INTERACTIONS BETWEEN RED CELL ANTIGENS AND CORRESPONDING ANTIBODIES, WITH SPECIAL REFERENCE TO ABO-HAEMOLYTIC DISEASE. STUDIES WITH LABELLED ANTIBODIES AND THE ELECTRON MICROSCOPE.

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ABSTRACT

It is known that even in moderately severe ABO-haemolytic disease of the newborn (hdn), the infant's red cells give either a negative or only weakly positive reaction, whereas in Rh-hdn the reaction is strongly positive. This discrepancy was investigated using radio-iodine-labelled antiglobulin sera together with labelled anti-A and anti-Rh. It was found that the minimum number of anti-A molecules detectable on newborn A cells was the same as the minimum number detectable on adult A cells, which in turn was similar to the minimum number of anti-Rh molecules detectable. The amount of anti-A on the red cells on infants with ABO-hdn was found to be between 0.25 and 3.5 µg per ml cells (usually 0.55 µg/ml or less).

It was concluded that IgG anti-A is much more effective than IgG anti-Rh in bringing about red cell destruction. Further experiments suggested that the mechanism by which IgG anti-A brings about red cell destruction in ABO-hdn is not complement-activation but may well be agglutination of red cells followed by sequestration.

In a complementary series of experiments a method of labelling antibodies with colloidal gold was adapted for use in the electron microscope to study the distribution of A and Rh antigenic sites. Purified horse anti-human IgG labelled with gold particles was found to be useful for this purpose.

D antigenic sites were found to be single entities randomly dispersed on untreated Rh positive cells. Conversely, a clustered distribution of D sites was observed in papain-treated RBC and on -D- RBC coated with IgG anti-D. A antigenic sites were found to be clustered in proportion to the amount of bound IgG anti-A; reasons are given for considering this pattern to be indirect evidence of mobility of A antigens. There is some evidence that D and c antigenic sites are located close together on cDE/cDE (R_2R_2) cells.

PREFACE

The investigations described in this thesis are those which I have been personally concerned with during the last three years in the MRC Experimental Haematology Unit at the Wright-Fleming Institute, St. Mary's Hospital Medical School. The principle aim of this research has been to gain further knowledge, either directly or indirectly, about the immunohaematological problems found in haemolytic disease of the newborn.

The thesis consists of 5 chapters. In chapters I and II studies concerning ABO-haemolytic disease of the newborn are reported. The central subject of chapter I is the search for an explanation of why the antiglobulin test is weakly positive or negative in cases of ABO-haemolytic disease.

In chapter II, observations are reported on the mechanisms of in vivo red cell destruction by low concentrations of IgG anti-A.

Chapters III, IV and V report experiments, using a gold-labelled anti-human globulin as electron-microscope marker, related to the distribution of A, D and c antigen sites on the surface of the human red cell. In chapter III there is a description of the development of the gold-labelling method. In chapter IV, observations on the distribution of A and D antigen sites are reported. Implications of the observed distribution in relation to the mobility of these antigens and to the serological properties of the antigen-antibody systems concerned are discussed. In chapter V evidence for a possible molecular proximity of D and c antigen sites of Rh system is presented.

Acknowledgements: I am deeply indebted to Professor P.L. Mollison and Dr. N.C. Hughes-Jones, for their excellent tutorship. Their clear and bright thoughts were a guidance of inestimable value for my investigations.

The technical aspects of the electron microscopy were carried out jointly with C. Stolinski, to whom I am very grateful. I would also like to thank Professor A.S. Breathnach for allowing the use of his electron microscope. I am also grateful to Mrs. Eileen Law for her excellent typing and cooperativeness and to my wife, Mirtha, for her continuous encouragement.

During the time in which these investigations were carried out, I was holder of a scholarship from the INSTITUTO VENEZOLANO DE INVESTIGACIONES CIENTIFICAS, IVIC, CARACAS, VENEZUELA, to whom I am greatly indebted for their support.

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CHAPTER I

THE DIRECT ANTIGLOBULIN REACTION IN ABO-HAEMOLYTIC DISEASE OF THE NEWBORN

QUANTITATION OF IgG ANTI-A (B) ON RED CELLS OF AFFECTED INFANTS

INTRODUCTION

When foetal red cells which carry antigens not present in the mother, gain entrance to the maternal circulation, they may stimulate the production of antibodies in the mother. In the human species, if these antibodies produced in the mother are of the IgG class, they can cross the placenta and produce immune destruction of the red cells of the foetus and the newborn infant. This process involving immune destruction of the newborn red cells by maternal antibodies was first demonstrated by Levine and Stetson (1939) and Levine et al. (1941).

Though IgG anti-A and anti-B are the commonest antibodies present in human serum which potentially could cause haemolytic disease of the newborn, this disease was first recognised within the Rh system, probably due to the fact that Rh-alloimmunization usually produces a severe haemolytic process with an incidence that used to be about 0.5 per cent before the introduction of the clinical use of IgG-anti-D for the prevention of Rh-alloimmunization. By contrast, severe cases of ABO-haemolytic disease of the newborn are very rare, in the vicinity of 1 in 2000/3000 (Mollison, 1972 p.163); milder cases of haemolytic disease of the newborn due to ABO incompatibility are however, relatively common.

It was precisely in relation to the diagnosis of the Rh-haemolytic disease of the newborn that Coombs, Mourant and Race (1945) described the use of anti-human globulin to detect Rh-"incomplete antibodies" in the serum of mothers with affected infants ("indirect antiglobulin test") or on the infant's red cells which were sensitized in vivo ("direct antiglobulin test", Coombs, Mourant and Race (1946).

The antiglobulin test has been an extremely valuable tool in the diagnosis of the Rh-haemolytic disease of the newborn, for it is the simplest and best way of showing IgG (anti-Rh) antibodies coating the infant's red cells. Furthermore, when the test is carried out properly, a negative result virtually excludes the diagnosis of haemolytic disease of the new born due to Rh-alloimmunization.

By contrast, in the case of ABO-haemolytic disease of the newborn, the situation is very different. When routine techniques are used, even in moderately severe cases of the disease the infant's washed red cells give either a weakly positive or a negative direct antiglobulin reaction. For example, Crawford et al. (1953) using a routine technique on a tile found that in a series of 11 affected infants all with signs of moderately severe disease, the direct antiglobulin reaction was weakly positive in 7 and negative in the remaining 4. Similarly, in a series of 9 cases, Shumway, Miller and Young (1955) found a weakly positive reaction in only 2 cases with negative reactions in the remaining seven. In 21 cases of mild or severe ABO-alloimmunization described by Hsia and Gellis (1954), the direct antiglobulin test was invariably negative.

Rosenfield (1955) described a method of improving the sensitivity of the antiglobulin reaction by increasing the ratio of antibodies to red cells and by centrifuging at 2000 rev min for 10 seconds. The reactions were read visually and were also read microscopically when necessary. With this procedure, the number of molecules of antiglobulin combining with each red cell is increased, moreover, the introduction of centrifugation brings the cells closer together thereby facilitating the formation of antibody - cross links between the cells. Rosenfield (1955), Rosenfield and Ohno (1955) and others have found that when this modified antiglobulin reaction ("Spin Coombs") is used, a positive reaction is obtained in a much higher proportion of cases of ABO-haemolytic disease of the newborn being on some occasion even positive with the red cells of only mildly affected infants.

The need to use a more sensitive technique to detect antibody-coating of red cell in ABO-haemolytic disease and the search for other tests for this purpose (Munk-Andersen, 1956) merely reinforces the fact that the direct antiglobulin reactions in cases of ABO-haemolytic disease are weakly positive or negative.

The weakness of the antiglobulin reaction in the ABO-haemolytic disease is difficult to understand especially because the haemolytic process can occasionally be quite severe. Hence, the paradox arises of IgG antibodies causing accelerated red cell destruction, but at the same time apparently being present on the red cells at only low concentration.

There seem to be several possible explanations for the observation of weak direct antiglobulin reactions in ABO-haemolytic disease of the newborn. The main possibilities are the following :

1) The amount of antibody coating the circulating red cells of the affected infant may be very small and, therefore, the antiglobulin reaction may be performed at the lower limit of the sensitivity of the technique. The finding that eluates, prepared from red cells of affected infants, may give strong indirect antiglobulin reactions when tested with adult A_1 cells, may suggest that the weak antiglobulin reactions are not due to small amounts of antibody coating the circulating red cells (Voak, 1968). However, as will be explained later, when antibodies are eluted from a certain volume of cells (usually 0.5 ml or more) and tested with a much smaller number of cells, an important concentration factor may play a part, as was correctly appreciated by Voak and Williams (1971) in a later paper. A low amount of antibody coating the red cells of affected infants could be explained by the fact that A and B antigens are present in many other cells of the human body besides the red cells. Moreover, A (or B) blood group substances are present in the serum of the newborn (Høstrup, 1963). These extra-erythrocyte antigen sites might compete (a possible "protective mechanism") with the antigens on the red cells for the available IgG anti-A (or B) transferred from the mother. (See Discussion).

One difficulty with this first explanation is that, if it was true, then it would imply that red cell destruction in vivo by IgG anti-A (or anti-B) is a much more efficient process than red cell destruction in vivo by IgG anti-D, an implication calling for yet further explanation.

2) Another possibility is that most of the antibody coating the cells of the affected infants may come off the cells on washing. However, the finding of equilibrium constants ranging from 0.6 to 13 x 10^8 l/mole for human anti-A (Economidou et al., 1967a) seems to rule out this possibility.

3) A further possibility may be the "weakness" of A and B antigens on cord cells in comparison with adult cells. Newborn A and B red cells are known to give weaker serological reaction than corresponding adult red cells perhaps due simply to the fact that newborn cells have only about one quarter of the total number of antigenic sites present in the adult cells of the same group (Economidou et al., 1967b). This "weakness" of the A and B antigens on cord cells or a "special quality" of these sites which may obstruct the reaction between the bound IgG antibody and the anti-IgG reagent, has also been postulated as a possible explanation for the weak antiglobulin reactions in ABO-alloimmunization. These possibilities however, seem to be ruled out by the fact that newborn A_1 cells may give a strong indirect antiglobulin reaction when incubated with enough IgG anti-A (Voak and Williams, 1971).

4) Voak and Williams (1971) suggested that the combination of the small size of IgG antibodies and the relatively greater distances between the A sites of A_1 infant cells compared to those of adult A_1 (A_1 infant cells have about 200,000-250,000 sites in comparison to 800,000-1,000,000 A antigenic sites per cell in adult cells, Economidou, et al., 1967b, Greenbury, et al., 1963) might be the cause of the weak antiglobulin reaction in ABO-haemolytic disease. However, it is difficult to explain why or how the greater distance separating antigen sites in infant cells would cause the "...A/B sites on infant cells to be relatively unbridgeable compared to those on corresponding adult cells..." As the number of D antigenic sites in infant and adult Rh positive cells is much smaller than the number of A_1 sites in infant cells, then the distance separating D sites is much greater than the distance between A sites. However, this greater distance separating D sites is no obstacle to give strong antiglobulin reactions in cases of Rh-haemolytic disease.

The search for an explanation for the weak antiglobulin reactions observed in cases of ABO-haemolytic disease of the newborn has been the basic aim of this chapter. In attempting to find a solution for this problem, it appeared that some useful information could be derived if quantitative studies were carried out with both radiolabelled IgG anti-A (or B) and anti-human IgG on the reactions of infant and adult A (or B) cells with the corresponding IgG antibodies and antiglobulin reagent.

MATERIALS AND METHODS

1) Red Cells

a) From normal newborn infants

Cord blood was obtained at the time of delivery and mixed with ACD (1.2 ml of ACD"A", prepared according to Mollison, 1972, p.686, plus 4 ml of blood) and kept at 4^{o} up to seven days before being tested. The red cells were washed 4 times in 10 g/l NaCl buffered with phosphate to pH 7.0 (PBS) before being used. The red cells were grouped using standard techniques and reagents obtained from the Blood Group Reference Laboratory. Group A red cells were further tested with an extract of Dolichos biflorus (Lectin-A_1, DADE, Division American Hospital Supply Corporation) and only red cells that were agglutinated by this reagent, (i.e. A_1 cells) were used.

b) Red cells from infants with ABO-haemolytic disease

Criteria for the diagnosis of the disease are described later.

Blood samples were obtained either clotted or anticoagulated in EDTA or in ACD. In clotted samples, the clot was broken by manipulation with wooden sticks and the red cells released were then suspended and washed in PBS at 4^{o}. Red cells from EDTA or ACD samples were washed 3 times before use.

c) Red cells from normal adults

Blood was mixed with ACD and stored at 4^{o} for up to 7 days. A_1 subgroup was determined by testing with an extract of Dolichos biflorus.

The cells were washed 3 times in PBS before being tested.

2) Antibodies

a) IgG anti-A

i) Source: a 23 year old group O volunteer was injected intramuscularly with 5 mg of purified A glycoprotein, kindly donated by Prof. Winifred M. Watkins. The purified human A substance was sterilized by heating for 30 minutes at 80^{o} on 3 consecutive days, followed by filtration through millipore filter. The glycoprotein was suspended in saline solution at a concentration of 2 mg/ml.

The volunteer was plasmapheresed at days 8, 11 and 12 post-injection. The ACD plasma was clotted with thrombin topical (Parke Davis) at a ratio of 5 units of thrombin per ml of plasma and pieces of wooden sticks were added to collect the clot. The resulting serum was then filtered through gauze, centrifuged at 1700g for 30 minutes and made up to 5×10^{-4}M with sodium azide 10 g/litre.

The indirect antiglobulin titre of the whole antiserum tested against adult A_1 cells was 1:1600.

ii) Fractionation: the anti-A serum was fractionated by DEAE-Cellulose (Whatman DE-52) chromatography. A column 45 x 3.5 cm was filled with about 150g of ion exchanger which was found to be suitable to fractionate about 100 ml of serum.

20 mM Tris/HCl pH 8.2 was used as starting buffer and the amounts of 100 ml of serum to be fractionated were dialysed for about 24 hours against 2 changes of 4 litres each of the 20 mM buffer before being fractionated.

After the first fraction was eluted, the concentration of the buffer was increased to 0.2 M, and after a second fraction was eluted, the rest of the proteins were eluted with 0.5 M Tris/HCl.

The fractions were then separately concentrated by pressure dialysis and tested for anti-A activity. The first fraction was found to have the bulk of the anti-A activity, as judged by indirect antiglobulin test and agglutination tests for A_1 adult cells. By double immunodiffusion, using a standard Ouchterlony technique (Ouchterlony, 1958) it was found that fraction I gave a single line of precipitation with an anti-whole human serum. This precipitation line showed a pattern of identity with the line obtained with an anti-human IgG serum. This first fraction was, therefore, identified as IgG anti-A and seemed to be free of IgM or other immunoglobulin contaminants.

iii) Radioiodination:

The IgG anti-A preparation was labelled with ^{125}I (or ^{131}I), by the iodine monochloride method (McFarlane, 1958).

100 µCi of ^{125}I and 0.075 ml of ICl (0.42 mg I/ml in 0.1 M HCl) were used for each 10 mg of IgG. 1 M glycine in 0.25M NaCl pH 9.0 was used to bring the pH of the reactants to about 9.0. The labelling reaction was carried out at 4^{o} for 5 minutes with continuous stirring. Then, the labelled protein was dialysed for 24 h against PBS and subsequently centrifuged at 90,000 for 30 minutes.

The efficiency of labelling was of the order of 60-80 per cent. There was no need for preoxidation of the -SH groups if labelling was carried out immediately after DEAE-chromatography. The total volume of the protein mixture was kept as small as possible, and was always less than 5 ml. At the ratio of ICl to protein used, it was estimated that about 0.4 atoms of Iodine were bound to each IgG molecule.

The specific activity of the preparation was determined by estimating the protein concentration by Lowry's et al. (1951) method and then by counting the radioactivity of a measured aliquot of the same solution used for the estimation of the protein concentration. As IgG standard, IgG preparations (kindly supplied by Mrs. B. Gardner) whose total N had been estimated by the modified micro-Kjeldahl technique of Melamed and Green (1963) were used. In terms of ct/min, the specific activity of the

newly labelled IgG was usually found to be between 4000 and 10,000 ct/min per μg of IgG anti-A. From the specific activity of the preparation, and assuming a mol. wt. of 160,000 for the IgG antibody molecules, it was also possible to estimate the number of ^{125}I-labelled IgG anti-A molecules from a particular radioactive count. Corrections for radioactive decay were performed by counting a ^{125}I standard under the same conditions. A Gamma Counter was used to estimate the radioactivity of the samples.

iv) Antibody Concentration

The method used was a variation of the procedure described by Hughes-Jones (1967) for the determination of the concentration and equilibrium constant of anti-D preparations.

20, 50, 100 and 200 μl of group A_1 red cells were incubated with a fixed volume of 50 μl of ^{125}I-IgG anti-A for 90 minutes at 37°. Controls for nonspecific uptake were set up using group O red cells. Unbound antibody was separated by centrifugation and the coated red cells were then washed in PBS at 4°, lysed and the radioactivity estimated. The results were analysed according to the derivation of the law of mass action derived by Scatchard : a plot of the ratio of bound antibody to free antigen against bound antibody was made and the result was extrapolated to the abscissa which gave the concentration of the antibody in the preparation tested. (see Fig. I-1) This antibody concentration was estimated to be about 84 μg IgG anti-A /ml and corresponded to a "purity" of about 1 per cent.

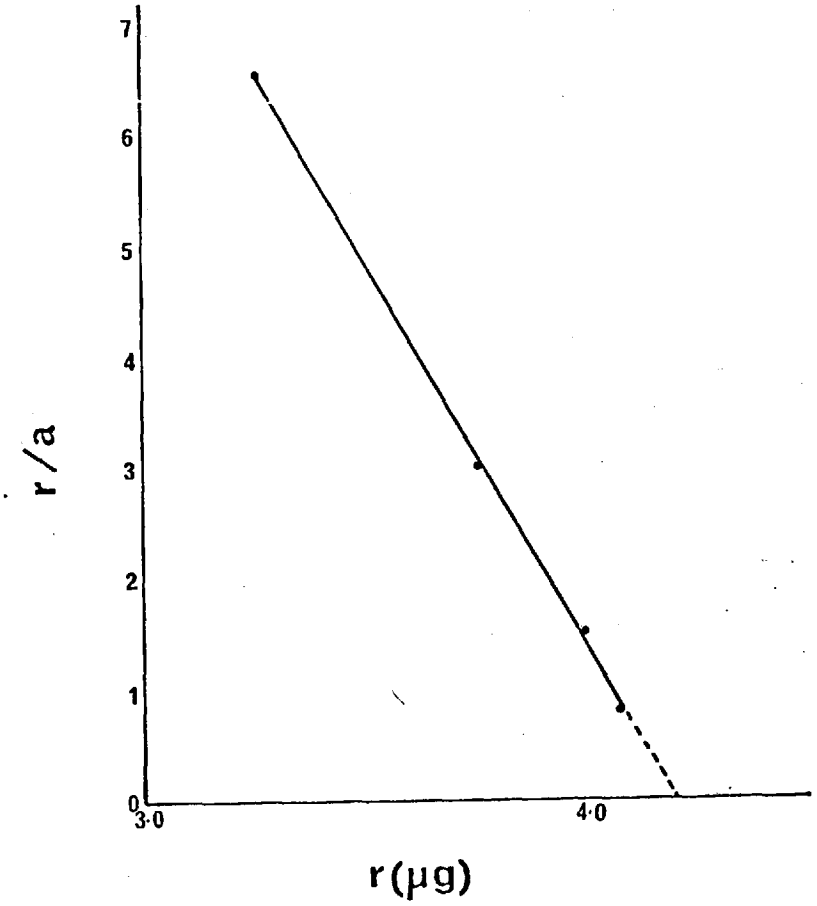

Fig I-1 : An example of the plot r/a against r.

The extrapolation to the abscissa gives the concentration of ^{125}I-anti-A in one ml of a 1:20 dilution. a = free antigen, estimated assuming a total number of 1×10^6 sites/cell (Economidou et al 1967b) and that 1 antibody binds to 1 antigen site.

As the nonspecific uptake, estimated by the uptake of ^{125}I-IgG anti-A on the group O cells, was in every case less than 10% of that on the test cells it was considered unnecessary to carry out any further purification of the ^{125}I-labelled IgG anti-A.

This labelled anti-A had an indirect antiglobulin titre of 1:8000 with A_1 adult cells.

b) HORSE ANTI-HUMAN IgG

This antiserum had been produced in hyperimmunized horses by injections of purified human IgG (Medical Research Council, 1966). The antiserum was kept frozen at -40°.

i) Purification: was carried out by adsorption onto an immunoadsorbant followed by elution at pH 2.8.

The immunoadsorbant was prepared by the cross-linking of purified human IgG (Human Normal Immunoglobulin, Lister Institute) by treating the IgG with glutaraldehyde, according to the method of Avrameas and Ternynck (1969). The procedure was as follows:

1. 250 mg of IgG were dissolved in 5 ml of 0.1 M Phosphate buffer pH 7.0
   while stirring, 1 ml of a .25 g/l solution of glutaraldehyde (BDH) was added drop-wise; a gel was formed, which was left overnight at 40°.

2. the completeness of the insolubilization was established by dispersing the material in water followed by separation by centrifugation of the insoluble material and estimation of the absorbance of the supernatant at 278 nm. The procedure was repeated until the supernatant reading at 278 nm was less than 0.040.

3. the insoluble protein was then re-suspended in 0.2 M phosphate buffer pH 7.3 and homogeneized in small portions in a loose fitting homogeneizer. This was followed by centrifugation and the supernatants were discarded. The same procedure was then repeated 4 times using 0.1 M Gly/HCl buffer pH 2.8 (the buffer to be used for the elution of the antibody). The absorbance at 278 nm of the last supernatant was essentially 0.

The immunoadsorbant was then incubated with 50 ml of horse anti-human IgG serum for 30 minutes at about 22°. The supernatant was separated by centrifugation. The immunoadsorbant with the bound anti-IgG was then washed twice with cold PBS. Elution of the antibody was carried out by re-suspending the immunoadsorbant with the bound antibody in 10 ml of 0.1 M Gly/HCl pH 2.8. This was mixed for 5 minutes and then separated by centrifugation. The pH of the supernatant, which contained the purified horse anti-IgG antibody, was quickly raised to 7.0 with 0.1 N NaOH. The absorption and elution procedures were repeated twice. The eluates were then concentrated to 2/3 ml by pressure dialysis. An alternative method of elution of the anti-IgG from the immunoadsorbant was performed

by the method of Edgington (1971) using 2.5 M KI pH 7.0 as eluting buffer in the place of 0.1 M Gly/HCl pH 2.8.

Most of the work described in this chapter was carried out with an anti-IgG preparation purified with this procedure. However, an alternative method, which was found to give a better yield and a better preservation of the antibody activity (see forward) was carried out by coupling covalently purified IgG on to CNBr-Activated Sepharose 4-B (Pharmacia). Briefly, the procedure was as follows :

5g of washed and swollen Sepharose reagent were incubated overnight with 50 mg of IgG dissolved in 10 ml of a buffer constituted of 0.1 M $NaHCO_3$ in 0.5 M NaCl pH 8.0. This was continuously mixed by rotation. Virtually all the IgG was coupled. Non reacted activated sites on the Sepharose were deactivated by treatment with 25 ml of 1M ethanolamine (BDH) pH 8.0. The immunoadsorbant was then washed with 3 cycles of alternating washes in buffers constituted of 0.1 M sodium acetate in 1 M NaCl pH 4.0 and 0.1 M sodium borate in 1 M NaCl pH 8.0. Next, a column procedure was used to absorb the antiserum, 25 ml each time, followed by washing with 0.1 M sodium borate in 1 M NaCl pH 8.0 and elution with 0.2 M Gly/HCl pH 2.8. The column procedure had the advantage of being faster and of offering the possibility of following visually the antibody elution with the aid of a spectrophotometer and graph plotter attached to a fraction collector (LKB-Ultrorac Fraction Collector).

ii) Radioiodination : The iodine monochloride method described for IgG anti-A was also used to label the anti-IgG preparations. However, it was observed that the anti-IgG was poorly labelled if preoxidation of the SH-groups was not carried out before labelling either with ^{125}I or ^{131}I.

The preoxidation of the sulphydryl groups was achieved as follows:

To one ml of a concentrated sample of purified anti-IgG, 0.5 ml of 1 M Gly/HCl pH 3.6 was added. Then 1 or 2 drops of iodine in potassium iodide solution (20 mg I_2 in 5 ml of 0.1 M KI), just enough to give a very light yellow colour to the solution, was added and the mixture was immediately run in a small column of Sephadex G-25 equilibrated with 1 M Gly/HCl pH 3.6. After elution from the column, the pH of the protein solution was adjusted to 8.5 with 0.5 M NaOH and then the protein solution was immediately labelled with ^{125}I or ^{131}I.

The efficiency of labelling with the ^{125}I preparation was always higher than with that of ^{131}I.

The specific activity of the labelled anti-IgG was estimated in a similar manner to that described for ^{125}I-labelled IgG anti-A.

iii) Concentration : was estimated by a similar method to that used to determine the concentration of IgG anti-A, that is by calculating the maximum amount of labelled antibody that could be bound by a molar excess of antigen, but in this case the antigen was composed by O R_1R_2 group red

cells coated with IgG anti-D. The coated cells were prepared by incubating 1 ml of red cells with about 150 µg of IgG anti-D. Controls for nonspecific uptake were prepared using Orr group red cells which were also incubated with IgG anti-D. The purified anti-IgG preparations were found to be about 7.5 per cent "pure" when prepared with glutaraldehyde-cross linked IgG and about 59 per cent "pure" when prepared with the Sepharose IgG immunoadsorbant.

Some information regarding the identification of the horse anti-IgG antibody is given in Chapter III.

3) SEROLOGICAL TECHNIQUES

a) INDIRECT ANTIGLOBULIN TEST

Two methods were used :

i) the standard technique described by Mollison (1972, p.422).

ii) Rosenfield's (1955) modification of the test, commonly known as "Spin Coombs".

i) In the standard procedure 1 drop (about 0.07 ml) of a 20% red cell suspension of 3 times washed red cells is incubated with 4 drops of a dilution of the antiserum to be tested. The incubation was carried out for 90 minutes at 37^{o} and then the cells were washed 4 times in cold PBS. After the last wash, the cells were gently resuspended and one drop of the resuspended cells was delivered to each of 2 squares of an opal tile: one was mixed with one drop of an antiglobulin reagent (from an optimally diluted rabbit anti-human globulin or from a commercial preparation), and the other drop was mixed with PBS. The tile was rocked

gently and cell suspensions were examined for agglutination within 5 to 10 minutes under a strong light. The reactions were recorded semi-quantitatively as ++++ or complete (2 or 3 solid clumps), +++ ("large clumps"), ++ ("medium size clumps"), + (small but clearly visible clumps) and ± or weak (very small just visible clumps).

ii) In the second method, the main features are an increased ratio of antiglobulin to sensitized cells and centrifugation which brings red cells closer together, hence facilitating the cross-linking by the antiglobulin. The first stage was carried out as in the standard technique or alternatively, with a weaker cell suspension. After the washing procedure, sufficient cold PBS was added to the pellet of sensitized cells to make a 1-2 per cent red cell suspension. Then 1 drop was delivered to each of 2 small plastic tubes. To one was added one drop of antiglobulin reagent and to the other, one drop of saline. The tubes were then spun for 10 seconds at about 660 *g*, the pellet gently broken and visual agglutination was recorded or if a negative result was obtained, the suspension was then examined for agglutination under the microscope. The presence of "clusters" of 4-5 cells or more was taken as indicative of a positive test.

It was usually observed that with the spin technique, the titre of the antibody under test was slightly greater, i.e. equivalent to one more doubling dilution.

b) DIRECT ANTIGLOBULIN TEST

This was carried out with 3 times washed red cells, using procedures similar to those described for the second stage of the indirect test.

c) AGGLUTINATION

This was carried out simply by mixing in a tube 1 drop of 5 per cent red cell suspension with 2 drops of dilutions of the antisera to be tested. After 90 minutes at 37°, the suspension was examined visually or microscopically for agglutination.

d) TESTS USING 2-MERCAPTOETHANOL (2-me) TREATED SERUM

The rationale of this test is that under mild reducing conditions, IgM and possible dimeric IgA are dissociated into sub-units and lose most of their agglutinating power, but IgG molecules are not affected. This test was found useful to determine the IgG anti-A and anti-B titres in the serum of group O mothers of infants affected with ABO-haemolytic disease without the interfering effect of the IgM or IgA anti-A or anti-B.

The modification of the test suggested by Reesink et al. (1972) was used for this purpose. Equal parts of serum to be tested and 0.2 M 2-me (Koch Light) in 0.2 M phosphate buffer pH 7.4 were mixed at 37° for 2 hours. Dilutions of this treated serum were used for serological tests without making any attempt to remove the 2-me or to alkylate the dissociated IgM sub-units to prevent their recombination. The presence of small quantities of 2-me has not been found to affect the antigen- antibody reaction.

4) QUANTITATIVE STUDIES WITH RADIO-LABELLED ANTIBODIES

a) MAXIMUM UPTAKE OF ^{125}I-IgG ANTI-A ON CORD AND ADULT RED CELLS

The estimation of the maximum uptake of ^{125}I-anti-A by A_1 red cells is a direct measurement of the total number of antigenic sites of these cells. Greenbury et al. (1963) and Economidou et al. (1967b) found that this total number of antigenic sites was about 0.8-1.2 x 10^6 per A_1 adult cell and about 0.25-0.37 x 10^6 for A_1 cord cell (Economidou, et al., 1967b). Similar studies to those of Economidou et al., were carried out mainly to check the anti-A antibody preparation. The procedure was as follows: aliquots containing a known number of red cells (usually 2 x 10^7) were incubated with decreasing serial amounts of ^{125}I anti-A to which 30 g bovine serum albumin/l was added. From 24 to 1.5 µg anti-A were used for experiments with cord cells and 48 to 3 µg anti-A for experiments with adult cells. After 90 minutes at 37°, the cells, which were strongly agglutinated, were separated from the free antibody by centrifugation; supernatants containing the free anti-A were saved for counting. Then the coated red cells were washed 3 times with 2 ml of cold PBS, transferred to clean tubes, lysed and counted.

The results were analysed by plotting the ratio of bound to free antibody (r/a) against bound antibody (r) and extrapolation to the abscissa, to estimate the maximum bound antibody (n) as applied by Hughes-Jones et al. (1967)*. The maximum bound antibody can be equated to the total number of antigenic sites if the assumptions are made that 1 antibody binds to only one antigenic site, which, in the case of IgG-anti-A Antigen system may be incorrect at low or medium saturation of the A sites because an anti-A molecule might be attached through both F(ab)s portions to 2 antigenic sites (Hughes-Jones, 1972) but is likely to be true in the region of antibody excess as in the present experiments.

With these studies, in which the maximum uptake of IgG anti-A on A red cells was determined, it was also possible to obtain the value of the equilibrium constant of the ^{125}I-labelled IgG anti-A (see footnote).

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* The expression of the law of mass action derived by Scatchard was used to analyse the results. Briefly,

$\frac{r}{a} = Kn - Kr$ Where r = bound antibody on the red cells.
a = free antibody.
n = maximum antibody that could possibly be bound on the red cells.
K = equilibrium constant.

The values of $\frac{r}{a}$ against r were plotted

When $\frac{r}{a} \rightarrow 0$, then $r \rightarrow n$, therefore, by extrapolating $\frac{r}{a}$ to the abscissa (r = 0) the value for n (r = n) is obtained.

The value for K at half saturation of the antigenic sites can be determined from the formula $K = \frac{1}{a}$ which is easily derived by substituting r for $\frac{1}{2}n$ in the equation $\frac{r}{a} = Kn - Kr$.

b) END POINT OF THE ANTIGLOBULIN REACTION WITH CORD AND ADULT RED CELLS

The minimum number of anti-A molecules detectable with the antiglobulin test on cord and adult A_1 cells was determined as follows:

a fixed number (approximately 2×10^8) of A_1 adult cells or cord cells was incubated at 37° for 90 minutes with serial dilutions of ^{125}I-labelled anti-A at a ratio of 1 volume of red cells to 6 volumes of anti-A. Group O red cells were used as a control. The cells were washed 3 times with ice-cold PBS and the uptake of ^{125}I determined within 30 minutes. The same samples of red cells were tested for agglutination by optimally diluted rabbit anti-human IgG both macroscopically (on a tile) and microscopically after 10 seconds of centrifugation at 660g (number 5 of the standard MSE bench centrifuge).

Similar experiments were carried out with O Rh positive (R_1R_2) red cells and ^{125}I-labelled IgG-anti-D.

From the specific activity of the preparation, the red cell count and PCV of the suspension, and assuming the molecular weight of IgG to be 160,000 daltons, the results were expressed either as molecules of anti-A per red cell or as µg of anti-A / ml red cells.

c) RATIOS OF UPTAKE OF ANTI-IgG MOLECULES TO IgG ANTI-A ON INFANT AND ADULT A_1 RED CELLS

The number of anti-IgG molecules which combines with IgG anti-A bound on A_1 adult or cord cells was estimated using the method described by Rochna and Hughes-Jones (1965).

One ml of washed A_1 red cells was incubated for 90 minutes at 37° with a dilution of ^{125}I-IgG anti-A containing about 10 µg of antibody. The cells were then washed 3 times with 4 ml of cold PBS, and resuspended in enough PBS to give a 50 per cent cell suspension and aliquots of 0.2 ml were delivered to each one of 8 tubes. One ml of ^{131}I-labelled anti-human IgG in concentrations ranging from 0.5 to 16 µg antibody / ml was then added to 6 of the 8 aliquots and 1 ml of cold PBS was added to the remaining 2. The reaction was allowed to proceed for 10 minutes at 37°. The suspensions were then centrifuged at 1700*g* for 2 minutes, the supernatants saved and the cells washed three times with 4 ml of cold PBS.

The following radioactive measurements were made, using standard techniques in a well-type scintillation counter :

i) ^{131}I content in the supernatant, representing the free anti-IgG.

ii) ^{131}I content of the cells, representing bound anti-IgG.

iii) ^{125}I content of the cells, representing bound anti-A.

The ^{125}I-anti-A content of the 2 aliquots of cells in which the anti-IgG was substituted for PBS, was used to check that the labelled anti-A was not eluted from the red cells upon combination with anti-IgG.

Controls using group O red cells were set up in parallel.

The ratios of the amount of ^{131}I-anti-IgG to ^{125}I-anti-A on A_1 adult red cells, were compared with those obtained when cord A_1 red cells were used.

Similar experiments were also carried out with O Rh positive red cells coated with ^{125}I-labelled IgG anti-D in the place of anti-A.

5) SEROLOGICAL STUDIES IN 15 CASES OF ABO-HAEMOLYTIC DISEASE OF THE NEWBORN. QUANTITATION OF THE ANTIBODIES COATING THE RED CELLS IN VIVO

a) DIAGNOSIS AND SEROLOGY

Blood samples of affected infants were kindly sent to us from various other hospitals and regional centres.

The diagnosis was made by the finding, in a full-term A or B infant born to an O mother, of a serum bilirubin concentration of 10 mg/100 ml or more on the second or third day of life and the demonstration of IgG anti-A or anti-B antibodies in the mother's sera. In 8 cases the concentration of bilirubin reached 18 mg/100 ml or more; 7 of these infants and 1 other were treated by exchange transfusion. In most cases, microspherocytes were prominent in films of peripheral blood.

There were 4 group B and 11 group A infants. The red cells of the group A infants were tested with an extract of Dolichos biflorus: 7 samples were strongly agglutinated, 2 were weakly agglutinated and 2 more were not agglutinated at all.

In all cases, eluates were prepared by the method of Landsteiner and Miller (1925). When enough cells were available, 1 ml of 3 times washed red cells was added to 2 ml of PBS preheated to 56°. The suspension was then incubated at 56° for 10 minutes with constant gentle shaking. Next it was centrifuged in a heated centrifuge for 2 minutes at 1700$\bar{g}$ and the supernatant was immediately separated. The eluates were then tested by the indirect antiglobulin technique using A_1 or B cells.

In order to discover how much anti-A was eluted from cord red cells on washing, 1 ml of red cells of 2 affected infants was washed 3 times and the supernatant wash solutions were saved, pooled and concentrated to about 0.5 - 1 ml and then tested by the indirect antiglobulin technique.

b) QUANTITATION OF IgG ANTI-A (B) ON THE RED CELLS OF AFFECTED INFANTS.

The amounts of IgG anti-A or anti-B on the red cells of affected infants were estimated using the ^{125}I-labelled antiglobulin technique described by Rochna and Hughes-Jones (1965). Hughes-Jones et al. (1967) had previously used this method for the quantitation of the amount of IgG anti-D on red cells of infants with Rh-haemolytic disease.

The uptake of ^{125}I-labelled anti-IgG by fixed amounts of A_1 red cell coated with known quantities of ^{131}I-anti-A and the free concentration of ^{125}I-labelled anti-IgG were estimated as described in the previous section, to study the ratios of uptake of anti-IgG to anti-A. In order to make a standard anti-IgG curve, the ratios (r) of bound anti-IgG to anti-A on the red cells were plotted against free anti-IgG: (see Fig. I-2). To determine the amount of anti-A or anti-B coating the cells, 2 aliquots of 100 µl of 3 times washed cells were incubated for 10 minutes at 37° with 1 ml of ^{125}I-anti-IgG containing about 15 µg antibody/ml. The free ^{125}I-anti-IgG was then separated by centrifugation and saved, and the red cells washed 3 times in cold PBS. The counts on the red cells (bound anti-IgG) and of the supernatant (free anti-IgG) were then determined. Nonspecific uptake, using group O cells and a similar amount of ^{125}I-anti-IgG, was substracted from the tests. Bound anti-A or anti-B, coating the red cells of the affected infant, was then derived from the standard curve by finding the ratio, r, of anti-IgG to anti-A(B) at the given free anti-IgG concentration and applying the relationship:

$$\text{anti -A(B) µg/ml red cell} = \frac{^{125}\text{I- anti-IgG µg/ml red cells}}{\text{r (at given free anti-IgG concentration)}}$$

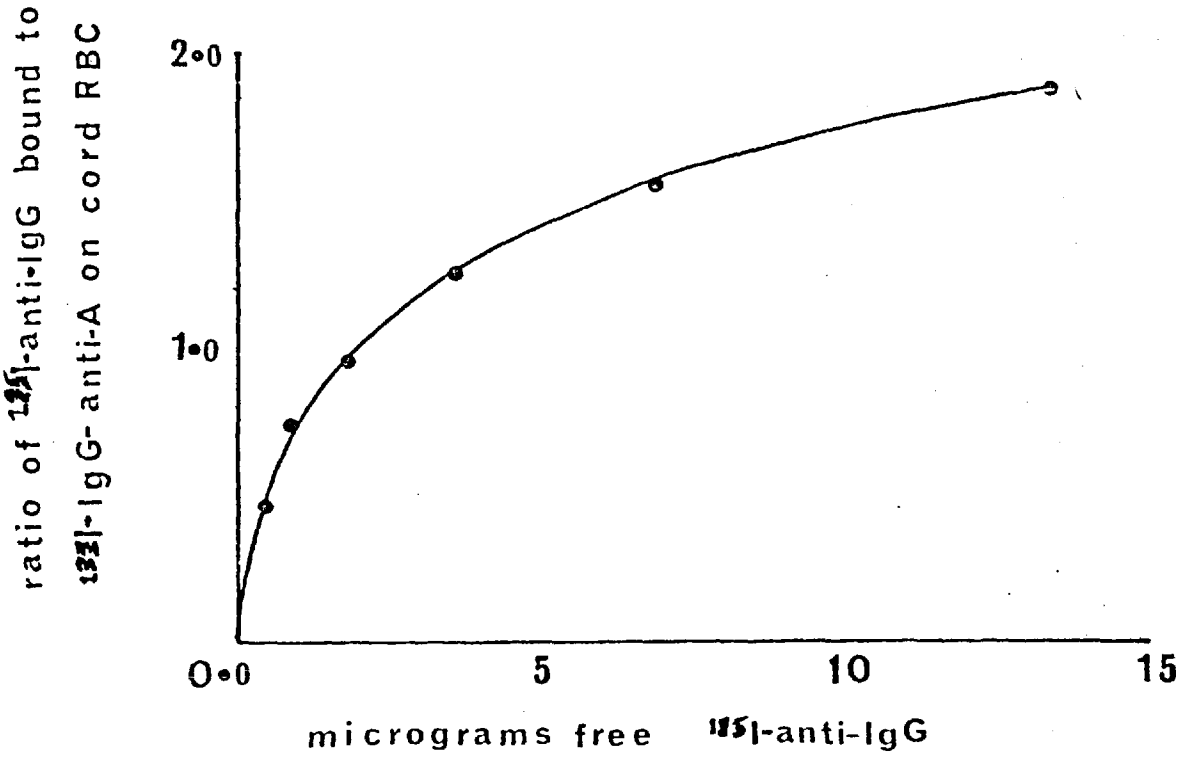

Fig I-2 : Standard curve for the estimation of IgG anti-A or anti-B on red cells of affected infants with ABO-haemolytic disease: ratio of the number of anti-IgG molecules combined with one anti-A (B) molecule at given free concentrations of anti-IgG in µg/ml.

RESULTS

1) MAXIMUM UPTAKE OF ^{125}I-ANTI-A BY CORD AND ADULT A_1 RED CELLS

Two A_1 cord samples and two A_1 adult samples were tested. The total number of antigen sites which correspond to the maximum number of IgG anti-A molecules taken up by the red cells was found to be approximately 0.3×10^6 antigen sites per A_1 infant and 1.2×10^6 per A_1 adult cell. Fig I-3 shows an example of this type of experiment. These estimates are of the same order as those previously reported by Greenbury et al. (1963) and Economidou et al. (1967).

The equilibrium constant of the IgG anti-A was found to be on average about 9×10^8 1/Mole.

2) END POINT OF THE ANTIGLOBULIN REACTION WITH CORD AND ADULT RED CELLS

The end point of the antiglobulin test was estimated by finding the minimum number of IgG anti-A molecules bound on red cells that was necessary to give a positive antiglobulin reaction.

The results for adult and cord A_1 red cells are shown in Table I-1. It can be seen that both for adult and for cord A_1 red cells, the least number of IgG anti-A-molecules which could be detected by the indirect antiglobulin method using an optimally diluted rabbit anti-human globulin, was approximately 150 antibody molecules per red cell. This figure

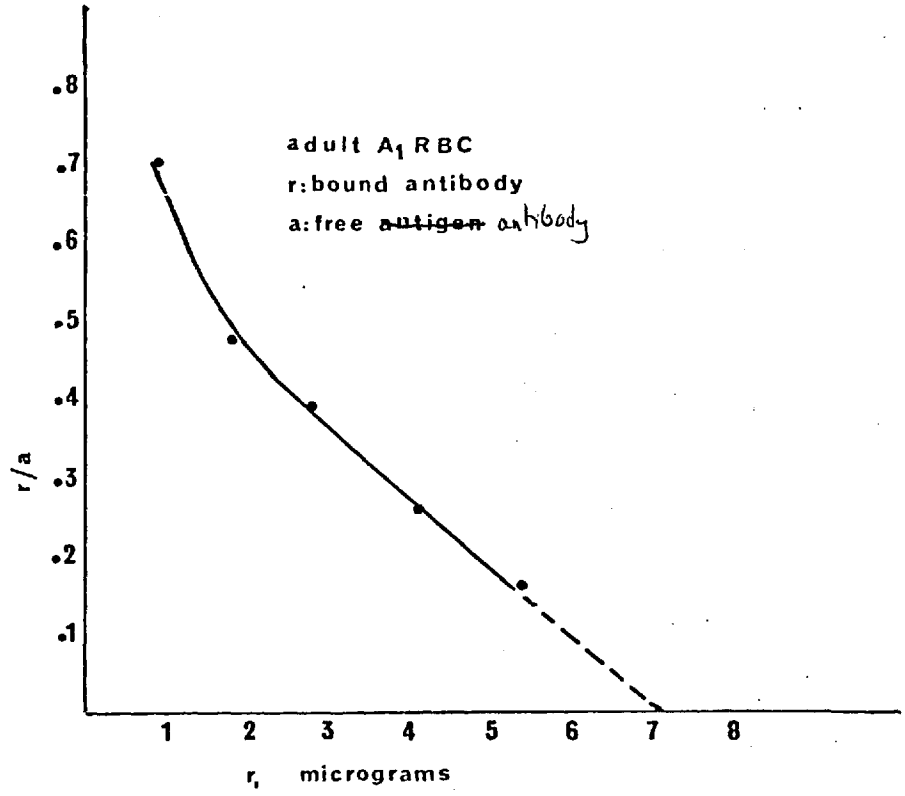

Fig I-3 : Estimation of the total number of antigen sites: maximum amount of ^{125}I-IgG anti-A combined at equilibrium with 2×10^7 group A_1 red cells from an adult donor.

TABLE I-1

END POINT OF THE ANTIGLOBULIN REACTION

Reactions with an anti-IgG serum of normal adult and cord A_1 red cells coated *in vitro* with similar numbers of IgG anti-A molecules.

ADULT RED CELLS			CORD RED CELLS		
No. of IgG anti-A molecules/cell	µg anti-A/cell	Reactions with anti-globulin serum	No. of IgG anti-A molecules/cell	µg anti-A/cell	Reactions with anti-globulin serum
2500	6.6	++	2140	5.7	++
970	2.6	++	1060	2.9	++
520	1.4	+	490	1.3	+
214	0.6	+	226	0.6	+
145	0.4	trace	161	0.4	trace
128	0.3	-			

The reactions with an antiglobulin serum are given as ++ or + to indicate the degree of agglutination; "trace" indicates that only a dubious reaction was present on macroscopic examination although microscopically there was definite agglutination

is quite similar to the estimated minimum number of IgG anti-Rh molecules detectable on Rh positive cells by the use of antiglobulin sera (Dupuy et al., 1964; Hughes-Jones et al., 1964) and is also in the same order of magnitude of the least number of complement molecules (C_4 or C_{3b}) detectable on red cells by immunoadherence or by anti-complement sera (Cooper, 1969; Dr. Margaret Polley, personal communication).

Table I-1 also shows that the reactions of adult and cord red cells coated with similar number of anti-A molecules were indistinguishable.

3) RATIO OF ANTI-IgG TO IgG ANTI-A ON CORD AND ADULT A_1 RED CELLS

When cord and adult red cells were coated with similar amounts of anti-A and then incubated with decreasing amounts of anti-IgG, it was found that the extent of the reaction between IgG and anti-IgG was the same (irrespective of whether the IgG anti-A was coating cord or adult red cells). Thus, when aliquots of 0.1 ml of cord or adult A_1 red cells coated with about 0.8 µg of labelled anti-A were incubated with decreasing amounts of labelled anti-IgG starting at about 16 µg per ml, it was found that the maximum ratio of anti-IgG molecules to IgG molecules was 1.8 to 2.0 for both types of cells. Furthermore, in a control experiment in which D-positive red cells coated with a comparable amount of labelled IgG anti-D was used, a similar ratio of anti-IgG to IgG anti-D was observed.

The reason for choosing the particular concentrations of anti-IgG was because near-saturation of antigen sites on the IgG molecules was obtained at a free equilibrium concentration of 10-15 µg of anti-IgG/ml, which was also observed in the original technique described by Rochna and Hughes-Jones(1965).

The observed maximum ratio of 1.8 - 2 anti-IgG molecules to IgG molecules was lower than those found by Kabat (1961, p.26): 6; Costea et al. (1962): 7 - 9; and Rochna and Hughes-Jones (1965): 6 - 8. However, Dr. N.C. Hughes-Jones (personal communication) in recent years, using an anti-IgG purified by the use of insolubilized cross-linked IgG by glutaraldehyde treatment, has also found the maximum ratios of anti-IgG to IgG to be about 2. To elucidate this problem, experiments were carried out using horse and rabbit anti-IgG preparations which had been purified by absorption and elution from an IgG-Sepharose 4-B immunoadsorbant (as opposed to the purification procedure in which the immunoadsorbant was prepared by glutaraldehyde cross-linking of IgG). With these different purified anti-IgGs, ratios of 6-7 were obtained, suggesting the possibility that in the IgG glutaraldehyde cross-linked preparation, fewer antigen sites were available and/or that there was a partial loss of antibody activity in the anti-IgG upon the purification procedure. For example, when anti-IgG was eluted from the Sepharose immunoadsorbant with Gly/HCl buffer at pH 1.8 - 2.0 instead of 2.8 - 3.0, then the maximum ratio obtained was only about 3.6 - 4.0.

4) NUMBER OF ANTI-A AND ANTI-B MOLECULES ON RED CELLS IN CASES OF ABO-HAEMOLYTIC DISEASE

The amount of anti-A or anti-B on the red cells of 15 affected infants was estimated by the use of a single batch of ^{125}I-labelled antiglobulin and a calibrated standard curve. The results of this estimation, together with other relevant findings are summarized in Table I-2. It can be seen that the amount of anti-A or anti-B on the red cells ranged from 0.25 to 3.5 µg antibody per ml red cells. It was estimated that these amounts in µg correspond to 90 to 1310 antibody molecules per single cell. These estimations were carried out assuming that human IgG has a mol. wt. of 160,000 daltons, that about 1×10^{10} red cells are present in one ml of cells and from Avogadro's number. The median value for the 15 cases is about 0.45 µg antibody / ml red cells or approximately 160 antibody molecules per single red cell.

The direct antiglobulin test when read macroscopically, was positive in 11 cases and when read microscopically, was positive in one furtner case. It can be seen that there was a close correlation between the amount of antibody coating the cells and the antiglobulin reaction: when read macroscopically, the direct antiglobulin reaction was negative in 4 of the 5 samples which had the smaller degree of coating. Furthermore, the results confirmed those found in Table I-1 regarding the end point of the antiglobulin reaction, showing that, with a routine technique, 0.35 - 0.40 µg of antibody per ml red cells is the least detectable amount of anti-A(B) on red cells.

In all 14 cases in which an eluate at 56° was prepared from the infants' red cells, the expected antibody (anti-A or anti-B) was demonstrated. As will be pointed out in the Discussion, this is not surprising in view of the concentration factor that results from the procedure used in the elution and testing of the antibodies.

As Table I-2 also shows the IgG anti-A (or -B) in the mothers' sera, as measured by the indirect antiglobulin reaction using 2-me treated sera ranged from 1 : 16 to 1 : 4000. Unfortunately, 6 of the sera were obtained more than 3 days post-delivery, therefore, they may not indicate the titre of IgG anti-A (or -B) at the time of delivery. It can also be seen in Table I-2, that there is a poor correlation between the IgG anti-A (or -B) titre in the maternal serum and, on the one hand, the amount of antibody coating the cells and, on the other, the severity of the disease as judged by the serum bilirubin concentration.

5) ELUTION OF ANTI-A FROM RED CELLS DURING WASHING

Only traces of anti-A were detected in the wash solution obtained from the red cells of 2 infants with haemolytic disease due to anti-A.

Similarly, in quantitative experiments with normal A cells coated *in vitro* with ^{125}I-labelled IgG anti-A, a maximum of 20 per cent of antibody was recovered from 4 consecutive washes.

TABLE I-2

Cases of ABO-haemolytic disease

Case No.	INFANTS					MOTHERS (all group O)	
	Blood Group	maximum recorded plasma bilirubin concentration		direct antiglobulin test		indirect antiglobulin test* (anti-A or -B)	
		mg/100 ml	time after birth	routine method	µg antibody per ml red cells	titre	time after delivery
1	A	19.4	48 h	neg	0.25	32	1 d
2	A	27.0	72 h	neg	0.3	16	3 d
3	B	18.0	60 h	+(microscop)	0.35	512	4 d
4	A	14.3	60 h	+	0.35	1000	-1 d
5	A	12.5	24 h	neg	0.35	128	2 d
6	B	11.0	48 h	+	0.4	4000	7 wks
7	A	27.0	24 h	+	0.4	2000	8 d
8	A	10.1	48 h	+	0.45	1000	4 wks
9	B	22.6	48 h	+	0.55	256	2 d
10	A	13.6	24 h	+	0.55	1000	6 d
11	B	18.0	24 h	+	1.1	2000	7 d
12	A	14.0	24 h	+	1.4	512	1 d
13	A	20.0	72 h	++	1.85	1000	3 d
14	A	..+		++	2.25	1000	1 d
15	A	24.3	72 h	++	3.5	2000	3 d

* anti-IgG

\+ cord bilirubin concentration 5.8 mg/100 ml; no later estimates

DISCUSSION

A) EXPLANATION FOR THE OBSERVED ANTIGLOBULIN REACTIONS IN ABO-HAEMOLYTIC DISEASE

The present results (small amounts of antibody on the cells of infants affected with ABO-haemolytic disease) show that the minimum number of IgG anti-A (or -B) molecules detectable on red cells by the antiglobulin test is about 150 per cell, both for cord and adult red cells. The number is about the same as that previously reported by Dupuy et al. (1964) and Hughes-Jones et al. (1964) for the detection of IgG anti-Rh by the antiglobulin test. As Table I-1 shows, the extent of the reaction between IgG anti-A and the anti-IgG was the same with adult and cord cells. Furthermore, the ratios of uptake of labelled anti-IgG to labelled anti-A by cord and adult cells were similar at comparable concentrations of free anti-IgG. These results clearly demonstrate that there is no "special quality" of the A antigen sites on the infants' cells, which affects the reaction of bound anti-A with an antiglobulin reagent. Also, despite the smaller amount of antigen sites on cord A_1 cells in comparison with adult A_1 cells, if there are enough antibody molecules coating the cord cells, the antiglobulin reaction will be positive, irrespective of the "greater distances" between the antigens. Since evidence was obtained that relatively little IgG anti-A is lost from 'coated' red cells on washing, it must be concluded that the weak positive direct antiglobulin test which is characteristic of ABO-haemolytic disease, indicates that, in this disease, relatively few antibody molecules are attached to the circulating red cells, and that the test is performed at the limits of its sensitivity. This conclusion was directly confirmed by measurement of the amounts of anti-A or anti-B coating the red cells of affected infants. Thus, in the present series of 15 cases, the amount of

anti-A or anti-B found on the cells was estimated to be between 0.25 and 3.5 µg ml cells; the median value was 0.45 µg/ml and only 5 values were above 0.55 µg/ml cells. These amounts of antibody-coating in cases of ABO-haemolytic disease are much lower than those found by Hughes-Jones et al. (1967) in cases of Rh-haemolytic disease and these estimations are directly comparable because of the use of a similar technique in both series. For comparison, both series are present in Fig. I-4. At levels of approximately 1 µg antibody per ml cells, the estimates carry a considerable error, because this amount of antibody on the cells is almost of the same order as the amount of nonspecific ^{125}I-labelled auto-IgG bound to the cells. However, the error is unlikely to be greater than ± 50 per cent, so that, in any case, the amount of specific antibody (anti-A or anti-B) bound in a typical case is unlikely to be greater than about 1 µg antibody per ml cells. This degree of coating is much less than that usually found in the Rh haemolytic disease, in which the amount in one series of cases reported by Hughes-Jones et al. (1967), was found to vary between 0.4 and 18 µg / ml, depending on the severity.

In Rh haemolytic disease, the amount of antibody on the infant's red cells, as µg antibody per ml cells, is very approximately the same as the concentration of anti-Rh in the mother's plasma in µg antibody per ml plasma (Hughes-Jones et al., 1971). On the other hand, in ABO haemolytic disease, the amount of antibody per ml on the infant's red cells may be as little as one-fortieth of the concentration of the corresponding antibody in the mother's plasma. For example, in the present series, the median value of IgG anti-A or anti-B in the mother's plasma was probably about 20 µg/ml (taking the median indirect antiglobulin titre in the series as 1000 and assuming that, as with anti-Rh, an indirect antiglobulin titre of 1 corresponds to about 0.02 µg antibody per ml). The smaller amounts of anti-A and anti-B on the infant's red cells are presumably due mainly to the competing effect of A and B substances widely scattered throughout the body tissues and secretions (Tovey, 1945). Thus,

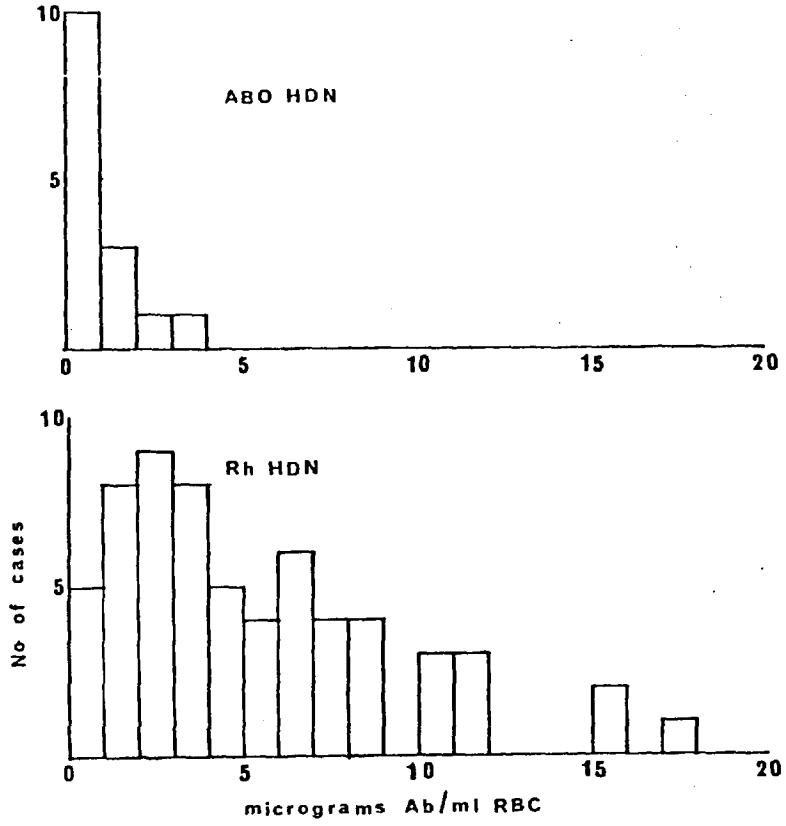

Fig I-4 : The amounts of antibody on the red cells of infants affected with ABO and Rh haemolytic disease. The amounts of anti-D were estimated from the data of Hughes-Jones et al., 1967.

soluble A (or B) substances and A (or B) antigen sites on leukocytes, platelets and on many other cells of the body (cf Mollison, 1972, p.230) by binding IgG anti-A (or -B) could effectively make 80-90 per cent of the antibody unavailable for the red cells and so constitute an effective protective mechanism.

B) SOME CONTRADICTORY OBSERVATIONS

Some observations which appear to contradict the present findings of only small amounts of antibody on the red cells of infants with haemolytic disease of the newborn, must be briefly discussed: Voak and Bowley (1969) and Voak (1969) reported that eluates from the red cells of infants with ABO-haemolytic disease might give strong indirect antiglobulin reactions with adult A_1 red cells, although the direct antiglobulin reactions on the infant's red cells were only weakly positive or even negative. This observation might seem to indicate that the infant's red cells were coated with considerable amounts of antibody. However, in a later paper,(Voak and Williams, 1971), it was pointed out that the elution procedure resulted in a considerable concentration of antibody so that the finding does not necessarily indicate that there is a substantial amount of antibody on each cell. If one considers some actual quantities, the degree of concentration involved in elution can be better appreciated. Suppose, for example, that antibody is eluted from 0.5 ml of red cells coated with 0.5 µg/ml of anti-A and 60 per cent of the antibody is removed during elution; the total amount of antibody eluted would then be 0.15 µg. Now, suppose that the eluate is incubated with 0.1 ml of a 5 per cent suspension of red cells and that 80 per cent of the antibody is taken up by the cells; there is then 0.12 µg on 0.005 ml cells or 24 µg/ml, sufficient to give a very strong antiglobulin reaction.

Haberman et al. (1967) in electron microscopic studies, using anti-A-ferritin conjugates, found that in the case of cord A cells, often the ferritin-tagged anti-A was observed in the cytoplasma within

pinocytic vesicles. They suggested that the weak antiglobulin reaction in ABO-haemolytic disease could be explained on the basis of inaccessibility of the antibody coating the cells. That this finding could not be the right explanation for the observed weak antiglobulin reactions was indicated by observations made by Voak and Williams (1971). These authors carried out similar studies and observed antibody-containing pinocytic vesicles only in cord cells containing ribosomes, i.e. immature cells, which constituted less than 10 per cent of the cells examined. Presumably, in cases of ABO-haemolytic disease, judging by the reticulocyte counts, the percentage of these immature cells is usually not higher than about 20 per cent.

Voak and Williams (1971), using electron microscopy and ferritin-labelled IgG anti-A and anti-B, found evidence of clustering of antigen sites, both on adult and infant red cells. They suggested that the reason for the relatively weak antiglobulin reactions of cord A cells sensitized with IgG anti-A, compared with the reactions of adult cells, might be the relatively greater distance between the A sites on the infant cells, compared with the shorter distance on adult A_1 cells, making cross-linking by anti-IgG molecules difficult. However, even on newborn red cells, the number of sites is about 10 times greater than the number of Rh(D) sites so that the distance between D sites must be much greater than that between A sites. Moreover, the present observations indicate that the ratio of anti-IgG molecules to anti-A molecules is in fact the same with adult and infant A_1 cells and, incidentally, is the same as that observed with anti-IgG and anti-Rh on Rh-positive red cells.

c) IS IgG ANTI-A (-B) MORE EFFECTIVE THAN IgG ANTI-Rh IN BRINGING ABOUT RED CELL DESTRUCTION IN VIVO?

Whereas weak positive direct antiglobulin reactions are associated with a definite haemolytic syndrome where anti-A and anti-B are concerned, a weakly positive direct antiglobulin reaction due to anti-Rh may be associated with no increase in red cell destruction whatever, (Mollison, 1951, p.382). Similarly, in a case in which, due to a misunderstanding, a dose of 150 - 300 µg anti-D was given to a Rh positive infant, instead of to its Rh negative mother, it was observed that when the infant's red cells were coated with approximately 1.5 µg anti-Rh per ml, only a very mild haemolytic syndrome was produced (Mollison, 1972, p.636). On the other hand, in our series, exchange transfusion was sometimes required even at a level of 0.3 µg anti-A per ml red cells.

In the search for an explanation for this difference, when the serological properties of the IgG anti-Rh (D) - Rh antigen (D) and IgG anti-A (or anti-B) - A (or B) antigen are compared, two striking differences are observed :

1) IgG anti-A (or anti-B), unlike anti-Rh, binds complement.
2) IgG anti-A (or anti-B), unlike anti-Rh, agglutinates red cells suspended in a medium of 10 g/l sodium chloride. (See Mollison, 1972, p.233 and p.279).

1) The binding of complement by anti-A (or -B) could explain a more effective red cell destruction, in comparison with anti-Rh, by complement mediated lysis, immunoadherence and erythrophagocytosis by the reticuloendothelial system (RES). However, is there enough IgG antibody on the affected cells to bind complement? In the case of IgM antibodies, only one rabbit or human IgM anti-A molecule is sufficient for the fixation of the first component of complement (C1) (Borsos and Rapp, 1965; Ishizaka, et al., 1966). But in the case of IgG complement-fixing antibodies like anti-A, then at least 2 molecules in juxtaposition are required, (Borsos and Rapp, 1965; Ishizaka et al., 1966), and it has been calculated from statistical considerations that if a cell has 600,000 binding sites for IgG molecules, about 800 must be present on the cell surface to provide an even chance that 2 will occupy adjacent sites and thus activate complement, assuming that the efficiency is 100 per cent (Humphrey and Dourmashkin, 1965); as an A_1 red cell from a newborn infant has about 250,000 to 350,000 binding sites, in this case even more than 800 IgG molecules are necessary to be present on the cell surface to have an even chance of two being in juxtaposition and so start complement-fixation. However, this reasoning would apply only if the A-anti-A antigen-antibody complexes are randomly distributed on the red cell membrane and would not apply if they were present in clusters, even at the low levels of antibody-coating observed in the ABO-haemolytic disease. Wang and Desforges (1971) found that complement titres, measured as C H_{50} units, were the same in normal newborns and in 10 newborns with ABO-haemolytic disease; no C_4 or C_3 was detected on the cells of the affected infants using anticomplement antisera (anti-β_{1E} and anti-β_{1C}). However, there are several objections

to this work: as they pointed out, not all the affected infants were tested with the anticomplement reagents and the fact that, β_{1E}(C4) and β_{1C}(C3b) were not detected, may not mean that there was no complement fixation: For example, in cases of cold agglutinin disease, Engelfriet, et al. (1970), could detect only α2D(C3d) coating the red cells. (Cold agglutinin disease is caused by an autoantibody with anti-I specificity, which is a potent complement-fixing antibody). It also should be pointed out that in the series of Wang and Desforges, only one infant was severely affected, the rest being only mildly affected.

Because of all the above-mentioned considerations, it was thought that a possible role for complement in ABO-haemolytic disease was yet to be clarified. Some studies on this subject were undertaken, and are reported in the next chapter.

2) The second serological difference between IgG anti-A (or -B) and IgG anti-Rh that might be important in the search for an explanation for the greater effectiveness of IgG anti-A in causing red cell destruction, is the greater agglutinating power of IgG anti-A (or -B) in comparison to IgG anti-Rh, i.e. IgG anti-A (or -B) agglutinates red cells suspended in a saline medium and IgG anti-Rh does not. (Some explanations for this different property are presented in the chapter on electron microscope studies of the distribution of D and A antigens in the red cell membrane). In quantitative studies with ^{125}I-labelled anti-A, it was commonly found that agglutination of cells was observed when the cells suspended in PBS and 30 g/l bovine serum albumin were coated with approximately 5000 antibody molecules, which is much greater than the degree of coating observed in our series of affected infants.

However, this finding does not rule out the possible role of agglutination of cells by anti-A (or -B) in causing red cell destruction in ABO-haemolytic disease because of the enhancement of the agglutination produced by IgG anti-A (or -B) observed when the cells are suspended in a medium of serum or plasma. In the next chapter, are described some experiments which were carried out to learn more about this possibility.

D) THE "WEAKNESS" OF THE A AND B ANTIGENS ON THE NEWBORN RED CELLS

It has been widely postulated that A and B antigens are weakly expressed on the red cells of newborn infants and that this fact explains why anti-A and anti-B very seldom cause severe haemolytic disease of the newborn (Mollison, 1972 pp. 619, 667). The expression of this "weakness" is reflected in the lower titres obtained when the same antibodies are tested with cord cells in relation to adult cells; or in the weaker positive reactions (antiglobulin test, agglutination tests) when both cord and adult cells are incubated with the same antibody dilution (Mollison, 1972, p.225). In this sense, it has been said that in comparison with adult A_1 cells, cord A_1 cells behave like adult A_2 cells. A rational explanation for this "weakness" is found in the smaller number of antigen sites present in cord A cells when compared to adult cells which are roughly, respectively, 250,000 and 1,000,000 per cell.

However, the present observations indicated that, when the amount of anti-A on cord or adult cells was the same, then similar anti-globulin reactions were obtained; The observations also corroborated Voak's (1971) finding that when cord A cells are incubated with enough anti-A antibody, a very strong reaction - formation of a solid clump of agglutinated red cells - can be obtained. Therefore, it seems fair to suggest that the expression "weak A antigens" for the A antigens on newborn cells is inappropriate because it suggests an antigen different in properties.

That the smaller amount of A antigen sites on newborn cells, i.e. the "weakness", could hardly be the right explanation for the low incidence of severe cases of ABO-haemolytic disease as suggested by Tovey (1945) and Wiener et al. (1949), seems to be indicated by the fact that the number of D sites on Rh positive cells from affected infants (obviously heterozygous) is less than 10 per cent of the total number of A sites present in newborn group A cells and it should be recalled that Rh-haemolytic disease is (or was) frequently a severe haemolytic process. In this sense, competition for the circulating antibody by the soluble A or B blood group substance in the circulation of the newborn (Høstrup, 1963), and by the A and B antigen present on the cells of other tissues (see Mollison, 1972, p.230 for a review), seem to be more important as a protective mechanism. In other words, the relative proportion of antigen to antibody may be the main factor in determing the amount of antibody bound on the cells and therefore in the determination of the extent of the haemolytic process.

When antibody is in excess, considerable amounts of antibody will be taken up by cord red cells, for example, Sieg et al., 1970, (quoted in Mollison, 1972, p.483) reported that in experiments in which 1 ml of group B cord cells was injected into a group A mother with an anti-B titre of 1:32, the red cells were destroyed within 20 min and usually within 5 minutes. Under similar circumstances, adult A_2 cells may be destroyed relatively rapidly. Mollison, (1972, p.483) found that 0.5 ml amounts of A_1 and A_2 cells were both removed within 3 minutes of the circulation when injected separately into a group O recipient. A haemolytic reaction has even been reported following the transfusion of A_x (very "weak" A) blood into a recipient whose serum contained a potent anti-A (Schmidt et. al., 1959). These observations are not in contradiction with the finding that when A_1 and A_2 cells are transfused in appreciable amounts into group O recipients, the A_1 red cells seem to be destroyed at a faster rate. In this situation, as A_1 red cells have about 4 times more antigenic sites than A_2, they will bind more antibody than A_2 red cells and consequently be preferentially destroyed. For example, using the same volume of cord and adult A_1 cells in the region of antigen excess, it can be demonstrated that in order to bind the same amount of anti-A, the cord cells should be incubated with 2 to 3 times more ^{125}I-anti-A than with the adult cells.

In trying to explain why A and B cord cells have less antigen sites than corresponding adult cells, the main possibilities that should be considered are: 1) a deficiency of the transferase enzyme involved in the last step of the synthesis of the A and B antigens;

2) lack of substrate, or 3) a combination of 1 and 2. The first possibility seems to be excluded by the finding of normal values of the αD-galactosyl-transferase and the αN-Acetylgalactosaminyl-transferase enzymes in the serum of newborn infants (personal communication from Mrs. M. Crookston, 1973). The αD-galactosyl and αN-Acetylgalactosaminyl transferases catalyze the addition, respectively of galactose and αN-Acetylgalactosamine to "H" substance receptors converting the H substance respectively in B and A antigens (Watkins, 1970).

Romano and Mollison (unpublished work) have carried out some experiments which indicate that the smaller number of antigen sites on group B cord cell (100,000 to 200,000 per cell) in comparison with adult B (approximately 800,000) is due to lack of substrate for the galactosyl-transferase. A brief account of these experiments is as follows:

B sites were generated on adult and cord group O cells using the method of Race and Watkins (1972a and 1972b) and the amount of B sites generated was estimated by the use of a ^{125}I-labelled IgG anti-B. For this purpose, 10 µl of group O packed cells were incubated for 4 h at 37° with 200 µl of serum from an adult group B donor, 1µM of UDP-Galactose (Sigma) and 4µM of $MnCl_2$. The number of generated B sites was then estimated with a ^{125}I-anti-B preparation, by methods similar to those used for the determination of A sites. In other experiments, B cord cells were incubated in similar mixtures to determine if the number of B sites could be increased to adult levels.

The results were as follows: between 8000 to 40,000 B sites were generated on adult group O cells, but only 300 to 800 B sites were generated on cord O cells. Similarly, an example of IgG anti-B that had an indirect antiglobulin titre of 128 with the adult treated cells converted to group B gave only a titre of 1 with the cord treated cells. When B cord cells were incubated with adult B serum and UDP-Galactose in the presence of Mn^{++}, no extra B sites were generated.

E) ARE A_2 INFANTS ALSO AFFECTED WITH ABO-HAEMOLYTIC DISEASE?

In the present series, as in the series reported by Gerlini et al. (1968), the red cells of some group A infants with haemolytic disease due to anti-A were not agglutinated by an extract of Dolichos biflorus. In these cases, a partial blocking of A antigen sites on the red cells of the affected infants, as an explanation for the negative reactions with Dolichos biflorus lectin, seems to be ruled out because of the low amount of anti-A on the cells; furthermore, there is no difficulty in grouping the cells of the affected infants with anti-A or anti-B grouping reagents. It can be concluded that, contrary to present belief, A_2 infants, as well as A_1 infants, may be affected with haemolytic disease due to anti-A. On theoretical grounds, there does not seem to be any reason why A_2 infants should be completely unaffected. Even if the number of A sites on the red cells of newborn infants who are genetically A_2 is as low as 50,000, A molecules (red cell antigen sites) would be in considerable excess over anti-A molecules in the plasma so that the number of antibody molecules taken up by A_2 cells should be scarcely less than that taken up by A_1 cells.

On the other hand, if there are qualitative differences between A_1 and A_2 antigens, as might be the case (Mollison, 1972, p.222), then IgG anti-A may bind better to A_1 antigen than to A_2 antigen. For instance, several examples of anti-A have been found to dissociate more easily from A_2 cells than from A_1, indicating that they have a higher binding constant for A_1 than for A_2 (Economidou, 1966 and present observations). This, together with a possible lower amount of A sites in A_2 cord cells may determine that under the same antibody concentration, A_1 cells may bind more antibody and, therefore, A_1 infants may be affected to a greater extent. Another point which is purely speculative is that A_1 antigen may be a better immunogenic stimulus than A_2 for eliciting antibody formation in the mother which would again favour disease in A_1 infants.

Summarizing the question of whether or not A_2 infants are expected to be affected with ABO-haemolytic disease, it can be said that although there seem to be some reasons why A_1 infants might be affected to a greater extent, there are also reasons for believing that A_2 infants should not be completely spared.

CHAPTER II

OBSERVATIONS ON THE REACTIONS IN VIVO AND IN VITRO OF A RED CELLS COATED WITH SMALL AMOUNTS OF IgG ANTI-A

Their relevance to red cell destruction in ABO-Haemolytic disease.

I) INTRODUCTION

In the previous chapter, studies were described in which the amount of IgG anti-A (or anti-B) present on red cells of infants affected with ABO-haemolytic disease was measured. The amount was found to be 0.55 µg or less per ml of red cells, in 10 out of 15 cases. By contrast, in Rh haemolytic disease, only 5 out of 62 infants had less than 1 µg anti-Rh per ml red cells (Hughes-Jones et al., 1967). Although it is true that Rh-haemolytic disease tends to be a more severe process than ABO-haemolytic disease, it seems that in cases of comparable severity, there is considerably more antibody on the red cells when anti-Rh is involved.

An important implication of this finding is that IgG anti-A and anti-B molecules are more effective than anti-Rh molecules in bringing about red cell destruction in vivo. In this chapter, studies are described, which were aimed at elucidating the mechanism by which red cell coated with small amounts of IgG anti-A are destroyed in vivo. For this purpose, the following main points were investigated:

A) Attempts were made to obtain direct evidence that IgG anti-A, at concentration of 1 - 2 μg per ml of cells or less, is more effective than IgG anti-Rh, at similar concentrations, to bring about red cell destruction in vivo. This was carried out by sensitizing ^{51}Cr-labelled-A red cells with known amounts of IgG anti-A and then following their survival in vivo, after re-injection into group A human volunteers. The results could then be compared with those obtained by Mollison, et al. (1965), on the rate of removal from the circulation of Rh positive cells sensitized with different amounts of IgG anti-D.

B) The possible role of complement was investigated in two ways:

1) Estimates were made of the least amount of ^{125}I-IgG anti-A (or -B) bound to red cells required for complement fixation as detected by tests with anti-β_{1E} (anti-C4) and anti-β_{1C} (anti-C3b) sera.

2) The red cells of infants affected with ABO-haemolytic disease were tested for complement components on the cells. For this purpose, the direct reactions of the cells with an anti-α_{2D} (anti-C3d) serum as well as with anti-β_{1E} and anti-β_{1C} were recorded.

C) The possible role of plasma or serum, in enhancing the agglutination of A (or B) cells by IgG anti-A (or anti-B) was investigated. Quantitative studies were carried out using ^{125}I-labelled IgG anti-A to estimate the relation between the amount of antibody on the cells and the agglutination observed with the cells when suspended in plasma serum and other media.

EXPERIMENTAL PROCEDURE

A) MATERIAL AND METHODS

1) IgG anti-A

a) Source:

Donations of plasma, totalling 5 litres, were obtained by plasmapheresis from two group O donors at the North London Blood Transfusion Centre. The donors were selected firstly, in that their plasma contained potent IgG anti-A and secondly, in that they had repeatedly had normal results in tests for liver function and negative results in tests for Australia antigen over a period of more than a year.

b) Fractionation

The plasma pool was fractionated by Dr. D. Ellis at the Blood Products Laboratory, Lister Institute, Elstree, using the cold ethanol fractionation method and the IgG fraction was further purified by elution from DEAE-cellulose. The final product contained 40 g protein per litre of which 92 per cent was estimated to be IgG by zone electrophoresis on cellulose acetate. It was suspended in 10g / l NaCl pH 7.5 and contained 1:10.000 thiomersal. The preparation was sterilized by millipore filtration and dispersed in sterile containers in volumes of about 1.5 ml per sample.

A sample of 2 ml of the IgG anti-A preparation (immunoglobulin fraction II) was spun at 90,000 g for 1 hour and then fractionated on a Sephadex G-200 column. A small amount of the protein was eluted

in a first peak and the rest was eluted in a second peak. The fractions forming the two peaks were pooled separately and concentrated to about 2 ml. Agglutination and indirect antiglobulin tests performed with the unfractionated and the concentrated peak I and II, using adult A_1 red cells, indicated that there was no anti-A activity present in the first peak and that all the activity present in the unfractionated preparation was recovered in the second peak. This result was interpreted to mean that there was no detectable IgM anti-A in the IgG anti-A preparation.

c) RADIOIODINATION

The iodine monochloride technique of McFarlane (1958) was used (see Chapter I).

Samples of the preparation were labelled with ^{125}I or ^{131}I, as convenient.

The specific activity of the labelled preparation was determined by the method described in Chapter I.

d) ANTIBODY CONCENTRATION

Aliquots of ^{125}I-IgG anti-A were incubated with increasing amounts of A_1 red cells to determine the maximum amount of antibody that could be bound at gross antigen excess. The details of the procedure are as described in Chapter I. The concentration was found to be approximately 60 µg IgG anti-A per ml. The equilibrium constant of this preparation was estimated to be in the order of 1.3×10^{-8} l/Mol.

2) HORSE ANTI-IgG

Anti-human IgG produced in hyperimmunized horses was purified by the use of a Sepharose-4B - IgG immunoadsorbant by a procedure similar to that described in Chapter I. The final purified ^{125}I-labelled anti-IgG had a protein concentration of 7.3 mg/ml and an antibody purity of 59 per cent. A calibrated, standard antiglobulin curve was made with ^{125}I-anti-IgG and ^{131}I-IgG anti-A bound on A_1 cells. The details of the methods were as given in Chapter I. The calibration curve was used for the estimation of the amount of unlabelled IgG anti-A on the red cells in the experiments of survival studies *in vivo*.

3) EXPERIMENTS IN VIVO. CLEARANCE OF RED CELLS SENSITIZED WITH SMALL AMOUNTS OF IgG ANTI-A.

a) PROCEDURE

Studies were carried out to find out the rate of destruction of autologous A cells coated with amounts of antibody of the order of 1 µg per ml of cells, when injected into human group A volunteers.

The design of the experiments was basically the same as that used in previous studies of the relation between the amount of IgG anti-Rh on a sample of red cells and its rate of clearance *in vivo*, (Mollison *et al.*, 1965).

The details of the techniques used were as follows:

20 ml of blood were taken from the subject and heparinized; an additional 4 ml were anticoagulated with EDTA and used for routine haematology studies. The heparinized sample was centrifuged and the

plasma discarded along with as much as possible of the buffy coat. The packed cells were then resuspended in a citrate-phosphate-dextrose solution (Mollison, 1972, p.682) and washed once. Finally the cells were resuspended in the same solution to give an approximate 50 per cent red cell suspension. The Hb and the PCV values of this suspension were estimated in a Coulter Counter. Next, using a tuberculin syringe, 1 ml aliquots were transferred to each of 4 sterile containers (A,B,C and D).

Sample A was labelled with ^{51}Cr by incubating the red cells at 37° for 10 minutes with a solution of $Na_2{}^{51}Cr\ O_4$ containing approximately 15 μCi in a volume of approximately 0.1 ml of saline. This sample was used for the determination of the total red cell volume.

Sample B was incubated with approximately 60 μCi of $Na_2{}^{51}CrO_4$ contained in a volume of 0.4 ml. 0.4 ml of saline was added to samples C and D. All four samples were then incubated 10 minutes at 37°. After incubation all four samples were washed twice with 20 ml of ice-cold saline and as much supernatant as possible was removed. Then a carefully measured volume of the sterile IgG anti-A was added to samples B and C (the same volume to each) and the same volume of saline was added to sample D. All three samples were then incubated at 37° for 30 minutes and washed thrice with 20 ml of saline each time.

Next, the antecubital veins of both arms were venesected and an indwelling needle provided with a 2-way tap was inserted and left in place. One vein was used only for taking blood, the other only for injecting the samples.

Sample A: The red cells were resuspended in 25 ml of saline. Exactly 20 ml of suspension measured in a glass syringe were injected. The residue was saved for the preparation of standards. At about 15 and 25 minutes blood samples were taken and mixed with EDTA.

Sample B: The red cells were resuspended in 25 ml of cold saline. At time "0", (when the injection was started) approximately 30 minutes after injection of sample A, 20 ml of the suspension were injected and the syringe was washed once with blood which was then injected back into the subject. (In one case the sample B was resuspended in about 6 ml of the volunteer's own plasma instead of saline and 5 ml of the suspension were injected). The time at which the injection was finished was recorded and the mid-point was taken as the time of injection. Blood samples (10 ml) were taken at about 3, 6, 12, 25 minutes and 1 hour and 4 hour post injection, and anticoagulated with EDTA.

Samples C and D were used for estimation of the amount of IgG anti-A on the cells injected. Sample B could not be used directly for the estimation of the amount of anti-A on the cells because the ^{51}Cr counts would have interfered with the ^{125}I counts of the labelled anti-IgG used for this purpose. Sample D was used as a control for nonspecific uptake of labelled anti-IgG. The uptake of anti-A was estimated by the use of a ^{125}I-labelled anti-IgG purified preparation which had previously been calibrated, using red cells labelled with known amounts of ^{131}I-IgG anti-A (Rochna and Hughes-Jones, 1965). The details of this procedure have already been described in Chapter I.

The amount of anti-A on the cells was expressed as µg of antibody per ml of red cells (and it was correlated to the rate of removal of the cells).

For the estimation of the ^{51}Cr counts, 2 ml of the blood samples, duplicates were made, were pipetted into plastic small bottles and lysed with saponin. A drop of octanol was added and the samples were counted in a gamma scintillation counter. Standards were made by diluting 1 : 25 a portion of the suspension used for red cell volume (A) and 1 : 100 an aliquot of the suspension of the sensitized cells (B).

In all the samples, Hb and PCV were estimated separately in a Coulter Counter.

b) ANALYSIS OF THE RESULTS:

The red cell volume was obtained from the average ^{51}Cr counts of the samples taken 15 and 25 minutes after the injection of sample A and the total counts injected as estimated from counting the standard. The results were expressed as ^{51}Cr counts per ml of red cells or as per gram of haemoglobin; these values were subsequently deducted from those found after the injection of the anti-A-coated cells in determining the survival of the latter. The survival values of the antibody-coated ^{51}Cr-labelled red cells were expressed as the observed percentage of the total counts expected following the second injection of cells (sample B) if there was no destruction of the anti-A coated cells. The latter expected counts, per ml of red cells or per gram of haemoglobin, were calculated from the estimate of red cell volume and the total counts injected in sample B, (estimated from the standard).

4) EXPERIMENTS IN VITRO

a) ENHANCEMENT OF AGGLUTINATION :

Red cells less than 1 week old and suspended in ACD were used. Most experiments were carried out with group A red cells from adults, but tests with group A red cells from newborn infants were also made. Samples were classified as A_1 or A_2 according to their reactions with an extract of Dolichos biflorus. The end points of agglutination reactions of A_1 red cells by IgG anti-A, when the sensitized cells were suspended in different media, were investigated. Similar experiments were also carried out using A_2, B and Rh positive (O R_1 R_1) adult cells and A_1 cord normal cells and IgG anti-A, anti-B or anti-Rh (anti-D) antibodies.

Some experiments were carried out with unlabelled antibodies and others with ^{125}I-labelled IgG antibodies. The techniques used were as follows:

i) In the experiments with unlabelled antibodies, red cells were washed 3 times in saline, the micro-hematocrit of the washed cells was determined and from this value, the amount of saline to be added to make a 20 per cent red cell suspension was calculated. 0.2 ml amounts of the red cell suspension were then transferred to each of a set of tubes which contained 0.8 ml of several antibody dilutions in saline. After 90 minutes at 37°, the cells were washed 4 times in cold saline and finally resuspended to about 5 drops dispensed from a "Pasteur pipette". Next, 2 drops

of the different media to be tested were delivered onto the squares of an opal tile and one drop of the washed sensitized cells was then added, mixed and spread out with a wooden stick, then the mixture was gently rocked over a lighted surface and observed for agglutination. The reactions were recorded at 5 and 10 minutes. Comparisons were always made with the reactions given with 2 drops of anti-human globulin (Ortho).

The media tested were as follows: saline (10 g/l), serum, plasma, serum to which albumin in a final concentration of 100 g/l had been added, serum to which human fibrinogen in a final concentration of 4 g/l had been added and also three fractions that were obtained by passage of 3 ml of ACD plasma from a group A_1 donor through a Sephadex G-200 column. The 3 fractions were eluted with 10 g/l NaCl in ACD then were concentrated to about 3 ml and their relative protein concentration roughly estimated by measuring the absorption of light at 278 nm.

ii) Similar types of experiments (but using radioiodinated antibodies) were also performed. In these experiments, ^{125}I-anti-A (or ^{125}I-anti-D) in the same volume and dilutions used for the test cells, was also added to O Rh negative cells to correct for nonspecific uptake. The amount of antibody coating the cells was estimated simply by counting the ^{125}I content of measured aliquots of known hematocrit and making corrections for nonspecific uptake. The results were expressed either in µg antibody per ml cells or in antibody molecules per cell.

It should be noted that with the technique used for visualization of agglutination (i.e. spreading a large drop on an opal tile and gently mixing by rocking and observation for about 10 minutes over a lighted surface), protein concentration of the suspending media increase due to evaporation of water, though no attempt to measure this increase was made.

b) UPTAKE OF COMPLEMENT BY IgG ANTI-A COATED CELLS

Experiments in this section were designed to find out the minimum amount of IgG anti-A bound on red cells which was necessary to produce detectable fixation of complement. The approach used was to mix red cells, sensitized with known amounts of antibody, with fresh, compatible serum and then try to detect complement components bound onto the cells by the use of anti-complement globulin sera.

Two methods were used :

i) The "two stage" test of Polley and Mollison (1961) in which in a first stage, cells and antibody are mixed in the presence of EDTA to prevent complement binding and in a second stage, the washed, antibody-coated cells were then incubated with fresh serum.

ii) A one-stage test in which aliquots of fresh defibrinated whole blood of group A_1 were mixed with IgG anti-A.

i) In the two-stage test, 3-times washed A_1 adult red cells were suspended in enough saline to give a 50 per cent suspension, then aliquots of 200 µl of the cells were incubated for 20 minutes at 37° with 0.8 ml of ^{125}I-anti-A dilutions containing from 10 to about 0.02 µg of anti-A per ml. The antibody-coated cells were then washed 3 times in saline and 0.5 ml of fresh, autologous serum (from defibrinated blood) was added to each tube and a further incubation for 20 minutes at 37° was carried out. Next, the cells were washed 3 times with 5 ml of cold saline and resuspended in a total volume of about 0.5 ml and the microhematocrits values were

determined. 0.2 ml amounts from each tube were then transferred to clean tubes, the cells were lysed and the ^{125}I content was determined. The volume of cells counted was determined from the microhematocrits values. The rest of the cells were used for serological tests with anti-complement globulin sera and anti-IgG.

ii) In the one-stage technique, aliquots of 0.2 ml of fresh, defibrinated A_1 whole blood of known hematocrit values were incubated for 90 minutes at 37^{o} with 0.8 ml of ^{125}I-labelled anti-A at antibody concentrations ranging from 10 to 0.02 µg/ml. After washing 3 times in saline, the cells were suspended in about 0.5 ml of cold saline and the hematocrit values for each tube were estimated. As in the 2 stage technique 0.2 ml were then transferred to clean tubes for counting and the rest used for serological tests.

The amount of anti-A either in µg/ml cells or in molecules / cells was calculated from the ^{125}I-content of the cells, the specific activity of the ^{125}I-anti-A and assuming a mol. wt. of 160,000 for the IgG anti-A molecules.

The anti-complement-globulin sera used were anti-β_{1E} and anti β_{1C}, produced in rabbits, and were obtained from Behringwerke. They were used at a dilution of 1 in 100, a dilution found to be optimal for the detection of both β_{1E} (C4) and β_{1C} (C3) on complement-coated red cells.

Tests to find out the percentage of hemolysis of a given number of cells (1×10^{8}) coated with varying amounts of ^{125}I-IgG anti-A in the presence of complement, were also carried out, but were found to be less sensitive than those previously described.

Most of the tests were carried out with the IgG anti-A preparation described in this chapter. However, it was thought that the efficiency of complement-binding within the same antigen-antibody system could vary, according, among other factors, to the equilibrium constant of the antibody involved and so different anti-A's might give different answers. Therefore, similar tests were carried out with the IgG anti-A preparation described in Chapter I, which had an equilibrium constant about 8 times higher than the one used in the experiments described in this chapter.

c) REACTIONS OF ANTI-COMPLEMENT SERA WITH RED CELLS OF INFANTS AFFECTED WITH ABO-HAEMOLYTIC DISEASE

Seven samples of cord blood from infants affected with ABO-haemolytic disease* were sent to us from various hospitals. The diagnosis of ABO-haemolytic disease was made by the finding, in a full-term group A or B infant born to a group O mother, of a plasma bilirubin concentration of 10 mg/ 100 ml or more in the first 72 hours of life, the detection of IgG anti-A (or anti-B) in the mother and the finding of IgG anti-A (or anti-B) in an eluate prepared from the infant's red cells.

The infant's red cells were washed 3 times in ice cold saline and a 20 per cent red cell suspension in saline was prepared. One drop of the red cell suspension was then mixed on a tile with 2 drops of optimally diluted anti-complement serum and observed for the development of agglutination. "Spin Coombs" tests (see chapter one) were also performed with a 1 per cent red cell suspension.

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* These cases were additional to those described in Chapter I.

The anti-complement sera used were anti-β_{1E} (anti-C4), anti-β_{1C}/β_{1A} (anti-C3b/C3c), both obtained from Behringwerke and anti-α_{2D} (anti-C3d) obtained from the Laboratory of Blood Product of the Netherlands Red Cross. Anti-complement sera was diluted 1:100 (anti-β_{1E}, anti-β_{1C}/β_{1A}) and 1:4 (anti-α_{2D}) before use.

d) REACTIONS OF RED CELLS OF INFANTS AFFECTED WITH ABO-HAEMOLYTIC DISEASE IN A MEDIUM OF PLASMA

The infant's red cells were washed 3 times, a 20 per cent red cell suspension was prepared and one drop was mixed on a tile with 2 drops of an ABO-compatible EDTA plasma from an adult donor. The mixture was then observed for the development of agglutination for 10 minutes. As controls, i) saline was substituted for plasma and ii) red cells from normal adults were tested in the place of the red cells from the affected infants. A direct antiglobulin test was always carried out simultaneously for comparison.

B) RESULTS

1) RATE OF CLEARANCE FROM THE CIRCULATION OF RED CELLS COATED WITH SMALL AMOUNTS OF IgG ANTI-A

Observations in 4 subjects are summarised in Table II-1 and in figure II-1. In cases 1 and 2, in which the red cells were coated with 0.6 and 2.2 µg anti-A / ml cells respectively and in which no agglutinates were seen on macroscopic observation of the suspension re-injected into the donor, all estimates of survival between 3 min. and 4 h. were within the range of 95-103 per cent and it was concluded that there was no appreciable sequestration or destruction of the red cells. On the other hand, in cases 3 and 4, in which agglutinates were seen on macroscopic examination of the suspension re-injected into the donor, samples taken at 3 min. provided evidence of definite sequestration of cells in case 3 and probable sequestration of cells in case 4. In case 3, there was a gradual return of cells to the circulation, so that survival rose from 57% at 3 min. to about 66% at 4 h. In case 4, survival increased from 89 per cent at 3 min. to 95 per cent at 12 min. and remained at that level over the 4 h. during which estimates were made. As Table II-1 indicates, red cells were resuspended in saline in cases 1-3 but in case 4 were resuspended in the subject's own plasma, as a consequence of which gross agglutinates were seen in the suspension which was re-injected. In case 3, the donor's red cells, coated with about 3.1 µg anti-A / ml cells and resuspended in saline, appeared agglutinated. This observation requires explanation, because to bring about agglutination of A or B cells incubated with IgG anti-A (or -B), approximately 12-14 µg antibody/ml of cells are required. The explanation for the observation of

TABLE II-1 Data on experiments in which red cells were coated with IgG anti-A *in vitro* and then re-injected into the donor

Subject	Group	Amount of anti-A on red cells (µg/ml)	Cells finally resuspended in	Agglutinates in injection suspension	Survival (%) at $2\frac{1}{2}$-3 min.	Survival (%) at 25-40 min.	Survival (%) at 4 h.
1. C.S.	A_1	0.6	saline	no	101	98	95
2. D.R.	A_1	2.2	saline	no	103	98	99
3. L.D.	A_2	3.1	saline	yes	57	62	66
4. P.L.M.	A_2	0.7	plasma	yes	89	95	95

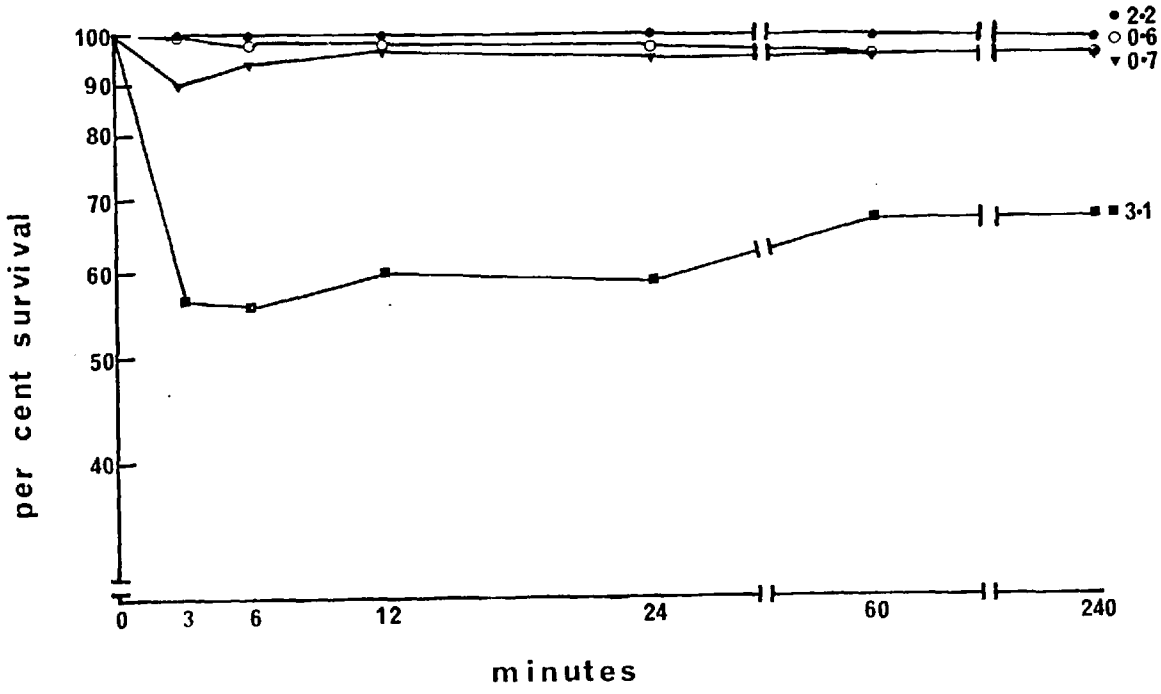

Fig II-1 : Survival of 0.5 ml of ^{51}Cr-labelled red cells from group A donors sensitized *in vitro* with varying amounts of anti-A and then reinjected into the donors. The figures against each curve are the estimates of the amount of antibody on the cells at the time of injection as µg antibody per ml red cells.

agglutination with only about 3.1 µg antibody / ml of cells in case 3, could be that the cells were agglutinated in the centrifugation step of the washing procedure and were afterwards only partially resuspended. Centrifugation enhances agglutination by bringing the cells close together and, therefore, making their cross-linking by antibodies easier.

It should be noted that it is difficult to interpret the results obtained with these in vivo experiments in which only 0.5 ml of antibody-coated cells were injected. Thus, for reasons to be discussed in the next section, the results of these tests almost certainly do not reflect the survival of the red cells in the circulation which would be observed if all the cells were equally coated with IgG anti-A.

2) EXPERIMENTS IN VITRO

a) MINIMUM NUMBER OF IgG ANTI-A AND ANTI-D MOLECULES REQUIRED TO AGGLUTINATE CELLS IN DIFFERENT MEDIA

It was found that A_1 adult red cells coated with as little as 0.3 - 0.5 µg IgG anti-A / ml cells (corresponding to about 110 - 190 IgG molecules per cell) were agglutinated when suspended in plasma and rocked on a tile. No significant difference was observed between heparinized - EDTA-treated or ACD- plasma from the same donor, although differences were observed between samples of plasma from different subjects. The degree of antibody-coating detected in this way was therefore about as small as could be detected with the antiglobulin reaction (see chapter one). It was observed that with the antiglobulin reagent agglutination of red cells occurred more rapidly than when the cells were suspended in plasma, which seems to indicate that the enhancing effect of plasma depends on the protein concentration - which

increases with time, due to evaporation of water. Thus, the plasma enhancing effect is stronger after 10 mins. of observation than at 5 mins. and is weaker or lost upon dilution of plasma with saline.

The enhancing effect of plasma was not affected by heating the plasma at 60° for 20 mins. or by freezing and thawing (2 times) or storing at 4° for 3-4 weeks.

With serum as a suspending medium, the agglutination of anti-A coated cells occurred only when cells were coated with at leat 2.2-3.0 µg/ml red cells and with saline, the minimum amount was 12-15 µg/ml.

When human fibrinogen in a final concentration of 4 g/l was added to the serum, the enhancing effect of the medium was identical to that of plasma; on the other hand, when bovine serum albumin in a final concentration even of 100 g/l was added to serum, the enhancing effect was no greater than that of serum alone. Similarly, when fractions of ACD plasma were eluted from Sephadex G-200 and tested at comparable protein concentrations, the first peak (containing fibrinogen) had a greater enhancing effect than the second, and that of the third was negligible.

Table II-2 summarizes the results given by an IgG anti-A preparation using A_1 red cells and autologous plasma and serum.

The augmentation of agglutination in a medium of plasma was the same for IgG anti-B-coated group B red cells from an adult, as it was for anti-A-coated adult group A cells. However, with A_2 adult red cells,

TABLE II-2

Comparison of the titre given by an IgG anti-A preparation by the indirect antiglobulin test with the agglutination titres obtained when the sensitized cells[1] were suspended in different media[2].

I)	Indirect antiglobulin test	2048
II)	Agglutination tests	
	a) Plasma	2048
	b) Plasma diluted with equal volume of saline	256
	c) Plasma fractions from Sephadex G-200	
	I	256
	II	128
	III	64
	d) Serum	256
	e) Serum plus human fibrinogen 4 g/l	2048
	f) Serum plus bovine serum albumin 100 g/l	256
	g) Saline	64

1 Adult group A_1 red cells

2 Autologous plasma and serum

the agglutination titres were found to be about 1 or 2 doubling dilutions less than with A_1 adult red cells (i.e. the A_2 cells had to be incubated with 2-4 times more antibody than A_1 red cells). Also, with A_1 or B red cells from newborn infants as with A_2 adult, the agglutination titre in plasma was about 1-2 doubling dilutions lower than with A_1 cells.

By contrast, no enhancing effect of plasma on agglutination was observed when Rh positive cells coated with IgG anti-D were suspended in plasma and observed on a tile. Thus, the minimum amount of IgG anti-D on red cells that would cause them to agglutinate in a medium of plasma was found to be about 50 µg/ml cells, i.e. more than 50 per cent saturation of D sites on R_1R_1 red cells.

The specificity of these enhancing reactions was always assessed by control tests which included: the reaction of corresponding red cells similarly treated but not coated with antibody; or the reaction of coated red cells suspended in ACD, EDTA-saline solution and heparin - saline solution. False positive reactions, due to rouleaux formation, were occasionally observed, when observation for agglutination on a tile was carried on for more than 10 mins. When in doubt about whether the reaction was positive or negative, it was found useful to add one drop of saline to the mixture on the tile, followed by redispersion of the cell suspension with the tip of a wooden stick: when the clumping of the cells was due to agglutination by antibodies, the clumping persisted but when it was due to rouleaux formation, the cell suspension became homogeneous and the clumps did not reform.

In conclusion, the present observations indicate that A_1 adult red cells sensitized with as little as about 0.5 µg anti-A / ml cells are agglutinated when suspended in plasma whereas under the same conditions, about 100 times more antibody is required in the case of Rh positive red cells sensitized with IgG anti-D.

b) MINIMUM NUMBER OF IgG ANTI-A MOLECULES PER CELL REQUIRED FOR COMPLEMENT-BINDING

In testing A_1 red cells which had been incubated as whole blood with IgG anti-A, positive reactions with anti-β_{1E} or anti-β_{1C} sera were obtained only when there was at least 14 µg anti-A/ml red cells, corresponding to about 5000 molecules of anti-A per red cell. Red cells coated with this amount of anti-A were agglutinated after being centrifuged during washing but the agglutinates were dispersed on shaking and reformed only very slowly (after about 8 mins.) when a drop of suspension was spread on a tile. On the other hand, the cells were rapidly agglutinated (within 1 or 2 mins.) when mixed on a tile with anti-β_{1E} or anti-β_{1C} serum; and the rapid agglutination observed with anti-β_{1E} and anti-β_{1C} was inhibited by the addition of one drop of a 1:100 normal serum dilution. When a two-stage technique was employed, in which the cells were firstly incubated with anti-A and then with complement, the results were essentially as before, though the one stage technique was found to be slightly more sensitive.

When another preparation of IgG anti-A, which had a higher equilibrium constant, was used for similar experiments, the results were the same: about 15 µg of anti-A / ml of cells were required to fix enough complement on the cells to be detectable with anti-β_{1E} or anti-β_{1C} sera. A trace of lysis (less than 5 per cent of 1×10^8 red cells)

was observed when the amount of bound anti-A was about 21 µg/ml red cells; and approximately 5 per cent lysis of 1 x 10^8 cells was observed only when there was about 45 µg anti-A / ml of red cells.

It should be noted that, within the A-anti-A system, complement-binding, as detected by the techniques described only occurs at the level of antibody binding which is also sufficient to bring agglutination of the red cells suspended in saline.

c) REACTIONS OF ANTI-COMPLEMENT SERA WITH RED CELLS OF INFANTS AFFECTED WITH ABO-HAEMOLYTIC DISEASE

Samples from 7 affected infants were tested. (See Table II-3). In all the cases of the present series, the infant's washed red cells gave negative reactions when tested with anti-β_{1E}, anti-β_{1C} and anti-α2D sera either on a tile or using a "Spin Coombs" technique.

d) REACTIONS IN A MEDIUM OF PLASMA OF RED CELLS OF INFANTS AFFECTED WITH ABO-HAEMOLYTIC DISEASE

As Table II-3 shows, in all of 5 cases in which the direct anti-globulin test was positive, the infant's cells suspended in plasma and mixed on a tile were agglutinated.

TABLE II-3

Case No.	Blood Group	Direct Anti-Globulin Test	Reaction in Plasma	Reactions with anti-$C4_d$, anti-C3 and anti-C3d sera	IgG anti-A and (or -B) titres in Mother Serum+	Severity
1	A_1	+	ND*	-	256	Clinical jaundice
2	B	-	ND*	-	16	Needed exchange transfusion
3	A_2	±	±	-	512	Needed exchange transfusion
4	A_1	+	+	-	64	Clinical jaundice
5	B	-	-	-	64	Bilirubin concentration: 13.8 mg/100 ml at 48 h
6	A_1	+	+	-	1000	Needed exchange transfusion
7	A_1	+	++	-	64	Bilirubin concentration: 9.5 mg/100 ml at 24 h

* ND : not done

+ 2me treated sera, reactions with anti-IgG

DISCUSSION

The series of experiments described in this chapter was aimed at: 1) assessing the greater efficiency of anti-A (or -B) in comparison to anti-Rh in causing red cell destruction *in vivo* and 2) investigating the role that the agglutinating and complement-fixing properties of IgG anti-A (or -B) but not of IgG anti-Rh, may have in the causation of red cell destruction *in vivo*.

The first point was investigated by estimating the rate of destruction *in vivo* of about 0.5 ml of A red cells, sensitized *in vitro* with small amounts of IgG anti-A ranging from 0.6 to 3.1 µg/ml cells. These *in vivo* experiments were only partially successful for reasons which are better appreciated if the enhancing effect of plasma on the agglutination of A cells by anti-A is discussed first; accordingly, the possible role of agglutination by anti-A in the destruction of red cells *in vivo*, the difficulties involved in the interpretation of the *in vivo* survival studies, and the experiments in relation to complement binding by anti-A, will be discussed in that order.

A) THE ENHANCING EFFECT OF PLASMA ON THE AGGLUTINATION OF A CELLS BY IgG ANTI-A. ITS POSSIBLE IMPLICATIONS

The amplification of the agglutination produced by "immune" antibodies with anti-Rh, anti-A, anti-M and anti-N specificities, by using serum as a diluent rather than 0.85 per cent NaCl, was reported by Boorman *et al.* (1945) and Boorman and Dodd (1947). These authors used a tube technique in which the sensitized cells suspended in serum were allowed to stand for 2 or 3 hours and the reaction was read macroscopically or microscopically.

On the average, titres were about 4 times higher in serum than saline. Plasma as a diluent (as opposed to serum and saline) was not tested. Witebsky, Rubin and Blum (1947) made similar observation to those of Boorman and Dodd; and Witebsky et al. (1947) described a test which they found useful to detect sensitization of red cells in cases of Rh haemolytic disease: about 0.01 ml of the infant's unwashed packed red cells were mixed on a glass slide with a large drop of serum or plasma from an adult, ABO compatible donor; the glass slide was then rocked and observed for 5-10 minutes; agglutination was observed with the red cells of infants affected with Rh haemolytic disease. Witebsky in a later paper (1954) reported that the washed cells of affected babies with Rh haemolytic disease fail to agglutinate when suspended in serum, and that sometimes even the unwashed packed cells of the affected infant were not clumped when suspended in serum but became clumped when suspended in a medium of serum plus 300g/l bovine serum albumin. By contrast, the red cells of an infant affected with ABO-haemolytic disease were clumped when suspended in serum but only weakly clumped in the serum-albumin mixture. Wiener et al. (1949) observed that, in a severe case of ABO-haemolytic disease, the infant's red cells, which appeared as a smooth suspension when suspended in saline, clumped spontaneously when suspended in plasma or acacia. Similar observations were made by Shumway, Miller and Young (1955), who mixed on a glass slide 1 drop of oxalated whole blood from the affected infant with one drop of AB serum heated at 56^{o} for 30 minutes. These authors recorded, within 60-90 seconds of observation, 8 positive reactions out of 9 cases tested. Similarly, blood freshly-drawn from an infant with ABO haemolytic disease was found to form large clumps which were easily seen when the blood was allowed to flow down the side of a tube or was examined on an opal glass tile (Crawford, Cutbush and Mollison, 1953).

In the present series of infants affected by ABO-haemolytic disease in 4 out of 5 cases the washed red cells or red cells in EDTA plasma clumped spontaneously within 5 or 10 minutes when suspended in 2 volumes of compatible plasma and rocked on a tile. This observation agrees with the results of in vitro experiments in which it was shown that A cells coated with only about 100-200 molecules of IgG anti-A per cell clumped spontaneously when suspended in a medium of plasma. This amount of anti-A was commonly found on the cells of infants affected with ABO-haemolytic disease and is also the minimum amount required to give a positive reaction using antiglobulin serum (see chapter I). On the other hand, in the case of Rh positive red cells coated with IgG anti-D it was found that a far greater amount of antibody namely, 40-50 µg/ml cell for R_1R_2 cells, had to be bound before the cells were agglutinated when one drop of a 20 per cent red cell suspension was mixed on a tile with 2 drops of plasma or serum. IgG anti-D will agglutinate Rh positive cells when suspended in 200-300 g/l bovine albumin (Diamond and Denton, 1945), especially if a good proportion of polymerized protein is present (Jones et al., 1969). The present observations, therefore, show that the efficacy of plasma and albumin in potentiating agglutination of red cells varies with antibody specificity. Thus, while plasma greatly enhances agglutination by IgG anti-A, it has little effect on agglutination by IgG anti-D. On the other hand, albumin is a better enhancing medium for IgG anti-D than for IgG anti-A.

That the optimum medium for agglutination differs for IgG anti-A and anti-D has also been observed by Munk-Andersen (1956). This author found that if one volume of a 5 per cent suspension of red cells was mixed with an equal volume of a medium containing, as final concentrations,

5 per cent dextran (mol. wt. about 70,000), 1.3% NaCl and 25-50% serum, agglutination occurred when the red cells came from an infant with ABO haemolytic disease, but not when they came from an infant with Rh haemolytic disease.

In the present studies, it was observed that plasma is more effective than serum in enhancing agglutination due to anti-A, and that serum becomes as potent as plasma when reconstituted with human fibrinogen to a concentration of approximately 4 g/l (incidentally, bovine fibrinogen was not effective). Furthermore, it was observed that the first fraction eluted when plasma was fractionated in a Sephadex-G 200 column and which contained most of the fibrinogen present in the plasma, showed a greater enhancement of agglutination than the second fraction, which contained most of the immunoglobulins; the effect of the third fraction, containing most of the albumin, was negligible. Therefore, the present observations indicate that fibrinogen is extremely important in producing the agglutination of red cells coated with IgG anti-A. A relatively high concentration of fibrinogen was present in the test used by Shumway et al. (1955) in their series of ABO-haemolytic disease (page 91) since oxalated whole blood was mixed with serum and presumably it was the presence of plasma, rather than the use of serum as an enhancing medium, which accounted for the sensitivity of their test. It should be noted that the effect of fibrinogen in potentiating the agglutination of antibody-coated cells was clearly described by Jandl and Castle (1956) although they were studying only the effect on Rh-sensitized cells.

The manner in which serum and plasma enhances the agglutination of red cells has yet to be clarified. The enhancing effect seems to be nonspecific in the sense that fibrinogen as well as gamma-globulin and albumin polymers have been found to be effective. As this phenomenon is more readily seen with IgG anti-A (or -B), which is known to bind complement, than with IgG anti-D which does not bind complement, it might be thought that complement was involved. However, it is likely that the phenomenon is not complement-mediated because 1) it occurs in the absence of ionized Ca^{++} (in EDTA or ACD plasma) which means that at least C1, C4 and C2 are not involved. 2) It is greater in plasma than in serum (Complement activity is maximum in serum). 3) It is not destroyed on storage or on freezing and thawing. An increase in the dielectric constant of the suspending medium facilitates agglutination by decreasing the repulsive forces between red cells (Pollack and Reckel, 1970); however, this factor alone would not explain the difference observed between plasma and serum in enhancing power on the agglutination of anti-A coated cells or the differences observed with anti-Rh coated cells as opposed to anti-A (or -B) coated cells.

An interesting explanation emerges from the work of Dr. J. Sirs and his colleagues (personal communications) based on the flexibility of the red cells. Plasma proteins interact with the red cell membrane, fibrinogen being the most active in this sense. This interaction gives rise to the formation of a "shaggy coat" of protein which surrounds the red cells in vivo or when suspended in plasma (and to a lesser extent in serum). This protein coat renders the cells more flexible. This increased flexibility allows the red cells to establish large surface areas of cell to cell contact which facilitates the formation of cell to cell cross-links, thus, bringing about agglutination of the antibody-

coated red cells. On the other hand, when the cells are suspended in saline, the absence of the protein coat makes the cells less flexible, so that the bridging of the cells by antibody-cross-links is more difficult or, when formed, the links may easily be broken by shear forces because of the smaller cell surface to surface contact areas involved. If this mechanism applies then it is easy to visualize that it might work more efficiently in the case of A or B adult cells, due to the greater number of antigenic sites present on the cell surface and less efficiently in the case of cord cells or Rh positive cells which have less antigen sites. However, it would not explain why with Rh positive coated red cells, a bovine albumin medium rich in polymers is more efficient than plasma or serum in bringing about agglutination of the cells; presumably a different mechanism may be involved in this latter case.

Summarizing the information previously discussed, A (or B) cells coated with numbers of IgG anti-A molecules as small as 100-200 per cell, when suspended in plasma can agglutinate spontaneously whereas Rh positive cells coated with similar numbers of IgG anti-Rh molecules are not agglutinated when suspended in plasma. Now, red cells are, of course, naturally suspended in plasma in the circulation so that it is pertinent to ask whether agglutination at low concentrations of anti-A also occurs in <u>in vivo</u> or is only an <u>in vitro</u> phenomenon. In trying to answer this question it is relevant to look at the information obtained when, accidentally or deliberately, subjects of group A or B (or AB) are

transfused with plasma or whole blood from a group O donor. Thus, many observations have been reported with passively-administered anti-A and anti-B, beginning with the work of Aubert, Boorman, Dodd and Loutit (1942). In reviewing published work and citing other unpublished observations, Mollison (1972, p. 532) pointed out that, following the administration of "immune" anti-A or anti-B, spontaneous agglutination of whole blood samples obtained from recipients was invariably seen.

From the previous considerations, it seems possible that agglutination might be important as a mechanism of red cell destruction in vivo by antibodies of the IgG anti-A-type. This possibility was discussed at some length by Wasastjerna (1951) who observed strongly agglutinated cells in the capillaries of the cheek pouch of the hamster after injecting potent haemagglutinating serum. Agglutination in vivo was also considered to be an important mechanism of red cell destruction by alloantibodies by Jandl et al. (1957). Although, like Wasastjerna, they were writing before the presence of receptors on macrophages for IgG had been described.

B) DIFFICULTIES INVOLVED IN THE INTERPRETATION OF THE PRESENT IN VIVO SURVIVAL STUDIES

Four experiments were carried out in which 0.5 ml of ^{51}Cr labelled autologous, red cells coated with IgG anti-A ranging from 0.6 to 3.1 µg/ml cells were injected into the circulation of human volunteers and their survival was followed in the first 4 hours. No sign of destruction was observed in two cases in which the cells were not

agglutinated when injected. Sequestration, within 2 or 3 minutes post-injection, of about 40 per cent of the tagged cells was observed in one case in which the cells were agglutinated when injected and about 11 per cent of immediate sequestration was observed in another case in which the cells were coated with only 0.7 μg/ml cell but were suspended in plasma and agglutinated when injected. In these latter two cases, a small percentage of trapped cells returned to the circulation as judged by a slight increase in the percentage of surviving cells.

The present observations are inconsistent in the sense that no destruction was observed in a case in which the cells were coated with about 2.2 μg/ml of cell whereas, some destruction was observed when the cells were coated with about 0.7 μg/ml., being the difference that the cells in the first case were agglutinated at the time of injection. However, as mentioned in the previous section, if low concentrations of IgG anti-A may cause destruction in vivo by producing intravascular agglutination, experiments in which small amounts of antibody-coated red cells are injected must be interpreted with caution. When antibody is distributed more or less evenly over the entire population of red cells, opportunities for agglutination will be altogether different from those obtaining when 0.5 ml of antibody-coated red cells are injected into the circulation of a normal subject in whom there are some 2000 ml of non-antibody-coated red cells. Thus, it was shown that, when a suspension of red cells moderately agglutinated by IgG anti-A was shaken vigorously to disperse the agglutinates and then centrifuged, the agglutinates reformed. On the other hand, when a suspension of similarly agglutinated group A red cells was shaken vigorously, mixed

with at least 20 times the number of unsensitized red cells, and then centrifuged, the agglutinates did not re-form. By the same token, if a small volume of antibody-coated cells is injected into the circulation, it is likely that, as the injected cells pass through the heart, turbulence there will tend to break up the agglutinates. Subsequently, the agglutinates will not re-form because of the large excess of unsensitized, unagglutinated cells even when the cells are flowing in a "quieter" part of the circulation simply because of the very slim chance of colliding together two or more antibody-coated cells. It is possible that very large agglutinates may not be dispersed during the first passage through the heart and may, therefore, be trapped in the liver, the lungs or elsewhere, and this may explain the results observed in case 3 in the present series and could also explain the smaller amount of trapping that may have occurred in case 4. It should be noted that in cases 2 and 3 in which the red cells were coated with 2-3 μg anti-A/ml cells, there was negligible destruction in the hour for which survival was followed, as expected if the mechanism of removal (in the absence of agglutination) is similar to that observed with IgG anti-Rh (cf. the results of Mollison et al., 1965). The above considerations may also explain the result observed in two experiments reported by Jandl, Jones and Castle (1957), in which group B red cells were labelled with ^{51}Cr and incubated with anti-D serum to produce "moderate" agglutination and were then re-injected into the donor's circulation. In one subject, 26 per cent of the cells were almost immediately removed, although about half were subsequently returned to the circulation. In a second subject, there was no apparent red cell destruction.

C) COMPLEMENT BINDING BY IgG ANTI-A

The present observations indicate that complement-binding by IgG anti-A can be detected only when approximately 4000 to 7000 antibody molecules are bound per cell, which is much more than the amount of antibody on the cells of infants affected with ABO-haemolytic disease (see chapter I). This result is in the same order of magnitude of the number of 7S rabbit Forsman antibody required to make 1 hole on the membrane of sheep red cells in the presence of guinea pig complement, which was estimated by Humphrey and Dourmashkin (1965) to be about 4000 on the average.

In the present experiments complement fixation was observed only when the red cells were at the same time, at least slightly, agglutinated. Agglutination of the cells might be of importance for complement fixation in the case of IgG anti-A because of the correlation observed (see chapter IV) between agglutination and aggregated distribution of A antigenic sites. When sites are aggregated there is evidently a greater chance of having two or more antibody molecules in juxta-position and hence a great chance of activation of C1 molecules.

On the other hand, no C4, C3b (Wang and Desforges, 1971) or C3d were detected on the red cells of infants affected with ABO-haemolytic disease by the use of anticomplement sera.

The present studies, therefore, indicate that complement plays no role in red cell destruction in cases of ABO-haemolytic disease. Moreover, since at the levels of antibody-coating required for C-fixation,

by IgG anti-A or-B agglutination of red cells is already present the red cells might in any case be destroyed preferentially by sequestration of the agglutinates rather than by complement-lysis or immune-adherence.

D) SPECULATIONS ON THE POSSIBLE MECHANISM OF RED CELL DESTRUCTION IN ABO-HAEMOLYTIC DISEASE

So far as explaining red cell destruction in ABO haemolytic disease is concerned, a mechanism has to be found which is capable of producing a moderately severe haemolytic process when the degree of red cell coating with IgG anti-A (or anti-B) reaches only 0.5 μg antibody/ml cells. The mechanism is most unlikely to be simply removal of red cells by macrophages since it is known that this degree of red cell coating with anti-Rh usually produces only a very mild haemolytic syndrome (Hughes-Jones et al., 1967). Moreover, in the present experiments, red cells coated with 0.5-2.5 μg IgG anti-A/ml underwent negligible red cell destruction in one hour, as with red cells coated with similar amounts of anti-Rh (Mollison et al., 1965). For the reasons previously given, it seems unlikely that complement plays any part in bringing about red cell destruction by IgG anti-A in haemolytic disease of the newborn. By exclusion, therefore, it seems that IgG anti-A probably causes red cell destruction in ABO haemolytic disease by producing agglutination in vivo which in turn presumably leads to trapping of cells, followed by metabolic damage.

OBSERVATIONS ON THE ABO-HAEMOLYTIC DISEASE OF THE NEWBORN (CHAPTERS I AND II) : SUMMARY AND CONCLUSIONS

The reactions between cord and adult group A cells with purified IgG anti-A preparations were studied and it was observed that when similar amounts of anti-A were on the cells, the reactions given with an antiglobulin serum were of comparable strength. When adult and cord cells coated with IgG anti-A were incubated with anti-human IgG, it was found that the reactions between anti-IgG and IgG anti-A were the same, whether the anti-A was coating adult or cord cells; furthermore, the reactions were similar to those observed when anti-IgG was reacted with IgG anti-Rh (anti-D) coating Rh positive red cells. It was concluded that the weak or negative direct antiglobulin reactions, observed with the red cells of infants affected with ABO-haemolytic disease, were probably due to the fact that a small amount of antibody was sensitizing the cells. That this was the case, was confirmed by measuring the amount of anti-A or anti-B on the red cells of 15 affected infants with haemolytic disease. The amount of anti-A in these cases was found to be between 0.25 and 3.5 µg per ml of red cells, being usually 0.55 µg/ml or less which was similar to the estimated minimum amount of anti-A necessary on red cells to give a positive antiglobulin reaction.

In cases of Rh- and ABO-haemolytic disease of the newborn of similar severity the amount of antibody on the cells is much smaller in the cells due to anti-A (or -B) indicating that IgG anti-A is more effective than IgG anti-Rh in bringing about red cell destruction *in vivo*. Studies were carried out to find an explanation for this higher effectiveness

of IgG anti-A in causing red cell destruction. Activation of complement was excluded because in *in vitro* studies, it was found that a relatively large amount of IgG anti-A, bound on the cells, was required to start C-fixation; moreover in affected infants, no C4, C3b or C3d was found on the red cells. On the other hand, it was observed that only 100-200 IgG anti-A molecules bound per cell were sufficient to cause agglutination when the cells were suspended in a compatible plasma. It was concluded that agglutination of red cells followed by sequestration and metabolic destruction by the reticulo-endothelial system may well be an important mechanism of red cell destruction in ABO-haemolytic disease. *In vivo* survival studies gave some support to this hypothesis.

CHAPTER III

AN ANTIGLOBULIN REAGENT LABELLED WITH COLLOIDAL GOLD FOR USE IN ELECTRON MICROSCOPY

I) INTRODUCTION

Labelling of antibody with suitable markers for electron microscopy has been found to be a useful tool in the identification of antigens or antibodies in biological material. Ferritin, first used by Singer (1959) and enzymes, first used in this way by Nakane and Pierce (1966), have been extensively employed as antigen and antibody labels. In both techniques, the label and antibody (or antigen) are conjugated by means of a covalent bond. These techniques are elaborate since they involve the conjugation procedure itself and the purification of the conjugates. Furthermore, when enzymes are used (peroxidases, phosphatases, cytochromes), histochemical reactions have to be carried out in order to visualize the site of the antibody complex.

Faulk and Taylor (1971) described a relatively simpler method in which antibody was labelled by combination with particles of gold, which are electron dense. No chemical conjugation was involved in the procedure. As this method seemed to be promising, it was decided to try to develop it in order to study the distribution of blood group antigens on the human erythrocyte membrane and also to study the reaction between the antigens and their specific antibodies.

In Faulk and Taylor's method, unfractionated antiserum for a specific antigen was mixed with an appropriate amount of colloidal gold. The

antigen-containing material was then treated with the gold-antibody complex and processed for electron microscopy using conventional thin-section techniques. It was thought that this method could be improved by investigating the reaction of gold particles with purified immunoglobulins, as opposed to unfractionated serum. Furthermore, it was decided that a method for preparing red cells for electron microscopy which would permit visualization of the entire surface of the erythrocyte membrane (as opposed to the use of thin sections in which only unidimensional information is obtainable) would give greater information concerning the distribution of the gold-labelled antibody.

SECTION I

ATTEMPTS TO OBTAIN GOLD-LABELLED HUMAN IgG BLOOD GROUP ANTIBODIES

The initial experiments were concerned with attempts to react gold colloid with human IgG anti-A and anti-D in order to obtain a suitable preparation for the study of the distribution of red cell antigen sites with the electron microscope. These experiments were not successful and the investigations were therefore extended to determine whether anti-IgG antibodies of animal origin could be labelled with gold. These latter experiments in which horse anti-human IgG antibodies were successfully labelled with gold particles are described in section II.

A) MATERIAL AND METHODS

1) PREPARATION OF COLLOIDAL GOLD

The colloidal gold was prepared according to the method of Weiser as described by Faulk and Taylor (1971), with minor modifications.

2.5 ml of 6 g/l chlorauric acid (Fisons Scientific Apparatus) was added to 60 ml of deionized water in a glass flask. 0.7 ml of 0.18M potassium carbonate was added and the mixture was stirred. After 10 minutes, 1 ml of a 1/10 saturated phosphorus solution in ether was added. This was stirred at room temperature for 15 minutes and then slowly heated until the solution developed a red wine-like colour. The final pH was found to be between 6.9 and 7.3. The requirement for deionized water was absolute. The addition of ethanediol, as recommended by Faulk and Taylor, was found to be unnecessary.

2) ANTIBODIES AND BOVINE SERUM ALBUMIN

a) Anti-A was produced by injection of A glycoprotein into a human group O volunteer. The IgG antibody fraction was separated by conventional DEAE chromatography, as described in chapter I.

b) Anti-D(IgG) preparations were used which were obtained from donors stimulated with Rh positive red cells and were similar to those used in the prevention of Rh-isoimmunization in Rh negative mothers. These preparations were donated by Dr. N.C. Hughes-Jones, to whom they were sent from commercial sources for the estimation of the concentration of anti-D.

c) Bovine serum albumin (BSA) was purchased from the Armour Pharmaceutical Co. The free amino groups on BSA were blocked by reacting the protein with an excess of formaldehyde. This modified protein had a slightly faster electrophoretic mobility at pH 8.1 than native BSA.

3) RADIOIODINATION OF IMMUNOGLOBULINS

was carried out according to the standard method of McFarlane (1958) as described in chapter I.

4) PREPARATION OF PROTEINS IN LOW IONIC STRENGTH MEDIUM

The human IgG anti-A and anti-D preparations were dialysed against low ionic strength medium. The dialysis was carried out in order to reduce the concentration of ions present in the antibody preparation which otherwise would aggregate and precipitate the gold colloid. The dialysis solutions used were: 1) 0.2M ethanediol, 2) 20 mM phosphate buffer at pHs in the range of 7.0 to 8.6. 3) 5 mM NaCl buffered to pH 7.0 with 20 mM phosphate buffer. Two ml of the antibody solution were dialysed for 3 hours against 2 l of the dialysing solution. In the dialysis procedure an appreciable amount of antibody-precipitate was observed when ethanediol was used. BSA was dialysed against distilled water buffered to pH 7.0 with 20 mM phosphate buffer. After dialysis, the protein solutions were ultra-centrifuged for 30 minutes at 90,000g to remove aggregates. The preparations were then divided in aliquots of 0.1 ml and kept frozen at -40°.

5) METHODS USED TO INVESTIGATE THE REACTION BETWEEN GOLD COLLOID AND HUMAN IgG ANTIBODIES

In the first series of experiments, attempts were made to directly label anti-A and anti-D with gold colloid.

The methods used were: a) that suggested by Faulk and Taylor (1971) to determine the optimum ratio of protein to gold colloid; and b) the use of ^{125}I-labelled human IgG and ultracentrifugation of the antibody gold particles mixture. These two methods were as follows:

a) In Faulk and Taylor's procedure, aliquots of 5 ml of gold colloid were added to different amounts of protein in the range of 1 µg to 1 x 10^{4} µg. After 10 minutes of incubation at room temperature, the gold-antibody solution was made up to 10 g/l with NaCl, using 100 g/l NaCl solution, and then centrifuged at 1700 g for 20 minutes. Gold particles adsorbed onto protein were not flocculated by NaCl and therefore remained in solution; non-adsorbed gold particles were aggregated and flocculated by the electrolyte and were separated from the solution by the centrifugation step. As the gold colloidal solution has a maximum of light absorption with a minimum of light scattering at a wavelength of 530 nm, it was possible to calculate the percentage of gold colloid stabilized in solution by measuring the decrease in light absorption at that wavelength; the percentage of "stabilized" gold colloid reflects the ability of the protein to interact with the gold colloid.

The influence of pH on the reaction between gold and protein was also investigated in the same way, using a constant ratio of protein to gold and varying the pH of the reactants.

b) The second method involved the mixing of various amounts of ^{125}I-labelled protein with a constant volume of gold colloid and then centrifuging the solution for 1 h. at 90,000g. The pellet (protein

coated with gold particles + non-coated and precipitated gold particles) and supernatant (free proteins) were then separated and the radioactivity estimated. The amount of ^{125}I-labelled protein coated with gold particles and the amount that remained free in solution could then be calculated.

6) REACTION OF GOLD COLLOID WITH BOVINE SERUM ALBUMIN

Varying amounts of BSA at a concentration of 10 g/l were added to 5 ml aliquots of gold colloid, the mixtures were incubated at room temperature for 10 minutes then made 10 g/l with NaCl using 100 g/l NaCl solution and finally spun at 1700 g for 20 minutes. The percentage of gold colloid stabilized by each amount of BSA was determined by measuring the absorption of light at 530 nm by the supernatants. The reactions between BSA and gold at pHs ranging from 6 to 9 were also studied. For these latter experiments, gold colloid of the desired pH was prepared by varying - by trial and error - the amount of 0.18 M potassium carbonate to be added to the chloroauric acid solution when the gold colloid was prepared; amounts smaller than 0.7 ml were used when the desired pH was lower than 7.0 and larger than 0.7 ml in the converse case.

B) RESULTS

1) PHYSICAL PROPERTIES OF GOLD COLLOID

It was confirmed that gold particles are negatively charged. This was done by electrophoresing untreated gold colloid in gels of 8 g/l agar and 0.05 N Tris maleate buffer at a pH varying between 5 and 9. This negative charge was observed at a pH higher than 5.5 - 6.0; below this range of pH the colloid was found to be unstable. At pH 6 to 9 gold colloid was found to be very stable when kept free of ions. Aggregation within a few seconds leading to flocculation can be brought about by the addition of electrolytes. Early stages of aggregation of the colloid were easily visualized by changes in the colour and aspect of the colloid; from transparent ruby-red it became turbid and bluish; subsequently a gross precipitate was formed.

Gold colloid was found to consist of round particles which had an average size between 2 to 5 nm; as seen at high magnification in the electron microscope, smaller or larger particles were occasionally seen.

By reducing the initial amount of water, the colloid could be prepared 4-6 times more concentrated than in the technique used by Faulk and Taylor.

It was estimated that about 1×10^{17} particles of gold were generated by each ml of chloroauric acid (6 g/l) used in the preparation of the gold colloid; this figure was estimated by assuming an average size of 3 nm, a spherical shape, a 51 per cent gold content in the chloroauric acid preparation and a density of gold of 19.3 g/cc. This order of magnitude was confirmed by calculations from dry weight results.

2) REACTIONS OF HUMAN IgG ANTIBODIES WITH GOLD COLLOID

When preparation of normal human IgG or preparations of IgG containing anti-D, anti-A or anti-B specificities, were reacted with gold colloid, no stable gold-labelled antibody preparations were obtained. Thus, after mixing human IgG and gold colloid, one of the following results were observed:

a) The gold colloid was aggregated and precipitated within 10 minutes by the simple addition of the IgG solution.

b) The second possibility was that no change in the appearance of the gold colloid occured within the 10 minutes of incubation with human IgG, but the colloid was flocculated after the mixture was made 10 g/l with NaCl.

c) The third possibility, frequently observed when the pH of the mixture was between 7.8 and 8.5, was that the human IgG protected the gold colloid against flocculation by NaCl. However, when experiments were carried out to label red cells with gold-labelled anti-A (or anti-D), then a high nonspecific uptake (in the order of 50 per cent or more of the specific uptake) was observed in the controls. The high nonspecific uptakes of the gold labelled antibodies were shown either in experiments in which the antibody was labelled with ^{125}I, or in experiments with the electron microscope. Therefore, it appeared that in this situation the gold colloid combined with the human IgG antibody but presumably the bond was very weak and the gold-protein complex was easily dissociated.

The weakness of the bond between gold and human IgG antibodies was established in experiments in which ^{125}I-IgG anti-D preparations were combined with gold colloid at pH 8.2. The anti-D apparently combined with the gold particles because no flocculation was observed upon addition of NaCl; however, when the preparation was ultracentrifuged at 90,000 g for 35 minutes, it was found that most of the gold was precipitated at the bottom of the tube and that only a small amount of this gold was combined with anti-D. Most of the radioactivity (40-80%), representing the free ^{125}I-anti-D, was found in the supernatant. The recovered gold anti-D complexes from several experiments were pooled and attempts were made to label Rh positive cells with the preparation, using Rh negative cells as controls. Nonspecific uptakes in the range of 40-70 per cent of the specific were obtained again in the negative controls.

It was thought that it was possible that the NaCl used to bring the solution to the tonicity of plasma was causing the weakening of the gold-IgG bond and therefore 80 g/l sucrose in the place of 1 g/l NaCl was used. Similar high nonspecific uptakes were obtained; moreover it was observed that sucrose reacted with gold colloid: i.e., sucrose by itself had a weak protective effect on the gold colloid (dextrans and ficoll fully protected gold colloid from electrolyte flocculation). Gold-IgG suspended in other buffers and solutions (glycine, Tris, phosphate, barbiturate, glucose) also proved unsuitable for labelling red cells.

3) REACTION OF BOVINE SERUM ALBUMIN WITH GOLD COLLOID

Both BSA and formaldehyde-treated BSA (in which free amino groups were blocked) were found to have a great affinity for gold particles as shown by their ability to protect gold colloid against flocculation by electrolyte. Similar results were found with dinitrophenylated-BSA.

Gold particles were firmly bound with BSA as demonstrated by the fact that even if the BSA-gold complex was made as high as 100 g/l with NaCl, no flocculation of the colloid was observed. Similarly, stable gold-BSA complex were formed at all pHs in the range of 6 to 9.

The minimum amount of BSA necessary to give almost complete protection against flocculation was estimated to be about 0.2 mg of BSA for each 5 ml of gold colloid.

C) DISCUSSION

Faulk and Taylor (1971) described a method of labelling antibodies with gold particles which it was thought could be applied for the direct labelling of human IgG blood group antibodies in order to study their distribution on the red cell membrane. All experiments carried out for this purpose proved to be unsuccessful. The present series of experiments indicated that human IgG antibodies did not combine firmly with gold particles. The bond established was easily dissociated by all buffers used to bring the protein-gold mixture to isotonicity or even by non-electrolytes like sucrose or glucose. Presumably, Faulk and Taylor's successful use of the method was because they were using whole rabbit antisera (rabbit IgG also failed to combine firmly with gold particles, see section II); however, it is likely that under those conditions gold particles are preferentially bound by the rabbit albumin present in the antisera; their apparent success in obtaining specific labelling might be due to the presence of antibodies of the IgA or IgM type, which in preliminary experiments we found to combine in a stable way with gold colloid. However, for studying the distribution of red cell antigen sites it is thought that either IgA or IgM antibodies were unsuitable.

Bovine serum albumin was found to form a stable bond with gold colloid. Thus, it was found that only 0.2 mg of BSA were enough to stabilize 5 ml of gold colloid. This figure gives a theoretical ratio of about 1 BSA molecule per 10-12 gold particles stabilized.

The precise mechanism by which hydrophobic colloids (such as protein solutions) stabilize hydrophobic colloids (such as gold colloid) (see Jirgensons and Straumanis, 1964) is not known. Gold particles are negatively charged in water and one might presume that they bind to the $-NH_3^+$ groups of the protein. It seems likely, however, that other charged groups or polar groups of the protein surface are more important in binding the gold particles, as demonstrated by the finding that the reaction of free amino groups of BSA with formaldehyde and dinitrophenyl groups has no effect on the ability of the protein to protect the colloidal gold against flocculation upon the addition of NaCl.

The great affinity of BSA for gold particles might be the basis for the development of a method in which BSA molecules are chemically conjugated to human IgG antibodies, and then the BSA part of BSA-IgG complex might be successfully labelled with gold.

SECTION II

GOLD-LABELLED HORSE ANTI-HUMAN GLOBULIN PROPERTIES AND USE IN ELECTRON MICROSCOPY

Following the failure to produce a useful gold-labelled human IgG reagent to directly label antigen sites of human red cells, it was decided to investigate the reaction between gold colloid and IgG antibodies from animal species. Rabbit IgG anti-A and anti-B and horse anti-human IgG were available in the laboratory and were tested for their affinity to combine with gold colloid.

Rabbit IgG antibodies, produced by immunization with A and B glycoproteins (kindly donated by Prof. W. Watkins), were purified by conventional DEAE chromatography. In experiments in which the rabbit antibodies were added to gold colloid no stable gold-antibody reagent was obtained, findings similar to those made with human IgG.

On the other hand, when purified horse anti-human IgG was added to gold-colloid, a stable gold-antiglobulin reagent was produced which was found to be useful for electron microscopic studies. Therefore, it was thought that by combining red cells with human IgG antibodies and then labelling the latter with gold-anti-IgG, useful information could be obtained regarding the distribution of the label on the cell surface. Thus, in this second section the preparation, analysis and use of the gold-labelled anti-IgG reagent are described. In addition, the techniques used for labelling and preparation of red cells for electron microscopy are also described.

A) MATERIAL AND METHODS

1) Horse anti-human IgG: This antibody had been produced in horses by injection of purified human IgG (Medical Research Council, 1966). The serum was kept frozen at -40°.

Before labelling with gold, the anti-IgG was purified by the procedure which was described in details in chapter I. Briefly, this consisted of

1) Preparation of a suitable immunoadsorbant which was achieved by coupling purified human IgG to CNBr-activated Sepharose 4-B (Pharmacia).
2) Absorption of the horse antiserum with the IgG-Sepharose immunoadsorbant.
3) Elution of the anti-IgG antibody from the immunoadsorbant with glycine buffer at pH 2.8.

The final protein concentration of this purified preparation was 10 g/l as estimated by light absorption of 280 nm and using an extinction coefficient of 1.35 for a 1 g/l solution. About 60 per cent of the purified anti-IgG labelled with ^{125}I could be absorbed by an excess of Rh positive red cells coated with IgG anti-D.

The purified horse anti-IgG preparation was dialysed against 5 mM NaCl pH 7.0, ultracentrifuged at 90,000 g for 30 minutes and divided in aliquots of approximately 0.1 ml containing about 1 mg antibody. The aliquots were kept frozen at -40° until use.

The reactions between horse anti-IgG and gold colloid were investigated by methods similar to those described in section I.

2) PREPARATION OF THE GOLD-LABELLED HORSE ANTI-HUMAN IgG

Gold colloid was prepared as described in section I of this chapter.

After several experiments it was found that the following was the best method.

Horse anti-human IgG and gold colloid were mixed in a plastic tube at a ratio of 1 mg of horse antibody to 2.5 ml of gold colloid, the protein was in a volume of 0.1 ml or less. This was reacted for 15 mins. at room temperature with frequent mixing by inversion. Then 10 mg. of BSA were added and the mixture was incubated for 5 mins. more. At this point, the mixture was made 10 g/l NaCl using 100 g/l NaCl solution at pH 7.0. Finally it was spun at 1700 g for 20 mins, there was usually either no precipitate or only a very little precipitate. The supernatant from this final preparation of gold-labelled anti-human IgG (gold-anti-IgG for short) was used without further dilution to label red cells coated with IgG antibodies.

The addition of BSA after the gold particles had reacted with the anti-human IgG antibody proved to be useful to further stabilize the gold-anti-IgG reagent i.e. helped to keep the labelling preparations free of aggregates. BSA itself did not displace the anti-IgG antibody from its combination with gold particles.

B) RESULTS

1) REACTIONS OF THE GOLD COLLOID WITH THE HORSE ANTI-HUMAN IgG

Horse anti-IgG was found to form stable complexes with gold colloid. The minimum amount of horse anti-IgG necessary to give almost complete protection against flocculation was estimated to be about 1.6 mg of horse antibody per each 5 ml of gold colloid. This figure gives a theoretical ratio of approximately 1 anti-IgG molecule / 4 gold particles.

When 1 mg of ^{125}labelled horse anti-human IgG was mixed with 2.5 ml of gold colloid for 10 minutes then made up to 10 g/l NaCl with concentrated NaCl and centrifuged at 90,000 g for 1 hour, it was found that between 10-20 per cent of the anti-IgG added remained free in the supernatant solution. As this free anti-IgG would not cause nonspecific uptake of gold particles on red cells, it was considered unnecessary to separate it from the gold-labelled anti-IgG although this could be achieved as follows: the gold-protein mixture was carefully placed onto a 1 ml layer of pure ethanediol and centrifuged at 90,000 g for 50 minutes. The gold protein complex entered the ethanediol layer but not the free anti-IgG which remained in the upper aqueous layer. Subsequently, the ethanediol molecules can be separated from the gold-anti-IgG by dialysis against 10 g/l NaCl. One setback is that the centrifugation and dialysis steps of this procedure produced some aggregation of the gold-anti-IgG complexes.

Horse anti-IgG was found to retain its antibody activity extremely well after labelling with gold. Thus, when aliquots of 0.1 ml of gold-labelled ^{125}I-anti-IgG complex, containing about 30 µg of radio-iodinated protein, were incubated with 0.1, 0.2 and 0.4 ml of red cells maximally coated with IgG anti-D, it was found that approximately 50 per cent of the ^{125}I-labelled anti-IgG gold complex could be absorbed by the human red cells coated with IgG anti-D.

The gold-labelled anti-IgG was usually prepared on the same day as it was used for identifying antigen sites on the red cell. However, it was found that the reagent could be kept for about 2 weeks; in this case it was spun 30 minutes at 1700g immediately before use. BSA at a concentration of 3 g/l added to the gold anti-IgG was found to keep aggregation of the gold-labelled anti-IgG complexes at a minimum.

At the ratio of 1 mg of anti-IgG to 2.5 ml of gold colloid, most of the gold particles were adsorbed onto protein as demonstrated by finding very little or no precipitate of aggregated gold particles in the presence of 10 g/l NaCl.

2) IDENTIFICATION OF THE HORSE ANTI-HUMAN IgG ANTIBODIES

a) Immunoglobulin type: Two ml of the horse antisera were fractionated on a Sephadex G-200 column using 10 g/l NaCl + 0.005 g/l sodium azide solution as eluant. Fig. III-1a shows the result. The 2 top 4 ml fractions from each peak were examined for anti-IgG activity by testing their capacity to agglutinate human O Rh positive red cells coated with human IgG anti-D at a concentration of about 60 µg IgG anti-D/ml of red cells. No anti-IgG activity could be detected in peak I, even when

all the fractions of that peak were concentrated to about 2 ml. Most of the anti-IgG activity was found to be present in peak II.

The absence of anti-IgG activity in peak I and the presence of the bulk of the activity in peak II, indicated that the horse anti-human IgG antibody was a 7S type antibody and not a 19S (IgM type) antibody (In gel filtration separation made with Sephadex G-200, macroglobulins are found in the first peak and 7Sγ-globulin in the second peak, Porath and Flodin, 1963).

Immunoelectrophoresis (IEP) was carried out using the following as antigens: concentrates from peaks I and II, purified anti-IgG and whole horse antiserum. Rabbit anti-whole horse antiserum was used as antiserum to the horse proteins. The IEP was performed on gels made of 10 g/l Difco Special Agar Noble and a barbiturate buffer of ionic strength 0.025 and pH 8.6 was used, the voltage applied was 5 V/cm. As shown in fig. III-1b the purified anti-IgG preparation had 2 major components which moved toward the cathode one with a γ2 mobility and the other with a γ1-β2 globulin mobility (which resembles the mobility of human IgA); these 2 major components are absent in the peak I of the G-200 fractionation which did not have any anti-IgG activity and are present in the peak II which had the bulk of the anti-IgG activity. These findings suggest that the 2 major components of the purified anti-IgG preparation are: 1) horse IgG (with γ2 mobility). 2) horse T-globulin (or IgG (T), with γ1-β2 mobility). The presumptive identification of the second component as horse T-globulin is based on:

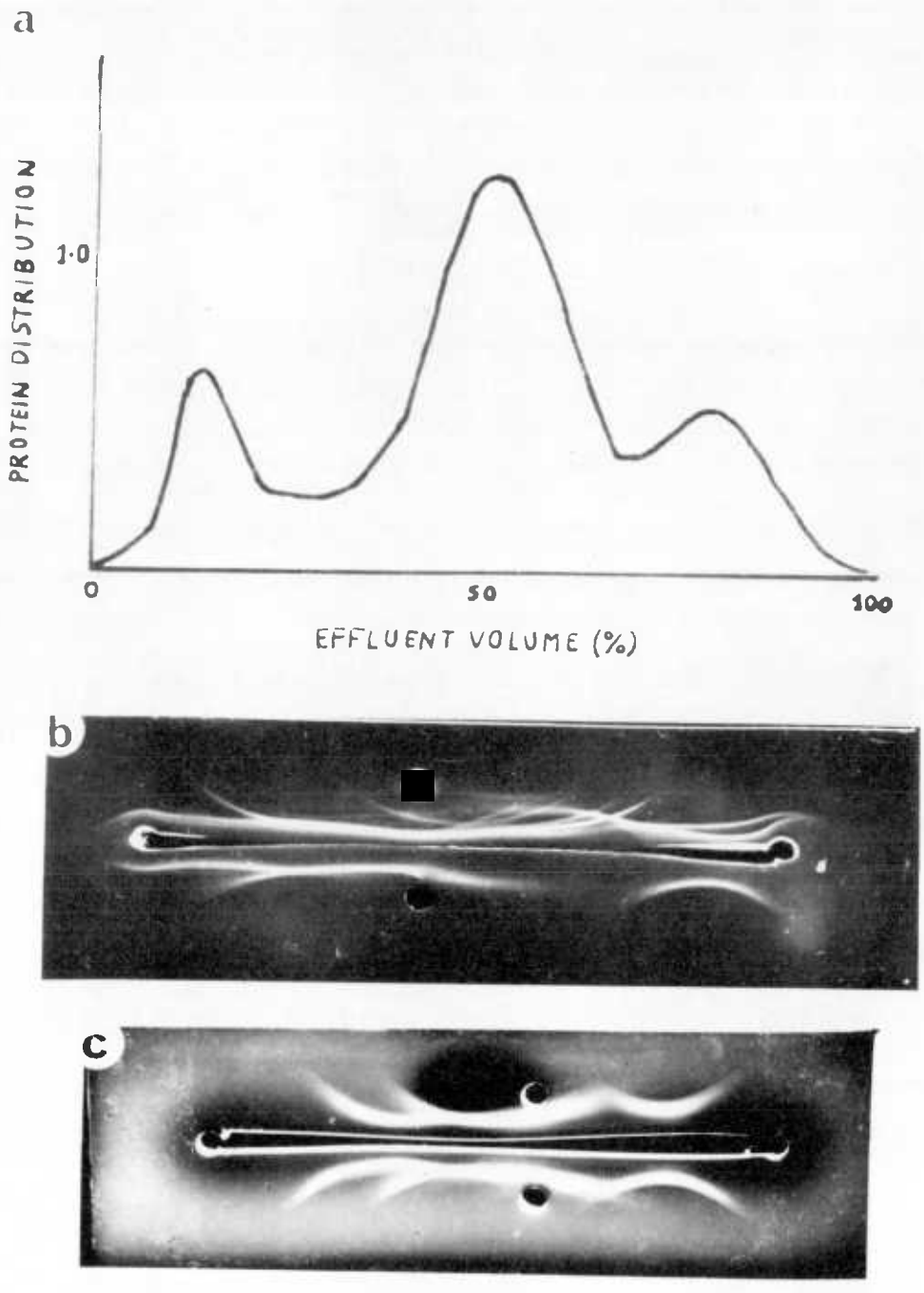

Fig III-1 : a) Sephadex G-200 separation of horse anti-human IgG.
b) Immunoelectrophoresis of whole horse anti-human IgG (top) and purified anti-IgG by affinity chromatography (bottom) against rabbit anti-whole horse antisera. c) Immunoelectrophoresis of gold-anti-IgG (top) and anti-IgG alone against rabbit anti-whole horse sera. The dark spot above the γ1-β2 line, IgG (T), correspond to the gold-protein complex which appeared as a bright red spot in the gel.

a) the γ1-β2 electrophoretic mobility observed (Johnston and Allen, 1968) and b) the fact that although this T-protein is barely detectable in normal horse serum, it increases in considerable amounts in horses hyperimmunized with protein antigens (as in this case, horse hyperimmunized with human IgG protein) and it may constitute the bulk of the antibody response (Kekwick and Record, 1941; Weir and Porter, 1966, and Weir et al., 1966). This horse T-globulin has a greater carbohydrate content than horse IgG (Weir and Porter, 1966), which might be of importance in the reaction with gold particles.

b) Identification of the horse antibody that combines with the gold particles as horse T-globulin : Three ml of gold-labelled anti-IgG to which the addition of BSA was ommited were concentrated by pressure dialyses to 0.2 ml. This concentrated reagent was electrophoresed along with the purified unlabelled anti-IgG and then reacted with an antisera against horse serum produced in a rabbit. It was observed, Fig. III-1c, that the gold-labelled anti-IgG, which could easily be seen in the gel because of its red colour, migrated to the γ1-β2 position and it showed a precipitation line with the rabbit anti-horse serum; on the other hand, another line of precipitation was observed in the IgG γ2 zone (horse IgG) which did not show any red colour (i.e. it had no gold attached). This result suggested that the horse T-globulin (which has γ1-β2 mobility) is the antibody that combines with the gold particles and that horse IgG, together with human and rabbit IgG may not form a stable bond with the colloidal gold.

3) ELECTRON MICROSCOPIC ANALYSIS OF THE GOLD-LABELLED ANTI-IgG REAGENT

The electron microscopy was carried out using a Phillips EM-300. One volume of gold-anti-IgG was diluted with 14 volumes of deionized water, a drop of the resulting gold-protein dilution was then touched with the carbon side of a carbon coated grid (see next subsection), and the attached gold-protein complexes were examined in the electron microscope. Fig. III-2 shows a representative area of an electron micrograph of the gold-anti-IgG reagent. The gold particles appeared as round electron dense particles, which were for the most part separate from each other, but some were found together in distinct groups containing 2, 3 or more gold particles, (single and grouped gold particles will collectively be called gold clusters, see later.).

All gold particles of the gold-anti-IgG preparation were adsorbed onto protein as indicated by the results of experiments in which one volume of a 1:15 dilution of the gold-anti-IgG was mixed with an equal volume of 10 g/l ammonium molybdate solution and analysed in the electron microscope. It was observed (fig. III-3) that all the gold particles were surrounded by a clear halo indicating that the particles had a protein coat which was not penetrated by the ammonium molybdate staining solution, the molybdate salt thus acting as a negative stain for the gold protein complex.

The size of the clusters and the distribution of the particles in the gold clusters, were studied in photographs which were taken at a basic electron microscope magnification of 21,500 and 44,000 times, and enlarged a further 3.75 times when printed (Ilfoprint photographic paper, Ilford, YR31P size 11" x 14").

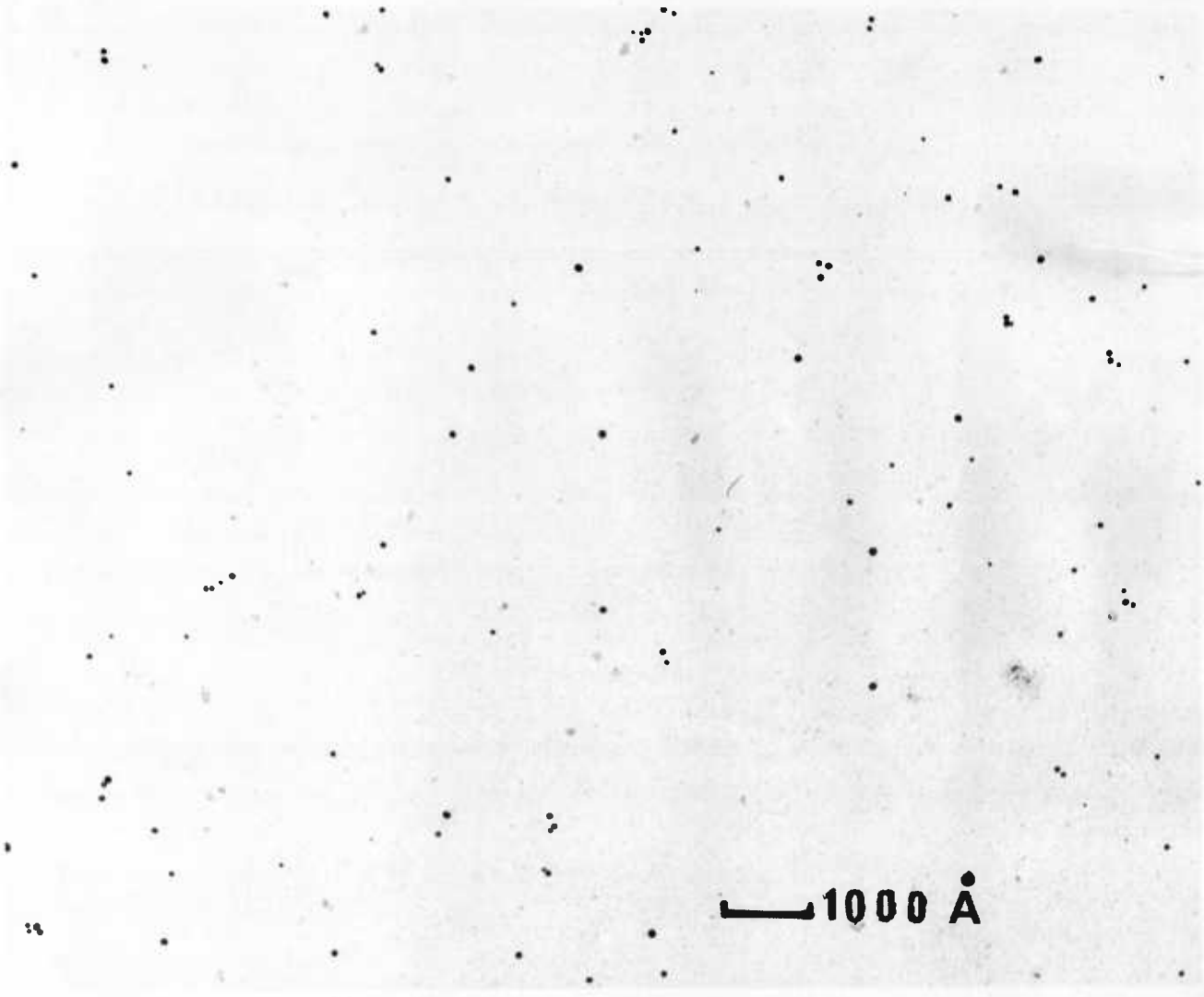

Fig III-2 : Gold-anti-IgG spread out on a carbon coated grid.

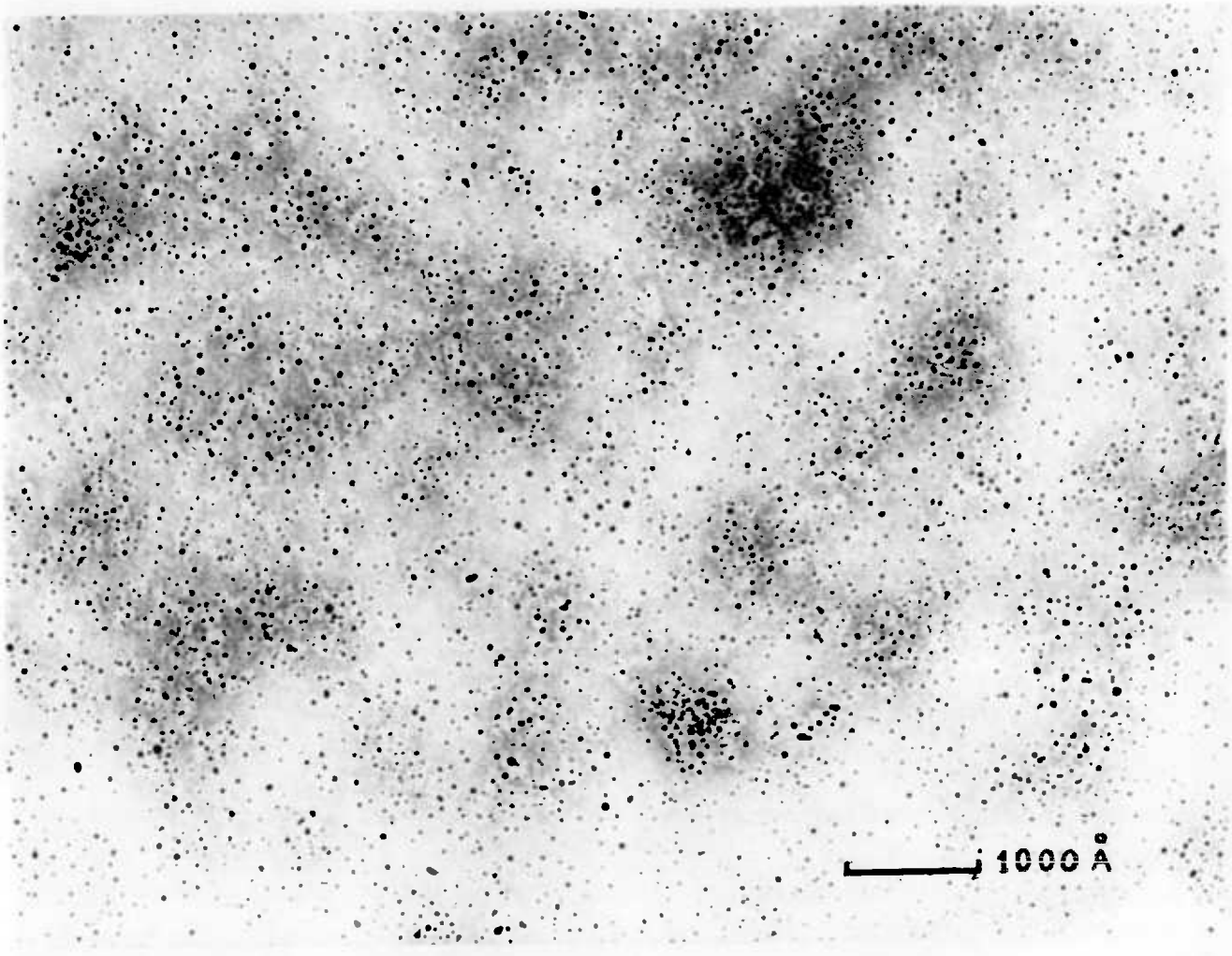

Fig III-3 : Gold-anti-IgG reagent negatively stained with ammonium molybdate showing that the gold particles are surrounded by clear halos (protein coat).

a) Size of the clusters of gold-anti-IgG: gold clusters were identified and demarcated with a felt pen and their maximum diameter was measured. In the identification and demarcation procedure of the gold clusters, the following general rules were observed:

1) Based on the size of the IgG molecules (horse anti-IgG antibodies were found to be IgG (T) and IgG), which according to Green (1969) do not exceed 140 ångström in any direction, the gold clusters were classified according to the measured diameters as: i) measuring less or equal to 140 ångströms, ii) measuring between 140-280 ångströms and iii) measuring more than 280 ångströms.

2) One or more gold particles within a distance of 140 ångströms, were taken as a gold cluster and it was assumed that they represented one labelled-anti-IgG molecule.

3) 2, 3 or more gold particles within a distance of 280 ångströms were also taken as a single cluster, and assumed to represent a dimer of gold-anti-IgG molecules.

4) 2 or 3 or more particles within a distance greater than 280 ångströms were taken as a cluster only if the distances separating nearest neighbouring particles within the clusters were less than 280 ångströms; these groups were assumed to represent polymers of gold-anti-IgG molecules.

As table III-1 shows, 83 per cent of the clusters were found to measure 140 angstroms or less; therefore the great majority of the gold-anti-IgG reagent was formed of gold-labelled monomers of anti-IgG. About 13 per cent of gold clusters measured between 140 and 280 angstroms; presumably this fraction represented the proportion of gold-labelled dimers of anti-IgG molecules. Only 4 per cent of the gold-anti-IgG molecules were found in aggregates of 3 or more molecules as judging by the proportion of clusters found to measure more than 280 angstroms.

Table III-1 : Size of the clusters and frequency distribution of the gold particles forming clusters of gold-anti-IgG

The data were estimated from 4 photographs of 2 gold-anti-IgG preparations made from two different batches of purification of horse anti-IgG antibodies.

A total of 970 clusters was analysed.

A) SIZE

Size in ångströms of the gold clusters	Percentage
≤140	83
140-280	13
>280	4

B) DISTRIBUTION

Number of gold particles per gold cluster	Frequency (per cent of the total number of gold cluster)
1	68
2	15
3	8
4	5
5 or more	4

b) Frequency distribution of the gold particles forming clusters of gold-anti-IgG : the frequency distribution of the number of gold particles in each gold cluster was studied to obtain information about the number of gold particles labelling each anti-IgG molecule.

Table III-1 shows the observed distribution for 970 gold clusters from 4 photographs of two different gold-anti-IgG.

As table III-1 indicates, about 83 per cent of the gold clusters were found to have either one single or 2 gold particles; in addition about 83 per cent were also found to measure 140 angstroms or less. Therefore it can be concluded that the majority of the horse anti-IgG molecules were labelled with only 1 or 2 gold particles. Only approximately 4 per cent of the gold clusters were found to have 5 or more particles, representing dimers or antibody aggregates.

The average number of gold particles per gold cluster was found to be 1.67 particles/cluster with a standard deviation of 0.18 (970 clusters studied). If the gold clusters larger than 280 ångströms are excluded, it can be estimated that on the average there were 1.5 gold particles per gold cluster and therefore per molecule of horse anti-IgG, that is, if the dimers of anti-IgG are included. The reason for including the dimers of anti-IgG in the estimation of the latter figure, is because normally a variable proportion of the IgG immunoglobulin population is found as dimers (Stanworth and Henney, 1967), especially in cases like this in which the antibody is dialysed against low ionic strength buffer and also because these dimer molecules preserve their antibody activity as individual IgG molecules.

4) LABELLING AND PREPARATION OF RED CELLS FOR ELECTRON MICROSCOPY

The results described in the previous subsections indicated that gold-labelled anti-IgG antibodies conserved their antiglobulin activity and that the gold-anti-IgG could be prepared reasonably free of aggregates.

Hence, gold-anti-IgG was considered a suitable reagent for use with red cells which were sensitized with human IgG blood group antibodies.

The methods used for sensitization, labelling and preparation of the red cells for electron microscopy are described next.

In the first place, 0.2 ml of packed red cells fresh or less than 36 hours old and of the appropriate blood group, were incubated at 37° with 1 ml of the purified IgG antibody preparations. IgG anti-D preparations were used at concentration ranging from 50 to 100 µg of antibody/ml, according to the particular experiment. IgG anti-A preparations were used at antibody concentrations ranging from 5 to 200 µg/ml, according to the particular experiment.

In the second place, the IgG antibody coated red cells were incubated with the gold-anti-IgG reagent in one of two ways: a) in a tube or b) on drops of the labelling reagent lying on parafilm. The procedures used were as follows:

a) When labelling was carried out in a tube, 0.1 ml of a 50 per cent sensitized red cell suspension was incubated with 0.5 ml of gold-anti-IgG for 10 minutes at 37°. Gold-anti-IgG which did not bind to the red cells was separated by washing with saline solution. About 50 µl of the labelled cells were then dropped into a glass bottle filled to the brim with 16 mM phosphate buffer at pH 7.0. Some cells lysed as they sank, others lysed and remained floating at the liquid-air interface These latter cells, which were flattened by surface forces, were picked up by touching them with the carbon

side of a carbon-coated electron microscope grid to which they stuck. The carbon coated grids were prepared (Stolinski and Gross, 1969), by evaporating carbon in a vacuum on electron microscope grids which had been coated with a thin cellulose film. The cellulose support film was then dissolved in amyl acetate, leaving a very thin carbon support film which was very useful to mount red cell ghosts on. Using this procedure, the sensitized red cells are labelled with gold-anti-IgG over the entire red cell membrane; when ghosts are viewed with the electron microscope, the depth of focus is such that the two superimposed surfaces cannot be separately focused and the true distribution of gold label on one surface only cannot be analysed. Because of this problem the procedure of labelling one surface only, which is described next, was generally preferred, although labelling of both surfaces has the advantage of being a very simple procedure.

b) The alternative way of labelling the red cells which results in the labelling of only one side of the ghosts was carried out by the method of Nicolson and Singer (1971), with minor modifications. Ghosts were produced by lysing already sensitized red cells, with IgG antibody, at the air interface of 1 g/l NaCl pH 7.0 which was preferred to 16 mM phosphate buffer pH 7.0 because it was observed that the rate of lysis of red cells in 1 g/l NaCl was slower than in 16 mM phosphate buffer. This slower rate of lysis was useful in these experiments because, as will be explained later, it was observed that red cells which lysed slowly formed ghosts which were intact (they had some haemoglobin left), whereas red cells which lysed quickly (virtually free of haemoglobin) were found to show disruption of the membrane (see chapter IV). Labelling was then carried out by placing the grid

with the ghosts onto a large drop of gold-anti-IgG labelling reagent. After 2 minutes the grid was picked up and excess gold-anti-IgG was drained by touching the grid with a piece of filter paper. It was then washed 3 times by quickly floating the grid with the ghosts on large drops of phosphate buffered saline and finally twice on drops of water at pH 7.0. Each time excess fluid was drained by touching the rim of the grid onto a piece of filter paper. If desired, fixation of the ghosts can be carried out before or after the labelling with gold-anti-IgG. This technique for labelling one side of the ghosts was preferred for experiments in which quantitative results were to be made since it has the advantage that only the side of the ghost away from the grid is in contact with gold-anti-IgG and therefore is the only one labelled.

5) FERRITIN MOLECULES CAN BE DISTINGUISHED FROM GOLD PARTICLES IN ELECTRON MICROSCOPE PHOTOGRAPHS

Ferritin molecules are extensively used as electron microscope labels for antibodies. In order to see if gold particles could be distinguished from ferritin molecules, a mixture of ferritin and gold particles was prepared and directly examined in the electron microscope Fig. III-4 shows a photograph of the ferritin-gold particles mixture. It was found that gold particles were denser, and on the average, smaller than ferritin molecules, therefore making it possible to differentiate between both labels. This distinction theoretically allows the simultaneous use of both markers which could be a potential useful technique.

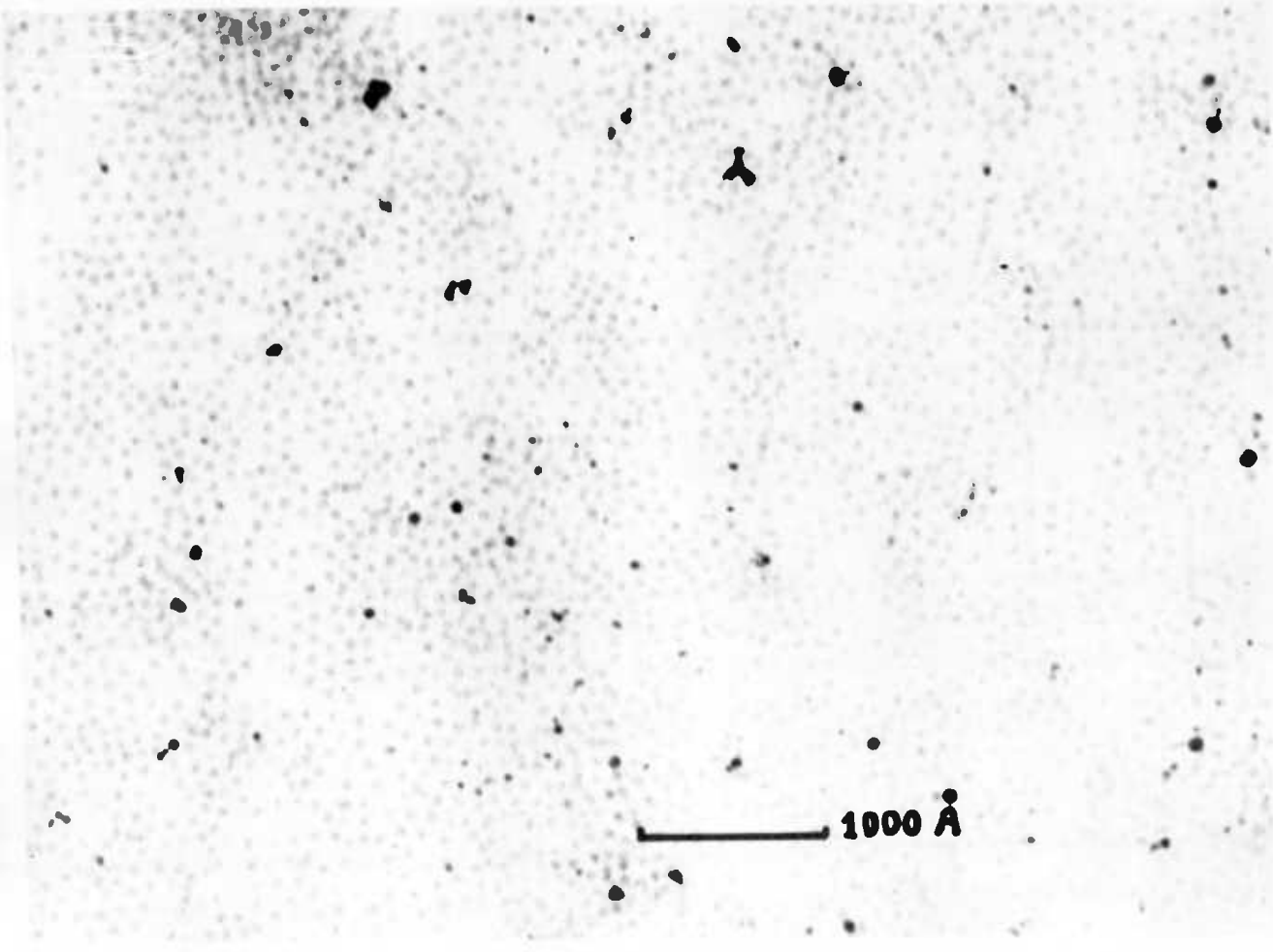

Fig III-4 : Mixture of gold particles and ferritin molecules. It can be seen that gold particles can be readily differentiated due to their higher electron density.

C) DISCUSSION

Faulk and Taylor (1971), described for the first time the use of gold particles as antibody markers. Their original method has been modified and adapted for the study of red cell membrane antigens. Some observations have been also made regarding the interaction between gold particles and some proteins.

Proteins should be relatively free of ions before being labelled with gold, otherwise the gold colloid is flocculated. In the original technique, dialysis against 0.2M ethanediol was used to solve this problem. In these studies it has been found that such dialysis resulted in a great deal of precipitation and aggregation of the protein. It was also found that dialysis against 5 mM NaCl pH 7.0 decreased the ion content to an aceptable level at the same time minimising the precipitation of the protein. Thus, dialysis against low ionic strength NaCl followed by ultracentrifugation before labelling the protein with gold was the procedure used to avoid flocculation of the gold colloid by electrolytes while keeping simultaneous aggregation of protein to a minimum.

The use of gold particles as antibody markers has several advantages. The method is simple, easy and inexpensive. Owing to the high atomic number of gold, the particles are very electron dense, therefore they are easier to visualize than ferritin on the electron microscope screen and also these particles are very suitable for focussing and for correction of astigmatism.

A disadvantage of this technique is concerned with the difficulties found when accurate quantitation of antigens is needed. The difficulty results from the fact that gold particles and anti-IgG molecules do not combine in a fixed ratio and also because one molecule of IgG (acting as antigen) can combine with a variable number of anti-IgG molecules. Thus, a single blood group antigen site might be represented by a variable number of gold particles which depends on how many gold particles were adsorbed on each anti-IgG molecule and on how many anti-IgG molecules were bound to the IgG antibody occupying the antigen site. However, from a practical point of view, this problem was only of minor importance because, as will be explained in the next chapter, discrete antigen sites were visualized in the majority of cases as small groups of gold particles containing usually 1 to 5 gold particles, for example, only in about 5 per cent of the clusters of label on Rh positive red cells was it difficult to ascertain whether they were indicative of more than one antigen site or of aggregated gold-protein complexes.

Another disadvantage of the gold-labelled antibody technique is that, at the present stage, only horse antibodies, presumably only horse T- globulin, possibly because of its higher carbohydrate content, has been found to have the ability to combine in a stable way with gold particles. However, the possibility exists that chemical modification of human or rabbit IgG or the conjugation of these immunoglobulins with BSA, may modify these proteins as to acquire the ability to combine with gold particles and therefore serve as the basis for a more general use of the gold labelling technique.

CHAPTER IV

DISTRIBUTION AND MOBILITY OF D AND A ANTIGENS ON THE HUMAN RED CELL MEMBRANE

I) INTRODUCTION

The studies presented in this section were a direct application of the gold labelling technique described in the previous chapter and were aimed at answering the following questions:

a) what is the distribution of the D and A antigens on the red cell membrane?

b) how mobile are these antigens?

c) what is the explanation for the different serological behaviour (i.e. in relation to agglutination, complement fixation and red cell destruction) between the A and D systems?

It was hoped that by finding suitable answers to the above questions, a better understanding of the immunology of haemolytic disease of the newborn and of immune haemolysis, could be derived.

II) METHODOLOGY

A) PREPARATION OF THE GOLD-ANTI-IgG LABELLING REAGENT

Horse anti-human IgG was prepared and labelled with colloidal gold as detailed in chapter III. The characteristics of the gold-labelled anti-IgG reagent have been also described in the previous chapter.

B) PREPARATION OF CELLS

Fresh red cells from donors of different Rh and ABO phenotype were used in the different experiments. 0.2 ml of cells were sensitized with their respective antibody at concentrations ranging from 50 to 100 µg/ml of anti-D and from 5 to 200 µg/ml of anti-A, according to the particular experiment.

The washed sensitized cells were then lysed in the liquid-air interface of a hypotonic (1 g/l) NaCl buffered to pH 7.0, picked up with carbon-coated electron microscope grids and labelled on one side with gold-anti-IgG (see Chapter III for details).

C) ELECTRON MICROSCOPY

Electron micrographs were taken usually at a basic magnification of 21,500, and then printed and enlarged 3.5 - 3.75 times on Ilfoprint (YR3.1P 3 11" x 14", from Ilford) photographic paper. To check the labelling reagent, micrographs of a 1 : 15 dilution of the gold-anti-IgG preparation used in a particular set of experiments were also taken together with the photographs of the gold-labelled cells.

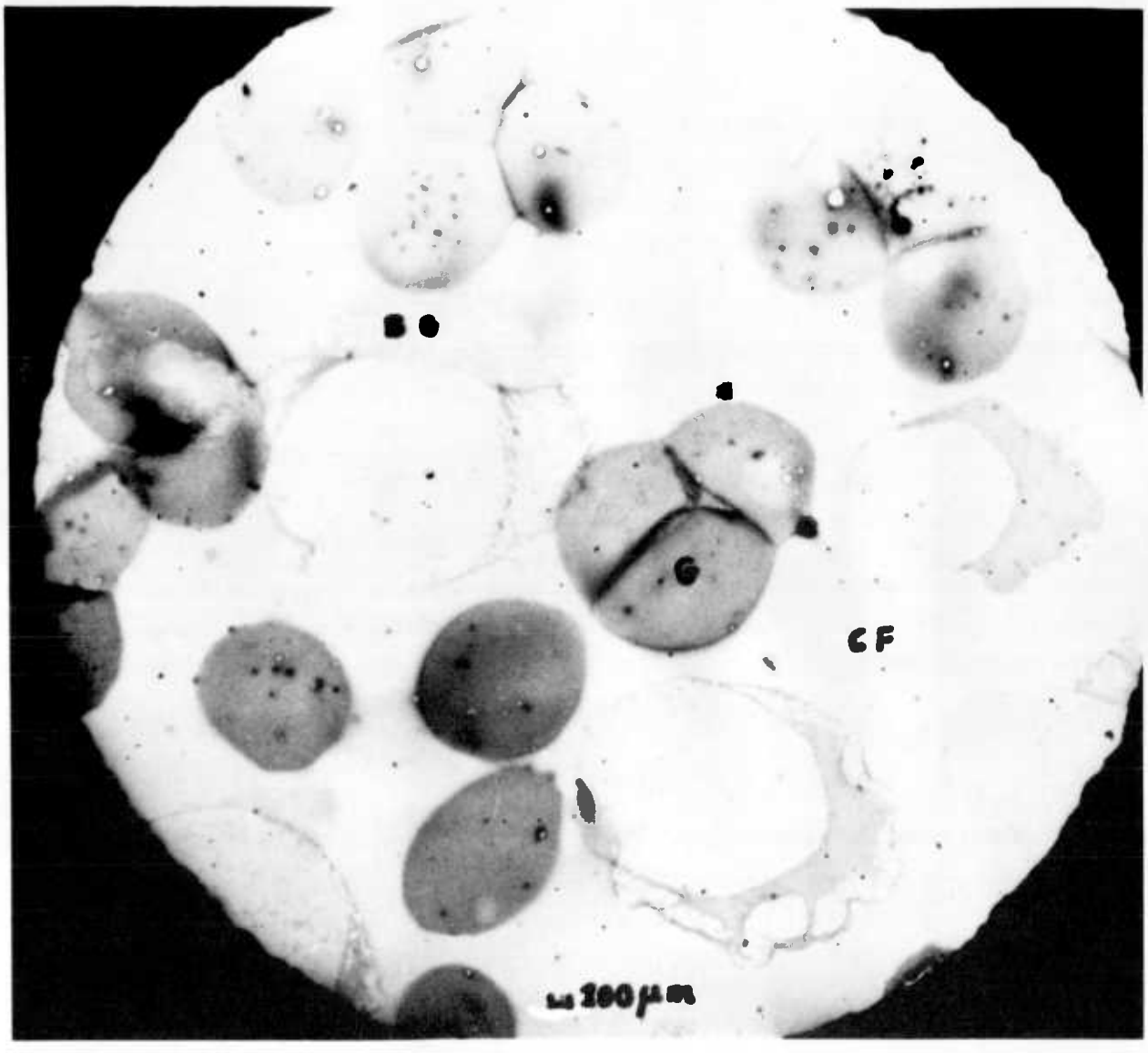

Fig IV-1 : General view of the gold-anti-IgG labelled ghosts. Some ghosts, usually the least electron dense, appeared broken up. CF = carbon film, G = ghost, BG = broken ghost.

with practically no haemoglobin left), the membrane frequently had an irregular "spotty" aspect with light and dark patches. Similarly, these haemoglobin free ghosts frequently appeared broken up in pieces and with irregular holes. These changes, which were interpreted as membrane disruption were rarely seen in ghosts of a darker aspect, i.e. of higher electron-density due to the remaining haemoglobin. The implications of this observation will be mentioned in the discussion. Low temperature (4-10°) of the lysing solution, and possible preincubation of the cells in 30-40 per cent glycerol, were observed to have a protective effect in the preservation of the integrity of the red cell membrane, though this aspect was not further investigated.

The membrane of intact ghosts were uniformly flattened as indicated by:

1) the regular and smooth aspect of the surface
2) the estimated total surface area of 120 to 150 μm^2 in agreement with previously estimated values (Houchin et al., 1958; Westerman et al., 1961).

Occasionally, some buckling of the membrane was observed in fixed ghosts which was probably associated with the increased stiffness of the fixed membrane. Therefore, in order to avoid irregularities on the ghost surface, fixation was restricted only to experiments in which additional information could be gained from this procedure.

B) ANALYSIS OF PHOTOGRAPHS

The number and distribution of gold particles on the ghosts was studied in material from several experiments. This analysis was carried out as described below:

1) FREQUENCY DISTRIBUTION OF THE GOLD LABEL

a) Gold particles present either as a single particle or grouped but forming a distinct group (i.e. "gold clusters") were encircled with a red marker and their frequency distribution was recorded.

b) A chart was constructed with the frequency distribution of the gold clusters containing 1 gold particle, 2 gold particles, 3 and so on. From this chart was estimated the average number of gold particle per cluster and the percentage of the clusters having 1, 2, 3 etc., gold particles.

c) A 1:15 dilution of the gold-anti-IgG reagent used for the particular experiment was analysed in a similar manner. From the average number of gold particles per cluster on the labelled cells and the average number of gold particle per anti-IgG molecule, the approximate number of anti-IgG molecules per gold cluster was estimated.

d) The total number of clusters, giving an indication of the level of saturation of antibody on the cells, was worked out from the number of gold clusters present on a measured area of the total surface and from the final magnification of the photograph. A total red cell surface of 145 μm^2 was assumed (Westerman et al., 1961).

2) SIZE OF THE GOLD CLUSTERS

The maximum size of the encircled gold clusters was measured and grouped in 3 classes (see forward):

a) clusters measuring 140 Å or less ($\leq$140)

b) clusters measuring more than 140 Å but less than 375 Å (140-375)

c) clusters measuring more than 375 Å ($>$375); most of these measured between 375-500 Å.

Based on Green's (1969) data of the size of the IgG immunoglobulins, it was assumed that the 3 classes represented :

class 'a' 1 gold-anti-IgG molecule bound to 1 IgG anti-D (or anti-A).

class 'b' 2 gold-anti-IgG bound in opposite directions to 1 IgG.

class 'c' 1 single and 1 dimer gold-anti-IgG bound in opposite directions to 1 IgG.

This classification implies that all 3 classes (a, b and c) represent one IgG blood group antibody (anti-D or anti-A) combined to a single antigen site. However, classes 'b' and 'c' may also be interpreted as representing 2 or more antigen sites, i.e. 2 or more D or A antigens close together; it is thought, however, that the latter analysis is not correct for reasons given in the Discussion section.

3) STUDIES ON THE RANDOMNESS OF THE SPATIAL DISTRIBUTION OF THE GOLD CLUSTERS

To find out whether or not the spatial distribution of the gold clusters on the red cell membrane was a random one, the observed distribution was compared with a theoretical, calculated, Poisson distribution. The use of such a distribution is justified in this case because what is being studied is the occurrence of the small gold clusters over the comparatively large surface of the red cell, which theoretically, if it is a random process, will be described by a Poisson distribution (see Croxton, 1959).

The gold clusters were encircled with a marker, a transparent grid with unit squares of 1.5 x 1.5 cm was placed over the photograph and the frequency distribution of the gold clusters per unit of area was determined. Thus, the arithmetic mean of the number of gold clusters per unit area was calculated and the expected Poisson distribution was estimated from this value. The X^2 test was used to test the fitness of the observed distribution to the expected one. A good fit, $P \geq 0.05$, was interpreted to mean that the observed distribution was a random one.

C) DISTRIBUTION OF D ANTIGEN SITES

Rhesus positive red cells sensitized with IgG anti-D and labelled with gold-anti-IgG showed a discrete characteristic labelling. The gold particles were found to be distributed on the red cell membrane in small groups usually containing 1-5 gold particles. This can be clearly seen in Figs. IV-2 and IV-3 in which are shown typical

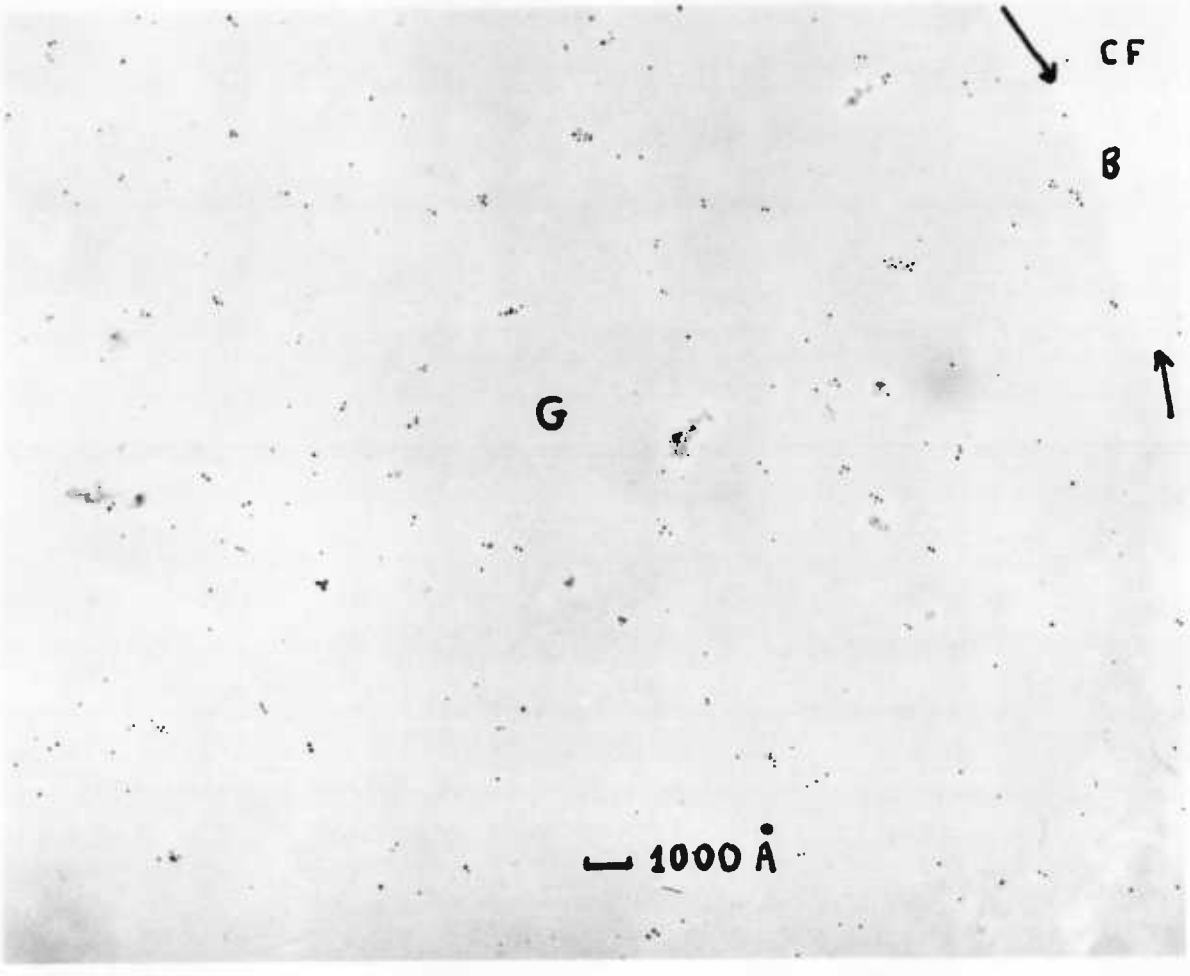

Fig IV-2 : Labelling pattern of an R_1r red cell ghost. It was estimated that approximately 2,800 D sites were combined to anti-D and gold-anti-IgG. CF = carbon film, G = ghost, B = border.

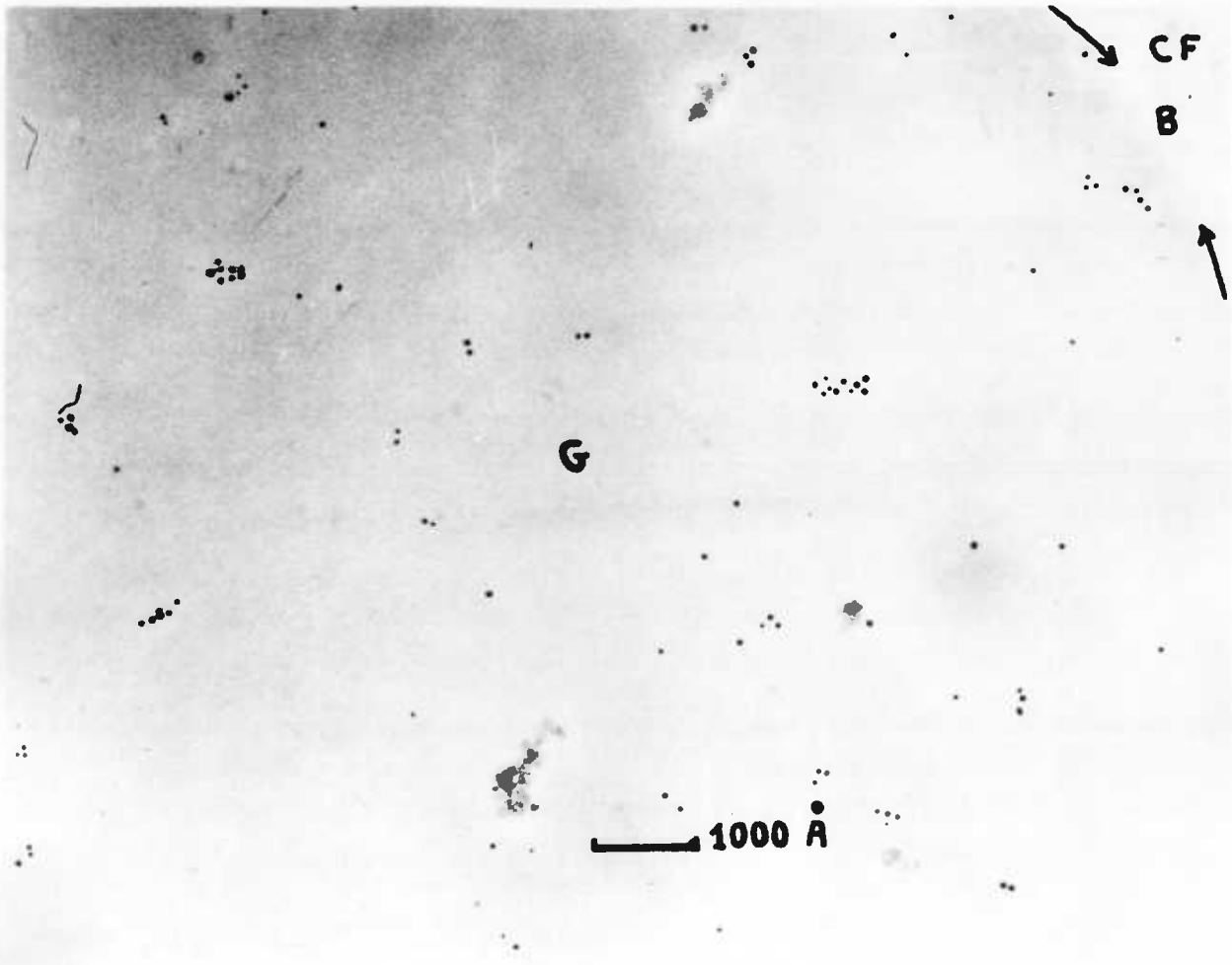

Fig IV-3 : Similar to Fig IV-2, higher magnification.

micrographs of this labelling pattern. In these Figures, 96 per cent of the clusters were found to have between 1 and 5 gold particles. The number of clusters per cell in Fig. IV-2 was estimated to be about 2800, which represents about 24 per cent of the total number of antigenic sites on an R_1r red cell (about 12,000, according to Rochna and Hughes-Jones, 1965). When the centre to centre distance between the nearest neighbouring clusters was measured, it was found that this distance was variable but a value of 0.10 - 0.13 μm was commonly observed. If 0.10 to 0.13 μm is taken as the average distance separating antigenic sites in an R_1r red cell, then it can be estimated that the total number of sites present on R_1r cells is about 10,000 - 15,000 (see Mollison, 1972), which agrees quite well with the estimated number from experiments using radioactive labelled antibodies determined by Rochna and Hughes-Jones (1965).

Fig. IV-4 demonstrates the indirect labelling of D antigenic sites in a R_2R_2 red cell. It was estimated that there were approximately 9,000 - 10,000 clusters per cell. When the number of gold particles forming each cluster was counted, it was found that, on average, 2.7 gold particles were present per cluster; assuming that an average of 1.5 gold particles were present on each anti-IgG molecule (see previous chapter), this corresponds approximately to 2 anti-IgG molecules per single IgG anti-D antibody. Ninety per cent of the clusters had between 1 and 5 gold particles. Sizing of the clusters was carried out by encircling them and measuring the maximum diameter. The results were

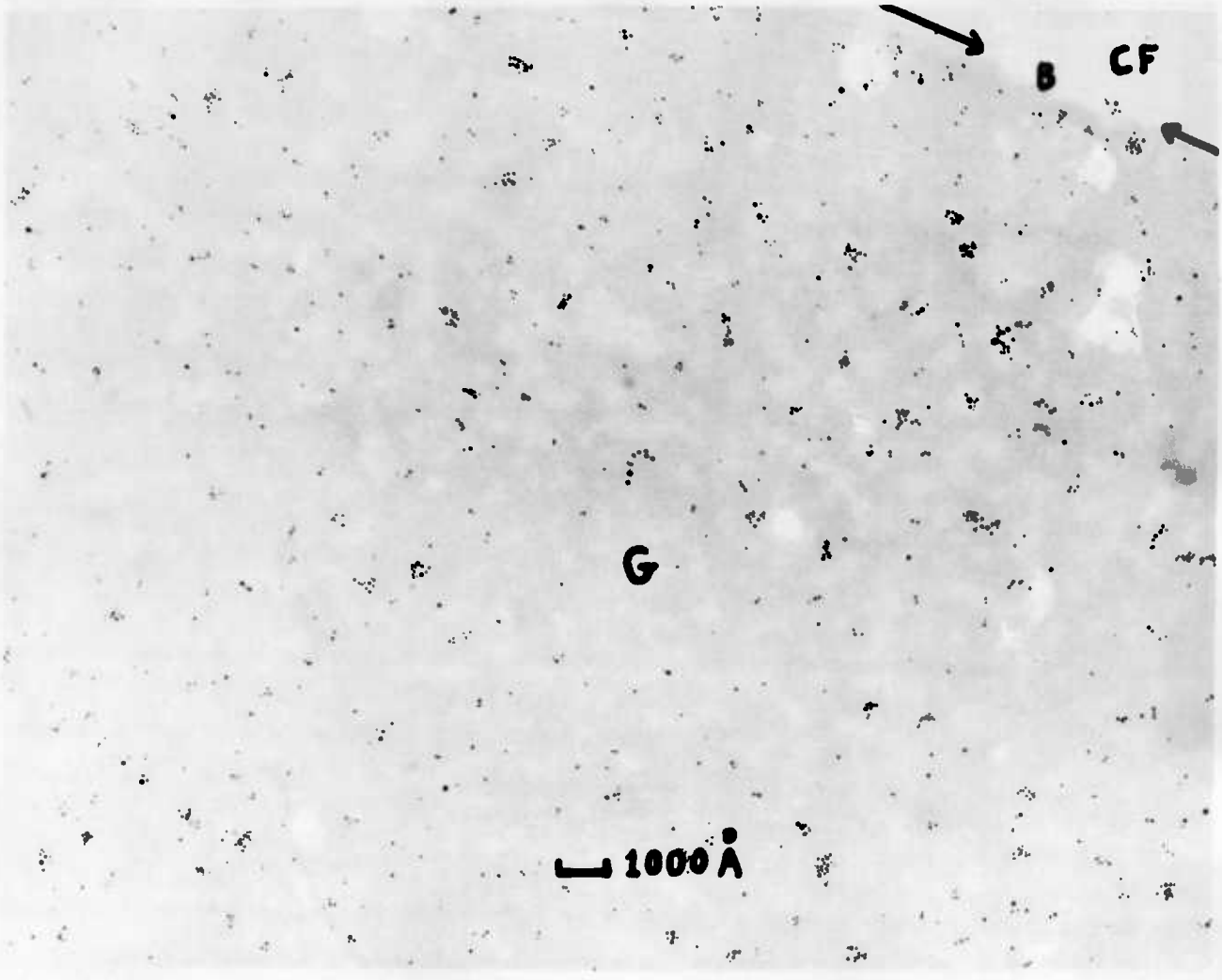

Fig IV-4 : R_2R_2 red cell combined with IgG anti-D and labelled with gold-anti-IgG (see also Fig IV-5). Approximately 9,000-10,000 gold clusters were estimated to be present on the cell. CF = carbon film, G = ghost, B = border.

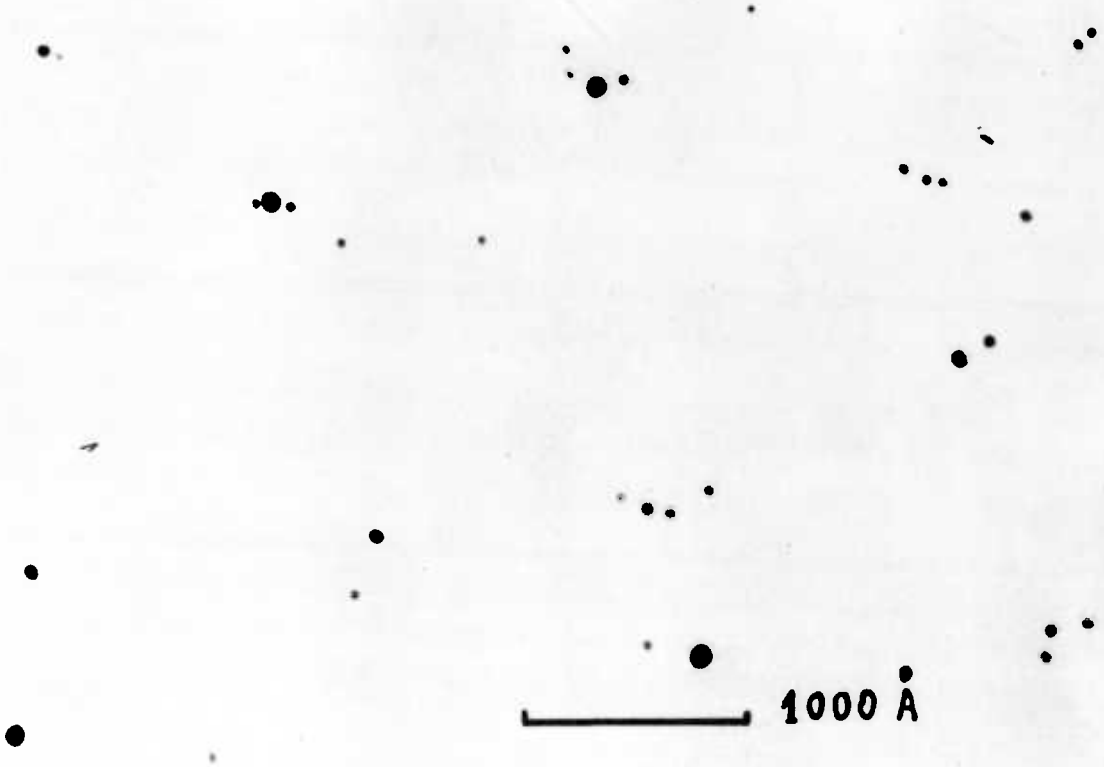

Fig IV-5 : R_2R_2 red cell combined with IgG anti-D and gold-anti-IgG. Approximately 10,000 sites were estimated to be combined. Note the distinct appearance of the gold clusters at very high magnification.

as follows :

SIZE IN ANGSTROMS	PER CENT
140	67
140-375	26
375	7

Several micrographs were analysed to see if the ultrastructural distribution of the D antigenic sites was random. The usual finding was a good fit between the observed and theoretical Poisson distribution, with a probability falling between 0.5 and 0.7. In some cases, at higher uptake of antibody, (more than 10,000 sites on each ghost), the fitn was not as good (i.e. $p = 0.05$). It was concluded that the D antigen sites were randomly distributed on the red cell membrane, when less than 10,000 of the sites were bound to anti-D, but that the evidence also indicated that there might be some aggregation of sites when more than 10,000 of the sites were bound. In Fig. IV-5 is demonstrated the appearance of the gold clusters at very high magnification. The red cell in the photograph was also estimated to have bound approximately 10,000 D sites.

The specificity of the indirect gold-anti-IgG method was demonstrated by the fact that very few gold particles were bound to Rh negative red cells or on Rh positive cells treated with "normal" human IgG without antibodies. One of the control red cells is presented in Fig. IV-6.

D) DISTRIBUTION OF A ANTIGEN SITES

When A_1 red cells were sensitized with IgG anti-A and labelled with gold-anti-IgG on one side only, the labelling characteristics varied according to the amount of antibody coating the cells. At very low

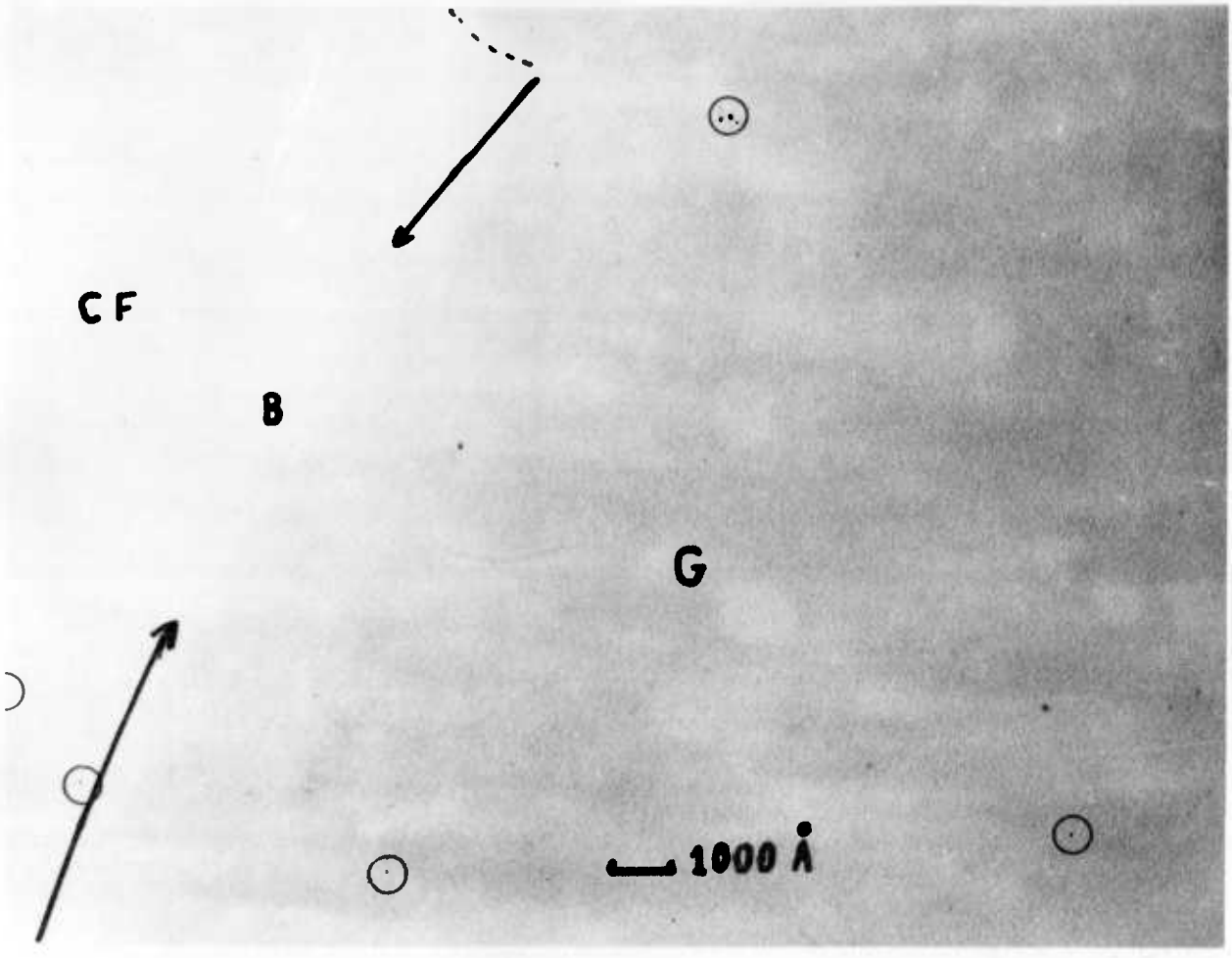

Fig IV-6 : Control ghost from an ORh positive red cell incubated with "normal" IgG and labelled with gold-anti-IgG. CF = carbon film, G = ghost, B = border.

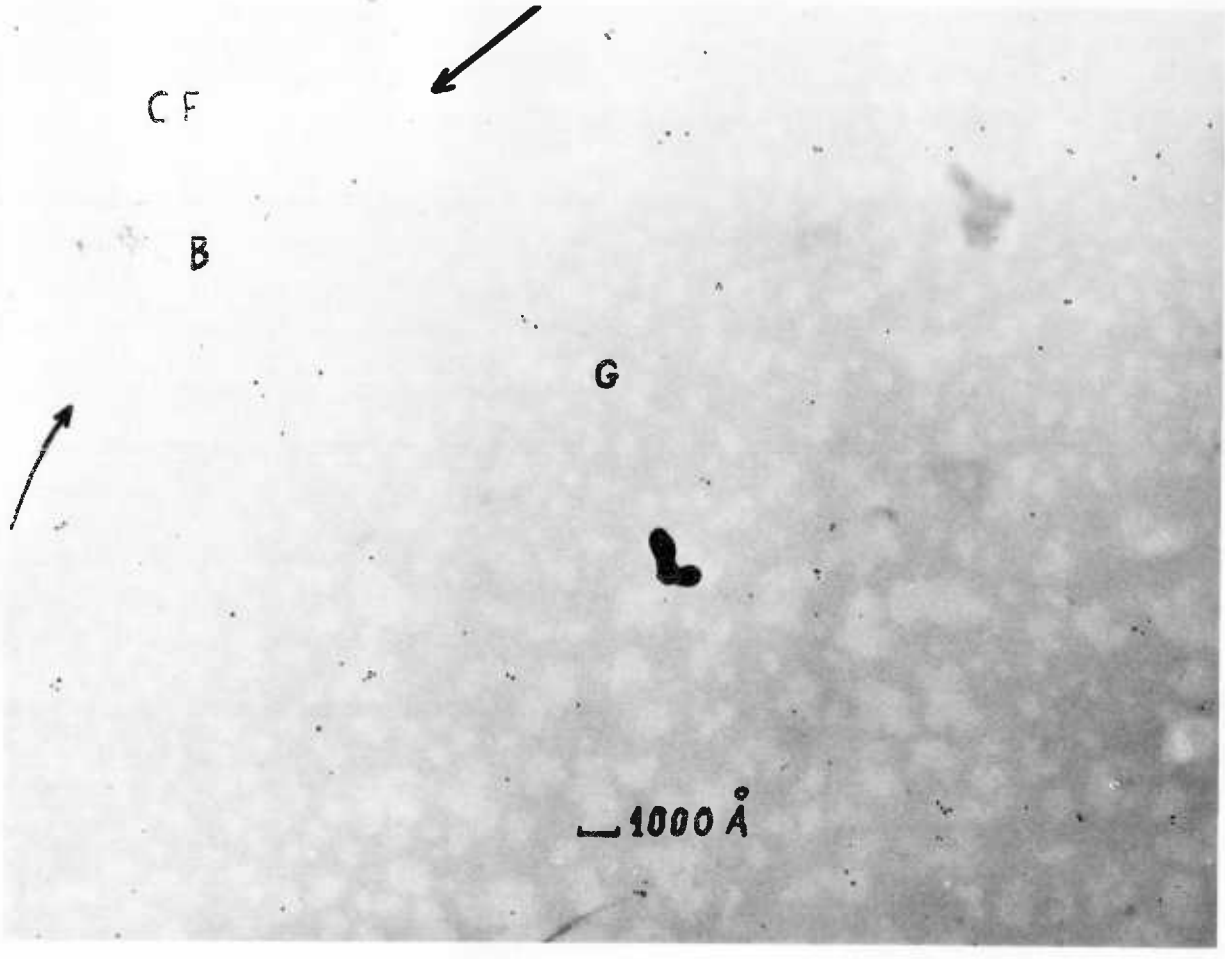

Fig IV-7 : A_1 red cell having approximately 1000 gold clusters. The red cells were not agglutinated by IgG anti-A. Note the similarities with the pattern of labelling observed with Rh positive cells.
CF = carbon film, G = ghost, B = border.

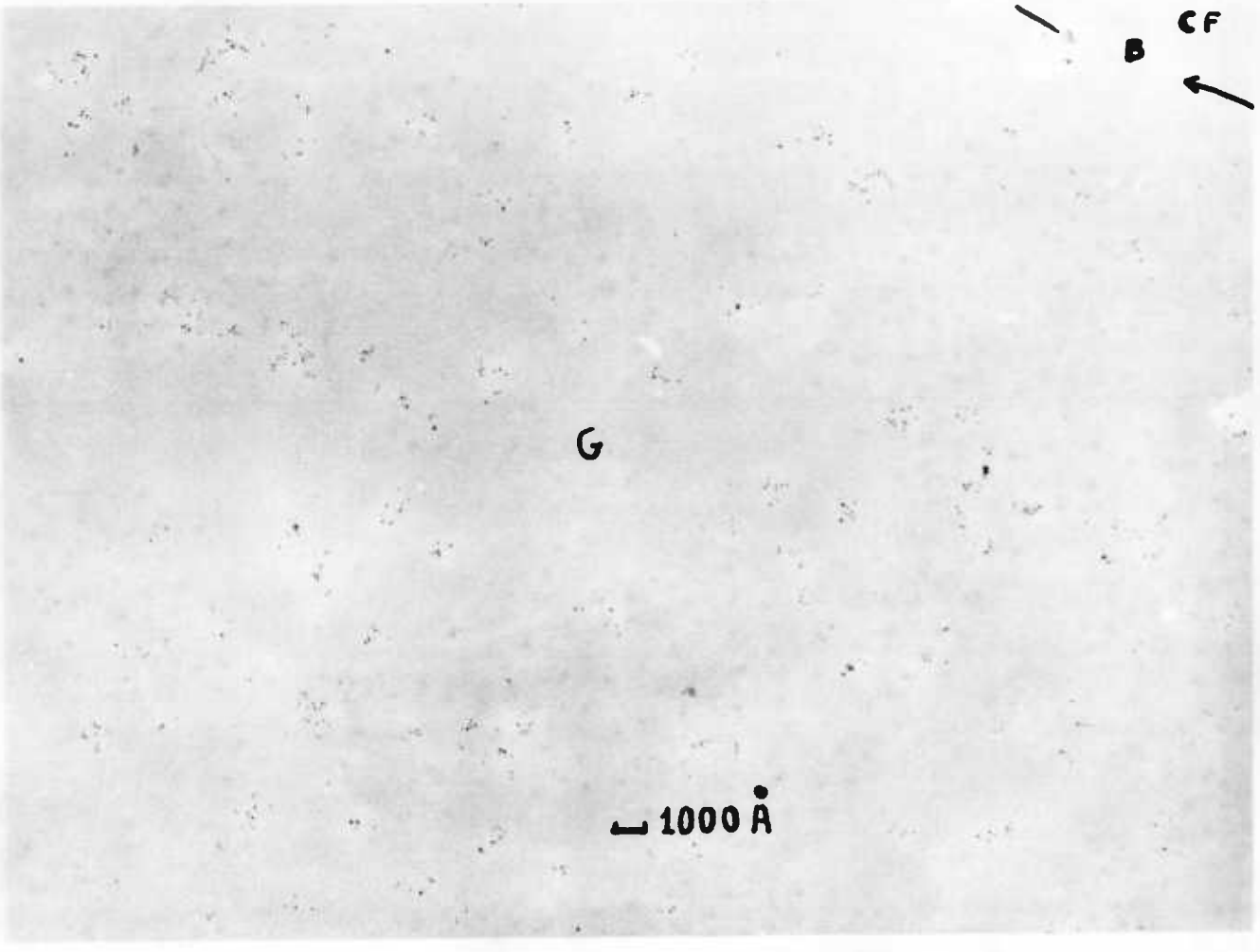

Fig IV-8 : A_1 red cell estimated to have approximately 3,000 to 4,000 gold clusters. The red cells from this experiment were not agglutinated by IgG anti-A. Note the increase in size and number of gold particles of the clusters. CF = carbon film, G = ghost, B = border.

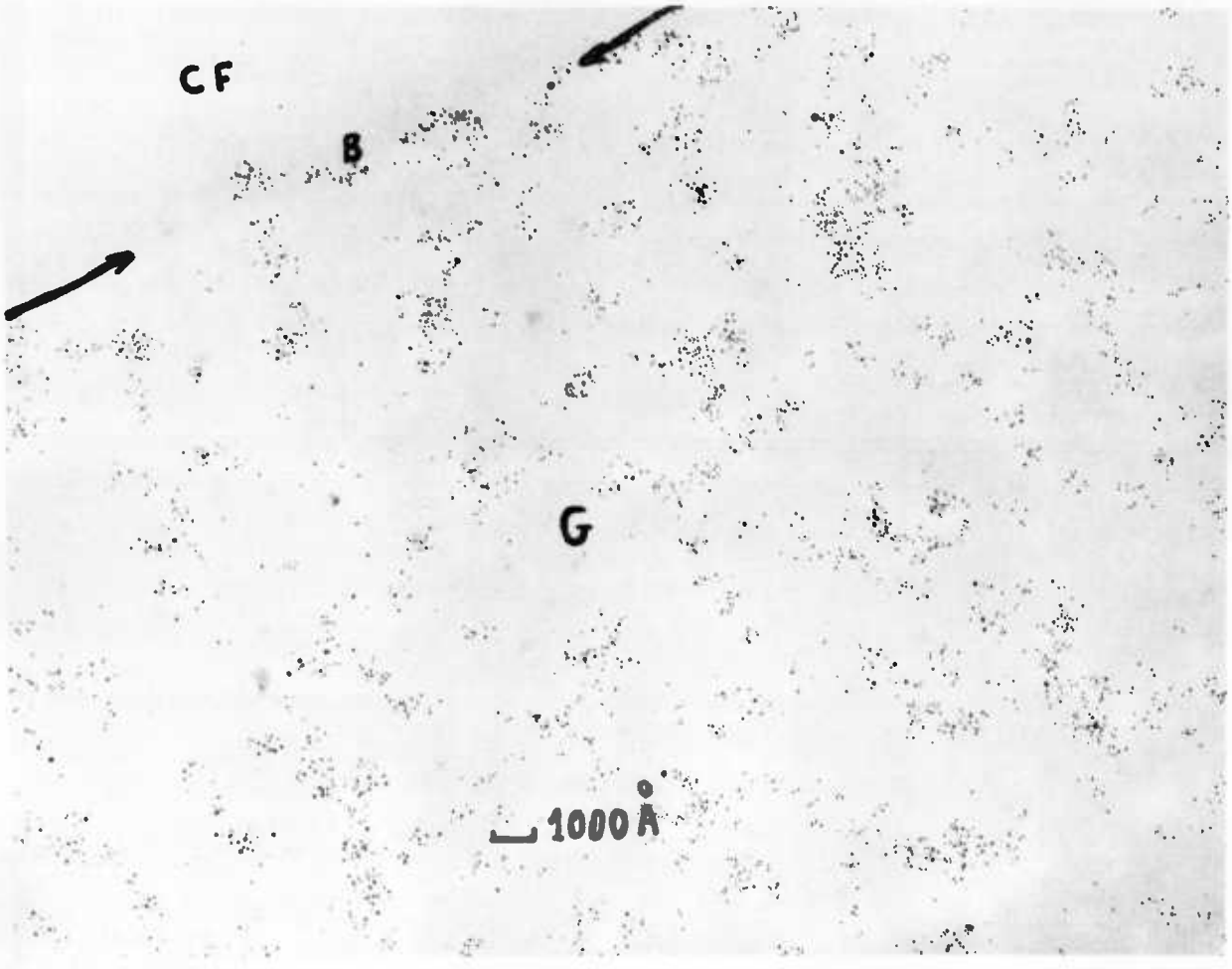

Fig IV-9 : A_1 red cells combined with approximately 15,000 to 20,000 anti-A molecules. The red cells from this experiment were agglutinated before labelling with gold-anti-IgG. Note the large patches of gold labels. CF = carbon film, G = ghost, B = border.

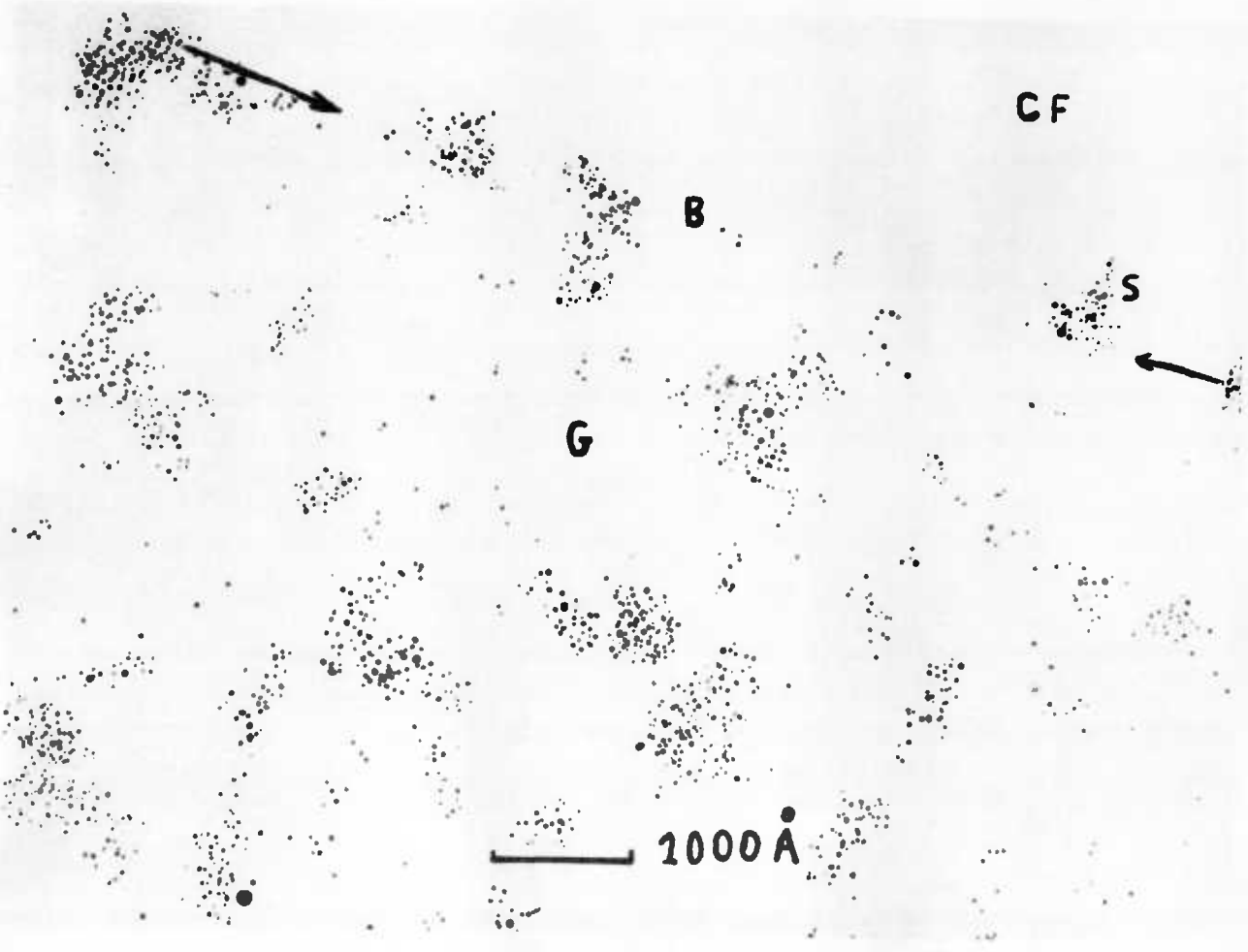

Fig IV-10 : Similar to Fig IV-9 but at higher magnification.

CF = carbon film, G = ghost, B = border, S = a protruding spicule.

uptake of anti-A i.e. 1000 molecules per cell, the label was found to be distributed in small groups of 1-4 gold particles on the red cell membrane, resembling the distribution of D antigen sites, the only difference being that in the case of A antigen sites the small gold clusters were not randomly dispersed on the red cell membrane as in the case of the D antigen sites. Fig. IV-7 demonstrates an A_1 red cell in which approximately only 1000 gold clusters per cell were found.

At 4-6 times greater uptake of anti-A (still insufficient to bring about agglutination) larger groups containing 10 or more particles were frequently seen indicating that probably some clustering of A antigen sites had occurred. This is shown in Fig. IV-8. When the A_1 red cells were sensitized with IgG anti-A at a concentration sufficient to cause agglutination, the label was found to be distributed in large patches over the red cell membrane. This is demonstrated in Fig. IV-9 and in Fig. IV-10, at a higher magnification.

With greater uptake of anti-A, the size of the clusters also increased up to the point in which the gold clusters fused with each other giving a reticular appearance and it was no longer possible to define clusters. At this stage, for obvious reasons, it was very difficult to decide the percentage of saturation of A sites with anti-A although an approximation could be obtained by assuming that gold-anti-IgG reacted with anti-A to the same extent as with anti-D. Hence, assuming that 2.7 gold particles represent the average number of anti-IgG molecules bound to a single anti-A molecule (and therefore to 1 A antigen site), by counting all gold particles on a given area of surface

and dividing the figure by 2.7, the approximate number of anti-A molecules in that area can be determined. Figs. 11 and 12 demonstrate the labelling characteristics at high concentration of IgG anti-A. From the previous assumptions it was estimated that 250,000 sites/cell were bound in Fig.11 and about 100,000 sites/cell in Fig.12. As both figures show, at these levels of saturation it is no longer possible to decide what is a cluster. On the other hand, the non random aggregated distribution of A_1 sites, can be clearly seen.

E) MOBILITY OF A AND D ANTIGEN SITES

1) MOBILITY OF A SITES

It is known that A antigens are located in (or associated with) the 85 ångströms membrane-intercalated particles of the red cell membrane, as seen with the freeze fracture technique (Pinto da Silva *et al.*, 1971). Pinto da Silva (1972) also demonstrated that these membrane particles were dispersed at pH 7.5 and that they could be aggregated by lowering the pH to 5.5. Pinto da Silva also found that the aggregation was reversible and that aggregation did not take place after glutaraldehyde fixation. It is also known that the intercalated particles contain acidic anionic residues which can be labelled with colloidal iron hydroxide (Nicolson, 1973a) and cationized ferritin (Pinto da Silva, *et al* 1973). Nicolson (1973a) confirmed Pinto da Silva's findings showing that the anionic sites were randomly dispersed under normal circumstances and that they could be aggregated by lowering the pH to 5.5 and also by treating the red cell membrane with proteases and phospholipase C.

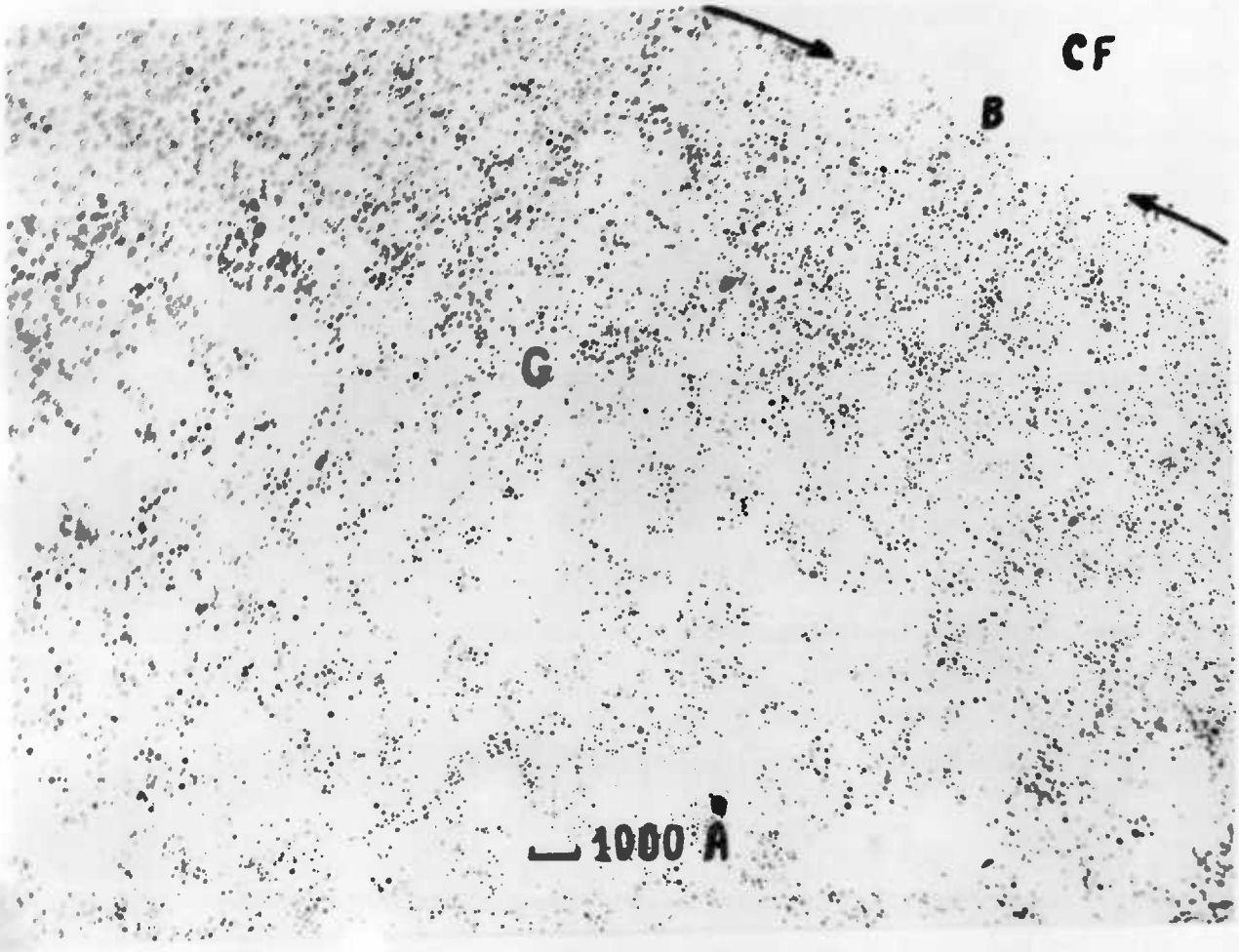

Fig IV-11 : A_1 red cells labelled with large amounts of anti-A (approx. 250,000 sites/red cell) followed by gold-anti-IgG.

CF = carbon film, G = ghost, B = border.

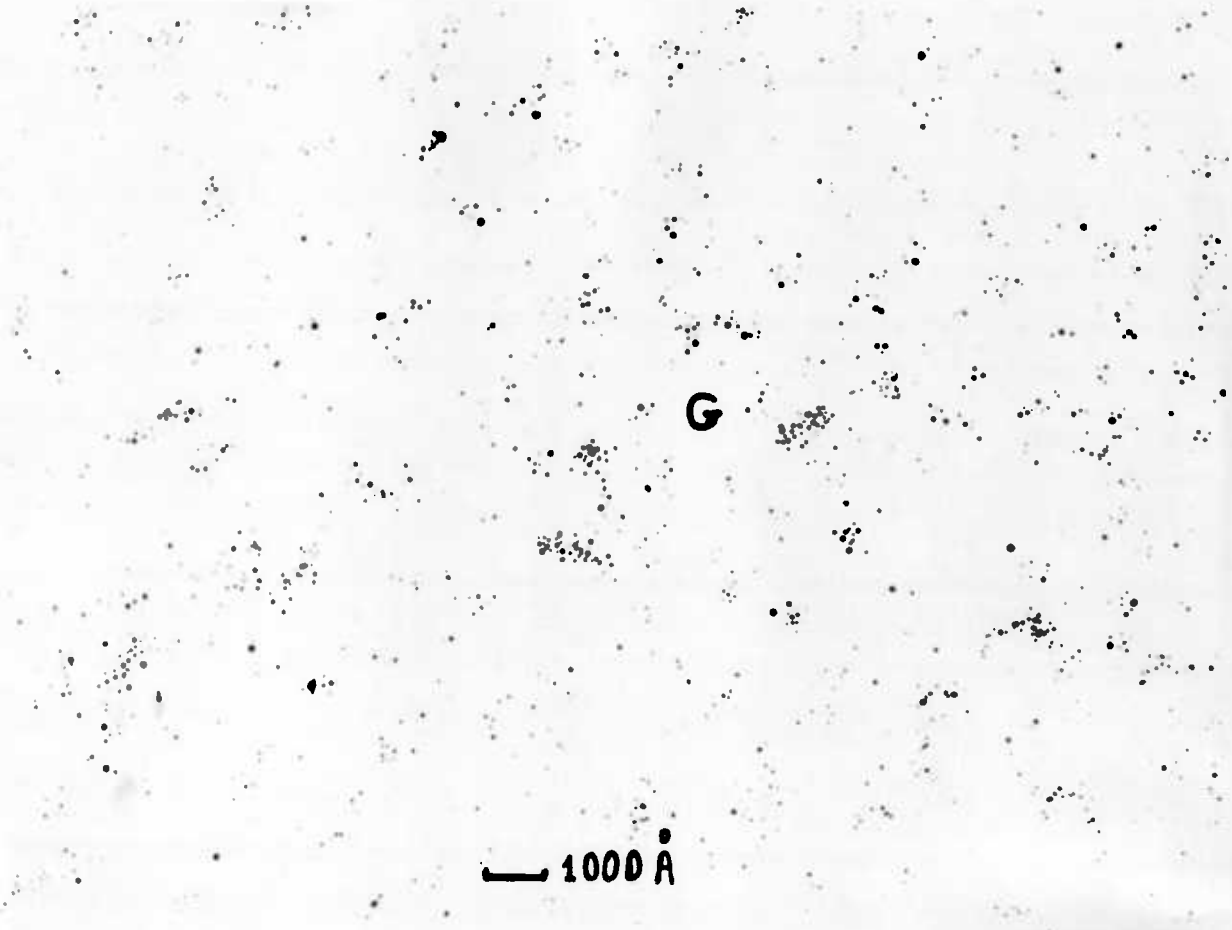

Fig IV-12 : A_1 red cell combined with approximately 100,000 anti-A molecules. Note the reticular aspect of the gold-anti-IgG .
G = ghost.

From the above considerations it can be inferred that under normal conditions A antigens are randomly dispersed on the red cell membrane. The experiments described here on mapping the A antigen sites revealed that the distribution was not random but aggregated and that the degree of aggregation was increased when the uptake of antibody was high. Therefore, it is concluded that the redistribution of sites observed at high anti-A uptake was antibody-induced. As we were using two antibodies, i.e. anti-A and anti-IgG, the question arises whether it was the anti-A or the anti-A plus gold-anti-IgG which produced the aggregation of the sites. Experiments in which A_1 cells coated with IgG anti-A were fixed with glutaraldehyde or methylglyoxal prior to the labelling with gold-anti-IgG, demonstrated that IgG anti-A alone could aggregate the A antigen sites. The result of one of these experiments in which red cells coated with anti-A were fixed before labelling with gold-anti-IgG, is demonstrated in Fig. 13. When the red cell and IgG anti-A were first incubated at pH 5.5, then fixed and labelled with gold-anti-IgG, the pattern of labelling was not different, as can be seen in Fig. 14. The same degree of aggregation was also obtained by indirectly labelling A sites on papain-treated (Löw, 1955) red cells.

2) MOBILITY OF D ANTIGEN SITES

There was no evidence of aggregation of D antigen sites induced by IgG anti-D or IgG anti-D plus gold-anti-IgG in untreated Rh-positive red cells; except when more than 10,000 sites were combined with anti-D in which case, evidence for aggregation of sites was occasionally obtained.

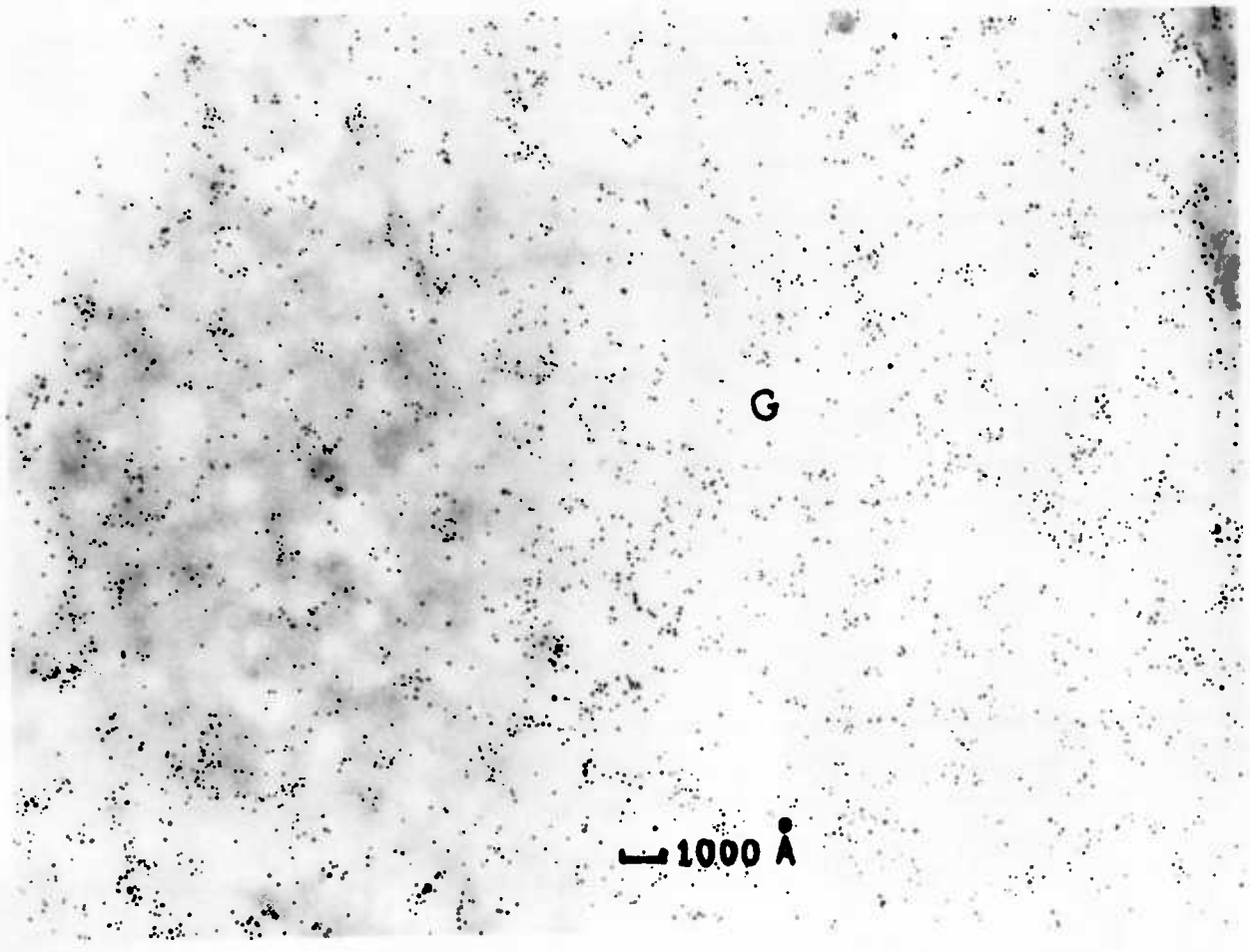

Fig IV-13 : A_1 red cell combined with IgG anti-A, and fixed with Methyl glyoxal before labelling with gold-anti-IgG. A sites were already aggregated by anti-A when the red cell ghost was incubated with gold-anti-IgG. G = ghost.

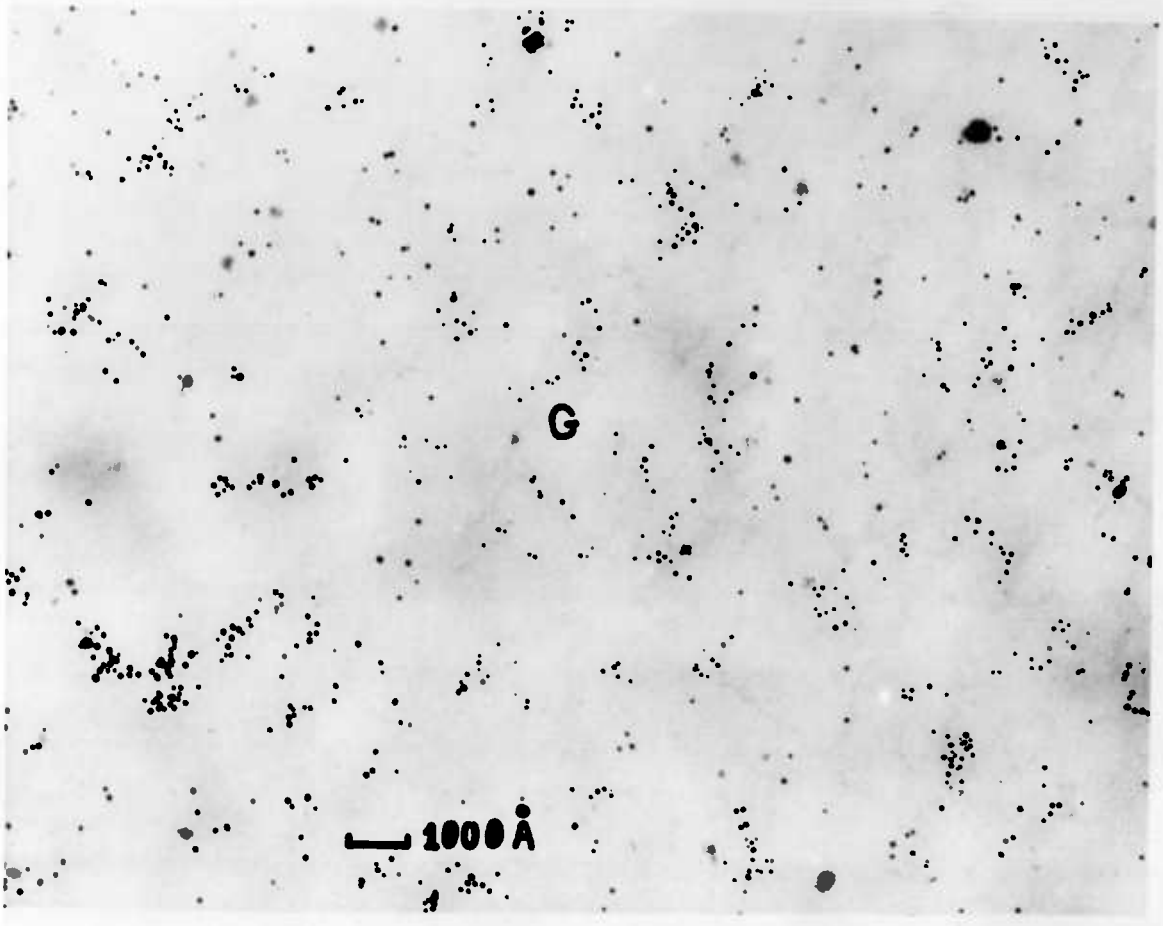

Fig IV-14 : A_1 red cell incubated at pH 5.5, combined with anti-A and fixed at that pH before labelling with gold-anti-IgG. The observed aggregation of A sites is of comparable degree to that observed in Figs. IV-12 and IV-13.

When R_1R_2 red cells were treated with papain followed by sensitization with IgG anti-D and labelling with gold-anti-IgG, the distribution of the label was found to be quite different: large patches of label were observed which clearly suggested that the papain treatment brought about the aggregation of the D antigen sites (Fig. 15).

DISTRIBUTION OF D-ANTIGEN SITES IN -D-/-D- CELLS

-D- is a very rare rhesus phenotype in which the only antigen expressed is D. The D antigen content of this type of cell is much higher than usual : 110,000 - 202,000 per cell (Hughes-Jones et al., 1971) and these cells are agglutinated in a saline medium by IgG anti-D (Race et al., 1950).

Cells from a -D-/-D- donor which had been kept frozen in glycerol at a temperature of -40° for 4 years were used to map the D sites. Glycerol was dialysed out against normal saline and the cells were washed twice in normal saline before being used.

The distribution of the D antigen sites in this type of cell is shown in Fig. 16. It can be seen that the label appears aggregated in large patches resembling the pattern of distribution of A sites. It was estimated that about 75,000 D antigen sites/cell combined with anti-D were indirectly labelled with gold-anti-IgG.

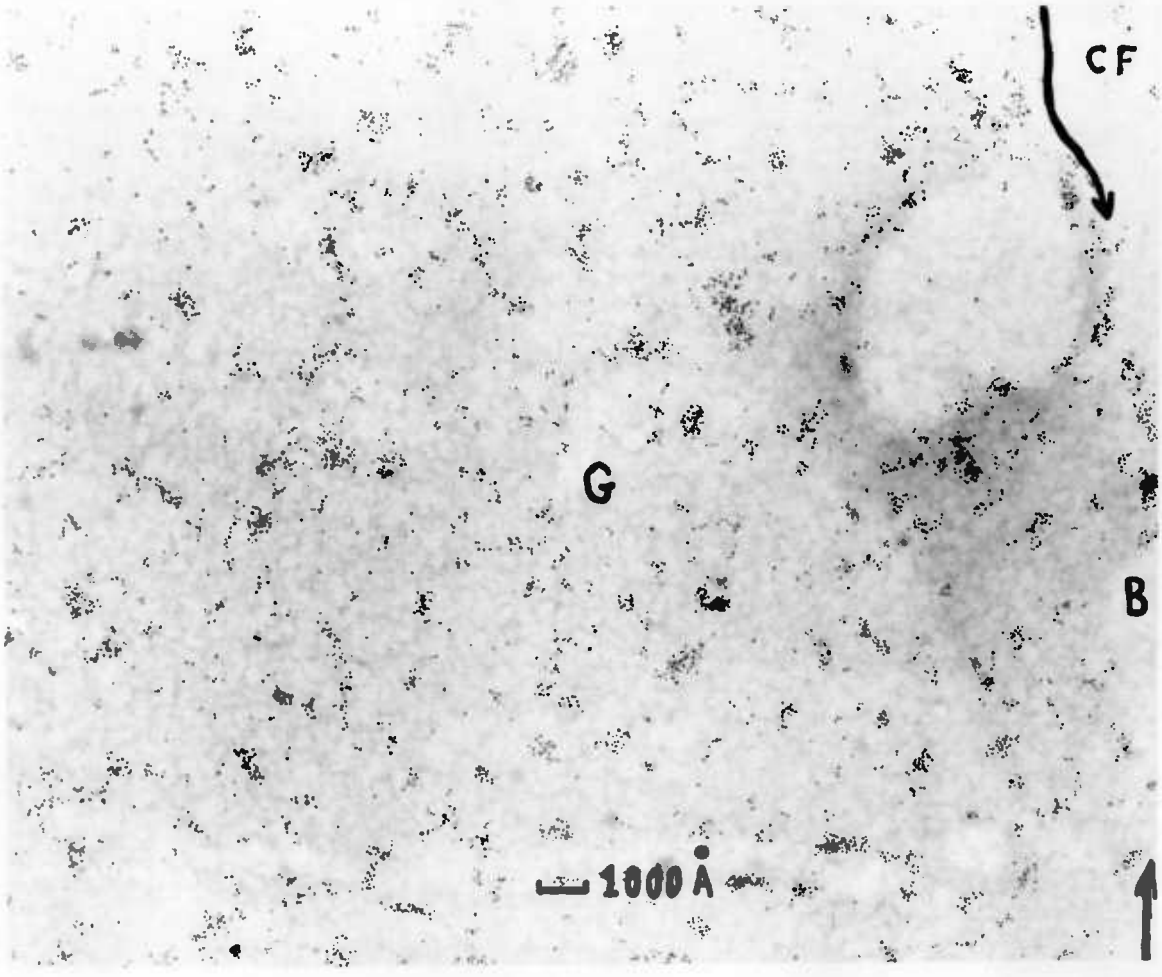

Fig IV-15 : R_1R_2 red cell treated with papain, combined with anti-D and labelled with gold. The observed aggregation of D sites contrast with the dispersed distribution observed in Figs IV-2 to IV-5. CF = carbon film, G = ghost, B = border.

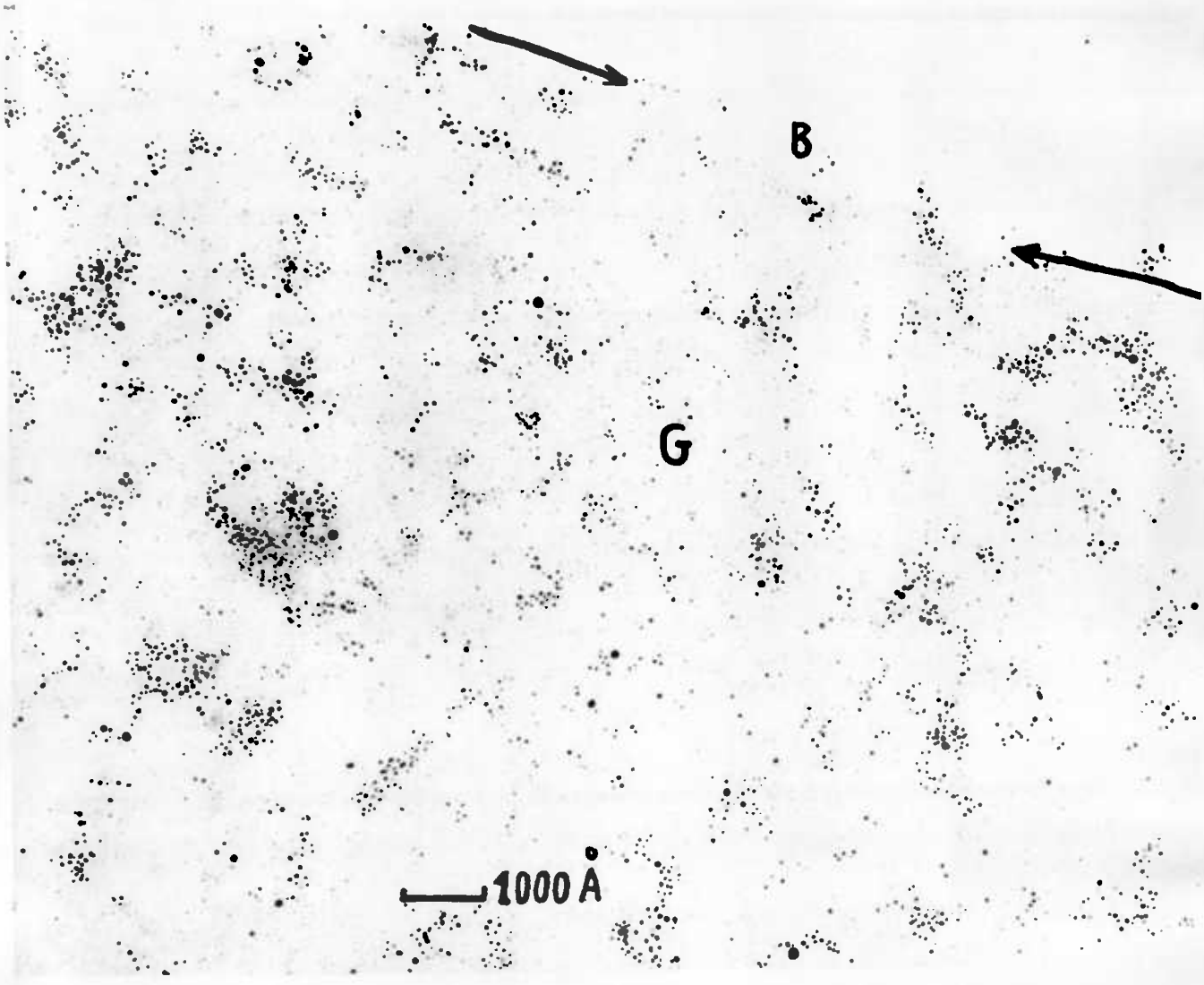

Fig IV-16 : Pattern of labelling of -D-/-D- red cells combined with IgG anti-D and labelled with gold-anti-IgG. The -D- red cells were agglutinated by anti-D. The observed aggregated distribution of D sites resembles the distribution of D sites in papain-treated cells and the distribution of A sites at a high degree of saturation with anti-A.

IV DISCUSSION

A) DOES HAEMOGLOBIN STABILIZE THE RED CELL MEMBRANE?

Red cell ghosts that were relatively free of haemoglobin (i.e. the least electron dense), had frequently a "spotty" aspect that was interpreted as membrane disruption because of the irregular appearance of the membrane and also because pieces of broken-up ghosts always appeared to be free of haemoglobin (Fig. IV-1).

Although there is no doubt that haemoglobin is not a structural component of the red cell membrane, it is very difficult to eliminate all the haemoglobin when preparing red cell ghosts, (for a review see Hanahan, 1973). Winterbourne and Carrell (1973) produced evidence that Heinz-bodies (aggregated and precipitated haemoglobin) are not attached to the red cell membrane by covalent or ionic bonds and they suggested hydrophobic interactions between haemoglobin and the red cell membrane as a likely explanation for the binding of the Heinz-bodies to the red cell membrane. Though our evidence is tenuous, the fact that ghosts which were free of haemoglobin were frequently broken suggests that haemoglobin stabilizes the red cell membrane by interacting with components of the membrane, probably with the protein spectrin. Spectrin, located in the cytoplasmic side of the red cell membrane, is known to be important in the regulation of the structural organization of the red cell membrane. For example, in ghosts, the major sialoglycoprotein of the red cell membrane can be aggregated by cross-linking anti-spectrin antibodies (Nicolson and Painter, 1973) or by lowering the pH to 5.5 (Nicolson, 1973a) which

is the isoloectric pH of spectrin. The redistribution of the sialoglycoprotein, or of other red cell membrane components, to my knowledge, have not been reported in intact red cells but only on ghosts. If haemoglobin by interacting with spectrin (or any other similar protein in the cytoplasmic side of the membrane) has a protective role in the structural organisation of the red cell membrane, then different results may be obtained when intact red cells, instead of ghosts, are used as in experiments similar to those described by Nicolson and Painter (1973) or Nicolson (1973a). Therefore, it would be interesting to find out whether or not the membrane-intercalated particles* can be aggregated in freeze-etching experiments when intact red cells are used; similarly it would be of interest to try to aggregate the anionic residues in intact red cells when the colloidal iron hydroxide technique is used.

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*The 85 ångström membrane-intercalated (or associated, or inner, or intramembranous) particles as observed with the technique of freeze fracture-or both freeze fracture and etching- (Pinto da Silva and Branton, 1970; Pinto da Silva, 1972), have been shown to correspond to the anionic residues by Pinto da Silva et al., 1973; and to the major sialoglycoprotein by Tillack et al., 1972. Therefore, membrane-intercalated particles (freeze-etching), anionic residues (labelled with cationized ferritin or colloidal iron hydroxide) and major sialoglycoprotein probably refer to the same molecular structure (see for a review, Steck, 1974).

An inability to bring about aggregation in the intact cell would provide further supporting evidence for a possible indirect role for haemoglobin on the structural organization of the erythrocyte membrane.

An alternate explanation for the apparent protective effect of haemoglobin on the red cell membrane structure would be that concomitantly with haemoglobin, some other important structural protein is lost in the process of making ghosts.

B) PREPARATION OF SPECIMENS FOR ELECTRON MICROSCOPY

The method used for preparation of red cells for microscopy was that described by Nicolson and Singer (1971), incorporating minor variations, as detailed in the previous chapter.

In this method, ghosts were first attached to a grid coated with a supporting material and then labelled with the gold-anti-IgG. This way of preparing the specimen has a great advantage over the conventional embedding and sectioning technique for studies of surface distribution: a two dimensional visualization of the label over the entire available surface of the cell is obtained allowing the analysis of large surface areas as opposed to the visualization of small surface areas when thin sections are analysed.

C) DISTRIBUTION OF D SITES

Lee and Feldman (1964) described for the first time the visualization of D sites using a conjugate of anti-D with ferritin. They noticed a fairly constant distance between each ferritin particle which

would suggest a regular pattern of distribution. However, as only a few ferritin particles were visualized in their preparations, no valid conclusion could be drawn from their experiments.

Davis et al. (1968) used anti-human gamma globulin conjugated with ferritin to visualize D sites. They concluded that in R_1R_2 red cells, D sites were regularly spaced with about 0.25 μm separating each other. As pointed out by Mollison (1972, p.270) this would mean that the total number of sites would be much less than the known figure from experiments using radioactive labelled antibodies.

Nicolson et al. (1971) pointed out how misleading was the analysis of micrographs of thin section due to the small number of sites visualized, essentially in only one dimension. These authors, also used anti-human gamma globulin conjugated with ferritin but their novel approach of looking at the distribution of the label over the entire available surface of the ghosts, allowed them to conclude that the distribution of D antigen sites was aperiodic (i.e. irregular spacing between antigen sites) and random.

In the present work, a gold-anti-IgG reagent was used to visualize D antigen sites on the side of the ghosts exposed to the labelling reagent. The results are essentially similar to those of Nicolson et al. (1971) in that the two-dimensional distribution of sites was random and that there was no regular spacing separating them, although when the distance separating nearest neighbouring gold clusters was measured, a value between 0.10 and 0.13 μm was commonly observed in R_1- cells.

This would explain why a periodic i.e. regularly spaced arrangement of sites could be seen when thin sections were analysed, as in the case of Davis et al. (1968).

Another important point to decide is if D antigens exist as single sites, or whether they are grouped in pairs or even larger groups; i.e. how many D antigen sites there are per each molecule carrying Rh antigen. From the results of Nicolson (1971) and the present work, it seems clear that the answer is one antigen site per Rh molecule. In the present work there are 2 pieces of evidence which would suggest that D-antigen sites exist as single discrete entities and not as groups of 2 or more. First, there is the evidence obtained from an analysis of the number of gold particles in each group as follows: About 96 per cent of gold groups on the ghost surface contained between 1 and 5 gold particles, with an average value of 2.7 particles per group. Now an analysis of the micrographs of the gold-anti-IgG itself (Table III-1) shows that about 91 per cent of the anti-IgG molecules were labelled with 1-3 gold particles, with an average of 1.5 gold particles anti-IgG molecule (exluding aggregates). There data suggest that there were an average of 1.8 (2.7/1.5) anti-IgG molecules present at each cluster of gold particles on the ghost surface. Further studies carried out with ^{131}I-labelled anti-IgG (the same anti-IgG as was used for gold labelling) showed that on average about 2 molecules of anti-IgG reacted with 1 molecule of ^{125}I-anti-D attached to the red cell surface under the same conditions. If we assume that the gold-anti-IgG reacts to the same extent with anti-D as the ^{131}I-labelled anti-IgG, then this quantitative analysis suggests that just one D antigen site is present under each cluster of gold particles.

Secondly, there is the evidence based on the size of the gold clusters. When the area covered by each group was measured, it was found that approximately 67 per cent had a maximum size of 140 ångströms or less, and about 93 per cent were found to have less than 375 ångströms. If it is assumed that the maximum size of an IgG molecule is about 140 ångströms (Green, 1969), then within an area of 0 to 375 ångströms there is room only for 1 to 3 anti-IgG molecules. As with radio-labelled antibodies, an average of 2 anti-IgG molecules were found to combine with one anti-D molecule, then it can be concluded that each gold cluster represent 1 to 3 gold-anti-IgG bound to 1 IgG anti-D, itself combined to a single D antigen site. The remaining 7 per cent of the groups which were larger than 375 ångströms in size are more difficult to interpret. It is thought that these groups represent aggregates of gold-anti-IgG or of IgG anti-D or both. Nevertheless, with the technique used here it is difficult to rule out the possibility that some D antigen sites may exist in pairs. It can also be argued that the sites are in pairs (or in small groups) but the binding of one antibody inhibits the binding of another to the neighbours antigen, because of steric hindrance. However, this is unlikely in view of the fact that red cells were found to bind the same number of the small univalent Fab anti-D fragments as the larger whole IgG anti-D molecule (Hughes-Jones, 1970).

D) THE DISTRIBUTION OF D SITES ON -D-/-D- RED CELLS

On cells of phenotype -D- the gold particles were found to be distributed in an irregular and aggregated fashion (Fig. 16) indicating that the D antigen sites are also aggregated. The question arises as to whether the D antigen is always present as aggregates in the -D- phenotype or whether aggregation is brought about by the action of the anti-D combined to the red cells as with the A antigen and anti-A (see page 158

One possible approach to answer this question would be to fix -D- cells which have been reacted with IgG anti-D prior to labelling with gold-anti-IgG. However, when an experiment of this kind was attempted, it was found that only a few gold-anti-IgG molecules remained bound to the cells, suggesting that most of the anti-D molecules came off the cells, probably due to inactivation of the D antigen activity by the fixative used (glutaraldehyde).

In the -D- cells, only the D antigen is expressed and the total number of antigenic sites is approximately the same as the sum of all the other antigens of the Rh system (Hughes-Jones et al., 1971). Now, if the theory that postulates that several antigens - "factors" - of the Rh system may coexist on the same molecule (Wiener, 1951) is correct, then it is possible that several D antigens are present on a single molecule in the case of -D- cells, and when these cells are labelled, this may result in the binding of more than one IgG anti-D antibody on each single molecule of Rh substance and to the binding of IgG anti-D through both Fab pieces which may lead to aggregation of the antigen-containing molecules. If the suggestion that D-antigen is present in groups in -D- cells is correct, then it will also offer a molecular explanation for the greater agglutinability of -D- cells, due to a "facilitated" cross-linking by IgG anti-D. This "facilitated" cross-linking, according to Nicolson (1973b) occurs when the antigen is distributed in clusters on the red cell surface. From the above considerations, it seems that an informative experiment, which we have not attempted yet, would be to investigate the distribution of the D sites on -D- cells which have been reacted with Fab anti-D as opposed to IgG anti-D.

DISTRIBUTION OF A SITES

Harris (1964) and Lee and Feldman (1967) used ferritin conjugated with anti-A globulin to visualize A antigen sites on adult group A red cells. The results obtained by Harris were difficult to interpret and no firm conclusions could be derived from his studies. Lee and Feldman produced micrographs of thin sections of group A and B labelled red cells with corresponding antibody-ferritin conjugates. Ferritin molecules were found to be distributed in clusters i.e. several ferritin molecules at each site, which were spaced as to suggest a regular pattern of distribution. However, looking at their figures 1 and 2 it can be seen that the spacing separating the ferritin clusters, was variable. Another point is that when figures 1 and 2 of their report are compared with figure 6 of the same paper (which is a print of a D cell exposed to ferritin anti-D) then it seems clear that each cluster of ferritin anti-A conjugate in figures 1 and 2 should represent several antigen sites because in the case of the D positive cells the antigen sites were molecularly dispersed (i.e. one D site per one ferritin particle, see fig. 6). Moreover, as the A sites were directly labelled with ferritin anti-A, if anti-A and ferritin were conjugated at a ratio of about 1 anti-A per 1-2 ferritin molecules then one A antigen site should be labelled by only 1 or 2 ferritin molecules, but Lee and Feldman's figs. 1 and 2 show many more. Thus, the distribution of ferritin seen in figs. 1 and 2 of their paper could also be interpreted as indicating that A sites exist as groups.

Voak and Williams (1971) studied the distribution of A sites in red cells from adults and infants. They used a ferritin IgG anti-A conjugate and a thin section technique for electron microscope specimen

preparation. These authors did a statistical analysis of their data and concluded that the distribution of sites was non-random and that this non-randomness appeared to originate from clustering together of sites. They did not observe large patches of ferritin presumably because of a rather scanty labelling. In a subsequent paper, Williams and Voak (1972) conjugated the lectin Dolichos biflorus with ferritin and studied the distribution of A sites in A_1 and A_2 red cells. They found essentially the same non-random, 'contagious', distribution of sites that they observed in their previous work.

In the present work, A antigen sites of red cells sensitized with IgG anti-A were indirectly visualized with gold-anti-IgG. At low uptake of anti-A, when about 1000-5000 gold clusters were present per cell, it was found that on the average there were about twice as many gold particles per cluster as in the case of the D antigen sites. As it is very likely that the D antigens are present as one site per Rh blood group substance, then it should be concluded that, even at that low saturation of A sites, on the average there were 2 A sites represented by each gold cluster. At greater uptake of anti-A (see Figs. IV-9 to IV-12) the distribution of sites became increasingly aggregated, being no longer possible to identify clusters. At saturation of 100,000 sites/cell or more, the distribution of sites appeared highly aggregated, indicating that the visualized A sites were close together in the red cell membrane.

Another point regarding the distribution of A sites was to assess if they were truly aggregated or whether the binding of one anti-A molecule was influencing the binding of subsequent ones so that the second antibody bound close to the first one, as suggested by Voak and Williams, 1971. To assess the matter further, we did the following simple calculations:

The total number of antigen sites in an A_1 red cell is about 1×10^6 (Greenbury, 1963; Economidou et al., 1967). If the assumptions are made that each antigen is in a unit square and that the total red cell surface is 145×10^6 nm^2, then the average distance 'a' separating the sites can be estimated as follows:

If

N = total number of sites = 1×10^6 = total number of unit squares

S = Surface = 145×10^6 nm^2 = LxL = L^2

a = average distance separating neighbouring clusters

Then,

$$N = \frac{L}{a} \times \frac{L}{a} = \frac{L^2}{a^2} = \frac{S}{a^2}$$

$$a = \sqrt{\frac{S}{N}} = 12 \text{ nm}$$

Thus, if there was no aggregation of A antigen sites i.e. they were distributed at random, then an average distance of 12 nm should separate them. However, examination of Figs. 9 to 12 shows that the distance which separates gold particles within the aggregates of label is frequently only 2 to 4 nm. This suggests that the A antigens are aggregated together under these conditions.

Matsukura (1972) demonstrated that A cells fixed with glutaraldehyde preserve their A antigenic activity as shown by their ability to bind with ferritin-anti-A conjugate. This author carried out his studies using thin sections and unfortunately gave no data regarding the distribution of the antigen sites. This would be of interest because when the cells are fixed with glutaraldehyde before labelling, the distribution of the membrane protein components is "frozen" and therefore one would expect a random, non-aggregated distribution of the A sites. Consequently, attempts were made to study the distribution of A sites on cells which were fixed with glutaraldehyde before being incubated with anti-A. Unfortunately, there was a very high nonspecific uptake of anti-A onto group O or B controls. Furthermore, it proved very difficult to produce suitable ghosts from glutaraldehyde-fixed cells because it was observed that in glutaraldehyde-fixed cells, haemoglobin is cross-linked to the membrane, making the resulting ghosts very electron dense and thus obscuring the visualization of the gold clusters.

A question that arises is how certain is the evidence that the A-antigen sites are located in the membrane-intercalated particles and therefore on the major glycoprotein. This point is brought up because both glycolipid (Ando and Yamakawa, 1973; Hakomori and Strycharz, 1968; Koscielak, 1963) and glycoprotein (Marchesi and Andrews, 1971; Hamaguchi and Cleve, 1972; Whittemore et al., 1969) preparations with A activity have been isolated from appropriate red cell stroma. Pinto da Silva et al. (1971) have produced evidence for the association between A_1 antigen sites and membrane-intercalated particles. Marchesi et al. (1972) also showed that the major glycoprotein of the red cell membrane, glycophorin, had A blood group activity and receptors for

Phytohemagglutinin (PHA). Using PHA-ferritin conjugate, Marchesi et al. (1972) also showed that the PHA receptors were associated with the membrane-intercalated particles as seen in freeze-etched human erytrocytes ghosts, thus suggesting an association between A-antigenic activity, membrane-intercalated particles and the major glycoprotein. In view of this association, an explanation should be found of how or why glycolipids molecules with A blood group activity can be associated with the major glycoprotein. As these A glycolipids cannot be a structural part of the major glycoprotein, it should be postulated that they are tightly associated with the major glycoprotein (and hence with the membrane-intercalated particles). Some evidence to support the view of a tight association between A glycolipids and major glycoprotein has been provided by the results of Ando and Yamakawa (1973) and Brennessel and Goldstein (1974). Ando and Yamakawa have isolated from human red cell polar glycolipid with A activity; the polar nature of their purified A cellular substance may well explain an association with the major glycoprotein. Similarly, Brennessel and Goldstein (1974), found tightly associated glycolipids with H activity to glycoproteins with M and N activity (M, N antigens are located on the major glycoprotein) which had been isolated either by Marchesi's and Andrews and Hamaguchi's and Cleve's methods for isolation of red cell glycoproteins.

MOBILITY OF RBC MEMBRANE ANTIGENS

Singer and Nicolson (1972) presented a model of membrane structure in which the lipid and protein components are in a fluid state. Interactions of a hydrophobic type between protein "mosaics" and lipids are possible because of an asymetric structure of the protein components which have a hydrophobic domain running into the lipid bilayer and a hydrophilic region directed to the aqueous phase; carbohydrates are attached to this latter region. This model has been widely accepted and one important prediction is the possibility of free diffusion of membrane components in the plane of the membrane.

Though the mobility of red cell membrane components may not be as dramatic as the capping phenomenom observed in lymphocytes, it has been shown that under various circumstances several membrane components are mobile, i.e., the major sialoglycoprotein (and therefore the membrane-intercalated particles with which it has been associated, Pinto da Silva et al., 1971; Marchesi et al., 1972; Tillack et al., 1972; Nicolson, 1973a), Con A receptors (Nicolson, 1973b), D antigens on -D- cells (this work) and D antigens on red cell treated with proteases (Nicolson, 1972; this work).

The present observations indicate that A sites, when coated with IgG anti-A and labelled with gold-anti-IgG, are distributed in an aggregated fashion and that this aggregation becomes more pronounced with increasing amounts of bound antibody. As A sites are either located on, or associated with, the major glycoprotein of the red cell

membrane, which is normally randomly distributed, then it can be concluded that these sites had been aggregated by the bound antibodies. This finding confirms Nicolson's observations (1973b) regarding a change in the surface distribution of the sialoglycoprotein in group B red cells ghosts after treatment with anti-B immunoglobulins. The author visualized the sialoglycoproteins by staining them with colloidal iron hydroxide. In the present work, the binding of antibody to antigen was performed by incubating intact, whole red cells with corresponding immunoglobulins whereas in Nicolson's work it was done on ghosts. In the present work the second stage of labelling with gold-anti-IgG was performed on ghosts immediately after they were produced, but it was found that the gold-labelling of sensitized ghosts (which were fixed with glutaraldehyde before labelling with gold-anti-IgG) did not alter signficantly the distribution of the A antigen sites. The combination of intact red cells with IgG antibodies as opposed to the labelling of ghosts may give different results in view of the findings of the variability in chemical composition of ghosts according to the method of producing them (Hanahan, 1973) and also because of the possible stabilizing role of haemoglobin on the red cell membrane structure.

The present observations confirm previous findings (Nicolson, Masouredis and Singer, 1971) of a random and dispersed molecular distribution of D antigen sites. In the present work, it was also found that the random and dispersed distribution of D-sites was changed to an irregular aggregated one in red cells treated with papain, suggesting that proteolytic treatment allowed redistribution of the D antigen sites. In addition, some aggregation of D sites was occasionally observed in cells having more than 10,000 sites combined with anti-D.

It was very interesting to observe that in -D- cells the topographic distribution of D sites was aggregated. We do not know what is the normal distribution of these sites without the bound antibody; the fluid model of membrane structure predicts a random distribution of membrane components (Singer and Nicolson, 1972) and if this is the case, then it is likely that the antigen sites were aggregated by the bound anti-D.

An interesting point to clarify would be the mechanism of aggregation of the antigen sites and other surface membrane components. In this respect, Nicolson and Painter (1973) found that it was possible to aggregate the sialoglycoprotein of the red cell membrane using whole immunoglobulin against spectrin but not with Fab anti-spectrin fragments, suggesting that spectrin molecules must be cross linked in order to produce the redistribution of the sialoglycoproteins. In the case of A sites, we have not tried to find out what is the distribution of sites using Fab fragments. However, it is known (Hughes-Jones, 1972) from thermodynamic data that IgG anti-A binds to red cells through both antigen binding sites and thus it is possible that sites on the same cells are cross-linked and in this way, aggregated. In the case of Rh positive cells, it is also known (Hughes-Jones, 1972) that each IgG anti-D binds to the D antigen through only one of the antigen binding sites and thus no cross linking occurs; this observation correlates with the lack of aggregation of sites seen with gold-anti-IgG labelling. On the other hand, in -D- cells, due to the greater number of antigen sites present and to the possibility that each molecule carrying Rh antigens

may have several D antigen sites, cross-linking of sites on the same -D- cell may happen. In the case of enzyme-treated cells, pH variation, etc., the mechanism of aggregation is obviously different and may be related to changes in the electrostatic charge and in the local physicochemical environment of the molecules involved.

RELATIONSHIP BETWEEN RED CELL AGGLUTINABILITY AND PATTERN OF DISTRIBUTION OF ANTIGEN SITES

It has been widely accepted that red cell agglutination by antibodies occurs when the forces that favour agglutination outweighs the forces that oppose agglutination (Pollack et al., 1965 and Pollack and Reckel, 1970). Ultimately, red cells in the agglutinates are held together by specific antibody bridging (Romano and Mollison, 1973).

Recently, Nicolson (1972 and 1973b) has shown that in several systems enhancement of cell agglutinability after protease treatment correlates with a change in surface distribution of the membrane components involved. Thus, trypsin treatment of 3T3 mouse fibroblasts increased agglutinability by Con A and correlated with a change in the distribution of Con A receptors from random dispersion to an aggregated state.

In the present work, it has also been observed that a correlation exists between agglutinability and clustered distribution of the antigen involved. For example, D sites on Rh positive cells are randomly dispersed under normal circumstances but acquire an aggregated distribution

on papainized and antibody-treated cells. This correlates with the fact that Rh positive cells, which are normally not agglutinated in 1 per cent NaCl by IgG anti-D, can be agglutinated after papain treatment. In addition, -D- cells which show a clustered distribution of D sites, in the absence of protease treatment, are also agglutinable by IgG anti-D in a saline medium (Race et al., 1950).

It is well known that untreated cells of A or B type are agglutinated by their corresponding IgG antibody in 1 per cent NaCl; the agglutinability in a saline medium may also be related to a grouped distribution of A or B antigen sites. Within the ABO system it may also be relevant that A or B cells when fixed with formaldehyde (Economidou, 1966) or glutaraldehyde (unpublished observations; also Matsukura, 1972) are 4-16 times less agglutinable than untreated cells. Clearly, fixation might "freeze" the distribution of sites preventing aggregation on combination with antibody. It should also be pointed out that in fixed cells the membrane is less deformable than in untreated red cells, and hence the formation of large areas of cell to cell contact in the places of multiple antibody bridging may be prevented.

One interesting point would be to decide if the clustering of sites itself "facilitates" agglutination or is a secondary consequence of agglutination. Nicolson (1972, 1973b) appears to believe that if antigen sites are distributed in clusters or are caused to "redistribute" into clusters by enzyme treatment, then the aggregated sites would be the sites at which multiple cross links can most readily form between the cells and overcome the repulsive forces that keep cells dispersed. In support of this possibility, Nicolson has found that in

regions of cell to cell contact in cell agglutinates formed by Con A-ferritin, the number of Con A-ferritin complexes is greater than in the rest of the cell surface. Though the junctions of the cells in the agglutinates were not particularly examined in the present work, on one occasion it was clearly seen that there were more gold clusters in the region of cell to cell contact. However, even if it is correct that aggregation of surface antigens is observed in the regions of cell to cell contact in agglutinates, this observation would not exclude the possibility that the aggregation of antigen sites (and therefore of antibody) occurred after a few cell to cell cross links had been established. Evidence provided by other workers does not, however, support entirely the concept that aggregation of antigen sites is a prerequisite for agglutination. Holburn et al. (1971), found that only about 120 IgM anti-D molecules per cell were enough to agglutinate R_1R_2 red cells. In this situation it would be very unlikely that either the D antigen sites or the IgM anti-D molecules were distributed in clusters on the red cell membrane because of the small number of antibody molecules involved. However, in this case the situation differs in that the larger size of the IgM antibody would permit the spanning of a greater distance between cells (Pollack, 1970).

In the case of the A - anti-A system, Salsbury and Clarke (1967) and van Oss and Mohn (1970), found that A cells agglutinated by IgG, IgM or by lectins with anti-A specificity, had a strong surface spiculation. In some of our electron microscope preparations, it was also observed that pointed-shape irregularities in the border of A cells in agglutinates

were associated sometimes with larger aggregates of gold particles. In view of these observations, it is tempting to speculate that, within the ABO system, agglutination by IgG antibodies may take place in the following way :

a) binding of antibodies or lectin to the antigen;

b) "local modification" of the properties of the red cell membrane;

c) aggregation of antigen sites and formation of spicules;

d) cell to cell contact facilitated by spicule formation;

e) formation of multiple cross-links and consequent agglutination.

The "local modification" of the properties of the red cell membrane referred to in b) could be, for instance, a neutralization by the antibody or lectin protein of the negative charges due to sialic acid residues which are located in the major sialoglycoprotein molecules of the red cell membrane to which the A antigens are associated. This in a sense, could be like 'chopping out' the sialic acid residues as occurs following treatment with proteases or neuraminidase. As a consequence of this charge neutralization, the electrostatic repulsion between adjacent glycoproteins, will be reduced facilitating the cross-linkage and aggregation of sites by combined antibodies.

MOLECULAR RELATIONS OF c AND D ANTIGEN SITES IN cDE / cDE (R_2R_2) RED CELLS

Are c and D antigen sites located on the same molecular structure?

I) INTRODUCTION

Though in recent years it has been established from indirect evidence that the Rh blood group substance is probably a protein or a lipoprotein (Kaufman and Masouredis, 1963; Green, 1967, 1968; Leddy, 1970), our understanding of the Rh blood group system in terms of molecular structure is very poor. The lack of chemical information about this system makes it very difficult to interpret the available serological and genetic data of this clinically important blood group system.

An interesting point to investigate is whether the antigen sites ("factors") responsible for the specificities (D, E, e, C, c) within the Rh system are part of a common antigenic structure ("agglutinogen") (Wiener's theory, see Mollison, 1972, p.267) or whether these antigen sites are located on different but closely related structures.

Some contradictory information exists on this subject. Nijenhuis (1961), by blocking various Rh specificities with "incomplete" i.e. non-agglutinating in saline, anti-Rh sera of different specificities and testing these cells with "complete", i.e. agglutinating in saline, anti-Rh sera, concluded that the different antigen specificities were

not independent but were part of a common structure. However, this approach does not seem an ideal one because of the difficulties involved in the interpretation of the technique itself (for example, how to interpret the various degree of "partial blocking" observed) and also because of the difficulties involved in defining precisely the specificities of the different antisera, i.e. anti-CE, anti-CD.

Rosse (1968) found that Rh positive red cells coated with IgG anti-Rh of at least 3 different specificities (i.e. anti-c + anti-D + anti-e) were capable offixing the first component of complement (C1). No C1 could be demonstrated on cells coated with only 2 different antibody specificities. It is thought that these results, although not conclusive, indicate that the different Rh antigenic sites are very close together.

Different results were obtained by Edgington (1971) who studied the inhibition of the binding of ^{125}I-Fab anti-D fragments by unlabelled antisera of different Rh specificities. This author found that IgG anti-D would effectively reduce the binding of ^{125}I-Fab anti-D but no competitive inhibition was found with antisera directed to other Rh specificities. According to the author, these findings would suggest a steric separation of the non-D determinants (E, e, C, c) on the erythrocyte surface by more than the combined molecular radi of the Fab anti-D and the IgG antibody to the non-D determinant. Unfortunately, Edgington did not give any details regarding the phenotype of the red cell used or if any combination of non-anti-D antibodies was tested for competitive inhibition of Fab anti-D which could have been relevant to the interpretation of the data.

As IgG anti-D and anti-c were available, investigations were started in order to look at the spatial relations of D and c antigen sites on red cells homozygous for both antigens. For this purpose, it was decided to study the distribution of gold-labelled anti-IgG on cells of cDE/cDE group which were coated simultaneously with IgG anti-D and IgG anti-c. By comparing this distribution with the distribution of the label obtained when anti-c or anti-D alone were coating the cells it was thought that it might be possible to decide if the c and D antigen sites were either: (1) very close together or (2) on a separated structure. If the former proved to be correct, then a proportion of gold clusters would have a greater number of gold particles than those observed for anti-D or anti-c alone, indicating the presence of 2 or more antigen sites together. If the latter proved to be the correct alternative, then each cluster would contain the same number of gold particles whether anti-D and anti-c were present together or separately on the red cell surface.

II) METHODOLOGY

A) Red Cells: fresh cells from a donor group cDE/cDE (R_2R_2) were used for these experiments.

B) Antibodies: IgG anti-D was used at an antibody concentration of approximately 100 µg/ml.

IgG anti-c was prepared from the serum of a woman whose baby had haemolytic disease of the newborn due to immune anti-c. The indirect antiglobulin titre was 1:500 using cde/cde red cells and 1:2 with CDe/CDe cells indicating that there was a small amount of contaminating anti-D present; therefore the anti-c antiserum was absorbed with CDe/CDe cells before use.

C) Sensitization and labelling procedure: 0.2 ml of a 50 per cent suspension of red cells of Rh group cDE/cDE were incubated at 37° with 0.5 ml of IgG anti-D, containing approximately 50 µg of antibody. After 40 minutes of incubation the cells were divided into two aliquots. Next, 4 ml of anti-c, containing about 40 µg of antibody were added to a first aliquot and 4 ml of autologous serum were added to the second aliquot. After a further 40 minutes at 37°, excess unbound antibody was washed off with 10 g/l ice-cold NaCl. The sensitized cells were then lysed at the surface of 16 mM phosphate buffer at pH 7.0. The resulting ghosts were picked up with support grids and then were labelled on one side only with gold-anti-IgG. The details of this procedure have been described in previous chapters.

To assess the distribution of c antigen sites, separate experiments were performed in which 200 µl of cde/cde red cells were incubated only with anti-c (approximately 45 µg) followed by lysis of the cells and labelling of the resulting ghosts with gold anti-IgG. Because of a limited availability of the anti-c antiserum, only two experiments in which labelling of c antigen sites alone were carried out, since experiments in which c and D antigen sites were labelled simultaneously were given priority.

III) RESULTS

In anti-D, anti-c and anti-D + anti-c antibody treated cells the label was found to be distributed in small clusters.

Fig. V-1 shows a micrograph of a cell treated only with IgG anti-D.

The pattern of labelling when only anti-c was combined with the red cells can be seen in Fig. V-2. Figs. V-3 and V-4 demonstrate the labelling characteristics when both anti-D and anti-c are combined with the red cells at the same time. Fig. V-5 shows again the simultaneous labelling of c and D sites but at higher magnification.

Tables V-1 summarizes the estimations that were made using data from several micrographs of cells coated with anti-D alone (column a) and of cells combined only with anti-c. These calculations were performed using the procedure described in previous chapters (see page 141).

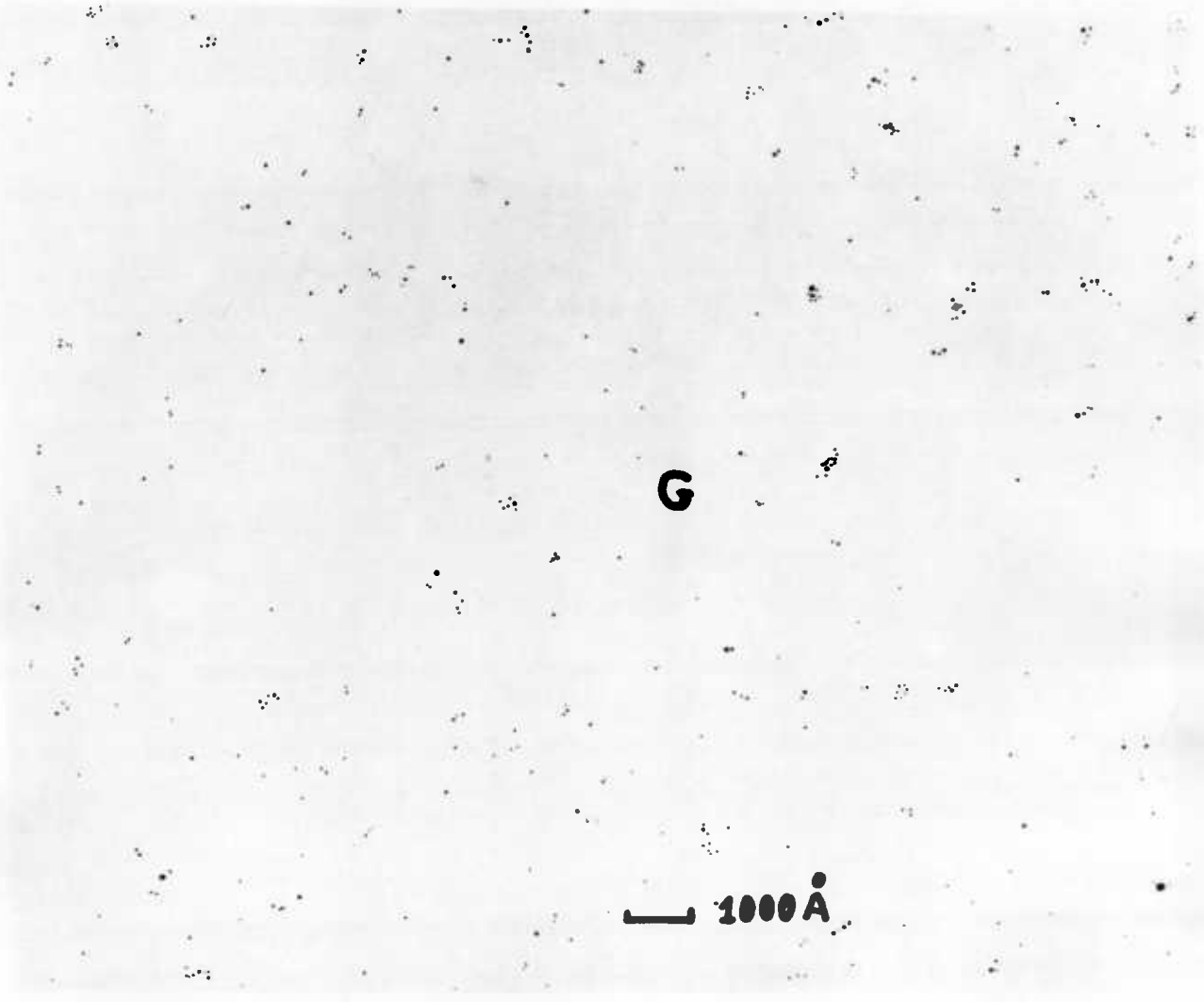

Fig V-1 : Pattern of labelling of D sites in R_2R_2 red cells combined with anti-D alone and labelled with gold-anti-IgG. See also Fig IV-2 to IV-5.

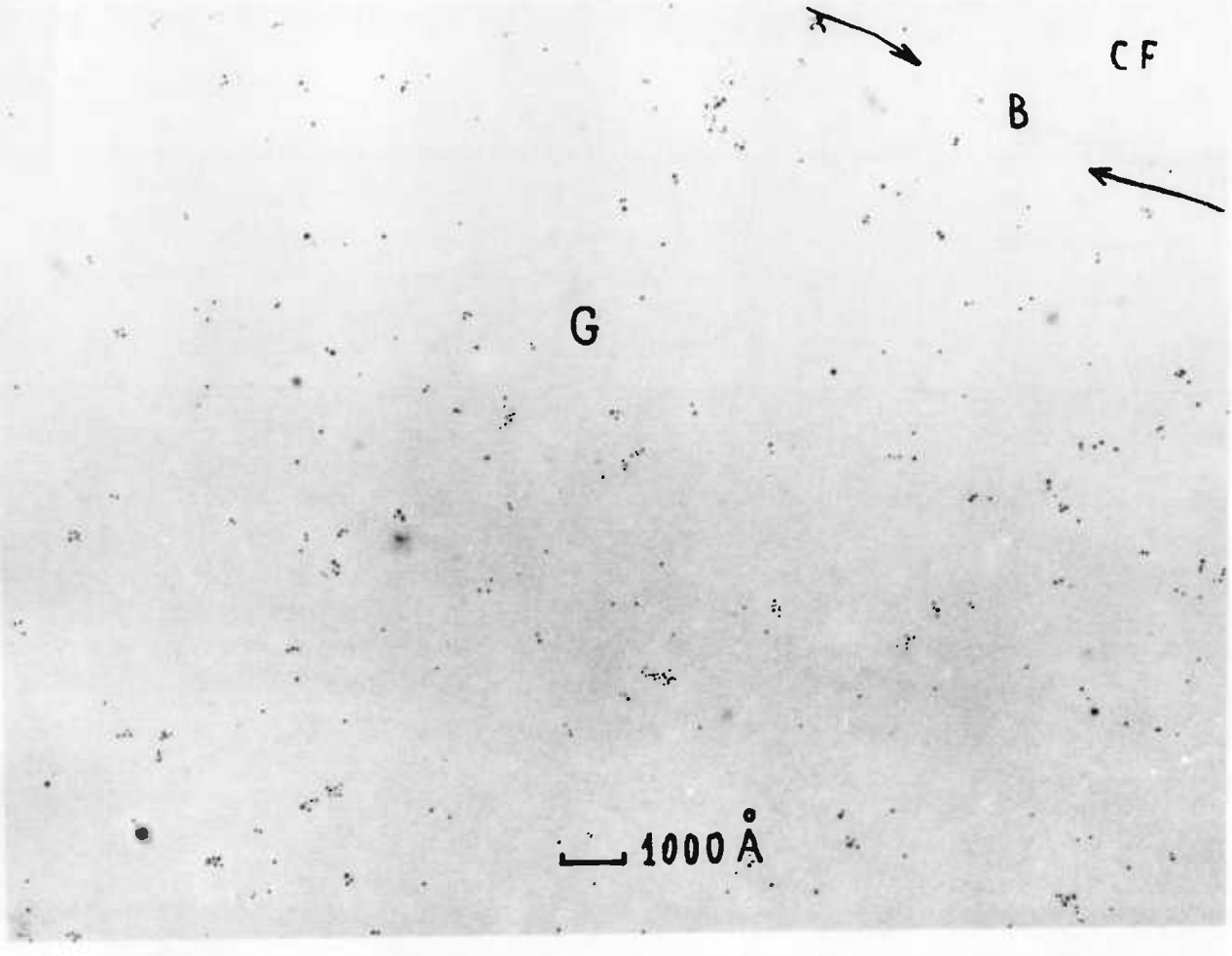

Fig V-2 : Pattern of labelling of c sites in R_2R_2 combined with anti-c alone and labelled with gold-anti-IgG. Note the resemblance with the pattern of labelling of D sites. CF = carbon film, G = ghost, B = border.

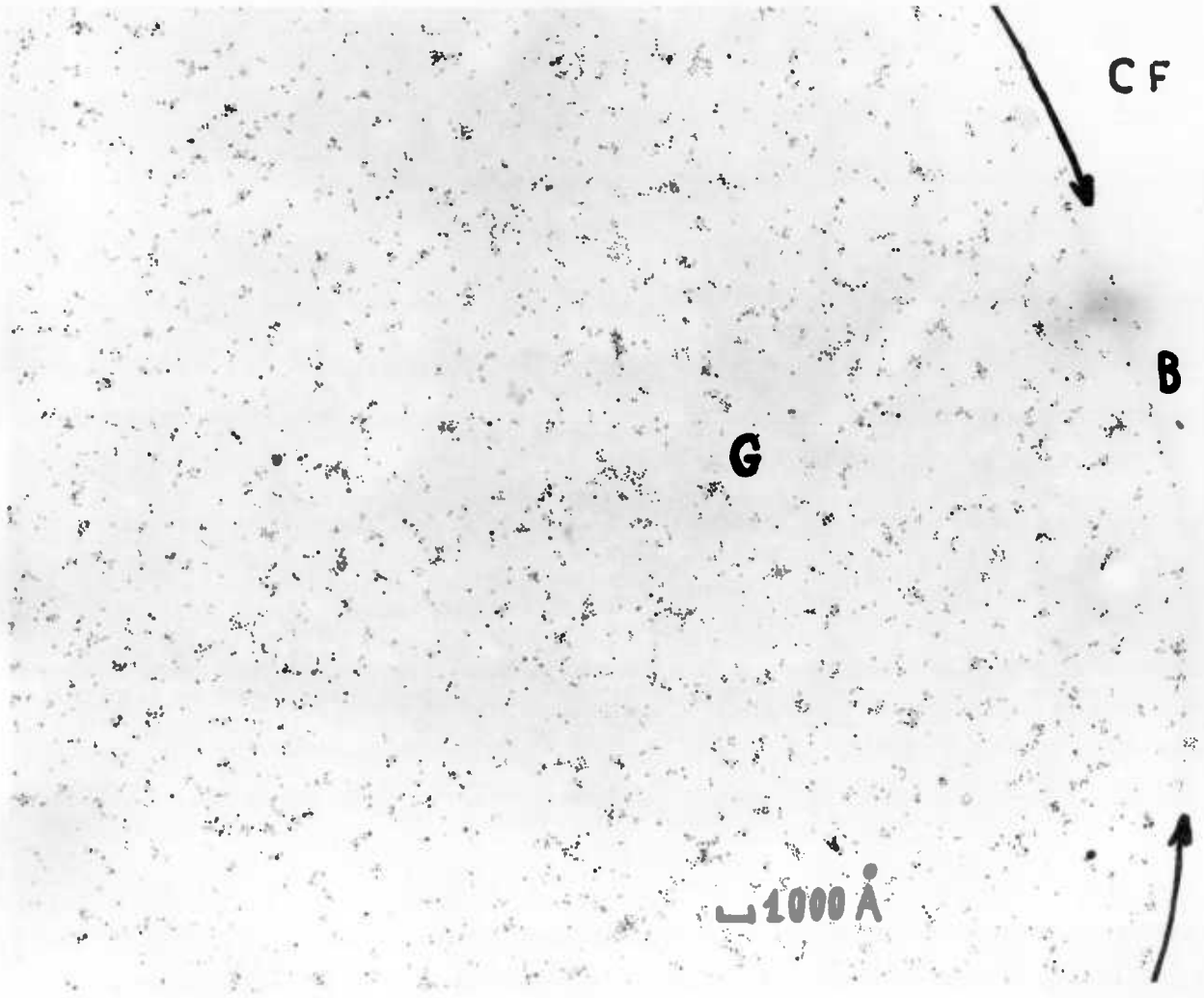

Fig V-3 : Pattern of labelling obtained when R_2R_2 red cells were combined both with IgG anti-D and IgG anti-c at the same time and labelled with gold anti-IgG. Note the greater proportion of large clusters in comparison with Figs. V-1 and V-2.

CF = carbon film, G = ghost, B = border.

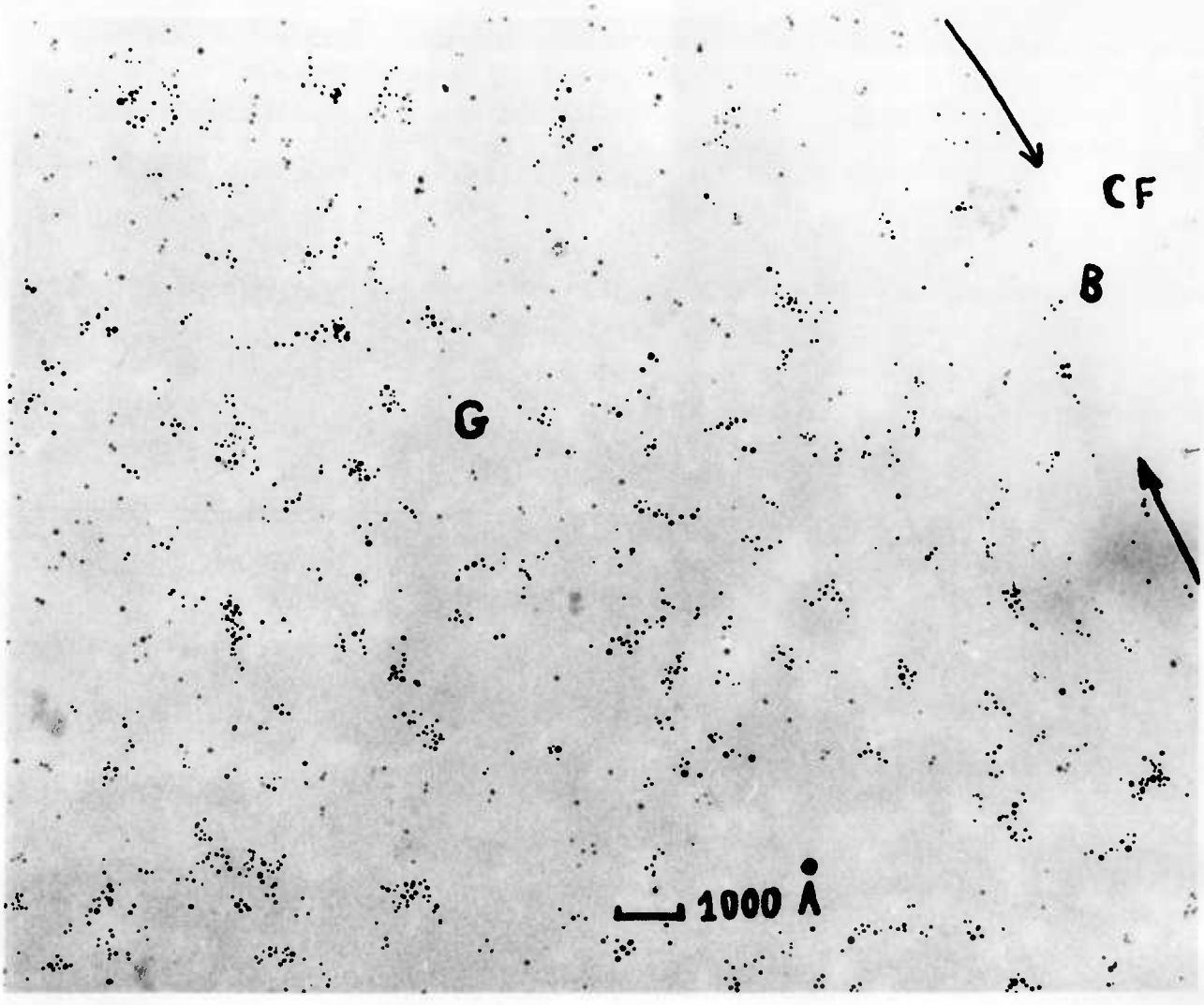

Fig V-4 : Labelling of D and c sites at the same time.

Higher magnification of Fig V-3. CF = carbon film, G = ghost,

B = border.

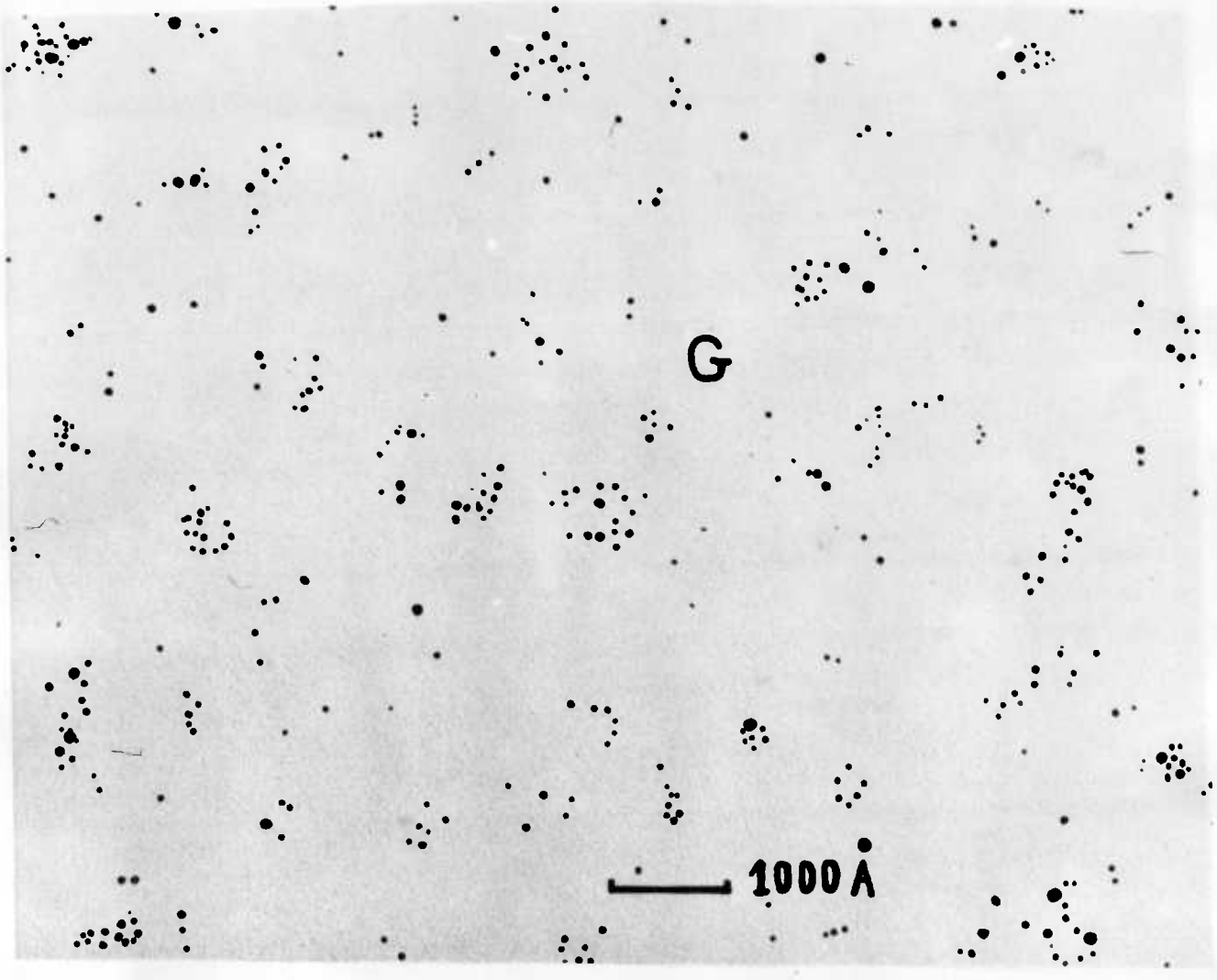

Fig V-5 : Labelling of D and c sites at the same time.
Compare with Figs. IV-4 and IV-5, (anti-D alone) note the greater proportion of large clusters in this case. G = ghost.

Table V-1

Summary of the characteristics of labelling of cDE/cDE red cells coated with IgG anti-D and of cde/cde red cells coated with IgG anti-c

		a anti-D	b anti-c
a)	Approximate number of clusters observed per cell	10,000	10,000
b)	Average number of gold particles per cluster	2.7	2.3
c)	Percentage of clusters having 1 to 5 gold particles	90	91
d)	Percentage of clusters measuring :		
	$\leq$140 ångströms	65	67
	140 to 375 ångströms	25	26
	$>$375 ångströms	10	7
e)	Test for randomness of the distribution of clusters. Goodness of fit to a calculated Poisson distribution	X^2 = 2.8 P = 0.5 (for n = 4)	Not Done

Red Cells coated with anti-D + anti-c: As can be appreciated in Figs.V-3 and V4, there are many more clusters of gold particles per cell than in the previous case. This greater number of labelled antigen sites made the analysis of these micrographs much more difficult because of the subjectivity involved in the demarcation of the clusters; on the other hand, it was necessary to keep the saturation of D and c antigen sites as high as possible otherwise it would be very difficult to derive valid statistical conclusions from the results.

Being aware of the limitations mentioned earlier regarding the subjectivity involved in the demarcation of the gold clusters, results are presented in Table V-2 for the characteristics of red cells combined with both anti-D and anti-c.

As Table V-2 indicates, the observed average number of gold particles per cluster in the case of red cells combined with anti-D + anti-c was found to be greater than either for anti-D or anti-c coated cells (approximately 4 in the former case against 2.7 and 2.3 in the latter). Similarly, the percentage of larger clusters (> 375 ångströms) was much higher in the case of red cells coated with anti-D + anti-c. In addition, some evidence suggesting a non random distribution of the anti-D + anti-c clusters was obtained.

Table V-2

Summary of the characteristics of labelling of cDE/cDE red cells combined with anti-D + anti-c

Data from 2 experiments

a)	Approximate number of clusters observed per cell	28,000 and 34,000
b)	Average number of gold particles per cluster	3.8 and 4.1
c)	Percentage of clusters having 1 to 5 gold particles	78
d)	Percentage of clusters measuring (ångströms) :	
	≤ 140	50 and 52
	140-375	27 and 29
	> 375	21
e)	Test for randomness of the distribution of clusters. Goodness of fit to a calculated Poisson distribution	$X^2 = 33$ $p = 0.001$ (for $n = 5$)

IV) DISCUSSION

The data for red cells coated with anti-D alone are compatible with a random distribution of D antigenic sites in which each cluster represents about 2 anti-IgG molecules bound to a single IgG anti-D therefore representing a single D antigen site, as discussed in the previous chapter. As can be seen in Fig. V-2 and Table V-1, similar results were obtained when only anti-c was combined with red cells of group Orr.

The data for red cells coated with a combination of anti-D and anti-c, within the limitations of the method, strongly suggest that a proportion of the clusters may represent more than one antigen site. For example, the average number of gold particles per cluster was estimated to be 3.8 - 4.1 in comparison with 2.7 for anti-D alone and 2.3 for anti-c alone. The observed average of approximately 4 gold particles per cluster implies that on the average there were about 3 anti-IgG molecules per cluster when both antibodies were on the cells, which is significantly higher than for IgG anti-D alone.

The average size of the clusters was also greater in the case of red cells coated with anti-D + anti-c; thus, 21 per cent of the clusters in the cells sensitized with both antibodies were larger than 375 ångströms in comparison with only 10 per cent and 7 per cent when anti-D + anti-c respectively were the only antibodies combined with the cells. There are many more c sites than D sites in red cells

homozygous for both antigens. Thus the total number of D antigen sites in cDE/cDE red cells has been estimated to be approximately 25,800 - 33,000 (Rochna and Hughes-Jones, 1965), in comparison with 70,000 - 85,000 for c antigen sites (Hughes-Jones, et al 1971). Therefore, if we accept the conclusion that a proportion of the clusters represent associated D and c sites, then, it seems logical to assume that all D sites are associated with c antigen sites and that a proportion of c sites is not associated with D sites. The other possibility would be that c antigen sites exist in doublets or in triplets on each Rh blood group antigen structure, but this possibility is not supported by the distribution of c antigen sites alone; data presented in Table V-1 indicate that the c antigen sites are single, i.e. discrete entities, dispersed on the red cell membrane.

Finally, the subjective difficulty of defining clusters in the analysis of the micrographs of cells coated with anti-D and anti-c simultaneously should be stressed. In the near future it is planned to pursue this problem with another approach. Based on the present observation that gold particles can be distinguished from ferritin molecules, it is hoped to label one antibody directly with gold particles and the other one with ferritin; this technique should allow the molecular proximity of both antigenic sites to be better assessed.

CHAPTERS III, IV AND V

SUMMARY AND CONCLUSIONS

Chapter III presents an adaptation and development of a method of labelling antibody with gold particles for the study of erythrocyte surface antigens. Purified horse anti-human IgG was found to combine with colloidal gold, producing a labelled-antiglobulin reagent which allowed the indirect visualization of the distribution of A, D and c blood group antigens on the red cell surface.

Chapter IV present information regarding the surface distribution of A and D antigens. The red cells were combined with IgG antibodies; ghosts were then produced which were mounted on a carbon coated E.M. grid and labelled only on one side with gold-labelled anti-IgG. In this way, it was possible to visualize the entire surface of the labelled side of the ghosts, allowing the study of the topographic distribution of the erythrocyte membrane antigens over large areas.

D antigen sites were found to be single entities dispersed on the erythrocyte membrane of normal Rh positive cells. Following papain-treatment of Rh positive red cells, a clustered distribution of D sites was observed. Clustering of D sites was also observed on red cells of the rare phenotype -D-.

A antigen sites were found to be clustered in proportion to the amount of IgG anti-A combined with the A_1 cells. Reasons are given for considering this pattern to be indirect evidence of mobility of A antigens.

It was observed that there was an association between agglutinability of red cells by IgG antibodies on the one hand, and clustered distribution of the antigen sites involved on the other hand.

The aggregated distribution of A (or B) antigen sites upon combination with IgG anti-A (or anti-B), also provides the molecular basis for the understanding of the complement-fixing ability of this antigen-antibody system. Similarly, the single dispersed distribution of D sites upon combination with IgG anti-D offers a molecular explanation for the absence of complement fixation by the D-IgG anti-D system. In the case of -D- cells combined with IgG anti-D, the observed clustered distribution of sites, and hence the molecular proximity of 2 or more IgG anti-D, allows for the prediction of complement fixation when sufficient IgG anti-D antibodies are combined with -D- red cells.

In chapter V, studies are presented in which there was some evidence suggesting that D and c antigen sites are located close together on R_2R_2 red cells.

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Direct Antiglobulin Reaction in ABO-Haemolytic Disease of the Newborn

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Summary

The minimum number of IgG anti-A (or anti-B) molecules detectable on A or B red cells by the antiglobulin reaction was found to be the same—that is, about 150 molecules per red cell—with newborn as with adult cells. Furthermore, the ratio of anti-IgG bound to IgG anti-A (or anti-B) molecules was the same whether the anti-A (or anti-B) molecules were present on newborn or on adult cells and was similar to that found for anti-IgG bound to IgG anti-Rh.

In 15 infants (11 group A, 4 group B) with haemolytic disease of the newborn due to ABO-incompatibility the amount of anti-A or anti-B on the red cells ranged from 0·25 to 3·5 μg antibody per ml red cells, corresponding to 90-1,320 antibody molecules per cell; only five infants had more than 0·55μg antibody per ml of red cells. These amounts are far smaller than those found in most moderate or severe cases of Rh-haemolytic disease.

It is concluded that the weak direct antiglobulin reactions observed in ABO-haemolytic disease are due simply to the fact that the number of anti-A (or anti-B) molecules on the infant's red cells is at the lower limit of sensitivity of the test. Since ABO-haemolytic disease can be quite a severe process it seems probable that IgG anti-A and anti-B molecules are more effective than anti-Rh molecules in bringing about red cell destruction.

Introduction

Even in moderately severe ABO-haemolytic disease of the newborn the infant's washed red cells give either a weakly positive or a negative direct antiglobulin reaction when routine techniques are used (Crawford *et al.*, 1953). Although more sensitive techniques such as the "spin Coombs" may consistently give positive reactions even in mildly affected infants (Rosenfield, 1955), this finding only stresses that the antiglobulin reactions are much weaker than those observed in Rh-haemolytic disease.

There seem to be several possible explanations for the finding of weak direct antiglobulin reactions in ABO-haemolytic disease; the amount of antibody on the circulating red cells may, in fact, be very small; most of the antibody may come off the red cells on washing; the A and B antigen sites on the red cells of newborn infants may be situated further from the cell surface than corresponding sites on adult cells, so that the anti-A and anti-B molecules become less accessible to antiglobulin serum after attachment to the sites; or there may be other steric effects which make cross-linking of antiglobulin molecules more difficult.

The questions posed above have been only partly answered by published work, mainly because no quantitative experiments with labelled antisera have been undertaken. It seemed that if quantitative experiments using both labelled anti-A and labelled antiglobulin serum were carried out some of the confusion which surrounds this subject might be dissipated.

Subjects and Methods

Normal Newborn Infants.—Cord blood was obtained at the time of delivery and mixed with acid-citrate-dextrose solution (ACD) (4 parts blood to 1 part ACD) and kept at 4°C for up to seven days before being tested. The red cells were washed four times in saline before being used. Only red cell samples which were agglutinated by an extract of *Dolichos biflorus* were used.

Infants with ABO-haemolytic Disease.—The diagnosis was made by the finding in a full-term A or B infant born to an O mother of a serum bilirubin concentration of 10 mg/100 ml or more on the second or third day of life; in eight cases the concentration reached 18 mg/100 ml or more; seven of these infants and one other were treated by exchange transfusion. In most cases microspherocytes were prominent in films of peripheral blood. There were 4 group B and 11 group A infants; the red cells of the latter were tested with an extract of *D. biflorus;* seven samples were strongly agglutinated, two were weakly agglutinated, and two were not agglutinated at all. In all cases eluates were prepared from the infant's red cells by the method of Landsteiner and Miller (1925) and tested against A_1 or B red cells by the indirect antiglobulin method.

Normal Adults.—Blood was mixed with ACD and stored at 4°C for up to seven days. Only samples which were agglutinated by an extract of *D. biflorus* were used. The cells were washed four times in normal saline before being tested.

IgG Anti-A.—Using standard diethylaminoethyl-cellulose chromatography IgG anti-A (free from IgM) was prepared from plasma obtained from a group O subject who had received injections of purified human A substance (kindly supplied by Professor Winifred M. Watkins). The IgG fraction was labelled with ^{125}I by the iodine monochloride method (McFarlane, 1958). The specific activity of the preparation was determined from the protein concentration using Folin-Ciocalteu's reagent. The concentration of IgG anti-A was 84 μg/ml and the indirect antiglobulin titre against A_1 red cells was 8,000.

Radioiodine-labelled Anti-IgG Serum.—Horse antihuman IgG was prepared by the method of Avrameas and Ternynck (1969). The product was labelled with either ^{131}I or ^{125}I as convenient.

Uptake of IgG Anti-A on to Cord and Adult A Red Cells.—The number of antigen sites on A_1 cord or adult cells was estimated using ^{125}I-labelled IgG anti-A. The minimum number of anti-A molecules detectable with the antiglobulin test on cord and adult A cells was determined as follows. A fixed number (approximately 2×10^8) of A_1 adult or cord red cells were incubated at 37°C for 90 minutes with serial dilution of ^{125}I-labelled anti-A at a ratio of 1 volume red cells to 4 volumes anti-A. Group O red cells were used as a control. The cells were washed four times with ice-cold saline and the uptake of ^{125}I was determined within 30 minutes. The same samples of red cells were tested for agglutination by optimally diluted rabbit antihuman IgG both macroscopically (using a tile) and microscopically after centrifugation for

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10 seconds at low speeds. The results were calculated from the specific activity of the preparation, the red cell count, and the packed cell volume of the suspension, and, assuming the molecular weight of IgG to be 160,000, were expressed either as molecules of anti-A per red cell or as μg of anti-A per ml of red cells. The ratios of anti-IgG molecules to IgG anti-A molecules on infant and adult A red cells and the number of anti-A or anti-B molecules on the red cells of infants affected with ABO-haemolytic disease were determined by the method of Rochna and Hughes-Jones (1965). In order to discover how much anti-A was eluted from cord red cells on washing, the red cells of two infants with ABO-haemolytic disease were washed four times in saline and the wash solutions were saved, concentrated, and tested by the indirect antiglobulin method. In addition, experiments were done with normal A red cells sensitized in vitro with ^{125}I-labelled IgG anti-A; the supernatant solutions from four consecutive washes were pooled and counted.

Results

Maximum Number of Anti-A Molecules taken up by Cord and Adult A_1 Cells.—Two A cord samples and two A_1 adult samples were tested; the average number of antigen sites, corresponding to the maximum number of anti-A molecules taken up, was found to be approximately $0{\cdot}3 \times 10^6$ and $1{\cdot}2 \times 10^6$ per cell respectively. These estimates are of the same order as those previously reported (Economidou *et al.*, 1967).

Minimum Number of IgG Anti-A Molecules detectable on Cells using Anti-IgG Serum.—For both adult and cord A_1 red cells the least number of anti-A molecules which could be detected by the indirect antiglobulin method was approximately 150 per cell (table I). The reactions of adult and cord red cells coated with similar numbers of anti-A molecules were indistinguishable.

TABLE I—*Reactions with an Anti-IgG Serum of Normal Adult and Cord A_1 Red Cells coated In Vitro with Similar Numbers of IgG Anti-A Molecules*

Adult Red Cells		Cord Red Cells	
No. of IgG Anti-A Molecules/Cell	Reactions with Antiglobulin Serum	No. of IgG Anti-A Molecules/Cell	Reactions with Antiglobulin Serum
2,500	+ +	2,140	+ +
970	+ +	1,060	+ +
520	+	490	+
214	+	226	+
145	Trace	161	Trace
128	—		

The reactions with an antiglobulin serum are given as + + or + to indicate degree of agglutination; "trace" indicates that only a dubious reaction was present on macroscopic examination although microscopically there was definite agglutination.

Ratio of Antiglobulin: Anti-A Molecules on Cord and Adult A_1 Red Cells.—Cord and adult red cells were coated with approximately 10 μg of ^{125}I-labelled IgG anti-A per ml of cells and incubated with decreasing concentrations of ^{131}I-labelled horse antihuman IgG at an initial concentration of about 15 μg of anti-IgG per ml. The extent of the reaction between anti-IgG and IgG anti-A on cord and adult red cells was about the same, the maximum ratio of anti-IgG to IgG molecules being 1·8 to 2·0. In a control experiment in which D-positive red cells coated with anti-D were used a similar ratio was observed.

Number of Anti-A and Anti-B Molecules on Red Cells in Cases of ABO-haemolytic Disease.—The amount of anti-A or anti-B on the red cells ranged from 0·25 to 3·5 μg/ml (table II); these amounts correspond to 90 to 1,320 molecules of antibody per cell. The direct antiglobulin test was positive in 11 cases when read macroscopically and positive in 1 further case when read microscopically. In this last case and in the three cases in which the test gave negative readings the amount of anti-A or anti-B on the red cells was always less than 0·4μg/ml. In all 14 cases in which an eluate was prepared from the infant's red cells the expected antibody (anti-A or anti-B) was shown to be present.

TABLE II—*Cases of ABO-haemolytic Disease*

Case No.	Infants: Blood Group	Infants: Maximum Recorded Plasma Bilirubin Concentration: mg/100 ml	Infants: Maximum Recorded Plasma Bilirubin Concentration: Time After Birth (Hours)	Infants: Direct Antiglobulin Test: Routine Method	Infants: Direct Antiglobulin Test: μg Antibody per ml Red Cells	Mothers (All Group O): Indirect Antiglobulin Test* (Anti-A or Anti-B): Titre	Mothers (All Group O): Indirect Antiglobulin Test* (Anti-A or Anti-B): Time After Delivery (Days)
1	A	19·4	48	Neg.	0·25	32	1
2	A	27·0	72	Neg.	0·30	16	3
3	B	18·0	60	+ (Microscop.)	0·35	512	4
4	A	14·3	60	+	0·35	1,000	−1
5	A	12·5	24	Neg.	0·35	128	2
6	B	11·0	48	+	0·40	4,000	7 weeks
7	A	27·0	24	+	0·40	2,000	8
8	A	10·1	48	+	0·45	1,000	4 weeks
9	B	22·6	48	+	0·55	256	2
10	A	13·6	24	+	0·55	1,000	6
11	B	18·0	24	+	1·10	2,000	7
12	A	14·0	24	+	1·40	512	1
13	A	20·0	72	+ +	1·85	1,000	3
14	A	†		+ +	2·25	1,000	1
15	A	24·3	72	+ +	3·50	2,000	3

*Anti-IgG.
†Cord bilirubin concentration 5·8 mg/100 ml; no later estimates.

Elution of Anti-A from A Red Cells during washing.—Only traces of anti-A were detected in the wash solutions obtained from the red cells of two infants with haemolytic disease due to anti-A. Similarly, in quantitative experiments with normal A cells coated in vitro with ^{125}I-labelled IgG anti-A a maximum of 20% of antibody was recovered from four consecutive washes.

Discussion

The present results show that the minimum number of IgG anti-A (or anti-B) molecules detectable on red cells by the antiglobulin test is about 150 per cell for both cord and adult red cells. The number is about the same as that previously reported (Dupuy *et al.*, 1964; Hughes-Jones *et al.*, 1964) for the detection of IgG anti-Rh by the antiglobulin test. Since evidence was obtained that relatively little IgG anti-A is lost from "coated" red cells on washing it must be concluded that the weak positive direct antiglobulin test reaction, which is characteristic of ABO-haemolytic disease, indicates that in this disease relatively few antibody molecules are attached to the circulating red cells. In the present series of 15 cases the amount of anti-A or anti-B found on the cells was estimated to be between 0·25 and 3·5 μg/ml; the median value was 0·45 μg/ml, and only five values were above 0·55 μg/ml. At levels of approximately 1 μg/ml the estimates carry a considerable error because this amount of antibody on the cells is almost of the same order as the amount of non-specific ^{125}I-labelled IgG bound to the cells. The error is unlikely to be greater than ± 50%, however, so that in any case the amount of specific antibody (anti-A or anti-B) bound in a typical case is unlikely to be greater than about 1 μg/ml. This degree of coating is much less than that usually found in Rh-haemolytic disease, in which the amount in one series of cases (Hughes-Jones *et al.*, 1967) was found to vary between 0·4 and 18 μg/ml, depending on severity.

In Rh-haemolytic disease the amount of antibody on the infant's red cells expressed as μg/ml of cells is very approximately the same as the concentration of anti-Rh in the mother's plasma in μg/ml of plasma (Hughes-Jones *et al.*, 1971). On the other hand, in ABO-haemolytic disease the amount of antibody per ml on the infant's red cells may be as

little as one-fortieth of the concentration of the corresponding antibody in the mother's plasma. For example, in the present series the median value of antibody on the infant's red cells was 0·45 μg/ml, whereas the median value of IgG anti-A or anti-B in the mother's plasma was probably about 20 μg/ml (taking the median indirect antiglobulin titre in the series as 1,000 and assuming that, as with anti-Rh, an indirect antiglobulin titre of 1 corresponds to about 0·02 μg of antibody per ml). The smaller amounts of anti-A and anti-B on the infant's red cells were presumably due mainly to the competing effect of A and B substances widely scattered throughout the body tissues and secretions (Tovey, 1945).

The fact that weakly positive direct antiglobulin reactions are associated with a definite haemolytic syndrome when anti-A and anti-B are concerned, whereas a weakly positive direct antiglobulin reaction due to anti-Rh may be associated with no increase in red cell destruction whatever (Mollison, 1951), indicates that IgG anti-A and anti-B are relatively more effective than anti-Rh in producing red cell destruction. One possible explanation of the difference would be that IgG anti-A and anti-B, unlike anti-Rh, bind complement. On the other hand, if A and B sites are distributed at random on the red cell surface it would be expected that a minimum of 800 molecules of antibody would have to be present on each red cell before there was an even chance that two antibody molecules would occupy adjacent sites and so activate complement (Humphrey and Dourmashkin, 1965). This reasoning would not apply if A and B antigen sites, when combined with their specific antibody, were present in clusters in the red cell surface as, in fact, they may be (see below).

Some observations which appear to contradict the present findings of only small amounts of antibody on the red cells of infants with haemolytic disease of the new born must be briefly discussed. Voak and Bowley (1969) reported that eluates from the red cells in infants with ABO-haemolytic disease might give strong indirect antiglobulin reactions with adult A_1 red cells, although the direct antiglobulin reaction on the infant's red cells was only weakly positive or even negative. This observation might seem to indicate that the infant's red cells were coated with large amounts of antibody. In a latter paper, however (Voak and Williams, 1971), it was pointed out that the elution procedure resulted in a considerable concentration of antibody, so that the finding does not necessarily indicate that there is a substantial amount of antibody on each cell. If one considers some actual quantities the degree of concentration involved in elution can be better appreciated. Suppose, for example, that antibody is eluted from 0·5 ml of red cells coated with 0·5 μg/ml of anti-A and 60% of the antibody is removed during elution; the total amount of antibody eluted would then be 0·15 μg. Now, suppose that the eluate is incubated with 0·1 ml of a 5% suspension of red cells and that 80% of the antibody is taken up by the cells; there is then 0·12 μg on 0·005 ml of cells or 24 μg/ml, sufficient to give a very strong antiglobulin reaction.

Voak and Williams (1971), using electron microscopy and ferritin-labelled IgG anti-A and anti-B, found evidence of clustering of antigen sites on both adult and infant red cells. They suggested that the reason for the relatively weak antiglobulin reactions of cord A cells might be the relatively greater distance between the A sites on the infant cells compared with the shorter distance on adult A_1 cells, making cross-linking by anti-IgG molecules difficult. Even on newborn red cells, however, the number of sites is about 10 times greater than the number of Rh (D) sites, so that the distance between D sites must be much greater than that between A sites. Moreover, the present observations indicate that the ratio of anti-IgG molecules to anti-A molecules is, in fact, the same with adult and infant A_1 cells and, incidentally, is the same as that observed with anti-IgG and anti-Rh on Rh-positive red cells.

In the present series, as in the series reported by Gerlini *et al.* (1968), the red cells of some group A infants with haemolytic disease due to anti-A were not agglutinated by an extract of *D. biflorus*, suggesting that contrary to present belief A_2 infants as well as A_1 infants may be affected. On theoretical grounds there does not seem to be any reason why A_2 infants should be spared. Even if the number of A sites on the red cells of newborn infants who are genetically A_2 is as low as 50,000, A molecules (red cell antigen sites) would be in considerable excess over anti-A molecules in the plasma, so that the number of antibody molecules taken up by A_2 cells should be scarcely less than that taken up by A_1 cells.

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Mechanism of Red Cell Agglutination by IgG Antibodies

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Abstract. It has been shown by others that agglutination of red cells by IgG antibodies depends on the density of antigen sites at the red cell surface, suggesting the possibility that agglutination by IgG antibodies might possibly be due to the cells being rendered 'sticky' rather than to specific cross-linking. However, the present experiments show that, when a mixture of purified IgG anti-A and anti-B is incubated with a mixture of A and B red cells, the clumps contain only A cells or only B cells, indicating that agglutination by these antibodies is, in fact, due to specific cross-linking.

Since normal red cells repel one another, cross-linking by antibodies is affected by the electric charge ('zeta potential') at the red cell surface and by the dimensions of the antibody molecule. Presumably, IgM molecules can bridge a larger gap than IgG molecules. It has been stated that the critical zeta potential above which agglutination of red cells occurs is –23 mV for IgM antibodies and –12 mV for IgG antibodies [4, 5].

It is generally accepted that agglutination is due to cross-linking by single antibody molecules of antigen sites having the same specificity, but although it seems certain that this is the mechanism as far as IgM molecules are concerned, evidence with regard to IgG molecules does not seem to have been provided. The fact that agglutination by IgG antibodies depends to some extent on antigen density (see Discussion) raises the question whether the mechanism of agglutination with IgG might be different. It seemed that the position could be clarified by carrying out experiments after labelling either

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A or B cells in some distinctive way and coating them with purified IgG anti-A and anti-B.

Drs. C. A. JANEWAY, jr. and J. H. HUMPHREY informed us [personal communication] that in an experiment which they had carried out with 'normal' anti-A and anti-B and A and B red cells, using phenylhydrazine to 'mark' one red cell population, agglutinates had been found to consist only of red cells of identical group (i.e. all A cells or all B cells). Since it was highly probable that in these experiments IgM antibodies had been used, it was felt to be worth while to repeat the experiment using purified IgG antibodies.

Materials and Methods

Human red cells were obtained from group A_1 and B donors.

Production of Heinz bodies. Phenylhydrazine HCl was used at a concentration of 0.01 g/100 ml in 0.15 M phosphate buffer, pH 7.0. 1 vol. of whole, freshly drawn blood mixed with EDTA was incubated at 37 °C for 10 min with 20 vol. of the phenylhydrazine solution. These conditions were found to be the minimal ones for the induction of Heinz bodies in 100% of red cells.

IgG anti-A and anti-B. IgG anti-A was prepared from the serum of a group O donor who had been immunised with purified human A substance, kindly provided by Professor W. M. WATKINS. IgG anti-B was prepared from a group A subject who had been found by screening to have a high titre of IgG anti-B.

IgG was separated from the sera by ion exchange chromatography (DEAE-cellulose, equilibrated with 0.02 M tris-HCl, pH 8.2). The anti-A preparation was absorbed twice with a large volume of B red cells and the anti-B was absorbed in the same way with A_1 cells. Indirect antiglobulin tests showed that the resulting preparations reacted only with A and with B cells, respectively. The final preparations had agglutinin titres, in a saline medium, of 16 (anti-A) and 8 (anti-B).

Agglutination tests. A_1 cells treated with phenylhydrazine and B cells untreated were washed separately three times with phosphate-buffered saline and 5% suspensions were then made. Equal volumes of the A_1 and B cell suspensions were mixed together and added to a mixture of IgG anti-A and IgG anti-B, each previously diluted appropriately in 2% bovine serum albumin. The whole mixture was incubated at 37 °C for 1 h and then centrifuged for 10 sec at low speed to concentrate the cells. Two drops of red cell suspension were drawn from the bottom of the tube and incubated with two drops of 0.5% methyl violet in isotonic saline at room temperature for 10–20 min to stain the Heinz bodies. Finally, films were made and examined. Appropriate controls were examined at the same time.

Results

It was found that the clumps of agglutinated cells were homogeneous from the point of view of A and B antigens. Figure 1 shows a typical result

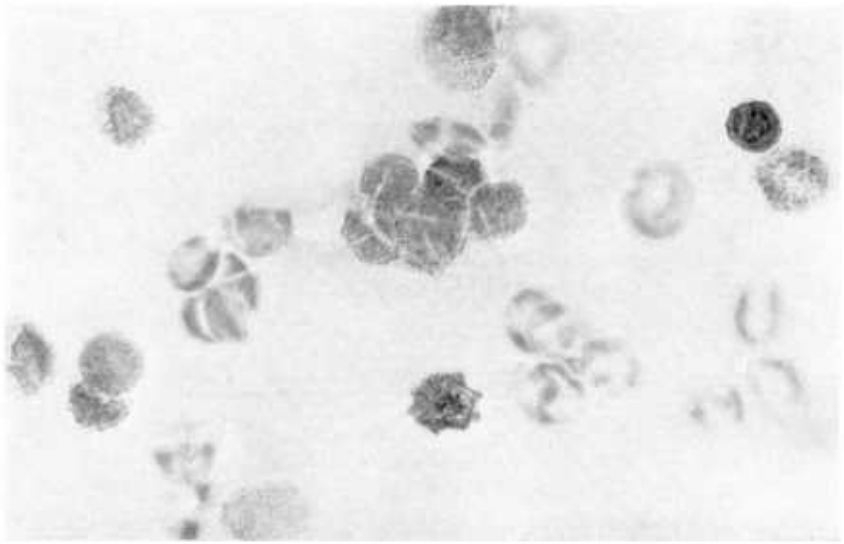

Fig. 1. Clumps containing only A_1 red cells (with Heinz bodies) or only B cells.

in which clumps are composed either of cells which all contained Heinz bodies or of cells which did not contain Heinz bodies.

It was noted that when red cells were incubated with higher concentrations of phenylhydrazine (e.g. 0.1 g/100 ml) for a longer period (e.g. 2 h), the clumps of cells dispersed immediately when methyl violet was added. Thus, within 1 min no agglutinates remained. When methyl violet was added to agglutinated but otherwise untreated red cells, agglutination was not disturbed. It seems probable that phenylhydrazine in relatively high concentration damages the glycoprotein moieties; the alternative explanation of elution of anti-A and anti-B, due to the low pH (3.7) of the methyl violet solution, seems unlikely since these antibodies are not eluted at low pH [1].

Discussion

Under the conditions of these experiments, the agglutination of A and B red cells by IgG anti-A and anti-B was due evidently to specific cross-linking between antigen sites of the same specificity located on different cells.

There appear to be at least two possible reasons why IgG anti-A and anti-B, unlike IgG anti-D, should agglutinate corresponding red cells suspended

in saline. First, A and B antigen sites are believed to be carried on the side chains of glycoprotein molecules which stick out from the red cell surface [3] whereas D sites may be carried on protein close to the cell surface. Second, since A and B sites are approximately 100 times more numerous than D sites, the chance of close contact between an anti-A or anti-B molecule, bound to an antigen site on one cell, with an A or B site on another red cell, is obviously very much greater than when anti-D and D are concerned. Alternatively, the uptake of much more IgG antibody onto A or B sites might reduce the surface charge on the cell below the critical level for agglutination. This reduction of surface charge might either facilitate specific agglutination or induce non-specific agglutination. However, the latter possibility seems to be excluded by the present observations. It has in fact been shown that when a hapten is covalently coupled to red cells in different amounts, a higher hapten density is required for agglutination by IgG antibodies than for IgM and that agglutination by IgG antibodies is determined by the hapten density at the cell surface [2], although this observation does not identify the precise mechanism of agglutination.

In any case, it seems to be clearly established by the present observations that agglutination of red cells by IgG antibodies, as by IgM, is due to the cross-linking of cells carrying antigen sites of the same specificity.

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AN ANTIGLOBULIN REAGENT LABELLED WITH COLLOIDAL GOLD FOR USE IN ELECTRON MICROSCOPY

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Faulk and Taylor (1971) described a method of labelling whole serum, which contained anti-salmonella antibody, with colloidal gold; this reagent was demonstrated to be useful as a means of investigating the distribution of antigen on cell surfaces by electron microscopy. We have attempted to label purified IgG preparations from humans and rabbits in the same way, but have found that the colloidal gold did not form stable complexes with the IgG, the label dissociating rapidly on addition of NaCl to isosmotic concentrations and also dissociating in a 7% sucrose solution. Furthermore, dialysis of the IgG preparations against ethanediol prior to labelling produced an appreciable amount of aggregation and precipitation of the protein. However, an antiglobulin reagent raised in a horse formed stable complexes with gold, and the loss of protein prior to labelling could be kept to a minimum by dialyzing against low molarity NaCl instead of ethanediol.

The colloidal gold was prepared as described by Faulk and Taylor (1971), with the following exceptions: the volume of deionized water was reduced to 60 ml; 0·7 ml of 0·18 *M* potassium carbonate was used, and the amount of phosphorus was reduced by half. The final pH was always between 6·9 and 7·3. Dialysis against ethanediol was omitted. The horse anti-human IgG was purified by adsorption followed by acid elution from human IgG coupled to CNBr-activated Sepharose 4B. It was dialyzed against 0·005 *M* NaCl (pH 7·0) and then centrifuged at 90,000 g for 30 min to remove aggregates. The final concentration of IgG was 10 mg/ml, as estimated by light absorption at 280 nm and using an extinction coefficient of 1350 for a 1 mg/ml solution. The preparation was then divided into aliquots of 0·1 ml and stored at −40°C.

Labelling was carried out by incubating 1 mg of the antiglobulin with 2·5 ml of the colloidal gold solution for 15 min at room temperature. To ensure that all the gold particles were adsorbed on to protein (to minimize non-specific uptake of gold), 10 mg of bovine serum albumin (BSA), previously dialyzed against water, was then added in a volume of 0·1 ml. After 5 min of further incubation at room temperature, NaCl (10 g/100 ml, pH 7·0) was added to give a final concentration of 1 g/100 ml. The mixture was centrifuged at 1700 g for 20 min; occasionally a small precipitate was found. The gold-labelled antibody was used without further dilution. The ratio of horse anti-human IgG to colloidal gold is about three times the minimum amount of protein that will stabilize the gold against flocculation by NaCl (1 g/100 ml). The amount of antiglobulin remaining unbound to gold was estimated by separation of the protein–gold complex by centrifugation; approximately 10–15 per cent of the IgG remained free in the supernatant, as estimated by ^{125}I-labelling of the IgG prior to labelling with gold.

The antiglobulin-gold complex was examined by electron microscopy on a carbon-coated grid. The gold particles have an average diameter of 3 nm and there were on average about 1·6 particles of gold to each molecule of antibody. Only about 3 per cent of the antibody molecules had five or more gold particles. Occasionally, dimers or aggregates were observed.

Bovine serum albumin was found to have an ability even greater than horse IgG to 'protect' colloidal gold against flocculation by NaCl, and it was estimated that one molecule of BSA was able to stabilize about 30 gold particles. Colloidal gold particles are negatively charged in water and one might presume that they bind to the NH_3^+ groups on the protein. It seems likely, however, that other charged groups or polar groups on the protein surface are more important in binding the gold particles, since we have found that the reaction of NH_3^+ groups on BSA with formaldehyde and dinitrophenyl groups has no effect on the 'protecting' ability of the protein against flocculation of the colloidal gold upon the addition of salt.

The antiglobulin retains its antibody activity extremely well after labelling with gold. Approximately 50 per cent of a ^{125}I-labelled antiglobulin–gold complex could be absorbed by human red cells coated with anti-D. Its use in localizing anti-D on human erythrocyte ghosts is illustrated in Fig. 1. Red cells (group O, R_1R_2) were reacted with human IgG anti-D and then, after washing, incubated with the gold-antiglobulin reagent. The cells were lysed at an air–water interphase and floating ghosts picked up on to a carbon-coated grid for examination with the electron microscope. This method of labelling is proving of value in the identification of the distribution of A and D sites on red cells.

Gold particles are denser than ferritin and thus they can be distinguished from the latter by electron microscopy. This allows for the simultaneous use of both markers for mapping the surface distribution of two antigens at the same time.

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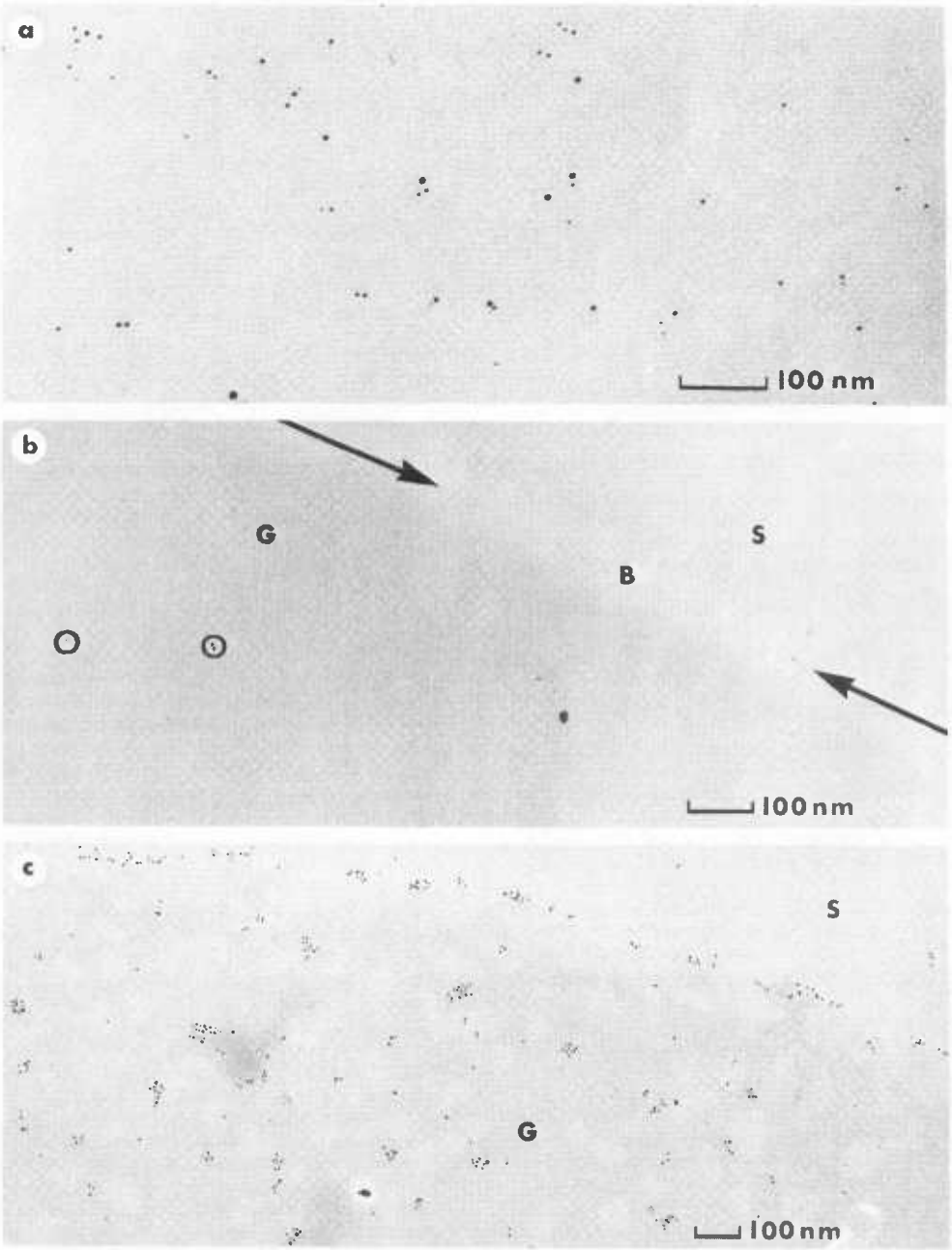

Fig. 1. Electron micrograph. (a) A dilute solution of gold-labelled anti-IgG. (b) Control. Rh-positive red cell and gold-labelled anti IgG only. Only a few gold particles are present over the ghost (G). B, edge of ghost; S, substrate. (c) Rh-positive red cell combined with anti-D and followed by addition of gold-labelled anti-IgG. G, ghost; S, substrate.

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