same series of solutions (see Fig. 5). Fractions 3 and 4, eluted with 0.08 M and 0.1 M phosphate buffer, contained relatively large amounts of anti-A although the same fractions contained less than 10 mg 7 S γ globulin per 100 ml (that is, less than 1% of the concentration in normal serum) and less than 1 mg 19 S γ globulin per 100 ml (less than 2% of the concentration in normal serum).

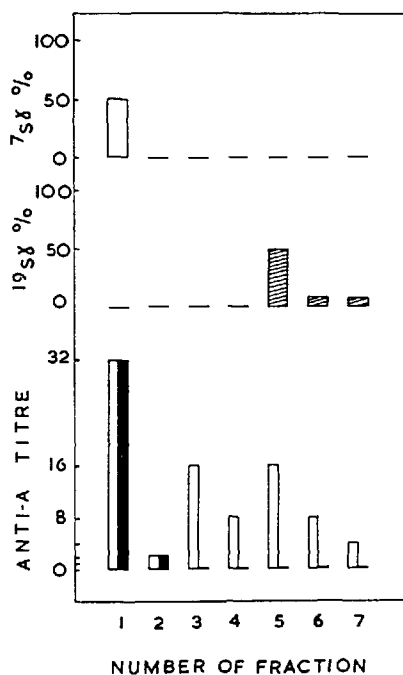


Fig. 5. Fractionation of serum from a group O mother (T. B.) of 2 infants severely affected with haemolytic disease of the newborn due to anti-A. Methods as in figure 4. As the figure shows, antibody activity (anti-A) was found in all fractions, including those containing very low concentrations of 7S γ and 19S γ .

Serological Characteristics of 19 S γ Anti-A and 7 S γ Anti-A

Since all examples of anti-A are composed at least partly of 19 S γ (see results with 2-mercapto-ethanol above), certain conclusions about the behaviour of 19 S γ anti-A could be drawn from the behaviour of whole serum. Many group B sera were found on fractionation to contain no anti-A activity in the 0.02 M fraction and these sera could be used to study the behaviour of "pure" 19 S γ anti-A. Less pure preparations were obtained by selecting the 0.2 M fraction from a serum containing both 7 S γ and 19 S γ anti-A; with the method used the 0.2 M fraction never contained more than 10% of the total amount of 7 S γ recovered from the column [3].

"Pure" preparations of 7 S γ anti-A were obtained by taking the 0.02 M fraction from selected group O sera. The cord sera from group O infants born to group O mothers could also be regarded as a potential source of pure 7 S γ anti-A. Samples of anti-A from fractions intermediate between 0.02 M and 0.2 M were also examined and are described below as "intermediate fractions".

Tests with A^p cells. Since all anti-A sera contain at least some 19 S γ anti-A and since about 30% of group O sera fail to agglutinate A^p cells (see Table I) and these sera do not react at all with A^p cells (see examples I. D. and J. J. in Table III), it follows that some 19 S γ anti-A have no anti-A^p specificity.

0.2 M fractions were not prepared from sera which failed to agglutinate A^p cells, but were prepared from more than 15 samples of serum which did agglutinate A^p cells. In every case the 0.2 M fraction agglutinated A^p cells.

0.02 M fractions prepared from more than 20 group O sera (all containing some 7 S γ anti-A) were tested against A^p cells. None of the fractions agglutinated A^p cells, but all of them reacted with A^p cells as shown by the fact that after absorption with A^p cells their titre against human A₁ cells was reduced. Similarly the 0.02 M fractions regularly sensitized A^p cells to agglutination by an anti-human globulin serum.

Table III shows some comparisons between the behaviour of 0.2 M and 0.02 M fractions prepared from the serum of the mother, T. B. This serum appeared to contain approximately equal amounts of 7 S γ and 19 S γ anti-A. Although the 0.02 M fraction did not agglutinate A^p cells it sensitized them to an antiglobulin serum. The 0.02 M and 0.2 M fractions were absorbed with A^p cells and then tested against

TABLE III

Reactions of 2 Different Group O Sera, and of Fractions from 1 of the Sera, with Human Group A and Pig Group A (A^p) Red Cells

Subject	Reactions ¹ with human group A cells			Reactions ¹ with A^p cells
	Unabsorbed	Absorbed with A^p cells		Agglutination
	Haemolysis	Agglutination	Agglutination	
T. B.	Whole serum	68	55	35
	0.02 M fraction ²	39	36	0
	0.2 M fraction ²	39	31	27
I. D.	Whole serum	0	13	0
J. J.	Whole serum	26	48	0

¹ The reactions are shown as titration 'scores'.

² Fractions from a DEAE-cellulose column.

human A cells; more anti-A was removed from the 0.2 M fraction than from the 0.02 M fraction.

As mentioned above, none of the 8 samples of serum from newborn group O infants of group O mothers agglutinated A^p cells. The 3 samples with the highest agglutinin titre against human A cells were tested against A^p cells using the indirect antiglobulin test and all gave positive results. All eight samples were absorbed with A^p cells and then re-tested against human A cells; in every case the anti-A titre was reduced.

In a small number of cases, the effect of 7S anti-A and 19S anti-A in haemolysing A^p cells was compared; it was found that either type of antibody, if sufficiently potent, would bring about haemolysis. For example, the 0.2 M fraction from the serum of volunteer D.B., after injection of A substance, haemolysed A^p cells although the less potent 0.02 M fraction from the same serum did not. On the other hand, the 0.02 M and 0.2 M fractions from T.B., which were about equally potent, both haemolysed A^p cells.

Effect of A substance on 7 S γ and 19 S γ anti-A. In 14 out of 15 sera tested, 7 S γ anti-A was far less easily neutralized by A substance than was 19 S γ anti-A; 2 examples are shown in Table IV. In com-

paring fractions from the first donor (D. B.) the 0.02 M fraction was diluted 1 in 5 and the 0.2 M fraction was diluted 1 in 100 because initially the anti-A titre of the 0.2 M fraction was far higher than that of the 0.02 M fraction. The degrees of agglutination produced by the diluted fractions was now similar, but it was found that the amount of A substance required to inhibit the 0.02 M fraction was about 20 times more than that required to inhibit the 0.2 M fraction.

The comparison in the case of donor T. B. was straightforward because the titre of anti-A agglutinin in the 0.02 M and 0.2 M fractions was similar. Again, the amount of A substance required to abolish the agglutinating activity of the 0.02 M fraction was about 20 times more than that required for the 0.2 M fraction.

Similarly the haemolytic activity of the 0.2 M fraction was far more easily abolished than that of the 0.02 M fraction. Thus in one example tested the amount of inhibition of a 0.2 M fraction produced by a 1 in 15,000 dilution of A substance was comparable to the effect on a 0.02 M fraction of a 1 in 500 dilution of A substance.

In one case only out of the 15 tested (subject R. P., see Table II) the 7 S γ anti-A was more easily neutralized than the 19 S γ anti-A

TABLE IV

Amounts of A Substance Required to Neutralize Anti-A in 0.02 M and 0.2 M Fractions Prepared from 2 Selected Group O Sera

Subject's initials		Dilutions of human A substance (reciprocals)						Saline
		10	100	1000	2000	5000	10000	
D. B.	0.02 M fraction							
	diluted 1 in 5	—	+	++	++	++	++	++
	0.2 M fraction							
	diluted 1 in 100	—	—	(+)	+	++	++	++
T. B.	0.02 M fraction							
	diluted 1 in 20	—	(+)	+	++	++	++	++
	0.2 M fraction							
	diluted 1 in 20	—	—	—	(+)	+	++	++

One volume of a fixed dilution of the fraction was added to one volume of various dilutions of A substance and the mixture was then tested for its ability to agglutinate human group A red cells.

— = negative reaction

(+), +, ++ = increasing degrees of agglutination

present in the same serum; thus when the dilutions of the 0.02 M and 0.2 M fractions were adjusted so that each gave a ++ agglutination reaction, an amount of A substance which would abolish the reaction of the 0.02 M fraction would not abolish that of the 0.2 M fraction.

Enhancement of agglutination by using AB serum as a medium. The Anti-A agglutinin titre in the 0.2 M and 0.02 M fractions prepared from group O sera was estimated, using saline and AB serum respectively as the diluting medium. As Table V shows, the titre of the 0.2 M fraction was the same with both media but that of the 0.02 M fraction was enhanced by using AB serum as a diluent. In a few cases 0.02 M fractions prepared from group B subjects were tested (see Table V) in case an antibody capable of agglutinating A cells in AB serum but not in saline should be revealed, but no such antibodies were found.

Indirect antiglobulin tests on partially-neutralized sera or fractions. As described in Methods, indirect antiglobulin tests were carried out on samples of serum, or on fractions from a DEAE-cellulose column, to which just sufficient A substance had been added to prevent the agglutination of saline-suspended red cells. Selected samples from

TABLE V

Effect of Using Serum as a Diluent in Titrating Anti-A in 0.02 M and 0.2 M Fractions from 2 Selected Sera

Subject		Dilutions of fractions (reciprocals)								
Initials	Group	Fraction	Medium	2	4	8	16	32	64	128
E. S.	O	0.02 M	Saline	++	+	—	—	—	—	—
			Serum ¹	+++	++	++	+	(+)	—	—
		0.2 M	Saline	++++	+++	+++	+++	++	+	(+)
			Serum	++++	+++	+++	+++	++	+	(+)
O. L.	B	0.02 M	Saline	—	—	—	—	—	—	—
			Serum	—	—	—	—	—	—	—
		0.2 M	Saline	+++	++	+	(+)	—	—	—
			Serum	+++	++	+	(+)	—	—	—

¹ The serum used as a diluent was selected for its very low content of A substance and was used at a dilution of 1 in 2.

— = negative reaction

(+), +, ++, +++, +++++ = increasing degrees of agglutination

group B subjects which had been shown to contain only 19 S γ anti-A, sensitized red cells to agglutination by an anti-19 S γ globulin serum and not to an anti-7 S γ globulin serum. Conversely, 0.02 M fractions from selected group O subjects, previously shown to contain substantial amounts of 7 S γ anti-A, sensitized red cells to an anti-7 S γ globulin serum. The reactions were always much stronger than those obtained with anti-19 S γ globulin serum (see Table VII).

Binding of complement. The amount of complement bound by 19 S γ anti-A and 7 S γ anti-A of equivalent titre was estimated using 0.02 M and 0.2 M fractions prepared from the serum of donor T. B. As Table VI shows, fresh serum absorbed with red cells which had been sensitized with the 0.02 M fraction had only a slightly lower titre (1/40) of complement than unabsorbed fresh serum (titre 1/80), showing that relatively little complement had been bound. On the other hand, fresh serum treated with red cells sensitized with the 0.2 M fraction had a titre of only 1/10 showing that much more complement was bound by the 19 S γ antibody.

The binding of complement by 19 S γ anti-A and 7 S γ anti-A was also compared using an anti- β_1 globulin serum to demonstrate the amount of complement on the sensitized cells (Table VII). Thus, group A red cells were sensitized with a 0.02 M fraction to which sufficient A substance had been added to inhibit agglutination and were tested with various antiglobulin reagents, before and after incubation with fresh serum as a source of complement. Similarly,

TABLE VI

Complement Titre of Fresh Serum after Absorption with Cells Sensitized with 7S γ Anti-A and 19S γ Anti-A Respectively

Fresh serum absorbed with group A cells sensitized with:	Dilutions of complement ¹ (reciprocals)			
	10	20	40	80
0.02 M fraction ² diluted 1 in 8	+++	++	+	—
0.2 M fraction ² diluted 1 in 8	+	—	—	—
Control: Unabsorbed fresh serum	+++	+++	++	+

¹ Serial dilutions were tested against sheep cells sensitized with bovine antibody and the degree of haemolysis noted (++ = approx. 50% lysis).

² Both fractions were prepared from serum T. B. The anti-A haemolysin titre of each fraction was 1 in 16.

TABLE VII

Complement-Binding by 7S γ Anti-A in a Fraction Prepared from a Group O Serum and by 19S γ Anti-A in a Group B Serum

		Results of antiglobulin tests with red cells sensitized with:							
Subject Initials	Group	Fraction	Dilution of A substance added	antibody alone ¹			antibody+complement ²		
				anti- 7S γ	anti- 19S γ	anti- β_1	anti- 7S γ	anti- 19S γ	anti- β_1
T. B.	O	0.02 M	1/10	++	—	—	++	—	—
		(diluted	1/50	++	—	—	++	—	(+)
		1 in 4)	1/100	+++	—	—	+++	—	+
A. S.	B	whole serum	1/50,000	—	(+)	—	—	(+)	+
		(diluted	1/100,000	—	+	—	—	+	++
		1 in 4)	1/500,000	—	+	—	—	+	++

¹ Fraction and serum were treated with E.D.T.A. (4 mg/ml) to prevent the uptake of complement.

² The source of complement was fresh serum from a normal group O donor which had been absorbed at 0°C with A₁ cells to free it from all anti-A activity.

group A red cells were sensitized with group B serum containing only 19 S γ anti-A; again, A substance was added to inhibit agglutination and the cells were tested with antiglobulin reagents before and after incubation of the sensitized cells with complement.

As Table VII shows, cells which gave a +++ reaction with anti-7 S γ globulin, after treatment with complement gave only a + reaction with anti- β_1 globulin. By contrast cells which gave only a + reaction with anti-19 S γ , gave a ++ reaction with anti- β_1 globulin after treatment with complement.

Reactions of Intermediate Fractions

As described above, anti-A activity was found in "intermediate fractions" obtained from only 1 out of 4 subjects stimulated with A substance but was also found in the selected mother (T. B.).

In both cases the serological characteristics of the anti-A in the intermediate fractions resembled that of the 19 S γ antibody (that is, the antibody recovered in the fraction containing the bulk of the 19 S γ globulin). For example anti-A in fractions 3 and 4 from donor

T. B. (see Fig. 5) had the following characteristics: it agglutinated A^p cells; it was inhibited by less A substance than that required to inhibit the anti-A in fractions 1 and 2; it did not agglutinate A cells more strongly in a medium of serum than in saline and its serological activity was completely abolished by treatment with 2-mercapto-ethanol.

Discussion

It has previously been shown that by using suitable stored red cells [8] or red cells from a patient with paroxysmal nocturnal haemoglobinuria [6] that all human anti-A sera will bring about specific haemolysis. The present observations suggest that all human anti-A sera will lyse fresh normal group A red cells provided that the ratio of serum:cells is sufficiently high.

There is evidence that the distribution of anti-A haemolysin is not unimodal. As Fig. 1 shows, the proportion of sera which contain extremely weak haemolysin (titre 0 or 1 when the ratio of serum:cells in the test is 40:1) is much greater than would be expected from a normal distribution with a single peak. As Table I shows, sera which are only very weakly lytic usually fail to agglutinate A^p cells.

The imperfect correlation between haemolysin titre and agglutinin titre noted in the present series has often been noted previously [5, 18, 7]. It seems to be due partly to the fact that anti-A agglutinin may be composed either of 19 S γ or of 7 S γ and that these antibodies vary in their ability to haemolyse red cells. Thus the least potent examples of 7 S γ anti-A are relatively more lytic than the least potent examples of 19 S γ anti-A; but when potent antibodies are considered, 19 S γ anti-A is more lytic than 7 S γ anti-A.

The present observations concerning differences between the anti-A in group B and group O subjects agree with those of ABELSON and RAWSON [2] and of KOCHWA *et al.* [10]. Thus in group B subjects, before and after stimulation with A substance, predominantly 19 S γ anti-A is produced, whereas in group O subjects even before stimulation some of the anti-A may be 7 S γ globulin and after stimulation 7 S γ anti-A as well as 19 S γ anti-A is produced in most subjects.

The present observations emphasize the considerable differences observed between different subjects. For example of the 5 group O subjects injected with A substance only 1 (R. P.) showed no increase in anti-A haemolysin (and relatively little increase in anti-A agglutinin). This particular subject had anti-A in "intermediate fractions"

as well as in those fractions containing the bulk of the 7 S γ and 19 S γ globulin. It may be relevant that the 7 S γ anti-A in this particular subject, unlike all other examples of 7 S γ anti-A tested in the present work, was easily neutralized by A substance.

ROCKEY and KUNKEL [17] drew attention to the fact that when anti-A sera were fractionated on DEAE-cellulose, a considerable amount of the antibody might be eluted in fractions intermediate between those containing the bulk of the 7 S γ globulin and 19 S γ globulin (the same point is suggested by the data of ABELSON and RAWSON [1]). ROCKEY and KUNKEL examined "intermediate fractions" in the ultracentrifuge and found that they contained material with a sedimentation coefficient of 11-12 S. The present observations confirm that when certain sera containing anti-A are fractionated on a DEAE-cellulose column much of the anti-A may be recovered in fractions intermediate between those containing the bulk of the 7 S γ and 19 S γ globulin respectively. The present work shows that the serological behaviour of this "intermediate" antibody is indistinguishable from that of 19 S γ anti-body.

The serological characteristics of 19 S γ anti-A and 7 S γ anti-A differ in many ways: thus 19 S γ anti-A of a given titre is neutralized by about 1/20th of the dose of A substance which is needed to neutralize 7 S γ anti-A of the same titre. KOCHWA *et al.* [10] found that anti-A in the 0.02 M fraction from a DEAE-cellulose column was often, but not always, difficult to inhibit with A substance but they did not report comparisons with anti-A in the 0.2 M fraction.

The fact that agglutination produced by 7 S γ anti-A is enhanced by the use of serum as a medium whereas agglutination produced by 19 S γ is not enhanced agrees with expectations. For example "incomplete" Rh antibody, now known to be made of 7 S γ globulin, may agglutinate red cells suspended in serum but fail to agglutinate saline-suspended red cells.

The "enhancing" effect of normal serum on agglutination produced by immune anti-A, first described by BOORMAN, DODD and MORGAN [4], suggests that these workers were dealing with selected group O sera containing substantial amounts of 7 S γ anti-A.

The method described by WITEBSKY [20] for revealing the presence of "immune" anti-A is evidently a very good way of demonstrating the presence of anti-A because the addition of A substance will preferentially neutralize 19 S γ anti-A and the use of AB serum as a diluent will enhance agglutination by the remaining 7 S γ anti-A.

When WITEBSKY's test is modified so that after the addition of AB substance to the anti-A serum the partially-neutralized serum is tested by the indirect antiglobulin method, it can be made a completely specific test for 7 S γ anti-A by simple using a specific anti-7 S γ globulin serum.

WINSTANLEY, KONUGRES and COOMBS [19] found a good correlation between the haemolysin titre of sera against human A₁ cells and the anti-A^p titre as measured by the indirect antiglobulin test with A^p cells and from this and other observations concluded that "so-called immune (haemolytic) anti-A human sera, as opposed to 'natural' (non-lytic) anti-A human sera, have an antibody (anti-A^p) which reacts with group A pig cells". In a further paper KONUGRES and COOMBS [11] concluded that the antibody in so-called "immune" anti-A human sera which is recognized by the Witebsky reaction is, like the haemolysin, anti-A^p in specificity.

In general the present observations confirm these findings; thus in the present series anti-A sera which failed to haemolyse human A₁ cells also failed to react with A^p cells; however there were minor discrepancies and some sera which weakly lysed human A₁ cells did not react with A^p cells.

The present observations show that the behaviour of 19 S γ anti-A and of 7 S γ anti-A with respect to A^p cells is different. Thus all examples of 7 S γ anti-A tested (including 7 S γ anti-A in cord sera) failed to agglutinate A^p cells suspended in saline but invariably sensitized them to agglutination by an antiglobulin serum. On the other hand most 19 S γ anti-A agglutinated A^p cells. Those 19 S γ anti-A which did not agglutinate A^p cells also failed to sensitize A^p cells to agglutination by an antiglobulin serum; moreover the anti-A titre of these sera was not decreased by absorption with A^p cells. The fact that some immune anti-A sera only weakly agglutinated A^p cells but strongly sensitized them to an antiglobulin serum is clearly shown by Table I (b) of WINSTANLEY *et al.* [19].

The present observations suggest that a test for the haemolysis of A^p cells would not be useful in distinguishing between 7 S and 19 S anti-A, since both antibodies, if sufficiently potent, bring about lysis.

The question now arises whether the term "immune" applied to human anti-A has any meaning—a question also considered by KOCHWA *et al.* [10].

One might define "naturally-occurring" anti-A as a type of 19 S γ

anti-A with a relatively low agglutinin titre, only very weak haemolytic activity and an inability to react with A^p cells. It may be of course that individuals who produce only this kind of antibody are ones who, for genetical or other reasons, respond differently to A stimuli in the environment and that the anti-A which they produce is simply a different kind of "immune" anti-A.

From a clinical point of view the important characteristics of anti-A are: the destructive power against group A red cells and the ability to pass the placenta. It seems certain that 19S γ anti-A when it is powerfully lytic, as it often is, can cause very rapid destruction of transfused A cells although when considering the transfusion of anti-A plasma to group A subjects the ease of neutralization of 19S γ anti-A might make it a relatively less dangerous antibody than 7S γ anti-A. From the point of view of haemolytic disease of the newborn it is evident that only 7S γ anti-A need be considered [10].

Tests which detect "immune" characteristics such as relatively strong haemolytic activity or the ability to react with A^p cells, which are associated both with 19S γ and with 7S γ "immune" anti-A are clearly not the best which can be devised for the prediction of haemolytic disease of the newborn due to ABO incompatibility and, for this purpose, tests which estimate only 7S γ anti-A must clearly be superior.

Summary

Examples of anti-A were obtained from various subjects: random donors; selected group O and B subjects, before and after the injection of A substance; the mother of an infant severely affected with ABO-haemolytic disease; and normal newborn infants.

Approximately 90% of the random group O sera would haemolyse group A red cells when the ratio of serum:cells was 40:1, and all group O sera tested were haemolytic when the ratio was 200:1. Approximately 30% of group O sera failed to agglutinate pig group A red cells and these sera were mainly those with a low haemolysin titre when tested against human group A red cells.

Selected sera were fractionated on a DEAE-cellulose column. In many cases elution was carried out only with 2 phosphate buffers, 0.02 M to obtain a fraction containing 7S γ globulin and 0.2 M to obtain a fraction containing 19S γ globulin, but in some cases intermediate fractions were obtained. The concentration of 7S γ and 19S γ in all fractions was estimated by an immunological method. It was confirmed that anti-A in group B subjects, before and after stimulation with A substance is predominantly 19S γ globulin whereas in group O subjects in addition to 19S γ anti-A there is often some 7S γ anti-A before stimulation and usually considerable 7S γ anti-A after stimulation. The finding of ROCKEY and KUNKEL [17] that considerable amounts of anti-A may be present in "intermediate" fractions obtained

from a DEAE-cellulose column, that is fractions containing only low concentrations of 7S γ and 19S γ globulin, was confirmed.

Various serological characteristics of these different fractions were studied: (1) Anti-A in the 0.2 M fraction (19S γ) was far more easily neutralized by A substance than the anti-A in the 0.02 M fraction (7S γ). (2) Whereas the agglutination produced by the anti-A in the 0.02 M fraction was greatly enhanced in a medium of serum, compared with saline, agglutination produced by anti-A in the 0.2 M fraction was not enhanced by serum. (3) Only the anti-A in the 0.2 M fraction was found to agglutinate A^p cells although the anti-A in the 0.02 M fraction would sensitize A^p cells to agglutination by an anti-7S γ globulin serum. (4) Anti-A in the 0.2 M fraction bound more complement than antibody of equivalent titre in the 0.02 M fraction.

In all these tests, antibody eluted in intermediate fractions and thought to correspond with the 11-12S antibody described by ROCKEY and KUNKEL [17] behaved in the same way as 19S γ anti-A recovered in the 0.2 M fraction.

It is clear that there is no single criterion of "immune" anti-A; an ability to haemolyse group A cells readily is possessed by most 19S γ anti-A and all 7S γ anti-A, whereas the ability of a serum, after partial neutralization, to sensitize A cells to an antiglobulin serum is almost confined to those containing 7S γ anti-A.

Résumé

Des anti-A ont été obtenus chez différents sujets: des donneurs pris au hasard; des sujets de groupe B ou O avant et après l'injection de substance A; de mères d'enfant atteints gravement de maladies hémolytiques ABO; de nouveaux-nés normaux.

A peu près 90% des sérums de groupe O testés hémolysent les érythrocytes de groupe A lorsque le rapport sérum/érythrocytes est de 40:1 et tous les sérums O testés sont hémolytiques quand le rapport est de 200:1. A peu près 30% des sérums de groupe O sont incapables d'agglutiner les érythrocytes A de porcs et ces sérums sont précisément ceux qui présentent un titre bas d'hémolysines lorsqu'ils sont testés contre les érythrocytes humains de groupe A.

Des sérums sélectionnés ont été fractionnés sur colonne de DEAE cellulose. Dans de nombreux cas, l'élution a été pratiquée seulement avec deux tampons phosphate, l'un à 0,02 M pour obtenir une fraction contenant les 7 S γ globulines, l'autre à 0,2 M pour obtenir la fraction des 19 S γ globulines, mais dans certains cas des fractions intermédiaires ont été obtenues. La concentration des 7 S et des 19 S γ globulines a été déterminée dans toutes les fractions par une méthode immunologique. Il a été confirmé que l'anti-A des sujets de groupe B, avant et après stimulation par la substance A, est surtout des 19 S γ globulines, tandis que chez les sujets de groupe O, avant la stimulation, en plus des 19 S γ anti-A, il y a souvent un peu de 7 S γ anti-A, et, d'ordinaire, après la stimulation, une grande quantité de 7 S γ anti-A. La découverte de ROCKEY et KUNKEL [17] selon laquelle une grande quantité d'anti-A peut se trouver dans la fraction «intermédiaire» obtenue à partir des colonnes de DEAE cellulose, et, que ce sont des fractions contenant uniquement de faibles concentrations de 7 S et 19 S γ globuline est confirmée.

Les différentes caractéristiques sérologiques de ces fractions ont été étudiées: (1) L'anti-A de la fraction 0,2 M (19 S γ) est beaucoup plus facilement neutralisée

par la substance A que l'anti-A de la fraction 0,02 M (7S γ). (2) Tandis que l'agglutination produite par l'anti-A de la fraction 0,02 M est fortement favorisée par un milieu sérique, l'agglutination produite par l'anti-A de la fraction 0,2 M n'est pas favorisée par un milieu sérique. (3) Seul l'anti-A de la fraction 0,2 M est capable d'agglutiner les érythrocytes A^p tandis que l'anti-A de la fraction 0,02 M sensibilise les érythrocytes A^p qui sont ensuite agglutinés par un sérum anti 7 S γ globuline. (4) L'anti-A de la fraction 0,2 M lie davantage de complément que les anticorps de même titre de la fraction 0,02 M.

Dans tous ces tests, l'anticorps élué à partir de la fraction intermédiaire et que l'on pense correspondre à l'anticorps 11-12 S décrit par ROCKEY et KUNKEL [17] se comporte de la même manière que l'anti-A 19 S γ retrouvé dans la fraction 0,2 M.

Il est clair que ceci n'est pas le seul critère de l'anti-A «immun»; l'aptitude à hémolyser les érythrocytes de groupe A est l'apanage de presque tous les anti-A 19 S γ et de tous les anti-A 7 S γ , tandis que l'aptitude d'un sérum, après neutralisation partielle, de rendre sensibles des érythrocytes à l'antiglobuline est presque le seul apanage de ceux contenant de l'anti-A 7 S γ .

Zusammenfassung

Es wurden Anti-A-Seren verschiedener Herkunft beschafft. Diese stammten von Blutspendern, ausgewählten O- und B-Individuen, vor und nach Injektion von A-Substanz, von normalen Neugeborenen sowie von der Mutter eines Kindes mit schwerem ABO-Morbus hämolyticus.

Ungefähr 90% der O-Seren hämolysierten A-Zellen, sofern das Serum-Zellverhältnis 40:1 betrug; bei einem Verhältnis von 200:1 erwiesen sich alle O-Seren als hämolytisch aktiv. Ca. 30% der O-Seren vermochten Schweineerythrozyten der Gruppe A nicht zu agglutinieren. Es handelte sich dabei in der überwiegenden Mehrzahl um jene Seren, die gegenüber menschlichen A-Zellen einen niederen Hämolyse-titer aufwiesen.

Einige ausgewählte Seren wurden auf DEAE-Zellulosekolonnen fraktioniert. In den meisten Fällen wurde die Elution nur mit zwei Phosphatpuffern vorgenommen. Mit dem 0,02 M-Puffer wurde die Fraktion, welche das 7S- γ -Globulin enthielt, eluiert. Zur Elution der Fraktion, in der das 19S- γ -Globulin enthalten war, wurde der 0,2 M-Puffer verwendet. In einigen Fällen wurden Zwischenfraktionen gewonnen. Der Gehalt der Fraktionen an 7S- γ - und 19S- γ -Globulin wurde immunchemisch überprüft. Es bestätigte sich, daß die Anti-A-Antikörper bei B-Individuen vor und nach der Stimulation mit A-Substanz vornehmlich den Charakter von 19S- γ -Globulinen aufweisen; bei O-Individuen findet man vor der Stimulation nebst 19S-Antikörpern auch eine gewisse Menge von 7S-Antikörpern. Nach der Stimulation mit A-Substanz steigt bei O-Individuen der Gehalt an 7S-Antikörpern meist stark an. Der Befund von ROCKEY und KUNKEL [17], die beträchtliche Mengen von Anti-A in Zwischenfraktionen mit geringem 7S- γ - und 19S- γ -Globulingehalt gefunden hatten, wurde bestätigt.

Die serologischen Eigenschaften dieser Fraktionen wurden untersucht. Dabei zeigte sich folgendes:

1. Die Anti-A-Antikörper der 0,2 M-Fraktion (19S γ) wurden wesentlich leichter durch A-Substanz neutralisiert, als diejenigen der 0,02 M-Fraktion (7S). 2. Die

Agglutination der Anti-A-Antikörper der 0,02 M-Fraktion wurde im Serummilieu im Vergleich zum Kochsalzmilieu wesentlich verstärkt; bei den Antikörpern der 0,2 M-Fraktion blieb dieser Effekt aus. 3. Lediglich die in der 0,2 M-Fraktion enthaltenen Anti-A-Antikörper vermochten A^p-Zellen zu agglutinieren. Die Antikörper der 0,02 M-Fraktion sensibilisierten A^p-Zellen, so daß diese durch Anti-7S- γ -Globulinseren agglutiniert wurden. 4. Die Anti-A-Antikörper der 0,2 M-Fraktion vermochten mehr Komplement zu binden, als titergleiche Antikörper der 0,02 M-Fraktion.

In all diesen Versuchen verhielten sich die in den Zwischenfraktionen enthaltenen Antikörper (11–12S-Antikörper von ROCKEY und KUNKEL [17]) wie die in der 0,2 M-Fraktion enthaltenen 19S-Antikörper.

Es ist klar, daß es kein verlässliches Einzelkriterium für den «Immuncharakter» von Anti-A-Antikörpern gibt. Die meisten 19S- γ -Anti-A- und alle 7S- γ -Anti-A-Antikörper vermögen A-Zellen zu lysieren. Die Fähigkeit nach partieller Neutralisation A-Zellen für die Wirkung von Antiglobulinseren zu sensibilisieren, findet sich fast ausschließlich bei Seren, die 7S-Anti-A-Antikörper enthalten.

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Authors' address: Prof. P. L. Mollison, Dept. of Haematology, St. Mary's Hospital Med. School, Paddington, London W 2 (England).

Serological Characteristics of Blood-Group Antibodies Made of γ and β_2 M Globulin Respectively

P. L. MOLLISON, M. C. ADINOLFI and MARGARET J. POLLEY

London, England

There are two main antibody proteins in human serum; the one which is quantitatively most important is referred to in this paper simply as γ globulin, but is often described as 7S γ globulin; the other is referred to here as β_2 M globulin, which corresponds with the terms 19S γ globulin or γ_1 M globulin.

In the present work, three methods have been used for classifying blood group antibodies as γ or β_2 M globulin. First, indirect antiglobulin tests using specific anti- γ and anti- β_2 M globulin sera. In order to make the anti- β_2 M globulin serum specific, it was necessary to absorb out anti- γ globulin present in the original rabbit serum; the resulting serum reacted well with red cells coated with β_2 M globulin, for example red cells sensitized with anti-Le^a, but failed completely to agglutinate cells coated with γ globulin, for example red cells sensitized with anti-Rh. Inhibition of specific antiglobulin reactions was used as a method of estimating the amount of γ or β_2 M globulin in various preparations; the method can detect amounts of less than 1 mg/100 ml (KEKWICK, VALLET, CUTBUSH, MOLLISON, THOMAS, GELL AND SOOTHILL, 1961; POLLEY, MOLLISON and SOOTHILL, 1962).

The second method used to distinguish between γ and β_2 M globulin antibodies was to fractionate sera on a DEAE-cellulose column, using a minor modification of the method of LEVY and SOBER, 1960. Only two eluents were used, 0.02 M phosphate buffer pH 8.1 and 0.2 M phosphate buffer pH 8.1. In the 0.02 M fraction more than 90% of the γ globulin was recovered without any trace of β_2 M globulin; in the 0.2 M fraction there was 3-10% of the γ globulin and most of the β_2 M globulin, as well as other serum proteins.

Sera were also treated with 2-mercapto-ethanol to inactivate antibodies made of β_2 M globulin (cf. GRUBB and SWAHN, 1958).

Those blood-group antibodies which are regarded as naturally-occurring were usually composed exclusively of β_2 M globulin. Although these antibodies are usually agglutinins at relatively low temperatures, they may fail to agglutinate cells at 37 °C, and they can then be tested by the indirect antiglobulin method. Anti-Le^a is a good example of an antibody which agglutinates cells at 25 °C but

not at 37 °C and at 37 °C sensitizes red cells to agglutination by an anti- β_2 M globulin serum. Blood group systems in which naturally occurring antibodies are found most commonly are ABO, MNSs, P and Lewis.

Anti-A and anti-B are almost always β_2 M globulin in group A and B subjects but are often partly γ globulin in group O subjects.

In the MNSs system the antibodies are usually β_2 M globulin but in the present work one example of anti-M and one of anti-N were encountered, both of which were γ globulin. The anti-M, which behaved as an agglutinin, was found in the serum of a patient who had given birth to an infant affected with haemolytic disease of the newborn due to anti-M (STONE and MARSH, 1959), but the anti-N, which was "incomplete", was obtained from a woman who had given birth to two normal, group M, infants (serum and details of history kindly supplied by Dr. ELOISE R. GIBLETT). Anti-S may also sometimes be a γ globulin antibody because examples which have caused haemolytic disease of the newborn have been described.

In the P and Lewis systems 11 of out 13 antibodies were wholly β_2 M globulin; the two exceptions were examples of anti-P + P₁ (anti-T)^a and anti-Le^a respectively; in both these cases, although the antibody was predominantly β_2 M globulin, a small amount was γ globulin.

One more naturally occurring antibody tested was the anti-I from the normal donor described by JENKINS *et al.* (1960); this antibody was also a β_2 M globulin.

Immune blood group antibodies are usually γ globulin but a minority are β_2 M globulin. It has been known for some time that agglutinating anti-Rh is a "heavy" (19S) globulin (CAMPBELL, STURGEON and VINOGRAD, 1955). Sera which contain Rh agglutinins almost always contain incomplete anti-Rh in addition and this "hidden" antibody can be revealed by fractionating the serum on a DEAE-cellulose column. Thus in one example tested in the present work, whereas the whole serum and the 0.2 M fraction strongly agglutinated red cells the 0.02 M fraction contained only incomplete antibody.

Although immune antibodies composed of β_2 M globulin are usually agglutinins, in some systems they may behave as incomplete antibodies. Thus we found that three out of 15 examples of incomplete anti-Jk^a tested were β_2 M globulin; one was a mixture of γ and β_2 M and the remainder were entirely γ globulin.

It appears that in some blood group systems there is a strong bias towards the production of β_2 M globulin as an immune response. Thus if there is an immune response to the Le^a antigen, the anti-Le^a produced is wholly or predominantly β_2 M globulin. Similarly, if a group B subject responds to the injection of A substance the anti-A produced is predominantly β_2 M globulin. By contrast group O subjects tend to produce γ anti-A as well as β_2 M anti-A (cf. RAWSON and ABELSON, 1960 and KOCHWA, ROSENFELD, TALLAL and WASSERMAN, 1961).

The question of what is a 'naturally occurring' anti-A and what an 'immune' anti-A is a difficult one, but two observations point to the idea that the distinction is a real one. First, if the haemolysin titre of a random sample is plotted against frequency it is found that the distribution is not unimodal and that too many sera

have a haemolysin titre of O or 1. Second, as shown by WINSTANLEY, KONUGRES and COOMBS (1957), only those anti-sera which readily lyse human A cells will react with pig group A cells suggesting that readily haemolytic anti-A sera contain an extra antibody, anti-A_P. We have confirmed these observations and added the point that whereas most β_2 M globulin anti-A agglutinate A_P cells, γ globulin anti-A do not. However, γ globulin anti-A invariably sensitize A_P cells to agglutination by an anti-7S γ globulin serum. Thus one may tentatively conclude that all γ anti-A but only some β_2 M anti-A are 'immune'.

It is possible to obtain anti-A which is pure β_2 M globulin by simply testing group B sera and finding one with no γ anti-A in it. Pure γ globulin anti-A can be prepared by fractionating a group O serum rich in γ anti-A and collecting only the 0.02 M fraction. With these preparations the behaviour of β_2 M anti-A and γ anti-A can be contrasted. β_2 M anti-A is found to be more readily neutralized by A substance than γ anti-A. Agglutination of γ anti-A but not of β_2 M anti-A is enhanced by using a medium of diluted AB serum. These observations show that WITBESKY's partial neutralization test (1948) is predominantly a test for revealing the presence of γ anti-A. One further difference between β_2 M and γ anti-A is that β_2 M anti-A is the better complement-binder.

After the work described here had been completed, ROCKEY and KUNKEL [1962] published their finding that anti-A may be composed of globulin with a sedimentation co-efficient of 11–12S, which is eluted from a DEAE-cellulose column in a fraction intermediate between those containing 7S and 19S γ globulin respectively. If any of the sera in the present work contained antibodies of this type, the antibodies would have been collected in the 0.2 M fraction and regarded as β_2 M globulin. Preliminary observations suggest that from a serological point of view, antibodies of 'intermediate type' behave like β_2 M globulin antibodies.

Not all β_2 M globulin antibodies bind complement better than γ globulin antibodies because there is undoubtedly a connection with blood group specificity. For example anti-Rh, whether made of β_2 M globulin or γ globulin, never or at most only very rarely binds complement, whereas anti-Jk^a whether made of β_2 M globulin or γ globulin seems invariably to bind complement.

Summary and Conclusions

Naturally-occurring antibodies are almost always made of β_2 M (19S γ) globulin. Such antibodies are usually agglutinins but at 37 °C they may behave as incomplete antibodies; naturally-occurring antibodies made of β_2 M globulin invariably bind complement and are often lytic.

Immune blood group antibodies are usually γ (7S γ) globulin but may be β_2 M globulin; immune antibodies made of γ globulin are usually incomplete but they may be agglutinins. Immune β_2 M globulin antibodies are usually agglutinins but may behave as incomplete antibodies, (e.g. some examples of anti-Jk^a).

In the ABO system β_2 M globulin antibodies bind more complement than γ globulin antibodies, but in some blood group systems (e.g. Rh) neither β_2 M nor γ globulin antibodies bind complement.

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**SURVIVAL OF ^{51}CR -LABELLED RED CELLS IN SUBJECTS WITH
THALASSAEMIA-TRAIT OR G6PD DEFICIENCY OR BOTH
ABNORMALITIES**

BY

**L. BERNINI, B. LATTE, M. SINISCALCO, S. PIOMELLI, U. SPADA,
M. ADINOLFI and P. L. MOLLISON**

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Survival of ^{51}Cr -Labelled Red Cells in Subjects with Thalassaemia-Trait or G6PD Deficiency or Both Abnormalities

L. BERNINI, B. LATTE AND M. SINISCALCO

Department of Genetics, Faculty of Science, University of Rome*

S. PIOMELLI AND U. SPADA

Institute of Medical Pathology, Faculty of Medicine, University of Naples

M. ADINOLFI AND P. L. MOLLISON

*Medical Research Council's Experimental Haematology Research Unit,
Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2*

THE distribution of thalassaemia and G6PD deficiency has been intensively studied in Sardinia and found to be positively correlated with the past incidence of malaria (Ceppellini, 1955; Siniscalco, Bernini, Latte and Motulsky, 1961). Thus among the isolated communities of the once malarious plains of the island it is easy to find villages where the frequency of each trait exceeds 20–30 per cent, and where, therefore, the presence of both the anomalies in the same individual is not uncommon. The concentration in a single locality of these elsewhere rare abnormalities offered an unusual opportunity to carry out red-cell survival studies in groups of subjects with one or other or both abnormalities, and in normal subjects, within a limited period of time and under controlled experimental conditions.

The reasons for wanting information about red-cell life-span in subjects with these abnormalities can be explained by considering some previous findings.

During studies in Sardinia it was observed that the red blood cells of individuals who had the thalassaemia-trait, but who were otherwise normal, had a G6PD activity about 30 per cent higher than that found in non-thalassaemic individuals of the same age and sex. Women who had the thalassaemia-trait and who were also heterozygous for G6PD deficiency had almost normal G6PD values, and so could easily be misclassified unless different discrimination thresholds were used for the diagnosis of the G6PD phenotypes in the presence of thalassaemia (Adinolfi, Bernini, Carcassi, Latte, Motulsky and Siniscalco, 1960).

Since it was known from the work of Marks, Johnson and Hirshberg (1958) that the enzyme activities of the red cells decrease with ageing, it was thought that the increased G6PD level in thalassaemia-trait individuals might be due to a younger mean age of their red cells due in turn to a shortened red-cell life-span. This hypothesis received indirect support from the finding that in the blood of thalassaemia-trait individuals the level of another enzyme—glutamic oxaloacetic transaminase—is also raised (Sass and Spears, 1961).

A shortened red-cell survival has recently been demonstrated in G6PD-deficient American negroes, even in the absence of drug administration (Brewer, Tarlov and Kellermeyer, 1961). The enzyme-deficiency observed in Caucasian subjects is known to be different from that

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observed in negroes because, at least in the red-cells, the deficiency is complete in Caucasians and only partial in negroes (Marks and Gross, 1959). Estimation of red-cell survival would confirm the effect of enzyme-deficiency on red-cell life-span in Caucasians and perhaps show whether it was more severe than the effect observed in negroes.

In all Sardinian villages so far studied the observed number of individuals carrying both thalassaemia and G6PD deficiency has been found to exceed slightly the expected figure, calculated from independent estimates of the frequencies of the two genes in each population (Bernini, Carcassi, Latte, Motulsky, Romei and Siniscalco, 1960). This excess, which becomes significant at a 5 per cent level when the data from all villages are pooled together (M. Siniscalco, unpublished data) may well be due to a higher fitness of the relevant genotype. It has, for instance, been observed that episodes of clinical favism are significantly less frequent (Siniscalco *et al.*, 1961) in this group of individuals, probably because of the above-described effect of thalassaemia on the G6PD level in the red cells. It was thus interesting to see if this increase in fitness is mediated through a shorter red-cell survival time than that observed in the presence of G6PD deficiency alone.

SUBJECTS AND METHODS

Population among whom the Study was Conducted

The village in which the study was carried out was Lodé in the province of Nuoro, Sardinia. This village may be regarded as a typical Mendelian isolate, which has existed for centuries in a highly malarious area. In previous surveys, blood examinations have been carried out on almost three-quarters of the inhabitants of this village, and genetical data have been obtained (M. Siniscalco, unpublished data). The frequencies of thalassaemia and G6PD deficiency among males are respectively 0.276 ± 0.01 and 0.282 ± 0.01 . The incidence of male carriers of both the traits is 0.079 (in contrast with the expected one of 0.067).

Haematological Techniques Used in Diagnosis

Red-cell counts and Price-Jones curves were performed with an electronic red-cell counter and pulse height discriminator (Lars Ljungberg, Celloscope).

Packed-cell volume was measured in duplicate with a capillary micro-method, at 8000 rev./min. for 15 minutes, using a Lars Ljungberg centrifuge (Cellocite).

Haemoglobin concentration was measured by the cyanmethaemoglobin method.

Haematological indices (MCV, MCH and MCHC) were calculated according to Wintrobe (1961). Reticulocyte preparations were made according to the method of Dacie (1956) and counts were made on return to Naples.

Red-cell osmotic fragility was usually estimated simply by noting, visually or colorimetrically, the degree of haemolysis in a buffered hypotonic solution corresponding to 0.375 per cent NaCl. This concentration was found to be optimal for discriminating between normals and thalassaemics (M. Siniscalco, unpublished observations). Whenever possible, this preliminary test was supplemented by estimation of the median osmotic fragility (Dacie, 1956) using only three hypotonic solutions corresponding respectively to 0.375, 0.40 and 0.425 per cent NaCl and using saponin as a haemolysing agent to obtain 100 per cent haemolysis.

Haemoglobin F was measured by the 1-minute alkali denaturation method of Singer, Chernoff and Singer (1951).

Haemoglobin A₂ was measured by starch-block electrophoresis at pH 8.6 according to the technique described by Kunkel, Ceppellini, Müller-Eberhard and Wolf (1957), as modified by Gerald and Diamond (1958) using a haemolysate prepared from red cells which had been best for 2–3 months at -20°C . in a glycerol-citrate-phosphate solution (see Mollison, 1961a).

Glucose-6-phosphate-dehydrogenase (G6PD) activity was estimated in the field with the screening method of Motulsky and Campbell-Krant (1960) and later measured quantitatively by the spectrophotometric method of Kornberg and Horecker (1955), on a sample of blood collected into ACD solution, frozen at -20°C . immediately after collection, and kept frozen until tested.

Haematological Diagnosis

The diagnosis of thalassaemia trait was based on microcytosis, as measured by the MCV and by the Price-Jones curve, a low MCH with a normal MCHC, and on a decreased osmotic fragility; these data were supplemented by the inspection of a blood film. In almost every case the propositus had at least one relative with a similar blood picture. Of the cases diagnosed as having the thalassaemia trait and finally included in the Cr survival study, 13 out of 16 were found to have an increased proportion of Hb-A₂. (In Lodé, out of a total of 90 subjects diagnosed as having the thalassaemia trait, 16 were found to have normal or diminished Hb-A₂, and of these two had readily detectable amounts of haemoglobin H; unpublished observations.)

The diagnosis of G6PD deficiency was established in the field with the Motulsky dye test and later confirmed by quantitative assay.

Normal subjects were those in whom all the above tests gave normal results.

Selection of Subjects, Coding and Randomization

The selection of subjects for the experiment was performed by two members of the team; each subject was given a serial number, and no other member of the team was aware of the diagnosis until the experiment, including the calculation of red-cell survival, was completed. All subjects were adult males between 18 and 60 years of age who were otherwise healthy and were following their usual lives. The labelling of all subjects was completed over a period of 6 days; the subjects to be injected on the same day were arranged in series of four, one belonging to each experimental group (whenever possible) and labelled at the same time. Two to four such series of individuals were handled each day. For the successive bleedings the subjects were given appointments, and each sample was coded with the serial number together with a serial letter corresponding to the day of treatment.

Experimental groups. Sixty-three individuals were studied; using the above-mentioned criteria, they were divided into the four following groups: (1) normal subjects (16 individuals), (2) thalassaemia-trait (14 individuals), (3) G6PD deficiency (23 individuals), (4) G6PD deficiency and thalassaemia-trait (10 individuals).

Labelling of Red Cells with ^{51}Cr

The citrate-phosphate-dextrose solution, pH 6.9, described by Mollison (1961b) was used throughout and is described here simply as citrate solution.

Eight to 9 ml. of blood were taken with a dry plastic syringe and placed in a screw-cap tube containing 17–18 ml. of citrate solution. After gentle mixing, the blood was centrifuged at 1500 rev./min. for 10 minutes, and most of the supernatant removed without disturbing the red cells. The red cells were finally suspended in 2–3 ml. of citrate solution before adding 200 μ c. of $\text{Na}_2^{51}\text{CrO}_4$ in 0.4 ml. of saline; the amount of chromate added was always less than 1 μ g. Cr per ml. red cells. The tube was then incubated for 15 minutes in a 37° C. waterbath and the red cells were then washed once in citrate solution and resuspended in citrate to a final volume of approximately 10 ml.

Injection of Labelled Red Cells and Taking of Blood Samples

The labelled red cells were immediately reinjected into the donor. Blood samples were taken at 20 minutes and at 2, 5, 9, 13, 17, 21, 15 and 29 days after the injection. Except for the labelling and injections of cells, all bleedings were effected in the evenings, with the individuals sitting. All samples of venous blood were collected without stasis and distributed into two tubes containing dry oxalate (Wintrobe mixture). Approximately 2.5 ml. of blood were taken into one tube and 12 ml. into the other. The tubes were stoppered and the blood was mixed with the anticoagulant by rapid inversion. Packed-cell volume and haemoglobin concentration were measured immediately, and the tubes were then stored at –20° C.

Estimation of Radioactivity of Blood Samples

As mentioned above, every blood sample was partitioned between two containers. The 2.5 ml. samples were shipped to Naples and the 12 ml. samples were shipped to London, and the radioactivity of all samples was thus measured independently in two laboratories. The samples were frozen within an hour or two of being taken and they remained frozen until an hour or two before being counted. The procedure for counting the samples was different in the two centres.

In Naples, exactly 2 ml. of blood were pipetted into a 16 × 100 mm. tube, the tube was stoppered and the radioactivity measured in a well-type crystal, using a pulse height analyser (Packard International Model 310 auto-gamma) with automatic sample charger. Each series of samples from a single individual was counted repeatedly to obtain a minimum of 30,000 counts (statistical error less than 1 per cent).

In London, sample cups with a capacity of approximately 6.5 ml. were filled with haemolysed blood, closed with a plastic cap and placed in a well-type crystal for counting. The cups used in this counter projected well above the top of the crystal, so that small differences in filling of the cup, of the order of ± 0.1 ml., affected the counting rate by not more than ± 1 per cent. For each sample 10,000 counts were recorded. In London, the haemoglobin concentration of each blood sample was estimated in duplicate by the cyanmethaemoglobin method. In a few cases, where the sample contained small clots, the sample was filtered through gauze and the radioactivity and haemoglobin concentration of the filtered sample determined.

Calculation of Cr Survival

Results were expressed as radioactivity per ml. of blood in the Naples series and as radioactivity per gramme of haemoglobin in the London series. With this exception, the calculations were the same in the two series. Thus, the count of the 20-minute samples was taken as 100 per cent, and all further counts were expressed as percentages of this count. Percentage survival, on a logarithmic scale, was then plotted against time on a linear scale.

The resulting curves showed the typical 'early-loss' phenomenon—that is, the early part of the curve during the first 2 or 3 days after the injection of labelled red cells was slightly steeper than the remaining part of the curve (Mollison and Veall, 1955). Between 5 and 30 days, the points could be well fitted by a linear equation $a + bx = \gamma$, where x is the time in

TABLE I
Cr SURVIVAL IN THE FOUR GROUPS STUDIED

Group	No. of Subjects	Regression coefficients*	
		Naples estimate	London estimate
Normal	13	0.00880 (± 0.000346)†	0.00871 (± 0.000284)
Thalassaemia trait	9	0.00828 (± 0.000275)	0.00861 (± 0.000248)
G6PD deficiency	18	0.01017 (± 0.000328)	0.00969 (± 0.000241)
Thalassaemia trait and G6PD deficiency	7	0.00977 (± 0.000524)	0.00953 (± 0.000356)

* Fraction of Cr disappearing per day between day 5 and day 30, ignoring the period of early loss.

† Standard error.

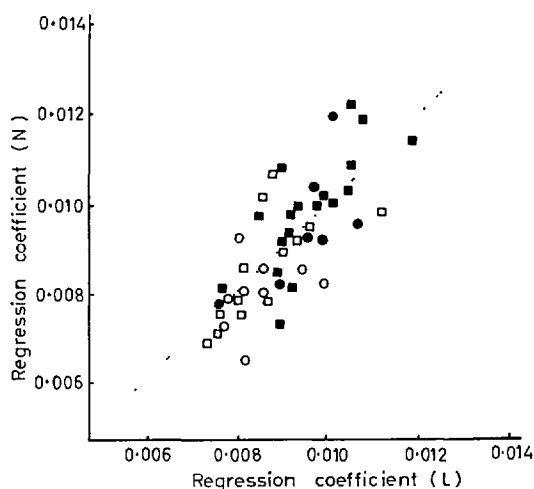


FIG. 1. Comparison between results obtained in individual cases in Naples (N) and London (L). The regression coefficient refers to the rate of loss of chromium between 5 and 30 days after labelling (see Methods). $\square$ = normal subjects; $\circ$ = subjects with thalassaemia-trait; $\blacksquare$ = subjects with G6PD deficiency; $\bullet$ = subjects with thalassaemia-trait and G6PD deficiency.

days, γ the percentage of residual radioactivity, b the regression coefficient of γ on x —i.e. the best possible estimate of the rate of disappearance of chromium from circulating blood—and a , the intercept of b on the γ axis, is a direct measure of the 'early-loss' due to the initial rapid elution of Cr.

RESULTS

It was decided that Cr survival could not be deduced reliably when less than seven blood samples had been obtained from a subject, and this led to the discarding of 15 subjects. One further subject, originally considered as normal, was found to have a slightly elevated (6 per

cent) level of Hb-F and also withdrawn. Thus there remained 47 subjects, distributed in the four groups as follows: (1) 13 normal subjects, (2) nine with thalassaemia-trait, (3) 18 with G6PD deficiency, (4) seven with G6PD deficiency and with thalassaemia-trait.

Cr Survival in the Four Groups

In Table I and Fig. 1 results are given simply as regression coefficients, as an index of the rate of loss of Cr between 5 and 30 days after injection of the labelled cells.

There is good agreement between the average results obtained in Naples and London (see Table I). The larger variability in the Naples series is probably due to two factors: first, the technique used in Naples demanded the pipetting of a precise quantity of blood into the counting vial, whereas the technique used in London did not. Second, the Naples results were based on Cr counts per unit volume of whole blood, and thus made no allowance for fluctuations in PCV, whereas the London results were expressed as Cr counts per gramme of haemoglobin.

In Fig. 1 the individual results from the two series are compared. Thus for each subject the regression coefficient as determined in Naples and London is plotted. As described in Methods, this coefficient applies only to the rate of loss of chromium in the period between 5 and 30 days after injection of the labelled cells. As the figure shows, results for the two series of measurements were in fairly close agreement; the correlation coefficient (r) was calculated to be 0.75.

Difference in Cr Survival between the Four Groups

Statistical analysis of the data was designed to evaluate the effects on Cr survival of thalassaemia-trait alone, G6PD deficiency alone, and of the combined abnormalities.

In assessing the effect of, for example, thalassaemia-trait, it was possible to use not only the comparison between the thalassaemia-trait subjects and the normal subjects but also between the subjects with thalassaemia-trait and enzyme deficiency on the one hand and those with enzyme deficiency alone on the other. The following method of analysis was suggested to us by Dr. C. A. B. Smith (personal communication). Using the term b for the regression coefficient in each group, and σ for the S.E., and using the sub-scripts N, T, R and TE for normal subjects, thalassaemia-trait subjects, enzyme-deficient subjects and subjects with the two abnormalities, the effect of thalassaemia-trait on the Cr survival curve could be expressed as:

$$\frac{\frac{b_T - b_N}{\sigma_T^2 + \sigma_N^2} + \frac{b_{TE} - b_E}{\sigma_{TE}^2 + \sigma_E^2}}{\frac{1}{\sigma_T^2 + \sigma_N^2} + \frac{1}{\sigma_{TE}^2 + \sigma_E^2}} \text{ with S.E. } \sqrt{\frac{1}{\frac{1}{\sigma_T^2 + \sigma_N^2} + \frac{1}{\sigma_{TE}^2 + \sigma_E^2}}} \quad (1)$$

Similarly, the effect of enzyme deficiency on the Cr survival curve could be written:

$$\frac{\frac{b_E - b_N}{\sigma_E^2 + \sigma_N^2} + \frac{b_{TE} - b_T}{\sigma_{TE}^2 + \sigma_T^2}}{\frac{1}{\sigma_E^2 + \sigma_N^2} + \frac{1}{\sigma_{TE}^2 + \sigma_T^2}} \text{ with S.E. } \sqrt{\frac{1}{\frac{1}{\sigma_E^2 + \sigma_N^2} + \frac{1}{\sigma_{TE}^2 + \sigma_T^2}}} \quad (2)$$

and the interaction between enzyme deficiency and thalassaemia as:

$$(b_{TE} - b_T) - (b_E - b_N) \text{ with S.E. } \sqrt{\sigma_{TE}^2 + \sigma_T^2 + \sigma_E^2 + \sigma_N^2} \quad (3)$$

The significance of these differences is assessed in the usual way by dividing each by its standard error and finding a 'P' value from the table of the Normal or Gaussian distribution. Although the distributions may not be exactly Gaussian, this cannot be very far wrong.

The results of the calculations, shown in Table II, lead to the following conclusions:

Thalassaemia-trait has no effect on Cr survival, but G6PD deficiency results in a small but highly significant shortening of survival.

TABLE II

CALCULATED DIFFERENCES IN REGRESSION LINES (A) AND S.E. OF THE DIFFERENCES (B) WEIGHTED ACCORDING TO THE FORMULAE (1), (2) AND (3) (SEE TEXT)

		A Calculated differences in regression lines	B S.E. of the differences	Differences divided by S.E. (A/B)	P*
Effect of thalassaemia on rate of decay	N	0.000482	± 0.000361	1.33	0.20
	L	0.000131	± 0.000284	0.46	0.70
Effect of G6PD defi- ciency on rate of decay	N	0.001422	± 0.000375	3.79	0.001
	L	0.000944	± 0.000283	3.33	0.001
Interaction	N	0.00012	± 0.000767	0.16	0.87
	L	0.00007	± 0.000572	0.12	0.90

N = Naples measurements; L = London measurements.

* Assuming nearly-Gaussian distribution.

TABLE III

Cr SURVIVAL EXPRESSED AS ' $T_{\frac{1}{2}}Cr$ ' IN THE FOUR GROUPS

Group	' $T_{\frac{1}{2}}Cr$ ' (days)	
	Naples estimate	London estimate
Normal	31.2	32.0
Thalassaemia trait	31.5	32.4
G6PD deficiency	28.8	28.9
Thalassaemia trait and G6PD deficiency	26.6	28.6

$T_{\frac{1}{2}}Cr$ is the time taken for Cr survival to fall to 50 per cent, taking as 100 per cent the Cr concentration in a sample taken 20 minutes after injection of labelled cells.

There appears to be no appreciable interaction between the effects of thalassaemia-trait and G6PD deficiency on Cr survival. Thus, the difference in survival between thalassaemic-enzyme deficient and pure thalassaemic red cells is similar to the difference between pure enzyme deficient and normal red cells ($P = 0.87$ and 0.90 in the two series). In other words, the red cells of carriers of both abnormalities have a survival which is shorter than normal but is no different from that of red cells from subjects with G6PD deficiency alone.

Amount of 'Early-Loss' and Conventional $T_{\frac{1}{2}}Cr$ in the Four Groups

No significant differences in 'early-loss' were observed. Thus, taking the London figures, the average 'early-loss' in the four groups varied between 4.5 per cent and 6.0 per cent. Differences in the conventional $T_{\frac{1}{2}}Cr$ were similar to the differences between the regression coefficients described above (see Table III).

DISCUSSION

In considering the present results, it will be assumed that Cr 'elutes' from the red cells of thalassaemia-trait subjects and of G6PD deficient subjects at the same rate as from normal red cells, so that true red-cell survival can be deduced from the Cr survival data.

The normal red-cell survival observed in the thalassaemia-trait subjects in the present series agrees with previous results obtained by Hamilton, Sheets and DeGowin (1950) and Kaplan and Zuelzer (1950). These authors used the differential agglutination method of Ashby, which is unsuitable for the detection of small departures from normality and which involves cross-transfusion. More recently, two studies using ^{51}Cr -labelled red cells have been published. The first refers, in an appendix to a study on erythrokinetics in thalassaemia major, to only two thalassaemia-trait subjects (Malamos, Belcher, Gyftaki and Binopoulos, 1961). The other contains data on nine adults with thalassaemia-trait; however, the subjects were relatively severely affected, all being anaemic and the most severe having a haemoglobin concentration of 7.6 g. per 100 ml. with a reticulocyte count of 10.6 per cent; of the nine subjects, four had a reduced Cr survival (Pearson, McFarland and King, 1960). One difficulty in evaluating these data is that no normal subjects were studied at the same time using the same technique of Cr labelling, and the data were not evaluated statistically.

The finding, in the present work, of a normal red-cell survival in subjects with thalassaemia-trait, although contrary to our expectation, is not very surprising; a parallel might be drawn with subjects who are heterozygous for the gene for sickle-cell haemoglobin who have a normal red-cell survival in contrast to subjects who are homozygous for this gene who have a greatly diminished red-cell survival (Callender, Nickel and Moore, 1949).

If it is confirmed that red-cell survival is usually normal in thalassaemia-trait subjects, it follows that the mild anaemia observed in these subjects must be due simply to diminished red-cell production. Support for this deduction comes from the consistent marked reduction of ^{59}Fe incorporation observed respectively in four and nine carriers of thalassaemia in the above-mentioned papers by Malamos *et al.* (1961) and Pearson *et al.* (1960). In thalassaemia major, erythrokinetic studies have demonstrated that the anaemia is due more to an extremely low production rate than to severe reduction of life span (Grinstein, Bannermann, Vavra and Moore, 1960).

If red-cell life-span is normal in thalassaemia-trait subjects, it follows that the proportion of young red cells in the blood must be normal and that there must therefore be some other explanation for the raised G6PD level in the red cells of these subjects.

The present data indicate that in the thalassaemia-trait subjects red-cell production could be increased sufficiently to compensate for the amount of red cells withdrawn in sampling so that red-cell volume was maintained more or less constant. Thus, in the London series Cr survival was expressed as counts per gramme of haemoglobin, so that if there had been a steady decline in total circulating haemoglobin during the observation period the Cr survival would have been over-estimated. On the other hand, in the Naples series Cr survival is expressed as counts per millilitre of whole blood, so that provided total blood volume was kept constant the progressive fall in circulating red-cell volume would not have produced an error. Thus, if total circulating red-cell volume had fallen during the observation period in the thalassaemia-trait subjects, the London figures for Cr survival should have been higher than the Naples figures, but in fact the Naples figures were slightly higher.

Although correction of the results for loss of labelled cells in blood sampling is not necessary in the present series, since tests and control subjects were treated alike, it should perhaps be

emphasized that the amount of blood withdrawn in sampling in the present series was probably a little greater than the amount usually withdrawn in Cr survival studies. Nevertheless, the difference between withdrawing approximately 135 ml. in blood samples as in the present series and withdrawing only 50 ml. would only make a difference of about 2 per cent in the apparent survival at the end of the study (e.g. a difference between 50 per cent and 51 per cent).

The present observations indicate that red-cell life-span in G6PD deficient males in Sardinia is only slightly reduced. Thus, if $T_4\text{Cr}$ is 32 days in normal subjects and 29 days in G6PD-deficient subjects, then, by applying corrections for Cr elution to convert the normal Cr survival curve into a linear curve corresponding to a mean life-span of 120 days, the same corrections give a life-span of about 100 days in G6PD deficiency. This reduction in life-span is less than that which was found by Brewer *et al.* (1961) using DF^{32}P as a label; thus, they found life-span in normals to be 132 days and in G6PD deficiency to be 96.6 days.

The present estimations can also be compared with those of previous workers who have used ^{51}Cr as a label (Brewer *et al.*, 1961; Kellermeyer, Tarlov, Schrier, Carson and Alving, 1961). Thus, Brewer *et al.* found $T_4\text{Cr}$ to be 18, 18 and 24.5 days in three subjects with G6PD deficiency, compared with a $T_4\text{Cr}$ of 25.5–31.0 days (mean 28.7 days) in normal subjects; these figures suggest a rather striking shortening of life-span, but the figures of Kellermeyer *et al.* do not support this; thus, they found $T_4\text{Cr}$ in three G6PD-deficient subjects to be 23, 25 and 26 days, figures which are just at the lower end of the normal range reported by the same group of workers, and are thus more in keeping with the present observations.

In using ^{51}Cr as a red-cell label in studies of this kind, it is evidently essential to have a control group of normal subjects and to label the red cells of test and control groups by a carefully standardized technique.

The present observations (Table III) confirm that when red cells are washed in citrate-phosphate solution (pH 6.9) before being labelled with ^{51}Cr , then the subsequent $T_4\text{Cr}$ in normal subjects is of the order of 32 days, rather than the 26 days found when red cells are labelled by adding ^{51}Cr to whole blood mixed with ACD (Mollison, 1961b).

Presumably, the longer $T_4\text{Cr}$ found when cells are washed in citrate-phosphate solution before labelling is due to the subsequent slower elution of Cr; the longer $T_4\text{Cr}$ is clearly advantageous in looking for such differences in Cr survival.

The mild reduction in red-cell life-span observed in G6PD-deficient subjects is associated with lowered plasma haptoglobin levels (unpublished data) but is not associated with anaemia. The fact that red-cell life-span is not further shortened in individuals who have the thalassaemia-trait as well as G6PD deficiency shows that the two abnormalities do not interact in their effects on red-cell survival. However, it is likely that they interact in other ways, because the incidence of clinical favism in G6PD-deficient individuals is lower when there are also carriers of thalassaemia (Siniscalco *et al.*, 1961) and the frequency in the general population of individuals with both abnormalities tends to be higher than expected (Adinolfi *et al.*, 1960).

SUMMARY

Red-cell survival was estimated by ^{51}Cr -labelling in 13 normal subjects, nine subjects with thalassaemia-trait, 18 subjects with G6PD deficiency and seven subjects with both G6PD deficiency and thalassaemia-trait. Samples were counted in Naples and London with good agreement.

In thalassaemia-trait alone Cr survival was normal, but in G6PD deficiency, with or without thalassaemia-trait, it was diminished. The differences were small, so that on the average $T_{\frac{1}{2}}Cr$ was about 3 days less in G6PD deficiency, but the difference was shown to be significant at the 0.01 level of probability.

Assuming that Cr elutes from thalassaemia-trait red cells and from G6PD deficient red cells at the same rate as from normal red cells, one may conclude that in subjects with thalassaemia-trait red-cell survival is normal, and the mild anaemia must therefore be due solely to diminished red-cell production; and that in subjects with G6PD deficiency red-cell life-span is of the order of 100 days, and that this slight reduction is fully compensated by a corresponding increase in red-cell production.

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SULLA ONTOGENESI DEGLI ANTICORPI « NATURALI »
DEI GRUPPI SANGUIGNI UMANI

M. ADINOLFI

Medical Research Council's Experimental Haematology Research Unit,
Wright-Fleming Institute of Microbiology, London.

INTRODUZIONE

Il neonato umano incomincia a produrre le γ -globuline con ritardo in confronto alla maggior parte delle altre siero proteine ed i suoi poteri di difesa antibatterici sono esclusivamente affidati, nelle prime settimane di vita, agli anticorpi materni del tipo γ G-globuline che hanno attraversato il filtro placentare. Oltre che gli anticorpi batterici di origine materna il siero del cordone contiene anche gli anticorpi dei gruppi sanguigni umani che hanno attraversato e superato la barriera placentare.

Queste ricerche sono state intraprese allo scopo di stabilire le modalità con cui, in rapporto al tempo ed al tipo di γ -globuline, il neonato umano inizia a sintetizzare gli anticorpi « naturali » del sistema A B O e del sistema I-i e l'anticorpo incompleto freddo a specificità anti-H simile.

METODI E RISULTATI

Anticorpi anti-A ed anti-B.

I campioni di sangue di 18 neonati dall'età fra 7 e 34 settimane sono stati ottenuti mediante incisione al calcagno o prelievo alla vena femorale.

I sieri sono stati esaminati per la presenza di anticorpi agglutinanti ed emolizzanti anti-A ed anti-B usando rapporti siero:emazie e siero:emazie:complemento come descritto in precedenza (POLLEY, ADINOLFI e MOLLISON, 1963). I sieri contenenti gli anticorpi aggluti-

TABELLA 1.
Anticorpi anti-A ed anti-B in sieri di neonati umani.

No.	Età	Gruppo A B O	Agglutinina anti-A		Agglutinina anti-B		Emolisina	
			prima	dopo 2-me	prima	dopo 2-me	anti-A	anti-B
1	7	B	0	0	...	...	0	...
2	8	O	0	0	0	0	0	0
3	9	A	...	...	0	0	0	0
4	9	A	...	...	0	0	...	0
5	13	O	0	0	0	0	...	0
6	19	O	15	0	8	0	0	0
7	19	O	28	0	8	0	0	0
8	20	O	21	0	0	0	8	0
9	20	O	20	0	0	0	3	0
10	21	A	...	...	62	0	...	15
11	24	A	...	...	22	0	...	n. t.
12	25	B	18	0	...	...	0	...
13	28	O	32	10	17	0	8	0
14	31	B	23	0	...	...	0	...
15	31	O	31	19	26	8	10	8
16	32	O	31	15	15	0	12	0
17	33	O	61	36	20	0	15	8
18	34	A	...	...	43	20	...	12

Nota: l'età è espressa in settimane; il titolo dell'anticorpo è espresso come « score »; n. t.: non esaminato.

nanti sono stati trattati con 2-mercaptoctanolo (2-me) e sono stati nuovamente titolati per la presenza di anticorpi anti-A ed anti-B del tipo γ G-globulinico (ADINOLFI, POLLEY, HUNTER e MOLLISON, 1962).

Nella tabella 1 sono riportati i risultati; i sieri dei neonati di età inferiore alle 19 settimane circa non contenevano anticorpi anti-A ed anti-B. Questi anticorpi incominciavano ad essere prodotti in neonati di circa 20 settimane ed erano completamente inattivati dal trattamento con 2-me; la produzione di anticorpi resistenti al trattamento con 2-me incominciava solo intorno alla trentesima settimana.

Cinque sieri di neonati (no. 9, 10, 15, 17 e 18) sono stati frazionati con cromatografia su colonna di DEAE-cellulosa usando 7 soluzioni tamponate di fosfati a pH 8,1 e molarità 0,02; 0,04; 0,06; 0,08; 0,12; 0,16 e 0,2. Il contenuto di γ G, γ A e γ M-globuline nelle singole frazioni era accertato mediante la tecnica di inibizione dei tests anti- γ G, anti- γ A ed anti- γ M-globuline (POLLEY, MOLLISON e SOOTHILL, 1962); le frazioni 0,02 M contenevano esclusivamente γ G-globuline; quelle 0,16 M contenevano la maggior parte delle γ M-globuline eluite (circa il 60-80 per cento delle γ M-globuline presenti nel siero originale) e tracce di γ G-globuline (meno del 10 per cento della quantità presente nel siero originale); le frazioni 0,04, 0,06 e 0,08 M di un siero normale di un individuo adulto contenevano il 10, 10 e 50 per cento rispettivamente delle γ A-globuline presenti nel siero originale.

I risultati dell'esame delle frazioni per la presenza di anticorpi anti-A ed anti-B sono riportati nella tabella 2. Tutti i sieri contenevano anticorpi anti-A ed anti-B nella frazione 0,16 M e questi anticorpi erano inattivati dal trattamento con 2-me; i sieri no. 15, 17 e 18 contenevano anche anticorpi nella frazione 0,02 M e questi anticorpi non erano inattivati dal trattamento con 2-me; infine i sieri no. 10 e 18 avevano anche anticorpi nella frazione 0,08 M che erano parzialmente inattivati dal trattamento con 2-me e sensibilizzavano le emazie ed un siero anti- γ A globuline umane.

Anticorpo anti-I.

Ventitre coppie di sieri materni e dei relativi cordoni sono state esaminate per la presenza dell'anticorpo agglutinante freddo a specificità anti-I. L'anticorpo era presente in 20 sieri materni (86%) ed in 14 sieri del cordone (61%); le medie dei titoli, espressi come «scores»

TABELLA 2.

Frazionamento con cromatografia su colonna di DEAE-Cellulosa degli anticorpi anti-A ed anti-B presenti in 5 neonati umani.

Siero No.	Anti-corpo	Titolo dell' anticorpo ⁽¹⁾							
		nel siero originale	nelle frazioni (DEAE-cell. cromatografia):						
			0,02	0,04	0,06	0,08	0,12	0,16	0,2 M
9	anti-A	22	0	0	0	0	0	18	0
10	anti-B	58	0	0	0	12	5	42	0
15	anti-A	36	15	0	0	0	7	28	0
	anti-B	24	0	0	0	0	0	18	0
17	anti-A	55	24	0	0	0	8	40	0
	anti-B	22	0	0	0	0	0	15	0
18	anti-B	50	15	0	0	15	3	28	3

(¹) Il titolo dell'anticorpo è espresso come « score ».

(RACE e SANGER, 1958), erano: 22,5 per i 20 sieri materni e 19,1 per i 14 sieri del cordone. In 7 casi l'anticorpo era presente nel siero della madre ed assente nel siero del relativo cordone; in 1 caso l'anticorpo era assente nel siero della madre ed era presente nel siero del cordone (tabella 3); nei casi in cui l'anticorpo era presente nell'uno e nell'altro siero non esisteva buona correlazione tra i titoli. Nella tabella 3 sono anche riportate le frequenze attese per le quattro possibili combinazioni in cui l'anticorpo poteva essere presente o assente nelle coppie dei sieri, assumendo che la sintesi dell'anticorpo fosse indipendente nella madre e nel neonato; cioè a dire le frequenze attese sono state calcolate sulle frequenze dell'anticorpo nei sieri materni e nei sieri del cordone.

Sette neonati sono stati sottoposti a prelievi di sangue 3 o 4 volte a distanza di circa 5 settimane; il primo prelievo era effettuato quando il neonato aveva circa 8-10 settimane e l'ultimo quando aveva 24-26 settimane. Il titolo dell'anticorpo era pressochè costante in 4

TABELLA 3.

Anticorpo anti-I ed anticorpo incompleto freddo (N.i.c.) in 23 coppie di sieri materni e del cordone.

Siero materno	Siero del cordone	anti-I		N. i. c.	
		No. oss.	No. atteso	No. oss.	No. atteso
Presente	Presente	13	12,0	17	16,6
Assente	Assente	2	1,2	1	0,5
Presente	Assente	7	8,0	2	2,5
Assente	Presente	1	1,8	3	3,4

neonati; in 2 neonati l'anticorpo era assente durante tutto il periodo di osservazione ed infine un neonato ha iniziato a produrre l'anticorpo quando aveva circa 18 settimane.

I sieri di 5 individui adulti e 3 sieri del cordone sono stati frazionati con cromatografia su colonna di DEAE-cellulosa usando solo due soluzioni tamponate di fosfati a pH 8,1, la prima 0,02 M e la seconda 0,2 M (ADINOLFI, POLLEY e MOLLISON, 1962); l'anticorpo era eluito nella frazione 0,2 M la quale conteneva le γM e γA -globuline e tracce di γG -globuline.

L'anticorpo anti-I, presente nei sieri materni o del cordone, era inattivato dal trattamento con 2-me.

Anticorpo incompleto freddo.

Questo anticorpo era messo in evidenza usando siero fresco di cavallo come sorgente di complemento ed immunoconglutinina di coniglio (MOLLISON e POLLEY, 1959).

Ventitre coppie di sieri materni e del cordone sono state esaminate; l'anticorpo incompleto freddo a specificità anti-H simile era presente in 19 sieri materni (82%) e 20 sieri del cordone (86%); le

medie dei titoli erano: 31,7 per i 19 sieri materni e 26,5 per i 20 sieri del cordone. L'anticorpo era presente in 2 sieri materni ed assente nei rispettivi sieri del cordone; in 3 casi l'anticorpo era presente nel siero del cordone ed assente nei rispettivi sieri materni. La distribuzione delle frequenze dell'anticorpo nelle coppie madri neonati è riportata nella tabella 3; le frequenze attese sono state calcolate come nella sezione « anticorpo anti-I ».

In sei dei sette neonati ai quali sono stati eseguiti prelievi di sangue ad intervalli regolari, il titolo dell'anticorpo era pressochè costante durante tutto il periodo di osservazione; in 1 neonato l'anticorpo era assente durante l'intero periodo di osservazione.

L'anticorpo era eluito con cromatografia su DEAE-cellulosa nelle frazioni 0,08 e 0,10 M; l'anticorpo era sempre assente nella frazione 0,02 M contenente almeno l'80 per cento delle γ G-globuline presenti nel siero originale.

Il trattamento dei sieri con 2-me inibiva l'anticorpo incompleto freddo.

Il titolo dell'anticorpo, espresso come « score », è stato messo in relazione al fenotipo ABO, al fenotipo Lewis ed al carattere « secreto- », « non-secreto- », usando 84 sieri di individui adulti.

Nella tabella 4 sono riportate le medie dei titoli in 45 sieri in rapporto al fenotipo ABO dei donatori; la media più alta è stata

TABELLA 4.

Media dei titoli (« scores ») dell'anticorpo incompleto freddo in 45 sieri.

Gruppo A B O	No. sieri esaminati	Media dei titoli
AB	3	37,6
A ₁	12	31,1
B	6	28,5
A ₂	5	14,6
O	19	15,5

osservata in individui di gruppo AB, quella più bassa in individui di gruppo A₂ e O.

Nessuna reazione è stata osservata tra medie dei titoli dell'anticorpo e carattere « secretore », « non-secretore » o fenotipo Lewis.

Preliminari ricerche, eseguite su un gruppo di sieri provenienti dai membri di 10 famiglie comprendenti 20 genitori e 42 figli, hanno consentito di mettere in evidenza una variabilità del titolo in individui con lo stesso genotipo A e B.

DISCUSSIONE

La presenza degli anticorpi anti-I ed incompleto freddo nei sieri del cordone è stata dimostrata per la prima volta da Crawford e Mollison (MOLLISON, 1956). Per analogia a quanto è stato osservato per gli anticorpi batterici e del sistema ABO anche gli anticorpi anti-I ed incompleto freddo presenti nel siero del cordone sono stati considerati di origine materna e quindi del tipo γ G-globuline. Tuttavia la presenza degli anticorpi in alcuni sieri del cordone in assenza di essi nei rispettivi sieri materni e la persistenza degli anticorpi a titolo costante in neonati esaminati ad intervalli regolari di tempo costituiscono altrettante prove in favore alla ipotesi che gli anticorpi presenti nel siero del cordone non sono di origine materna.

Questi anticorpi, inoltre, non hanno le caratteristiche delle γ G-globuline, cioè a dire delle uniche γ -globuline capaci di attraversare il filtro placentare (HEMMINGS e BRAMBELL, 1961); essi infatti non sono eluiti con cromatografia su colonna di DEAE-cellulosa nella frazione 0,02 M insieme alle γ G-globuline e sono inattivati dal trattamento con 2-me, un composto che, nelle condizioni sperimentali usate, inattiva le γ M e γ A globuline ma lascia inalterate le γ G-globuline.

Gli anticorpi anti-I ed incompleto freddo presenti nel siero del cordone sono quindi prodotti dal neonato verosimilmente durante l'ultimo periodo della vita embrionale.

La sintesi degli anticorpi anti-A ed anti-B ha inizio invece quando il neonato ha almeno tre mesi (MORVILLE, 1929). Nella pre-

sente serie la sintesi aveva inizio quando il neonato aveva almeno 20 settimane ed i primi anticorpi ad essere prodotti erano del tipo γ M-globuline; essi infatti erano inattivati dal trattamento con 2-me ed erano eluiti nella frazione 0,16 M con cromatografia su colonna di DEAE-cellulosa. Anticorpi del tipo γ G-globuline, resistenti al trattamento con 2-me ed eluibili nella frazione 0,02 M, incominciavano ad essere prodotti intorno alla trentesima settimana. Inoltre due sieri (no. 10 e 18), su cinque esaminati, contenevano anticorpi del tipo γ A-globuline.

La produzione degli anticorpi anti-A ed anti-B del tipo γ M-globuline precede quindi l'inizio della sintesi degli anticorpi del tipo γ G-globuline; questa successione è simile a quella osservata per gli anticorpi immuni antibatterici in neonati umani stimolati durante le prime settimane di vita (SMITH, 1960, 1964). Sembra inoltre che le macro- γ -globuline siano le prime γ -globuline ad essere prodotte non solo ontogeneticamente ma anche filogeneticamente (UHR, FINKELSTEIN e FRANKLIN, 1962).

La precoce sintesi degli anticorpi anti-I ed incompleto freddo in confronto agli anticorpi del sistema ABO conferma che il neonato umano non attraversa un periodo di completa immaturità immunologica (SMITH, 1964) e suggerisce che il processo di maturazione immunologica, analogamente a quanto è stato osservato per altre specie animali (SHAUL, 1962; SILVERSTEIN, 1964), non ha luogo simultaneamente per tutte le cellule immunologicamente competenti, ma avviene « a salti ».

Poichè gli anticorpi anti-I ed incompleto freddo sono degli autoanticorpi, sorprende che essi siano i primi anticorpi di gruppo sanguigno ad essere prodotti: la presenza dei rispettivi antigeni I ed H nell'embrione e nel neonato dovrebbe infatti inibire la maturazione immunologica delle cellule potenzialmente capaci di produrre gli anticorpi specifici (BURNET, 1959; TALMADGE, 1959; LEDERBERG, 1959).

Per quanto riguarda l'anticorpo anti-I è necessario tener presente la particolare ontogenesi degli antigeni del sistema I-i; MARSH (1961) ha infatti dimostrato che le emazie del neonato sono simili, alla nascita, a quelle dei rari individui a fenotipo i (emazie i_{cord}) e che solamente all'età di 18 mesi circa, riducendosi progressivamente la sintesi dell'antigene i ed aumentando quella dell'antigene I, il neo-

nato acquisisce il fenotipo « adulto » I. Ulteriori ricerche sono quindi necessarie per stabilire se la sintesi dell' anticorpo anti-I non preceda l' inizio della produzione dell' antigene I. Va inoltre ricordato che l' anticorpo anti-I è presente ad alto titolo in pazienti affetti da anemia emolitica da auto-anticorpi freddi. In questi pazienti l' anticorpo è del tipo γ M-globuline, ha struttura molto omogenea, ha caratteristiche antigeniche individuali ed ha le proprietà di una proteina « monoclonale ». (HARBOR e DEREVILL, 1964).

Per quanto riguarda l' anticorpo incompleto freddo è invece necessario rilevare che questo anticorpo non ha le caratteristiche dei classici anticorpi dei gruppi sanguigni; è infatti presente nei sieri dei pazienti affetti da severa ipogammaglobulinemia ed ha alcune proprietà in comune con la properdina (ADINOLFI, DANIELS e MOLLISON, 1962).

Dai risultati riportati in questo lavoro risulta inoltre che il titolo di questo anticorpo dipende dal fenotipo ABO nel senso che la media dei titoli dell' anticorpo è più alta in individui di gruppo AB che in soggetti di gruppo O; poichè le emazie di gruppo O hanno più sostanza H che le emazie di individui di gruppo AB, nell' ordine: $O > A_2 > A_2B > B > A_1 > A_1B$ è molto probabile che il titolo dell' anticorpo incompleto freddo sia inversamente proporzionato alla quantità di sostanza H presente in ogni soggetto. Sembra quindi che, in analogia a quanto osservato per altri anticorpi di gruppo sanguigno (CINADER, 1963) i meccanismi genetici che controllano la sintesi dello anticorpo incompleto freddo sono mediati dai meccanismi genetici che controllano la sintesi dell' antigene autologo.

SUMMARY

The pattern of the production of anti-A and anti-B agglutinins and haemolysins was followed in 18 newborn infants whose ages ranged from 8 to 34 weeks.

The newborn infants started to produce anti-A and anti-B agglutinins between the age of about 18 and 20 weeks; the first antibodies appeared to be γ M-globulin since they were eluted with DEAE-cellulose chromatography with the fraction containing mainly γ M-globulin and were inactivated by treatment with 2-mercaptoethanol (2-me); γ G anti-A and anti-B, eluted with

0.02 M fraction and not inactivated by treatment with 2-me, were found in newborn infants more than 26 weeks old.

Five sera were tested for the presence of anti-A and anti-B γ A-globulin; these sera were fractionated with DEAE-cellulose chromatography and two out of the five sera were shown to contain antibodies in the 0.08 M fraction. The antibodies present in this fraction sensitized human red cells to an anti- γ A-globulin serum and were inactivated by treatment with 2-me.

Evidence was produced that the anti-I and the normal incomplete cold antibodies present in cord serum were synthesized by the foetus in utero; thus these antibodies were not eluted with the γ G-globulin with DEAE-cellulose chromatography, were inactivated by treatment with 2-me and did not disappear from the circulation of the newborn infants with time as do γ G antibodies.

RIASSUNTO

Poichè tracce di γ M-globuline, prodotte dal neonato, sono presenti nel siero del cordone e la sintesi delle γ G e γ A-globuline ha inizio solamente dopo qualche settimana dalla nascita, il neonato umano è considerato immunologicamente immaturo ed incapace quindi di produrre anticorpi dei gruppi sanguigni. L'A. dimostra, tuttavia, che almeno due anticorpi dei gruppi sanguigni sono prodotti durante l'ultimo periodo della vita fetale e sono presenti nel siero del cordone umano. Questi anticorpi sono: l'anticorpo agglutinante freddo a specificità anti-I e l'anticorpo incompleto freddo a specificità anti-H.

La precoce sintesi dei due anticorpi è discussa in rapporto alle correnti ipotesi della tolleranza immunologica, in vista del fatto che i due anticorpi sono auto-anticorpi e la loro sintesi non sembra essere influenzata dalla presenza, nel neonato umano, dei rispettivi antigeni eritrocitari.

L'anticorpo incompleto freddo ha inoltre la caratteristica che il suo titolo strettamente dipende dal fenotipo ABO o meglio dalla quantità di sostanza H presente sulle emazie di ciascun soggetto.

La sintesi di questi anticorpi è stata infine paragonata a quella degli anticorpi agglutinanti ed emolizzanti del sistema ABO ed è stato osservato che il neonato umano inizia a produrre anticorpi anti-A ed anti-B solamente verso la 18-20 settimana. Dapprima gli anticorpi sono del tipo γ M, infatti sono eluiti nella frazione cromatografica contenente solo γ M globuline e sono inattivati dal trattamento con 2-mercaptoetanolo; solamente quando il neonato ha 26-30 settimane di vita ha inizio la sintesi degli anticorpi del tipo γ G. Preliminari risultati sono riportati sulla sintesi degli anticorpi con la struttura delle γ A-globuline.

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Some Serological Characteristics of Normal Incomplete Cold Antibody

M. ADINOLFI

Medical Research Council Experimental Haematology Research Unit, St. Mary's Hospital, London

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Summary. The finding that the presence of normal incomplete cold antibody in cord serum is independent of its presence in the corresponding maternal sample suggests that the antibody is produced by the newborn infant. The observation that the antibody has a constant titre in newborn infants aged between a few weeks and 6 months points to the same conclusion.

The normal incomplete cold antibody differs from other blood group antibodies in its thermolability, in being present in the serum of patients with severe hypogammaglobulinaemia and in its interaction with some polysaccharides and bacteria. In many respects the antibody resembles properdin, but markedly differs from properdin in its specificity and other serological characteristics.

INTRODUCTION

Human red cells left in contact with normal human serum at 0° for 2 hours and then washed with warm saline, in order to elute the cold agglutinins, give a positive reaction when tested with a suitable anti-globulin serum. Dacie (1950) who described this reaction, termed the antibody responsible the 'normal incomplete cold' antibody.

The normal incomplete cold (n.i.c.) antibody is firmly bound to the red cells only in the presence of complement (Dacie, 1950) and cannot be detected directly in the anti-globulin test but only indirectly by the ability to bring about the binding of complement; thus red cells sensitized with n.i.c. antibody are not agglutinated by an anti- γ -globulin serum but are agglutinated by antisera which react with complement components (Dacie, Crookston and Christenson, 1957). Under appropriate conditions the antibody will lyse normal human red cells (Mollison and Thomas, 1959) and sensitize them to an immunocglutinin (I-K) serum (Mollison and Polley, 1960).

It has been shown that the antibody is present in cord serum (Crawford and Mollison, 1956) but that it is not a γ G-globulin and indeed has properties different from classical blood group antibodies (Adinolfi, Daniels and Mollison, 1963).

This paper reports some further observations on the nature of the n.i.c. antibody and its incidence in the serum of newborn infants, normal adult subjects and patients with hypogammaglobulinaemia.

MATERIALS AND METHODS

Human sera

The frequency of the n.i.c. antibody in a normal population was studied by testing serum from forty-five random donors; the serum, separated as soon as possible from fresh

clotted blood, was stored at -50° for not more than 1 week before being used. In relation to the ABO blood groups the forty-five donors were distributed as follows: A₁B: 2; A₂B: 1; A₁: 12; A₂: 5; B: 6; O: 19.

In studying the relation between the titre of the n.i.c. antibody and the ability to secrete A, B and H substances, sera from twelve additional group O donors were tested. The sera were selected in order to obtain two equal groups of 'secretors' and 'non-secretors'. Further sera and samples of saliva were obtained from ten families with twenty parents and forty-one children.

Twenty-three pairs of maternal and cord sera were tested; the cord blood was obtained from the umbilical vein; the maternal samples were taken by venepuncture within 2 days of delivery.

Eleven infants, from 3 to 15 weeks old, were bled by heel prick, at least 1 ml of blood being collected; six of these infants were born prematurely, but were otherwise normal. One of the infants was bled three times within a period of 6 weeks.

Seven normal infants, born at term, were bled by venepuncture three times at intervals of about 5 weeks. These infants were from 7 to 26 weeks old and they were receiving a normal course of vaccination with oral poliomyelitis vaccine (Sabin) and with a combined diphtheria, tetanus and pertussis vaccine, following the schedule and the dosage described by Butler, Benson, Urquhart, Goffe, Knight and Pollock (1964) (see section 'Study 1, group B').

The sera from fifteen patients with hypogammaglobulinaemia were kindly supplied by the Medical Research Council Working Party; six of these sera were tested only a few weeks after being collected and had been stored at -50° ; the other eight sera had been stored at -20° for many years; estimates of the concentration of γ -globulins were kindly supplied by Dr J. F. Soothill. All sera were tested for anti-A, anti-B and anti-I agglutinins. Some sera were also tested for bacterial antibodies.

Polysaccharides and simple sugars used in inhibiting the n.i.c. antibody

Zymosan samples (type A) were obtained from Fleishman Laboratory, U.S.A.; Light & Co., England and I.S.M., Italy. The soluble dextran was produced by Bengers Laboratories Ltd, England (Dextraven; 6 per cent dextran in saline); potato starch was obtained from B.D.H., U.K. and Griffin & George, U.K.; D-glucose, L-fucose were obtained from B.D.H. and Inulin (from Dahlia) from Difco Lab., U.S.A.

Bacteria

The samples of bacteria were kindly supplied by Dr A. A. Glynn. *E. coli* C3; *E. coli* A55; *Streptococcus aureus* 80; *Salmonella enteritidis* variety Danysz and *Pneumococcus* type XIV were grown on nutrient agar, killed by heating at 60° for 1 hour and washed three times with distilled water. The bacteria were then weighed (wet weight), re-suspended in buffered saline to give approximately 10 mg of bacteria in 1 ml and were stored at 4° , if not used immediately. Before being used the bacteria were washed again with buffered saline.

Sera used as a source of complement

Horse serum was separated within a few hours from freshly drawn blood and absorbed at 0° three or four times with previously washed human red cells. The absorbed serum was partitioned into small aliquots and stored at -50° . Fresh human serum was absorbed three times at 0° for 3 or 4 hours with group O red cells.

Immunoconglutinin (I-K) serum

Two sera were prepared in rabbits by giving repeated injections of a killed suspension of *Salmonella pullorum*; the doses were those described by Mollison and Polley (1960), based on Coombs and Coombs (1953).

Serological tests for detecting the n.i.c. antibody

Ten volumes of each of a series of doubling dilutions of the antibody-containing serum were incubated at 0° for 2 hours with one volume of a 50 per cent suspension of fresh group O red cells and one volume of complement; the red cells were then washed three times with warm saline, in order to elute the cold agglutinins, and finally tested with a suitable immune serum on an opal tile.

In many experiments fresh horse serum, previously absorbed with human red cells, was used as a source of complement and the uptake of complement was detected by using an (I-K) serum. In a few experiments fresh human serum absorbed three times with group O red cells was used as a source of complement and the antiglobulin test was performed using an anti-human- β -globulin serum. When it was desired to make the test more sensitive, twenty volumes of serum were mixed with two volumes of complement and one volume of a 50 per cent suspension of red cells.

Owing to the limited amount of serum available, the samples obtained by heel prick from the eleven newborn infants were tested using the following dilutions: 1 in 2, 1 in 5, 1 in 10, 1 in 20 and 1 in 40. As controls, normal adult and cord sera were used at the same dilutions.

In this paper the amount of antibody is expressed as 'score' (Race and Sanger, 1958).

Bacterial antibody test

The indirect agglutination method was used (Neter, 1956). Suspensions of bacteria in buffered saline were heated for 1 hour at 100° and the supernatant was then recovered by centrifugation at 15,000 rev/min for 30 minutes. One volume of the supernatant was mixed with one volume of a 10 per cent suspension of group O red cells, previously washed with buffered saline, and incubated at 37° for 20 minutes. The 'modified' red cells were washed three times and resuspended in saline to make a final 5 per cent suspension.

The bacterial agglutinins were tested by adding one volume of the modified red cell suspension to each of a serial, doubling, dilution of the serum; after 1 hour at room temperature the deposited red cells were examined microscopically and the degree of agglutination scored.

Treatment of sera with zymosan, other polysaccharides and simple sugars

Aliquots of 1 ml of serum were added to 3 mg of zymosan; the mixtures were incubated for 1 hour at 0°, 17° and 37°; zymosan was maintained in suspension by frequent mixing. The supernatants were recovered by centrifugation at 15,000 rev/min, at the same temperature as used for the absorption stage. In the same way, fresh serum was treated with different amounts of potato starch.

In the experiments with dextran, inulin, D-glucose and L-fucose, two aliquots of 1 ml of serum (samples A and B) were used for each substance. After incubation at room temperature for 20 minutes the B series was dialysed at 0° for 2 hours against two changes of buffered saline; the A series was used undialysed. The n.i.c. activity in the treated sera was tested using human serum as a source of complement and an anti- β -globulin serum. Each sample was also titrated against sheep red cells at 37° for 1 hour and the degree of haemolysis scored.

Inhibition in vivo of the n.i.c. antibody

This experiment was performed by injecting a normal subject with 50 ml of a 6 per cent w/v solution of dextran in 0.9 per cent sodium chloride solution (Dextraven). Samples of blood were taken 3 days before the injection, 10 minutes before and 10 minutes after the injection; further samples were collected 1 hour, 3 hours, 1 day and 2, 5 and 8 days after the injection. The serum was separated as soon as possible and stored at -50° . The samples were all tested at the same time using the same source of complement and I-K serum.

The sera were also tested against sheep red cells at 37° and the degree of haemolysis scored.

Absorption with bacteria

Aliquots of 1 ml of serum were absorbed with different amounts of bacteria (wet weight) at 0° for 30 minutes. The serum was then centrifuged at 0° at 15,000 rev/min for 30 minutes and the precipitate discarded. In the same way aliquots of serum containing an anti-H agglutinin and a plant extract with anti-H specificity (*Ulex europaeus*) were absorbed with *E. coli* C3 and, as a control, *E. coli* A55.

Heating at 56° and 63°

One sample of serum from a group A₁B donor, one serum containing an anti-P₁ agglutinin and one containing an incomplete anti-D antibody were used. Preliminary investigations using DEAE-cellulose chromatography and treatment of the serum with 2-mercaptoethanol (for methods see Adinolfi, Polley, Hunter and Mollison, 1962) had shown that the anti-P₁ agglutinin was a γ M-globulin and the anti-D antibody a γ G-globulin.

Aliquots of serum previously diluted with equal volumes of buffered saline were heated at 56° and 63° for 30 minutes, 1, 2 and 3 hours. The n.i.c. antibody was detected using horse serum as a source of complement and an I-K serum; the anti-P₁ by agglutination in saline at 12° and the anti-D antibody by the indirect antiglobulin test.

RESULTS

FREQUENCY OF THE N.I.C. ANTIBODY IN RANDOM ADULT DONORS; ITS RELATION TO THE ABO GROUPS

Using the test in which the ratio of serum to red cells was 20 to 1 the n.i.c. antibody was detected in forty-two out of the forty-five samples tested (93.3 per cent). The three sera apparently free of the antibody were retested using a ratio of serum to red cells of 40 to 1 and the antibody was then detected at a low titre in two of the samples. When the serum free of n.i.c. antibody was tested against sheep red cells at 37° , the degree of haemolysis was found similar to that of the serum used as controls. Thus failure to determine n.i.c. antibody was not due to any anticomplementary property of the serum.

The score of the antibody in the forty-five samples, in relation to the ABO blood groups is given in Fig. 1. The mean score of the antibody in the four ABO blood groups was calculated and the results are shown in Table 1; the titre of the n.i.c. antibody (expressed as the score) in the twenty-six AB, A and B subjects was compared with that in the nineteen group O donors and the data were analysed using the *t* test (Snedecor, 1950). The value of *t* obtained was 3.43 with 43 d.f.; $P < 1$ per cent.

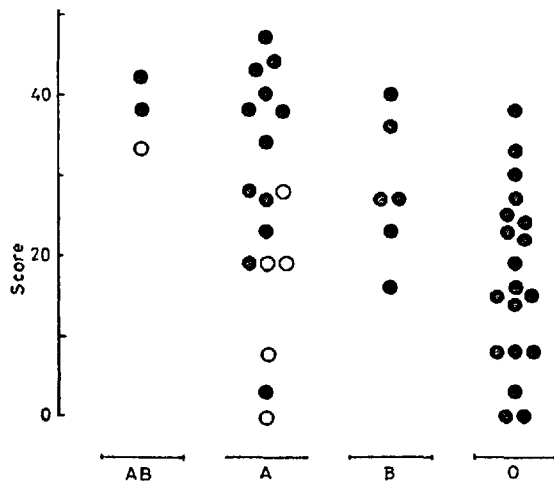


FIG. 1. Distribution of the n.i.c. antibody in forty-five sera from normal adult donors in relation to the ABO blood groups; O = A₂B or A₂ subgroups.

TABLE 1
N.I.C. ANTIBODY IN FORTY-FIVE SERA FROM RANDOM ADULT DONORS*

	Blood group				
	A ₁ B or A ₂ B	A ₁	B	A ₂	O
Number tested	3	12	6	5	19
Antibody mean score	37.6	31.6	28.5	14.6	15.5

* Mean score of n.i.c. in relation to the ABO blood groups.

The family data were analysed to see whether the amount of n.i.c. antibody in the serum was related to the ABO genotypes or to the ability to secrete A, B and H substances.

Sibs heterozygous for the ABO genes showed a considerable variation in the antibody titre; thus, the scores of the n.i.c. in five sibs of genotype A₁O were 34, 5, 3, 39 and 36.

There was no difference between 'secretors' and 'non-secretors' with regard to the amount of antibody in the serum. This observation was confirmed testing six group O 'secretors' and six group O 'non-secretors'; thus the mean scores of the antibody were respectively 45.5 and 47.8.

MATERNAL AND CORD SERA

The n.i.c. antibody was present in nineteen out of twenty-three maternal sera (82.6 per cent) and in twenty out of twenty-three cord sera (86.9 per cent). The mean score of the antibody, calculated only for those samples in which antibody was present, was 31.7 for the nineteen maternal samples and 26.5 for the twenty cord sera. Fig. 2 shows the score of the antibody in each maternal sample compared with that in the relevant cord serum. In twelve out of the seventeen pairs of maternal and cord sera in which the antibody was present in both samples, the antibody had the same score or the difference did not exceed

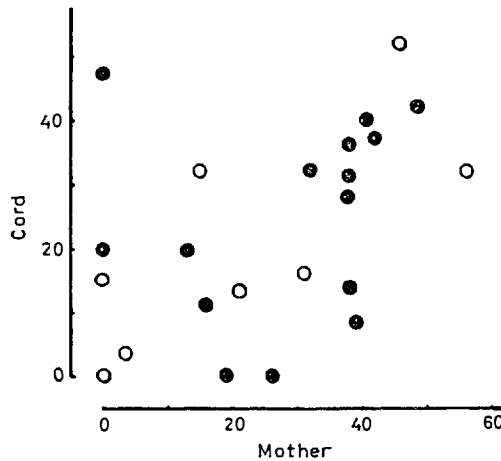


FIG. 2. N.i.c. antibody in twenty-three maternal sera and corresponding cord sera; results are expressed as scores. ● = Mother and newborn infant of same ABO blood group; ○ = mother and newborn infant of different ABO blood group.

10 units; in one case the antibody had a definitely higher score in the maternal sample; in four cases the antibody had a higher score in the cord serum.

TABLE 2
N.I.C. ANTIBODY IN TWENTY-THREE PAIRS OF MATERNAL AND CORD SERA

	Mother	Cord	Observed	Expected*
(i)	Present	Present	17	16.6
(ii)	Present	Absent	2	2.5
(iii)	Absent	Present	3	3.4
(iv)	Absent	Absent	1	0.5

* The expected frequency was calculated assuming that the presence of the antibody in cord serum was independent of its presence in the corresponding maternal sample.

Table 2 gives the distribution of the n.i.c. antibody in twenty-three pairs of sera in relation to the four possible combinations in which it may occur. The observed frequency for each of the four possible combinations was compared with the expected frequency calculated on the assumption that the presence of the antibody in the mother's serum did not influence its presence in the infant's serum. In three cases, as expected on statistical grounds, the antibody was present in the cord serum and absent from the corresponding maternal serum.

THE N.I.C. ANTIBODY IN NORMAL INFANTS

The antibody was present in the serum of all the eleven newborn infants whose blood was collected by heel prick (Fig. 3). The score of the antibody ranged from 3 to 45 score units and was similar to that of the adult and cord sera used as controls. The score of the antibody in one infant bled three times within 6 weeks showed no significant change.

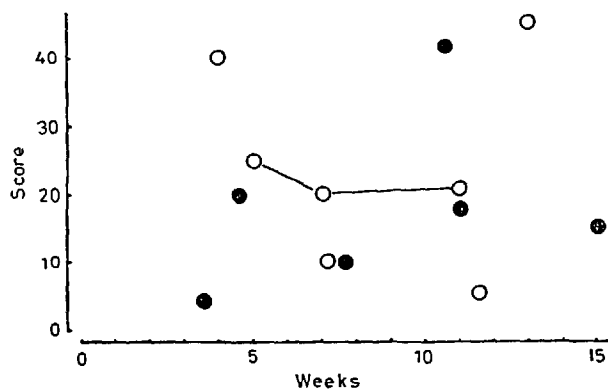


FIG. 3. N.I.C. antibody in eleven newborn infants bled by heel prick. ● = Premature infants. The three observations joined by a line were made on the same infant.

The antibody was present in the serum of six of the seven infants bled three times with intervals of about 5 weeks between bleedings (Fig. 4). The antibody had a constant score in two infants; in another three infants the score of the second and third samples was a little higher than that in the first sample; in the remaining case the antibody rose progressively from 22 to 43 'score' units between the first and the third samples.

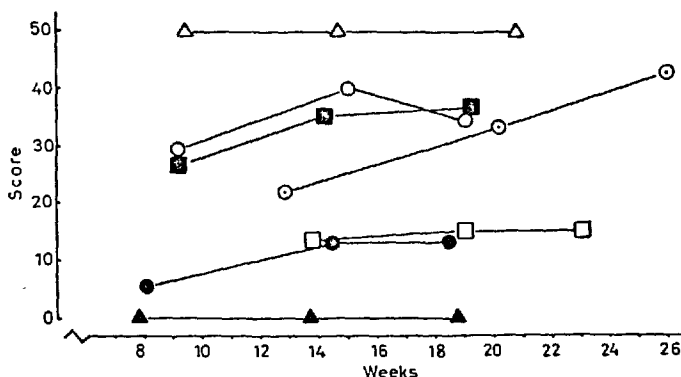


FIG. 4. N.I.C. antibody in seven newborn infants bled by venepuncture at intervals of about 5 weeks.

N.I.C. ANTIBODY IN PATIENTS WITH HYPOGAMMAGLOBULINAEMIA

Table 3 shows the results of testing fourteen sera from patients with hypogammaglobulinaemia. The antibody was found in all but one serum (No. 13). Owing to the limited amount of serum available the antibody could be titrated only in one serum stored at -20° for 2 years and in six sera stored at -50° for a few weeks after being collected. The titre of the n.i.c. in these sera was similar to that of the normal sera used as controls. Anti-A, anti-B and anti-I antibodies were absent from all sera; sera No. 3 and No. 4 were proved to be free of bacterial agglutinins against *E. coli* C3, *E. coli* A55, *Salmonella enteritidis* variety Danysz, *Staphylococcus aureus* 80, and *Pneumococcus* type XIV.

TABLE 3

N.I.C. IN SERUM OF PATIENTS WITH HYPOGAMMAGLOBULINAEMIA SYNDROME*

No.	Initials	γ -Globulins			Blood group	Anti-A	Anti-B	Anti-I	n.i.c.†
		γ G (mg/100 ml)	γ A† (% of normal serum conc.)	γ M†					
1	H.H.	6	2	62	A	...	nil	nil	32
2	D.H.	17.5	3	100	A	...	nil	nil	16
3	S.S.	12.8	0.5	17	O	nil	nil	nil	64
4	T.W.	25	0.2	0	B	nil	...	nil	64
5	A.A.	300	...	...	O	nil	nil	nil	32
6	A.W.	20	8	6	B	nil	...	nil	16
7	C.W.	140	3	6	A	...	nil	nil	16
8	J.A.	40	2	6	O	nil	nil	nil	++
9	J.A.	2.5	0	25	O	nil	nil	nil	+
10	A.G.	25	0	300	AB	...	...	nil	++
11	M.B.	80	...	...	A	...	nil	nil	++
12	J.C.	30	2	200	A	...	nil	nil	+
13	B.W.	40	4	18	O	nil	nil	nil	-
14	E.H.	200	0	0	A	...	nil	nil	+++

* The first six sera were stored at -50° for only a few weeks; the others were stored at -20° for periods between 2 and 6 years.

† The concentrations of the γ A and γ M globulins are expressed as percentages of the amounts in 'standard' normal sera.

‡ The n.i.c. antibody was titrated in seven samples (titre expressed as reciprocals). The degree of agglutination of the other seven sera was compared with that of a normal serum which gave a +++ agglutination.

REACTION OF N.I.C. WITH SOME POLYSACCHARIDES AND SIMPLE SUGARS

Samples of serum absorbed at 0° , 17° and 37° with different batches of zymosan type A (1 ml serum per 3 mg zymosan) were proved to be free of n.i.c. antibody. The antibody was also absorbed with two batches of potato starch; thus 0.3 g (wet weight) of potato starch incubated at 37° for 1 hour with 1 ml of serum completely removed the n.i.c. activity present in a serum containing the antibody at a titre of 1 in 64. The antibody could also be inhibited by mixing 1 ml of serum with the following amounts of polysaccharides and simple sugars: 60 mg dextran (Dextraven, Bengers Lab. Ltd), 100 mg inulin, 100 mg D-glucose and 100 mg L-fucose.

When 50 ml of a 6 per cent solution of dextran (Dextraven) was injected intravenously into a normal subject the score of the n.i.c. antibody fell from 30 to 3 units within 10 minutes and the pre-injection score was not regained for more than 2 days (see Fig. 5).

In all the experiments in which polysaccharides and simple sugars were used, controls were included to provide evidence that the negative results obtained in testing the n.i.c. antibody in the treated sera were not due to an interference between the substances used and complement. Thus, for example, the n.i.c. activity in the samples collected after the injection of dextran, was tested before and after adding fresh complement to the sera (see Methods); the same sera, and one sample of serum taken before the injection, were also tested against sheep red cells at 37° and the degree of haemolysis compared; thus it was found that whereas the dextran solution reduced the titre of the n.i.c. antibody, it did not alter appreciably the titre of complement.

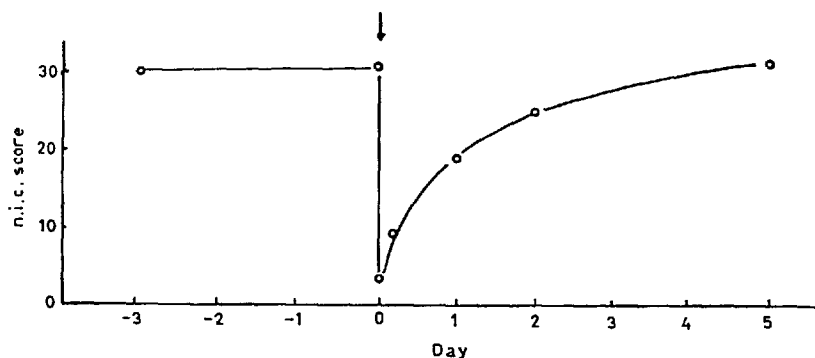


FIG. 5. Titre of n.i.c. antibody before and after an intravenous injection of 50 ml of a 6 per cent solution of dextran (Dextraven).

Absorption with bacteria

The n.i.c. antibody of three normal adult sera and two sera of patients with hypogammaglobulinaemia was completely removed after one absorption with *E. coli* C3 whereas absorption with the other bacteria was ineffective. Preliminary experiments showed that the nutrient agar used in growing *E. coli* C3 did not inhibit the antibody.

TABLE 4
EFFECTS OF ABSORPTION OF SERA UPON N.I.C. ANTIBODY

		Normal serum		Serum of a patient with hypogammaglobulinaemia	
		n.i.c.	Anti- <i>E. coli</i> C3	n.i.c.	Anti- <i>E. coli</i> C3
(i)	Untreated serum	+++	+++	+++	—
(ii)	Heated at 56° for 30 minutes	+	+++	...	...
(iii)	Absorbed at 0° with human red cells	—	+++	—	—
(iv)	Treated with H substance	—	+++	—	—
(v)	Absorbed with <i>E. coli</i> C3	—	—	—	—
(vi)	Absorbed with <i>E. coli</i> A55	+++	+++	+++	—
(vii)	Absorbed with <i>Staphylococcus aureus</i> 80	+++	+++	+++	—
(viii)	Absorbed with <i>Salmonella enteritidis</i>	+++	+++	+++	—
(ix)	Absorbed with <i>Pneumococcus</i> type XIV	+++	+++	+++	—

Table 4 gives the results of testing the n.i.c. antibody and *E. coli* C3 agglutinin in a normal serum and a serum of a patient with hypogammaglobulinaemia before and after absorption with bacteria. The two sera were also tested after heating at 56° for 30 minutes, after absorption with group O red cells at 0° for 2 hours and after adding H substance present in the saliva of a 'secretor'. From the above results it appears that *E. coli* agglutinin is not inhibited by H substance. On the other hand it was observed that these bacteria did not absorb the anti-H agglutinin present in a human serum or a plant agglutinin with anti-H specificity extracted from the *Ulex europaeus*.

Effect of heating at 56° and 63°

Fig. 6 shows the activity of the n.i.c. antibody and of the anti-D and anti-P₁ antibodies after heating at 56° and 63° for different periods of time. Heating at 56° for 30 minutes

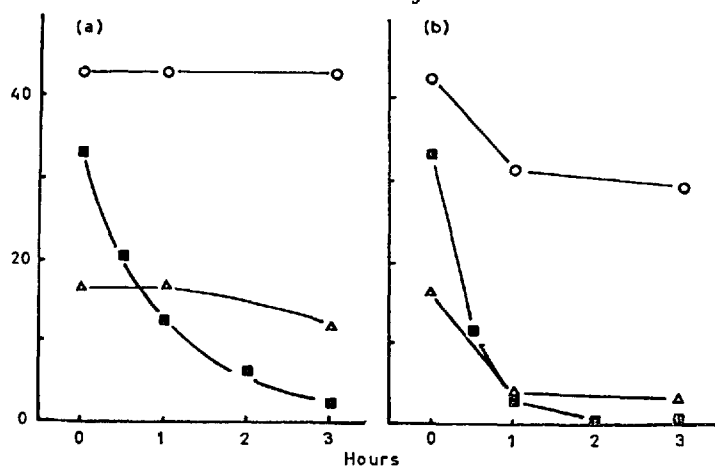


FIG. 6. Effect of heating anti-D (○), anti-P₁ (△) and n.i.c. (■) antibodies at (a) 56° and (b) 63°; titre are expressed as scores.

produced a marked fall in the titre of the n.i.c. antibody; this was completely destroyed after 3 hours; by contrast heating at 56° for 3 hours did not alter the titre of the incomplete anti-D, whereas the titre of the anti-P₁ agglutinin was only slightly reduced. When the experiment was repeated at 63°, both n.i.c. and anti-P₁ antibodies were destroyed after 1 hour; after 3 hours at 63° the titre score of the anti-D antibody fell from 43 to 31 units.

DISCUSSION

The n.i.c. antibody has a specificity closely related to anti-H; thus it reacts more strongly with group O red cells than with A₁B cells; the serum of group A₁B donors usually contains more antibody than the serum of group O donors and the reactions are inhibited by the saliva of 'secretors' but not by the saliva of 'non-secretors' (Crawford, Cutbush and Mollison, 1953). Furthermore it has been shown that the antibody does not react with Bombay red cells (Mollison, 1961).

The present results confirm that the titre of the antibody is higher in A₁B donors than in group O donors (Fig. 1 and Table 1) and show that the difference between group O donors and those of other groups is highly significant ($t = 3.43$; d.f. = 43; $P < 1$ per cent). It thus appears that the amount of the antibody in normal sera is inversely related to the amount of H substance on the red cells, as follows: A₁B > A₁ > B > A₂B > A₂ > O.

In view of this relation between the titre of the n.i.c. antibody and the ABO phenotypes, preliminary investigations were carried out on a limited number of families to see whether any difference in the amount of the antibody in the serum could be detected in subjects homozygous or heterozygous for ABO genes. The family data were also analysed to see whether the ability to secrete A, B and H substances had any influence on the amount of antibody. Clarke, McConnell and Sheppard (1960) have demonstrated that group O subjects secrete more H substances than group A donors, who, in turn, secrete more than groups B and AB. They have shown also that the amount of A, B and H substances secreted by an individual is in part inherited and that probably there is a difference between homozygous and heterozygous group A and B donors with regard to the amount

of H substance secreted in their saliva. In view of this evidence and the fact that the n.i.c. antibody has anti-H specificity, it seemed possible that the titre of the antibody in individuals might depend on an interaction between the ABO genotype and the 'secretor' status.

The present data showed that the titre of the antibody was variable in sibs of the same ABO genotype and that there was no relation between the amount of the antibody in serum and the ability to secrete A, B and H substances.

It appears that the n.i.c. antibody is produced by infants before birth; thus in three cases the antibody was detected in the serum of infants whose mothers lacked the antibody. Furthermore, n.i.c. antibody was found in almost all infants between 4 and 26 weeks of age and in many cases its titre remained approximately constant during this period. If the antibody had been of maternal origin (γ G-globulin) a temporary disappearance of the antibody or fall in titre after birth would have been expected. The antibody is apparently not a γ G-globulin (see also Adinolfi *et al.*, 1963) and the observation that it is present in the serum of thirteen out of fourteen patients with hypogammaglobulinaemia (Table 3) gives further support to this view.

The n.i.c. antibody differs from blood group antibodies also in its thermolability; thus it is relatively heat-labile at 56° and is rapidly destroyed at 63°; in this respect the n.i.c. antibody behaves differently from incomplete anti-D antibody (γ G-globulin) and anti-P₁ agglutinin (γ M-globulin). On the basis of the above results striking similarities may be drawn between n.i.c. and properdin; properdin, as well as n.i.c. is present in cord serum (Koch, Schultze and Schwick, 1958) in the serum of patients with hypogammaglobulinaemia (Barandum and Isliker, 1956) and it is thermolabile (Pillemer, Blum, Lepow, Ross, Todd and Wardlaw, 1954). Furthermore, properdin does not react with anti- γ G globulin serum (Hinz, Wedgewood, Todd and Pillemer, 1960).

In view of these similarities further investigations were carried out to see whether n.i.c. antibody and properdin had other serological characteristics in common. It has been shown that zymosan, potato starch, dextran and inulin react with properdin (Pillemer, Schoenberg, Blum and Wurzburg, 1955; Lepow, Pillemer, Schoenberg, Todd and Wedgewood, 1959; Turk, 1959) and in the present work these polysaccharides were found to remove n.i.c. antibody from human serum. With regard to the absorption with polysaccharides and simple sugars the antibody resembles the properdin-like factor described by Turk (1959) in rabbits. Nevertheless n.i.c. antibody and properdin differ in many respects; for example whereas the n.i.c. antibody is absorbed by zymosan at 0° as well as 37°, properdin is absorbed only at 17° or higher temperature. Very recently a properdin-like factor which is absorbed by zymosan at 0° has been described by Blum (1964) but it is not yet known whether this factor is related to n.i.c. antibody.

N.i.c. antibody also differs from properdin in its reaction with bacteria; thus whereas a wide range of bacteria absorbs properdin (Pillemer *et al.*, 1955) only *E. coli* C3 was found to absorb n.i.c. antibody. Incidentally, although *E. coli* C3 bacteria inhibited the antibody, these bacteria did not react with a human anti-H antibody or with a plant extract with anti-H specificity (*Ulex europaeus*) and H substance present in the saliva of a secretor did not inhibit anti-*E. coli* C3 agglutinins (Table 4).

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Anti-I Antibody in Normal Human Newborn Infants

M. ADINOLFI

Medical Research Council Experimental Haematology Research Unit, St. Mary's Hospital, London

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Summary. A cold agglutinin with anti-I specificity is present in a high percentage of cord sera. Evidence is produced that this antibody, like the 'naturally-occurring' anti-I present in adult serum, is not a γ G-globulin; thus the antibody, both in cord serum and in adult serum, is inactivated by treatment with 2-mercaptoethanol, is not eluted with γ G-globulin using DEAE-cellulose chromatography and is recovered in the fast sedimenting fractions after ultracentrifugation. Furthermore, the antibody does not disappear from the circulation of newborn infants as do γ G antibodies which cross the human placenta.

These findings indicate that the antibody in cord serum is a γ M-globulin and that its production often begins before birth.

INTRODUCTION

The occurrence of a cold auto-agglutinin in human serum was described by Landsteiner and Levine in 1926; the antibody was later shown to have anti-I specificity by Jenkins, Marsh, Noades, Tippet, Sanger and Race (1960).

Cold agglutinins have been shown to be present in serum from cord blood (Crawford and Mollison, 1956). This finding is somewhat unexpected since 'naturally-occurring' agglutinins, as a rule, seem to be γ M-globulin* and γ M-globulin is not transferred across the human placenta. Thus if it could be shown that the cold agglutinins in cord blood are γ M-globulin the inference would be that the foetus makes at least one antibody of recognizable specificity.

MATERIAL AND METHODS

Human sera

Twenty-three pairs of maternal and cord sera were collected; the cord blood was obtained by syringe from the umbilical vein; the maternal samples, taken by venepuncture, were collected within 2 days of delivery; twenty adult male donors were also bled by venepuncture.

Fifteen infants, from 3 to 22 weeks old, were bled by heel prick, at least 1 ml of blood being collected; eight of these infants were born prematurely but were otherwise normal.

Seven normal infants, born at term, were bled by venepuncture three or four times at intervals of about 5 weeks. These infants were from 7 to 26 weeks old and they were receiving a normal course of vaccination with oral poliomyelitis vaccine (Sabin), containing types 1, 2 and 3 strains, and with a combined diphtheria, tetanus and pertussis

* According to the recommendation suggested by the W.H.O committee (Prague, 1964) the following terminology was adopted to designate the three classes of γ -globulin: γ G (Synonymous γ_1 , γ_2 , γ_{SS} , 7S); γ A (Synonymous γ_1 , γ_2 , γ_{SA}); γ M (Synonymous γ_1 M, β_2 M, 19S, γ -macroglobulin).

vaccine. The schedule and the doses were those described by Butler, Benson, Urquhart, Goffe, Knight and Pollock (1964) in the section Study 1, group B. A blood sample was obtained from all infants immediately before the first dose of the vaccine.

In all cases the serum was separated from the clotted blood as soon as possible and stored at -50° if not used immediately.

Red cells

In testing the anti-I antibody, group O, P₂ red cells, selected for having a strong I antigen, were used. In most experiments group O, P₂, i₂ red cells stored at -50° in glycerol-citrate-phosphate solution were used as a control; in some cases, instead of stored red cells, cord red cells, selected for their weak I antigen were used. Before use, the red cells were always washed in buffered saline (0.17 M NaCl) solution to which sodium phosphate buffer was added to bring the final molarity to 0.18 and the pH to approximately 7.

Horse serum

Fresh horse serum was absorbed three or four times at 0° with previously washed human red cells. The absorbed serum was stored in small aliquots at -50° .

Immuno-conglutinin (I-K) serum

Rabbit I-K serum of autostimulation type (Coombs and Coombs, 1953) was used.

Serological tests

The anti-I antibody was titrated by making serial, doubling dilutions of serum or fractions in buffered saline; to each tube an equal volume of a 5 per cent suspension of red cells was added. The mixtures were left at 0° for 2 hours and the deposited red cells were then examined microscopically and the degree of agglutination scored.

Anti-A and anti-B agglutinins were titrated at room temperature using the same procedure. In testing seven selected cord sera the test was made more sensitive by increasing the ratio of the serum to the red cells; thus eight volumes of serum previously heated at 56° for 20 minutes, were mixed with one volume of a 5 per cent suspension of red cells.

The normal incomplete cold (n.i.c.) antibody was tested as described previously (Adinolfi, 1965). The amount of antibody is expressed as 'score', using the procedure described by Race and Sanger (1958).

Bacterial antibody test

E. coli C3 bacteria were used; the indirect haemagglutination method (Neter, 1956) was that described previously (Adinolfi, 1965).

Ion-exchange chromatography

In most cases the method used was a minor modification of that described by Levy and Sober (1960); two phosphate buffers, both pH 8.2, the first 0.02 M and the second 0.2 M were used in eluting the plasma proteins (Adinolfi, Polley, Hunter and Mollison, 1962).

Treatment of sera with 2-mercaptoethanol (2-me)

One volume of undiluted serum was mixed with an equal volume of 0.1 M 2-me in phosphate buffer pH 7.4 and incubated for 2 hours; the mixture was then dialysed at 4° against frequent changes of buffered saline. As a control, undiluted serum was treated with phosphate buffer.

The amount of γ G and γ M-globulins in the chromatographic fractions and in the fractions obtained after ultracentrifugation was assayed by the inhibition of antiglobulin serum method (see Polley, Mollison and Soothill, 1962).

RESULTS

ANTI-I ANTIBODY

Twenty-three pairs of mother and infant (cord) sera were tested for anti-I antibody using the same group O, I, P₂ red cells. The antibody was present in twenty out of twenty-three maternal sera (86.0 per cent) and in fourteen out of the twenty-three cord sera (60 per cent). The mean score of the antibody, calculated on the total of the sera in which the antibody was present, was 22.5 for the twenty maternal samples and 19.1 for the fourteen cord samples. The frequency of the anti-I antibody in the sera of twenty adults was 85 per cent and therefore similar to that in the mothers sera. In those cases in which the antibody was present both in the mothers' serum and in cord serum, the anti-I agglutinin was weaker in the cord serum than in the corresponding maternal serum with four exceptions in which the antibody had the same score or was slightly higher (Fig. 1).

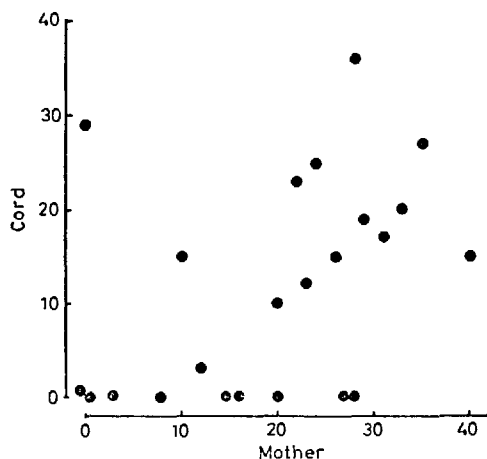


FIG. 1. Anti-I agglutinin in twenty-three pairs of maternal and corresponding cord sera; the degree of agglutination in each case is expressed as a 'score'.

Table 1 gives the incidence of the anti-I antibody in twenty-three pairs of maternal and cord sera. The expected frequency for each of the four possible combinations was calculated

TABLE 1
ANTI-I ANTIBODY IN MATERNAL AND CORRESPONDING CORD SERUM

	No. obs.	No. exp.*
(i) Present in mother, present in cord	13	12.0
(ii) Absent in mother, absent in cord	2	1.2
(iii) Present in mother, absent in cord	7	8.0
(iv) Absent in mother, present in cord	1	1.8

* Assuming that the incidence in cord serum is unaffected by the incidence in maternal serum.

on the assumption that the presence of the antibody in the mothers serum did not influence its presence in the infant's serum. There was good agreement between the observed and the expected frequencies; in one case, the antibody was present in the cord serum and absent from the corresponding maternal sample, as expected on statistical grounds.

The anti-I antibody present in cord serum usually did not react with cord red cells. In testing ten cord sera each against its own red cells, only three sera gave a weak positive reaction.

The frequency and the score of the anti-I antibody in fifteen newborn infants were similar to those of fifteen adult (mother) and fifteen cord sera selected at random (Fig. 2).

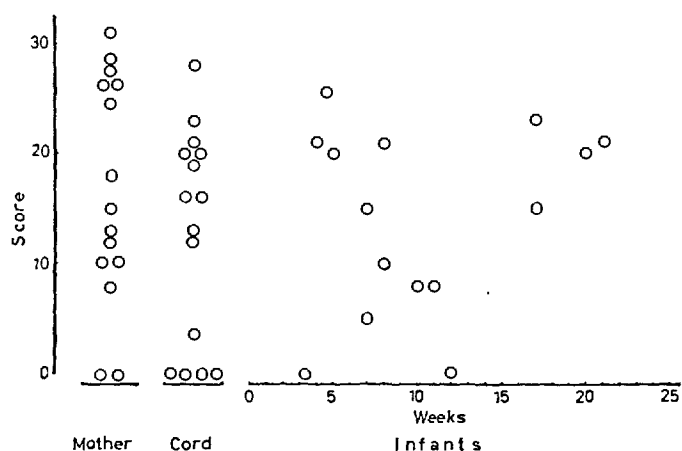


FIG. 2. Anti-I agglutinin scores in the sera of fifteen infants compared with those of fifteen adults (mothers) and cord sera.

In the seven normal infants bled periodically the antibody was regularly present in four (see Fig. 3 and Table 2); in two of them (Nos. 1 and 4 in Table 2) the antibody had a constant titre during the whole period of observation; in the other two (Nos. 5 and 7) the score increased by about 10 units between the first and the second blood samples taken 5 weeks apart. In two other infants out of the seven, the antibody was absent throughout the period of observation (Nos. 3 and 6); in the last case (No. 2) the antibody was absent when the infant was 8 and 14 weeks old but was present when the infant was retested at the age of 18 and 20 weeks.

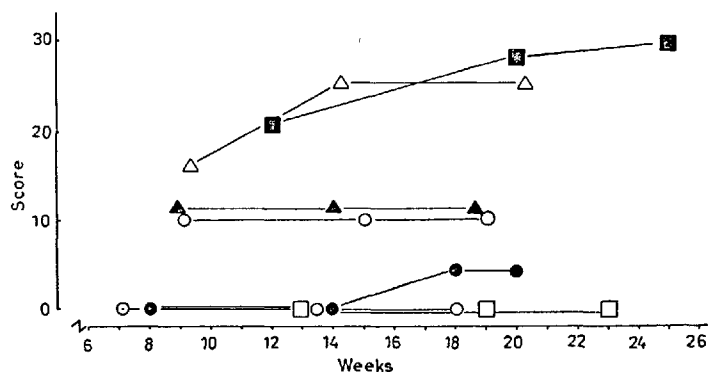


FIG. 3. Anti-I agglutinin score in seven infants bled on several occasions in the first months of life.

TABLE 2

ANTI-A, ANTI-B, ANTI-I, N.I.C. AND ANTI-*E. coli* C3 ANTIBODIES IN SEVEN INFANTS TESTED ON SEVERAL OCCASIONS IN THE FIRST WEEKS OF LIFE

No.	Age (weeks)	ABO group	Antibody score*				
			Anti-A	Anti-B	Anti-I	n.i.c.	Anti- <i>E. coli</i> C3
1	9	A	—	0	10	29	0
	15		—	0	10	40	0
	19		—	0	10	44	0
	21		—	75	—	—	0
2	8	O	0	0	0	5	0
	14		0	0	0	13	0
	18		0	0	5	13	0
	20		18	0	7	15	0
3	14	O	0	0	0	13	0
	19		0	5	0	15	0
	24		12	7	0	15	0
	29		22	17	—	—	27
4	9	O	0	0	12	18	19
	14		0	0	12	35	18
	19		5	0	12	37	27
5	9	A	—	0	17	50	12
	14		—	0	25	50	17
	20		—	0	25	50	14
6	8	B	0	—	0	0	18
	13		0	—	0	0	5
	18		0	—	0	0	0
7	13	O	0	0	20	22	18
	20		21	0	28	39	27
	26		21	13	30	43	13

— Not tested

* See text.

Three pairs of maternal and cord sera, two normal adults sera and two cord sera were fractionated on DEAE-cellulose using 0.02 M and 0.2 M phosphate buffers (see Methods). The anti-I antibody in adult and maternal sera was always recovered in the 0.2 M fraction which contained most of the γ A and γ M-globulins and very little γ G-globulin; the 0.02 M fraction, containing the bulk of the γ G-globulin, but no γ A or γ M-globulin, was free of antibody. The recovery of the antibody from cord sera fractionated with DEAE chromatography was not always satisfactory. When the antibody was recovered it was always found in the 0.2 M fraction, but in some cases the antibody was absent from both fractions.

Ten maternal and ten cord sera, all containing anti-I were treated with 2-me and then retested; in every case the agglutinating activity of the serum was destroyed.

The anti-I antibody present in one adult and two cord sera fractionated by ultracentrifugation was always recovered in the fast sedimenting fraction (fraction No. 6, see Methods); this fraction contained mainly γ M-globulin and only traces of γ G-globulin as tested by the inhibition of the antiglobulin serum method; γ G-globulin was present mainly in fraction Nos. 3 and 4; in these two fractions the anti-A γ G-globulin present in one cord serum was recovered.

ANTI-A, ANTI-B AND NORMAL INCOMPLETE COLD (N.I.C.) ANTIBODIES

Seven cord sera were selected which, on the basis of the maternal ABO blood groups, could not contain anti-A, or, in one case, anti-B antibodies unless the foetus itself had made the antibody. In relation to the ABO blood groups the samples were distributed as follows: in five cases the mother was group A and the infant group O, in one case the mother was group A and the infant group B and in the last case the mother was group AB and the infant group A; therefore in six cases anti-A, and in one case anti-B, if present in cord serum, would have had to be produced by the foetus. The antibodies were never found even after increasing the ratio of serum to red cells to 80 to 1.

Anti-A and anti-B agglutinins were also looked for in the serum of the infants bled periodically at about 5 week intervals. In all infants there was a 'null period' during which no anti-A or anti-B were produced (Table 2); the antibodies were first detectable when the infants were about 20 weeks old.

The n.i.c. antibody was present in six out of the seven infants who were bled at intervals; the antibody had a constant score in two (Nos. 3 and 5); in three other infants (Nos. 1, 2 and 4) the score increased from the first to the second sample and then was constant; in the remaining case (No. 7) the score increased from 22 to 43 in 13 weeks (Table 3). The n.i.c. antibody has been shown to be present in cord serum (Mollison, 1956) and not to be a γ G-globulin (Adinolfi, Daniels and Mollison, 1963; Adinolfi, 1965).

BACTERIAL ANTIBODIES

Twenty pairs of maternal and cord sera were tested for antibodies against *E. coli* C3; all twenty maternal samples contained the agglutinin; twelve out of the twenty cord sera had the antibody, always at a titre lower than the corresponding maternal serum.

The *E. coli* C3 antibody was present in three out of the seven infants bled periodically; in two the agglutinin was absent during the whole period of observation; in one case it was absent until the baby was 14 weeks old and was detected when the infant was 19 weeks old; in the last case the antibody had a score of 18 when the infant was 7 weeks old, of 5 at 13 weeks and 0 at 18 weeks (Table 2).

The twelve cord sera containing the antibody and the corresponding maternal sera were treated with 2-me; in the cord serum *E. coli* C3 antibody was never inactivated by

the treatment, while in the maternal serum the antibody was always partially inactivated.

Using 2-me treatment, the *E. coli* C3 antibody in the babies bled periodically was found to be inactivated by the treatment in some sera but not in others. Thus, of the three infants in whose serum the antibody was present throughout the whole period of observation, two (Nos. 5 and 7 of Table 3) had antibodies which were completely inactivated by 2-me; in the third (No. 4) the antibody was not inactivated. In the infant (No. 6) whose *E. coli* C3 agglutinin was disappearing slowly from the circulation during the period of observation, the antibody was not inactivated by the treatment; in the infant (No. 3) that started to produce the antibody only at the age of 14 weeks the *E. coli* C3 agglutinin was completely inactivated.

DISCUSSION

Cold agglutinins with anti-I specificity occur in human sera under three different circumstances (Race and Sanger, 1962).

(a) As an auto-antibody in normal subjects of I phenotype; this antibody has a thermal range of approximately 0–12° and usually has a low titre not exceeding 64 when one volume of titrated serum and one volume of a 2 per cent suspension of red cells is left at 0° for 2 hours (Dacie, 1954).

(b) As an iso-antibody in the rare subjects of I phenotypes (i_1 or i_2); this antibody reacts at 37° as well as at lower temperatures and its specificity varies slightly between different donors (for references, see Race and Sanger, 1962).

(c) As an auto-antibody in most patients suffering from acquired haemolytic anaemia of the cold antibody type (for references see Dacie, 1963).

Studies on the physical properties of the anti-I antibodies have so far been restricted to the agglutinins present in patients suffering from acquired haemolytic anaemia of the cold antibody type.

In 1953 Gordon performed an ultracentrifugal analysis of the cold autoantibody present in a patient with acquired haemolytic anaemia; the antibody was previously purified by absorption onto, and elution from, red cells and it was shown to be associated with γ M-globulins. This finding has been repeatedly confirmed by others using different methods of plasma protein fractionation (Weber, 1956; Fudenberg and Kunkel, 1957; Mehrotra and Charlwood, 1960; Mehrotra, 1960; Killander and Högman, 1963).

No studies have been reported so far on the anti-I that occurs as an iso-antibody, but in two cases in which this antibody was present in pregnant women no anti-I could be detected in the sera or on the red cells of the newborn infants (Marsh 1961; Wallace, cited by Race and Sanger, 1962) suggesting that this type of anti-I is probably a γ M-globulin.

The present experiments show that the anti-I present in normal adult serum is not a γ G-globulin; thus it was recovered in the 0.2 M fraction from DEAE-cellulose chromatography, was completely inactivated by treatment with 2-me and, after ultra-centrifugation was detectable only in the fractions containing the fast sedimenting serum proteins. Similar results were obtained with cord serum.

Evidence was also obtained that the antibody was produced by foetuses and newborn infants; in fact, the range of the antibody score in infants from 3 to 22 weeks old was similar to that of normal adults and the antibody titre varied very little in the infants bled repeatedly; if the anti-I had been an antibody of maternal origin it should have disappeared from the circulation of the newborn infant as do antibodies that cross the human

placenta. Furthermore, the titre of the antibody in cord serum was apparently unrelated to the titre of the corresponding maternal sample and in one case, the antibody was present in the cord serum while it was absent from the relevant maternal sample.

Anti-A and anti-B of γ M type were not produced by the newborn infants until about 20–22 weeks of age (Table 2) whereas *E. coli* C3 antibodies, inactivated by treatment with 2-me, were observed in newborn infants 9 and 13 weeks old (Table 2, cases Nos. 5 and 7). Fig. 4 shows a typical case (No. 7) selected from the seven infants bled periodically, in which the anti-I and the n.i.c. antibodies were both present in the serum during the whole period of observation, while the anti-A and anti-B antibodies were absent when the infant was 12 weeks old and were produced later. The *E. coli* C3 antibody was, in this case, inactivated by treatment with 2-me.

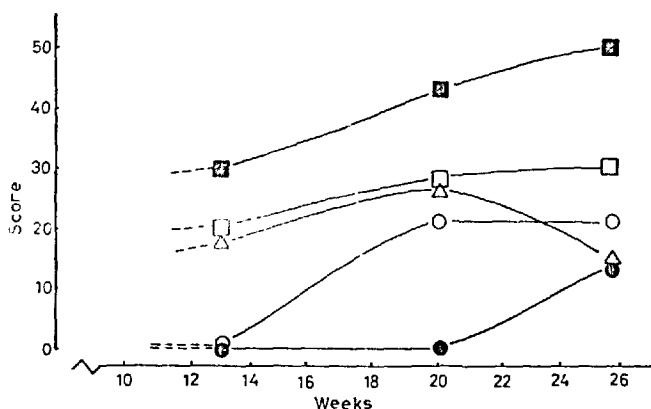


FIG. 4. Anti-A (○), anti-B (●), anti-I (□), normal incomplete cold (■) and *E. coli* C3 (△) antibody scores in an infant (No. 7) tested on several occasions in the first weeks of life.

The finding that a blood group antibody is produced in very young infants, and evidently by the foetus, is in good agreement with recent observations that newborn infants do not pass through a period of complete inability to produce globulins and therefore antibodies (Evans and Smith, 1963). In fact it has been shown that γ M-globulin is already present in cord serum (Franklin and Kunkel, 1958; Derrington and Soothill, 1961; Polley *et al.*, 1962) and that the synthesis of γ M and γ A-globulins increases rapidly in the first weeks of life (West, Hong and Holland, 1962). It has also been possible to immunize very young infants against a number of bacterial antigens and according to Smith (1960) the antibodies formed by such infants in response to stimulation with H antigen of *Salmonella* type consisted at least initially, of γ M-globulins.

Recently Fink, Miller, Dorward and Lo Spalluto (1962) observed that premature infants produced γ M and little or no γ G-globulins after stimulation with typhoid-paratyphoid antigens; typhoid O agglutinins were found only in the macroglobulin fraction and no change to γ G-globulin occurred with time or on secondary stimulation. When bacteriophages were used for immunization by Uhr, Dancis, Franklin, Finkelstein and Lewis (1962), it was observed that newborn premature infants produced antibodies as promptly as did older children and that antibodies formed after the stimulation were initially γ M-globulin. It has also been demonstrated that stimulation of lambs *in utero*

by a variety of antigens induces an active antibody response (Silverstein, Thorbecke, Kraner and Lukes, 1963). It is not surprising therefore that some γ -globulins produced by the newborn infant have blood group specificity.

The question arises as to whether the production of anti-I *in utero* is stimulated by maternal I antigen which has crossed the placenta or whether the anti-I is to be regarded as a 'naturally-occurring' antibody whose production is determined by purely genetic, rather than environmental factors. In the absence of any direct evidence on the subject one can only point to observations with the ABO system; here the evidence is strongly in favour of the idea that the blood group substances do not cross the placenta. Thus anti-A and anti-B are never found in the infant's serum unless the antibody is present in the maternal serum (as a γ G-globulin); if A and B substances cross the placenta an occasional infant would be expected to respond by producing γ M anti-A or anti-B, but this has not been observed so far. In addition there is the direct observation that A and B substances do not appear to cross the placenta (Høstrup, 1963).

Thus the occurrence of the anti-I agglutinin in the newborn infants seems unlikely to be due to stimulation *in utero* by specific maternal blood group antigen and may be regarded as perhaps the best example at present of the production of an antibody in the absence of specific stimulation.

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