THE COMPLEMENTARY HEMOLYSIS OF NORMAL ERYTHROCYTES

A Thesis

Presented to

The Department of Medical Microbiology

Faculty of Medicine

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Kenneth Howard Allen Jacob

August 1967

ABSTRACT

Normal washed human erythrocytes are susceptible to lysis, usually to the extent of 5 to 15%, when treated with guinea pig complement (C^1). The amount of lysis rises with initial washings to a peak and successive washings bring about a decrease.

The lytic system consisted of a total volume of 6.0 ml., with a final concentration of 3.5% erythrocytes and 16.5 HD_{50} guinea pig C^1/ml. The incorporation, in the system, of serum from rheumatoid arthritis patients, which is known to inhibit immune hemolysis, prevented lysis of normal washed erythrocytes.

The osmotic fragility of these erythrocytes was also tested as the cells were being washed. The fragility did not change. Heat inactivated C^1 did not produce lysis. Testing of concentrates of the wash waters revealed the presence of IgG or 7S globulins.

This lysis could theoretically be due to an antibody in the C^1 or to an autoimmune antibody on the washed cells. Various considerations suggest that the latter is the case and that antibody against γ-globulin which is present normally in blood prevents *in vivo* lysis.

Author - K.H.A. Jacob.

August, 1967.

ACKNOWLEDGEMENTS

To Dr. J. A. Romeyn, the author would like to express special thanks for his constant support and valuable guidance.

The author also extends thanks to Dr. J. C. Wilt and the staff of the Department of Medical Microbiology of the University of Manitoba for their advice and assistance.

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

This study arose out of the finding that normal human erythrocytes and erythrocytes from rheumatoid arthritis patients were susceptible to lysis by complement (C').

Romeyn and Onysko (1964) showed that antibody against rabbit γ-globulin would inhibit the lysis of sheep red blood cells sensitized with rabbit-produced amboceptor. This inhibition did not involve the destruction of C'.

Arguing that rheumatoid factor was an antibody against γ-globulin, Bowman (1966) titrated the ability of sera from rheumatoid arthritis patients to inhibit the complementary lysis of such sensitized cells. The titres correlated closely with the results of the latex fixation tests and the sensitized sheep cell agglutination tests for rheumatoid factor.

Bowman then suggested that the presence of a material in rheumatoid sera which would inhibit the complementary lysis of sensitized cells might mean that the cells of rheumatoid arthritis patients were in fact sensitized by an autoimmune antibody and required protection from complement. He therefore washed the red blood cells from both rheumatoid arthritis patients and normals in an effort to remove the protective activity. He then treated the cells with guinea pig C'. By using high concentrations of cells and complement, he detected between 0.5 and 15.5% lysis of the cells, but found no evidence that rheumatoid cells differed from normal ones.

These findings led to the present study which involves the

examination of the lysis of washed human erythrocytes by guinea pig complement, and the mechanism by which it is produced.

CHAPTER I

REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

As indicated in the Introduction, the present thesis involves a study of the lysis of normal human erythrocytes by guinea pig complement. Since the evidence suggests that this lysis is not due to the presence of an antibody in the guinea pig complement, the phenomenon suggests that normal human erythrocytes have combined with an auto-immune antibody.

In the early 1900's, Ehrlich enunciated the classic doctrine of "Horror autotoxicus". Some of the experiments on which this idea was based were reported by Ehrlich and Morgenroth in 1901. They were convinced that there existed "certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements and so giving rise to 'autotoxins' " (Ehrlich and Morgenroth, 1901). They immunized a "strong male goat" with nearly a litre of blood obtained from three other goats. The serum which they obtained following immunization lysed the cells of all but one of the nine goats they tested. Significantly, the cells of the immunized goat itself were completely unaffected by the haemolytic serum. The concept of "Horror autotoxicus" still stands today, and the breakdown of the "internal regulating devices" has been shown to lead to great dangers to the individual as Ehrlich suggested.

These classic studies of Ehrlich and Morgenroth carried out at

at the beginning of this century were of great importance in investigating the ability of the individual to react immunologically against its own material.

The present results may or may not be an example of "autotoxicity" in Ehrlich's sense. If they are not, at least we may suspect that they are due to a reaction of fresh guinea pig serum with constituents of the red cell membrane. Furthermore, the reactive constituents of the membrane might be part of the membrane itself or some material absorbed by the membrane from the plasma. Finally, if the phenomena are due to autoantibody, it should be of help to study the known instances of autoantibody production against red cells.

Accordingly, the literature review will be considered under the following headings:

1. The red cell membrane.
2. Serum components which bind to the red cell membrane.
3. The detection, properties and mechanism of production of red cell autoantibodies in autoimmune disease.

1. The red cell membrane

The red cell membrane is made up of a variety of complexes, including lipids, proteins, mucopolysaccharides as well as electrolytes. Since there appears to be a vast array of each of these components, they will only be discussed generally here. All these categories have been reviewed by Bishop and Surgena (1964).

All of the blood group antigens must be considered to be

components of the red cell membrane. These are comprised mainly of protein. The surface charge of the erythrocyte membrane is primarily associated with neuraminic acid (Eylar, 1962), probably involving the carboxyl group of this acid (Cook, 1961).

Abderhalden (1908) first reported that the lipid concentration of the red cells differed from that of the plasma. The phospholipids were shown to be cephalin, lecithin and sphingomyelin by Hack (1942). Because the lipids of the red cell are primarily structural in function and are concentrated at the surface (Ponder, 1954), the amount of lipid is related to the surface area of the cell itself. For this reason, the total lipid content of the cell is thought to be in the cell membrane (Boyer, 1961). These lipids are varied and play a predominant role in the selective permeability of the cell wall. Hughes-Jones and Gardner (1962) have demonstrated the exchange of I^{131} labelled lipid with that found in the cell wall.

Blagarazumova (1959) has shown that cholesterol bound in the cell wall is associated with protein, although there is exchange between the protein bound cholesterol and the cholesterol of the plasma. This was a confirmation of the work of Brun (1939), in which he showed that cholesterol existed in the free form rather than esterified. This factor of exchange with plasma components has been shown to apply to other constituents as well.

In the membrane of the red cell are also found electrolytes and amino acids. The amino acids have been assayed by Corsini and his co-workers (1959b). The electrolytes have also been shown to exist

exist in the cell membrane and to exchange with free electrolytes in the plasma (Hughes-Jones and Gardner, 1962).

2. Serum components which bind to the red cell membrane

The components of the plasma are varied and more numerous than those found in the membrane of the red cell itself.

Most of the red cell membrane components are found in the plasma, but besides these there are also various globulins, components of the complement system, as well as other factors such as conglutinins and opsonins.

Plasma cholesterol has been shown to freely exchange with cholesterol in the red cell membrane. Other components have also been shown to do this, components such as fatty acids, iodine, globulins and certain components of the complement system.

Hughes-Jones and Gardner (1962) by means of labelled I^{131} fatty acids showed definate exchange between the red cells and the plasma. Their experiments also showed the ability of inorganically labelled plasma iodine to exchange with inorganic iodine in the red cell membrane.

Stratton and Jones (1955) found that normal cells had a globulin-like substance attached to their surface and these cells gave a positive anti-globulin (Coombs) test when mixed with antihuman globulin serum produced in the rabbit. This evidence suggests that normal globulins can be bound by the surface of

normal human erythrocytes.

Chaplin and Cassell (1960) found that the eluates from normal erythrocytes contained a substance which was able to elicit an antibody against globulin in rabbits. This was probably the strongest evidence that normal cells existed in the state of "partial sensitization" with a globulin-like substance attached to their surface.

The term opsonin is generally applied to any protein in the plasma which will enhance the overall protein composition of a bacterium or any other particle rendering the particle more susceptible to phagocytosis. The exact nature of the opsonin or protein deposited on the surface of the particle has not as yet been analysed, but these substances are in the serum and are free to attach or adhere to particles.

Coombs and Coombs (1953) proposed that conglutinin, a substance present in serum which will agglutinate certain complexes of antigen, antibody and complement, is an antibody. This antibody is directed against fixed complement.

Certain complement constituents have been shown to exist on the surface of erythrocytes, namely β_{IE} and β_{IC} (Harboe, et al., 1963).

3. The detection, properties and mechanism of production of red cell autoantibodies in autoimmune disease:

Detection

The various methods used to detect autoimmune hemolytic

antibodies have been reviewed by Casey (1966).

Most laboratories today use three basic tests to detect autoantibodies to erythrocytes. These tests form the basis of the diagnosis of autoimmune haemolytic anemia (AIHA), and they are also applicable to other diseases. The antibodies may be attached to the red cell or may be free in the circulation. The tests used are the direct Coombs test, the indirect Coombs test and the tests using red blood cells which have been pretreated with various enzymes, such as bromelin (Dacie, 1960a).

A. Direct Coombs Test

This test (Coombs, Mourant and Race, 1945) is used to detect antibodies attached to red cells. These may be "complete" or "incomplete" antibodies. Complete antibodies are those which will agglutinate red cells in a saline medium. The incomplete antibodies will not agglutinate red cells in a saline medium but will in a protein rich medium. The Coombs test is also known as the antiglobulin test.

The Coombs test makes use of the fact that red cells coated with globulins will be agglutinated by an anti-γ- globulin serum. A suitable anti-globulin serum can be prepared by immunizing rabbits with either human serum or human γ-globulin. The serum produced is then treated with A, B and O cells to remove anti-A, anti-B and other non-specific anti-human factors. This absorbed serum will agglutinate red cells coated with incomplete antibody, due to the anti-γ- globulin in it. This ability is removed when

γ-globulin is added to the Coombs serum, but not when other globulins are added (Dacie, 1962).

A positive direct Coombs test means that the red cells are coated with protein that reacts with the Coombs serum. This is usually due to the presence of a warm incomplete antibody on the red cell surface. It reacts with the red cell optimally at 37 C. Sometimes, there are mixed γ and non-γ globulins on the cells, while in other cases there are globulins of the cold variety (Dacie, 1960b).

Dacie stresses that a positive direct antiglobulin test does not necessarily mean that the patient is suffering from AIHA. False positive results may be due to blood being refrigerated, when an incomplete cold non γ-globulin antibody usually present in human sera is adsorbed onto the red cells.

At the same time (Dacie, 1960a), there are three causes for false negative results occurring: first, an impotent Coombs serum; second, failure to wash the cells enough to remove all the serum (this would leave γ-globulins present which would negate the results); and lastly, inappropriate dilutions of the antiglobulin serum itself.

The complete or saline acting antibodies were regarded as being bivalent and consequently able to lead to agglutination <u>in vitro</u>, whereas the incomplete or blocking antibodies were considered monovalent and therefore incapable of causing agglutination. This incomplete antibody is predominant in hemolytic disorders and is able to cross the placental barrier. When both complete and

incomplete forms are present, the latter form seems to adsorb preferentially on the red cells which possess the antigen.

It has been shown that when in an apprpriate medium, the incomplete antibodies will cause agglutination. Such a medium is 20% bovine albumin. The ability of incomplete antibodies to cause agglutination in such a medium probably accounts for the destruction of erythrocytes in AIHA. Such antibodies are thought to cause small agglutinates in the blood which are removed and destroyed mainly in the spleen (Dacie 1960b).

In humans the types of autoantibody found usually are regarded as lacking specificity, although there may be a relationship with the Rh systems. This led to speculations about the antibody being directed against some fundamental precursor of blood group substance (Dacie, 1959, 1960b and 1962).

The γ-globulin of an animal comprises many specific antibodies but these heterogeneous components do not have different antigenic properties when injected into other species (Abrahams, 1962). It is on this fact that the efficiency of the Coombs test rests. This test will detect a wide variety of antibodies as well as those involved in AIHA. The Coombs test has helped considerably to elucidate the mechanisms of Rh sensitization, the historical aspects of which have been reviewed by Dacie (1962) and Race and Sanger (1962). The latter authors point out that in addition to the Rh mechanisms, the Kell system of blood groups has also been demonstrated by the test. This test has further demonstrated

practically all the subsequently discovered blood group systems by detecting antibodies to the antigens involved.

B. Indirect Coombs Test

The indirect Coombs test is used to demonstrate the presence of free, circulating incomplete antibodies in the serum. The test serum is incubated with normal cells which contain the appropriate antigens. After washing the cells to remove any serum globulins, non-specifically adsorbed, Coombs anti-human globulin serum is added. Agglutination indicates that γ-globulin antibodies are on the test cells, having been adsorbed from the serum to be tested.

This test is of less importance in the diagnosis of AIHA than is the direct Coombs test. It is however, essential in identifying the donor blood least likely to cause hemolysis if such a patient requires a transfusion.

C. Pretreatment of Erythrocytes with Enzymes

About the same time that Coomb's et al (1945) developed their method for detecting incomplete antibodies, Pickles (1949) found that cells previously exposed to a filtrate of a broth culture of Vibrio cholerae would show specific agglutination with incomplete anti-D sera. He also demonstrated that similar results could be obtained by enzymatic treatment of the red cells using trypsin. Pickles doubts if the mechanisms involved in these two cases are the same. Some structural alteration of the erythrocyte surface renders it agglutinable by incomplete antibodies in a saline medium.

Dacie (1964) notes that various enzymes may be used in place of trypsin, enzymes such as papain and ficin as well as bromelin.

Marrack (1963) has stated that although the indirect Coombs test is very sensitive, detecting antibody levels of about 54 µgms per ml., it is about four times less sensitive than the various enzyme techniques.

A ficin enzyme technique has been used by Helyer and Howie (1963) to show the presence of free circulating antibody in the serum of mice with AIHA and their work has been confirmed by Holmes and Burnet (1964) using cells treated with the enzyme papain.

Properties of Autoimmune Hemolytic Antibodies.

In a review by Leddy (1966), the characteristics of autoantibodies are outlined. The properties of autoantibodies are similar to those of the various immune globulins. Anti-erythrocyte antibodies are generally of the 7S size, these may be either γA or more commonly γG. The γA antibodies have been demonstrated by Ishisaka (1965), Kunkel (1963) and Rawson (1964). The more common γG antibodies have been shown by Mollison (1961) as well as Polley (1962). However, sometimes antibodies to erythrocytes are of the larger variety, either γM or as polymers of γA (Fudenberg, 1957).

There are two basic types of antibodies, those which agglutinate and those which are non-agglutinating. The non-agglutinating antibodies appear to be nearly always γG globulins. Recently, non-agglutinating γA globulin Rh isoantibodies have been demonstrated (Prager, 1964).

Some isoantibodies and certain types of autoantibodies are capable of directly lysing normal human erythrocytes, in vitro, in the presence of human C'. Both γG and γM antibodies may be hemolysins, but γA antibodies appear to be incapable of producing in vitro hemolysis, even in the presence of heterologous C' (Heremans, 1963 and Polley, 1963). A hemolysin may also agglutinate erythrocytes and in the absence of C' that is all one would observe.

In order for these antibodies to be classified as hemolysins,

they must have the capacity to activate the complete sequence of C' components at the erythrocyte membrance (Leddy, 1966).

Pirofsky (1965) worked on the structural properties and specificity of the erythrocyte autoantibodies from AIHA. The warm acting erythrocyte autoantibodies were studied using I^{131} kinetic techniques, 2-mercaptoethanol procedures and streptomycin inhibition (streptomycin inhibits the agglutinating activity of anti-Rh antibodies). This study indicates that the reaction between the erythrocyte surface and the autuantibodies was similar to the reaction seen with the incomplete Rh antibodies. Also, the molecular conformations of the erythrocyte autoantibodies and the incomplete Rh antibodies were similar. When the tertiary structure of both was altered, a similar number of reactive groups were exposed. Pirofsky postulates that the erythrocyte coating material is an antibody with its specificity directed against the entire Rh complex.

Warm reacting incomplete antibodies are detachable in vitro from red cells or stroma (Evans,1957), suggesting that there is a dynamic equilibrium between the antibodies and the red cell membrane and that of the serum. Evans et al (1961) have shown that antibody from patient's cells can be transferred to normal cells, in vitro, in a saline medium, and that this process may involve C'.

In the cases of the warm type of antibody associated with AIHA, the red cells seem to be coated with an antibody which reacts best at 37 C and there are no abnormal cold agglutinins in the serum.

In most cases, the patient's cells appear to be coated with only

γ-globulin because they fail to react with an antiglobulin serum to which γ-globulin has been added. In other cases, the red cells do react with the antiglobulin serum to which γ-globulin has been added indicating that the red cells have adsorbed some "non-γ-globulin; it now appears that this material is of the C' system and is namely β_1C and β_1E (Eyster, 1966).

Mechanism of Production of
Autoimmune Hemolytic Antibodies in Disease

How and why an apparently healthy individual should suddenly revolt against his own red cells, leukocytes or platelets still remains a mystery. In recent years, however, there have been several hypotheses put forth and some relevant experimental studies carried out.

There are two main ideas regarding the formation of autoantibodies. First, it is thought that red cells, leukocytes or platelets undergo some changes which render them antigenic. This might be caused by unmasking of hidden antigenic sites. On the other hand, it could be accomplished by the body, or possibly bacterial enzymes, by adsorption of viruses, or perhaps by adsorption of bacterial products or drugs. This latter consideration certainly explains drug induced hypersensitivity with the associated hemolytic anemia, thrombocytopenia and leucopenia, but it does not explain disorders which occur "spontaneously", as many of them do. There has been no support of this hypothesis gained through

experimental work on animals.

The second hypothesis attributes the primary fault to the antibody producing tissue itself. Burnet (1959) has supported this idea, particularly with respect to the hemolytic anemias, and explains the results as failure of normal homestatic mechanisms and the emergence of "forbidden" clones of antibody-producing cells. These"forbidden" clones do not have the capacity of self recognition. The normal antigens therefore appear "foreign" to these cells, stimulating the production of autoantibodies. These clones emerge as a result of spontaneous somatic mutations or as the result of viral or other forms of infection. Somatic mutations are most clearly evidenced in the hemolytic anemia complicating lymphoid neoplasms, particularly lymphosarcoma and chronic lymphatic leukemia, wherein autoantibodies are presumed to be synthesized by the neoplastic tissue itself (Dameshek, 1961).

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

General

All sera were inactivated at 58 C for thirty minutes when used for the first time and subsequently at 58 C for ten minutes before each use.

Centrifuging was done in either the International Portable Refrigerated Centrifuge Model PH-2 or the International Centrifuge Size 1 Model 5VB.

All optical densities were measured using a Hausch and Lomb Spectronic Twenty. Due to the reduction of some oxyhaemoglobin to methahaemoglobin when sensitised cells are lysed in the presence of complement, absorption is maximal at a wave length of 540 mμ. Therefore, optical densities were read at this wave length, with the following exception: the initial standardization of cell suspensions was performed at 550 mμ as cells lysed in distilled water show maximal absorption at this wave length (Kabat and Mayer, 1964).

Erythrocytes and Cell Suspensions

Sheep's blood was collected in a Baxter Transfuso-Vac bottle (Baxter Laboratories of Canada Ltd., Alliston, Ont.) containing 2.30 gm. per cent dextrose and 1.70 gm. per cent sodium citrate.

It was stored in the refrigerator at least four days prior to using.

Rabbit erythrocytes were obtained by cardiac puncture of normal laboratory white rabbits, mixed with an equal volume of sterile Alsever's Solution (see Appendix) and refrigerated.

Human erythrocytes were collected from normal donors. Immediately following venipuncture, the blood was added to a heparin solution at the rate of 10.0 ml. of blood to 0.1 ml. of 1% heparin sodium U.S.P. The blood was used immediately.

Human Group O Rh negative cells were obtained in the form of whole blood in standard transfusion bottles from the Winnipeg Branch of the Canadian Red Cross. These were kept in the refrigerator at 6 C until used.

The washing and standardisation procedure was identical for the different types of cells unless otherwise specified. Before use, the blood was centrifuged at 1000 x G for five minutes and the plasma discarded. The packed cells were then washed three times in modified Dulbecco Phosphate-Buffered Saline (DBS) (see Appendix) in 15.0 ml. centrifuge tubes, using a centrifuge speed of 1000 x G. Each of the first two washings were for five minutes while the third washing was for ten minutes. The packed washed erythrocytes thus obtained were used to make a cell suspension in DBS, such that 1.0 ml. of the suspension, plus 9.0 ml. of distilled water had an optical density of 0.225 at a wave length of 550 mμ. This is equivalent to approximately a one per cent cell suspension, and will be referred to subsequently as "2.25

cells". A blank consisting of 1.0 ml. of DBS and 9.0 ml. of distilled water was used to zero the machine. Cells were prepared freshly in this manner each day of an experiment and kept in the refrigerator until used.

Sensitizer

Rabbit -produced anti-sheep cell sensitizer was obtained from Markham Laboratories, Chicago 20, Illinois.

Anti-rabbit erythrocyte sensitizer was prepared by injection of fifty per cent suspensions of rabbit red blood cells into normal guinea pigs. These pigs were injected intraperitoneally with 1.0 ml. of the cell suspension from five to seven times over a period of two to three weeks and then bled for serum on two successive days following a five day interval after the final injection.

Anti-human red blood cell sensitizer was prepared by injection of washed erythrocytes in rabbits. Human Group O Rh negative cells were washed three times as previously described. The washed packed erythrocytes were used to make up a fifty per cent cell suspension in DBS. Normal rabbits were injected by a method analogous to that described above for the anti-rabbit erythrocyte sera.

Complement

The complement source was dehydrated guinea pig serum (Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ont.) which was reconstituted with the requisite volume of cold distilled water immediately before use, and kept in an ice bath until used.

Complement was also obtained by bleeding normal laboratory

guinea pigs. Cardiac punctures were performed on anaesthetized guinea pigs. The blood was placed in universal bottles and allowed to clot. After refrigeration overnight the serum was collected, pooled and centrifuged at 1000 x G for ten minutes at 4 C. The serum was distributed in 5.0 ml. amounts and kept at -76 C until used.

Titration of Sensitizer for Complementary Lysis.

Doubling dilutions of sensitizer were made in DBS in 1.0 ml. amounts in serological test tubes. To each tube was added 1.0 ml. of 2.25 cells, and the tubes were incubated at 37 C in a water bath for twenty minutes. After the incubation the tubes were transferred to an ice bath. To each tube was added 1.0 ml. of cold DBS and 1.0 ml. of 1:15 complement in DBS. The tubes were then incubated at 37 C for twenty minutes with intermittent shaking. After incubation the tubes were centrifuged in the cold at 1000 x G and 5 C for five minutes. The supernatants were then transferred to clean test tubes and the optical densities read at 540 mμ. The blank to which the spectrophotometer was zeroed consisted of the supernatants of the tubes put through the mechanics of the above procedure and containing 1.0 ml. of 2.25 cells, 1.0 ml. of 1:15 complement (in DBS) plus 2.0 ml. of DBS. Appropriate controls were included to determine the effect on the cells of DBS, complement and sensitizer alone. Duplicate tubes were prepared using 1.0 ml. of 2.25 cells plus 3.0 ml. of distilled water.

The optical density of these tubes was read at 540 mμ, with the machine being zeroed to a blank consisting of 1.0 ml. of DBS plus 3.0 ml. of distilled water. The mean values were then calculated thus giving a reading for one hundred percent lysis. The per cent lysis produced in any tube in the titration was calculated using the formula:

$$\% \text{ lysis} = \frac{\text{Optical density of the supernatant at 540 m}\mu}{\text{Optical density of one hundred per cent lysis}} \times 100$$

Results were plotted on logarithmic-probability paper with the reciprocal of sensitizer dilutions as abscissae and the per cent lysis as ordinates. An average straight line was drawn through the points, and the dilution of sensitizer producing fifty per cent lysis (1 HD_{50}) was noted. Three fifty per cent hemolytic doses (3 HD_{50}) were used in routine sensitizations.

Preparation of Sensitized Cells for Complementary Lysis

Equal volumes of 2.25 cells and 3 HD_{50} of sensitizer were mixed in a 50.0 ml. centrifuge tube and incubated at 37 C for twenty minutes with intermittent shaking. The tubes were centrifuged at 1000 x G and 5 C for ten minutes. The supernatants were decanted, DBS was added to the packed cells without resuspending and centrifugation was repeated. This procedure will be termed "washing briefly once". The supernatant was pipetted off and the cells were reconstituted to their original volume with DBS. After thorough mixing with a Vortex Junion Mixer, the cells were pooled

and stored in the refrigerator until used. They were well vortexed again prior to use to ensure an even suspension.

Titration of Complement

Complement was titrated each day of an experiment. A dilution of 1:30 complement was made in DBS after the serum was allowed to melt. This was further distributed in 0.9, 0.8, 0.7 ml. amounts in two sets of 10 tubes. Amounts of DBS to make up to 1.0 ml. volumes were added to give a harmonic series in the duplicate rows, A and B. Next, 2.0 ml. of cold DBS was added to each tube, followed by the addition of 1.0 ml. of sensitized 2.25 cells to each tube of Row A and 1.0 ml. of unsensitized 2.25 cells to Row B. Controls were included containing sensitized cells and DBS, unsensitized cells and DBS and unsensitized cells, complement and DBS. The tubes were then incubated at 37 C for twenty minutes with intermittent shaking. Immediately following the incubation, the tubes were centrifuged at 1000 x G and 5 C for five minutes. The supernatants were decanted into clean tubes and the optical densities at 540 mμ of each tube of sensitized cells in Row A read against the corresponding tubes of unsensitized cells in Row B. The mean optical density of two duplicate supernatants, each obtained from 1.0 ml. of sensitized cells plus 3.0 ml. of distilled water and put through the mechanics of the above titration procedure, was used as the optical density at one hundred per cent lysis. The per cent lysis in each tube was

calculated using the same formula described under Titration of Sensitizer for Complementary Lysis. The results were plotted on logarithmic-probability paper with volume of undiluted complement per tube as abscissae and per cent lysis as ordinates. An average straight line was drawn through the points, and the amount of complement corresponding to fifty per cent lysis taken as one fifty per cent hemolytic dose (1 HD_{50}). In routine experimentation four times this amount of same complement was used, unless otherwise specified.

Rheumatoid Sera

The "rheumatoid sera" were obtained from patients with rheumatoid arthritis. The serum was collected, and kept at -20 C when not in use. Prior to being used for the first time, they were allowed to melt completely and were inactivated at 58 C for thirty minutes. During successive uses the sera were again allowed to melt and inactivated at 58 C for ten minutes.

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

CHAPTER III

PROCEDURES AND EXPERIMENTAL RESULTS

1. WASHING OF NORMAL ERYTHROCYTES

Bowman (1966) found that when he washed fresh human erythrocytes three times in DBS, a mean per cent of 6.5 lysis occurred when high concentrations of C' were added. To see if any change in lysability would occur, the cells were repeatedly washed. The design of this experiment was as follows:

Procedure

The donor was bled and the blood heparinized. The ratio of blood to heparin was 10.0 ml. of blood to 0.1 ml. of heparin 1%. The tubes were centrifuged at 1000 x G for five minutes at 5 C. The supernatant was then removed and an aliquot of packed cells, 0.7 ml., was removed and made up to 7% suspension in DBS. This suspension was called "zero washed cells". The remaining packed erythrocytes were mixed with DBS, and centrifuged. The supernatant was again removed and an aliquot of packed cells taken and made up to a 7% suspension in DBS. This suspension was called "once washed cells". This procedure of washing was continued and the erythrocytes washed repeatedly.

The 7% cells from each wash were then added in 3.0 ml. amounts

to fresh tubes. DBS in 1.0 ml. amounts was added. Finally 2.0 ml. of C' containing 100 HD_{50}, was added. This C' was previously titrated prior to each experiment and was adjusted to contain 100 HD_{50} (see Materials and Methods). The mixture was then incubated at 37 C for sixty minutes.

The tubes were then removed from the water bath and 0.5 ml. of the mixture were added to 4.5 ml. of distilled water. This provided a measure of 100% lysis. The incubated tubes were then centrifuged at 1000 x G for five minutes at 5 C. The supernatants were transferred to fresh tubes and were read on the spectrophotometer at an optical density of 540 mμ. The machine was blanked to 4.0 ml. of DBS and 2.0 ml. of the pooled C' used.

The readings were recorded and the per cent lysis occurring at each wash calculated.

Results

The results for three different donors are given in Fig. 1 and Table I. The experiment was performed a total of fourteen times on the three donors. Observations indicate that when the cells are washed the amount of lysis rose to a peak after two or three washes. After the peak was reached each successive wash resulted in a decrease in the amount of lysis. The amounts of lysis, at the maximal level, ranged from 3 to 5%. This level decreased gradually as the number of washings increased and 0.5% lysis occurred after the

cells had been washed eighteen times in DBS.

From time to time during these experiments, washed cells were treated with heat inactivated complement and no substantial degree of lysis was ever observed. Samples of such readings are shown in Table III, and represent of the order of 0.1 or 0.2% lysis. Thus, Table I shows a startlingly large percentage of lysis.

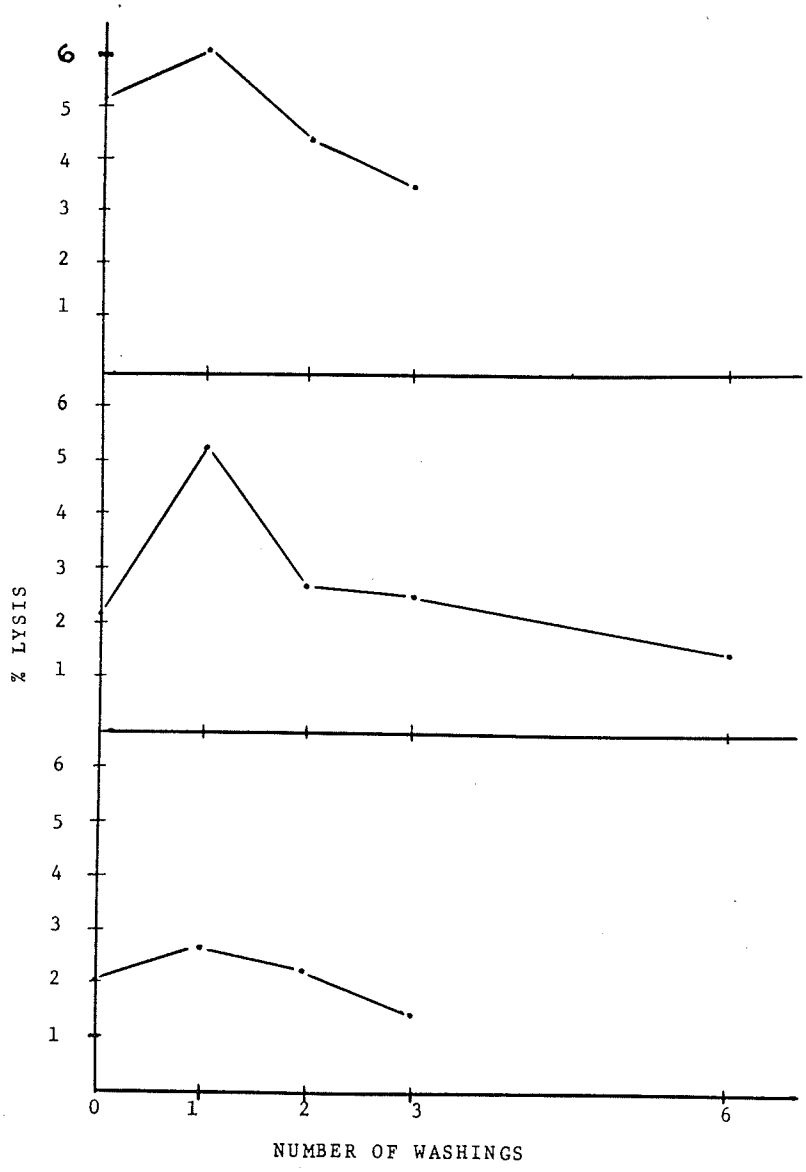

Fig. I. Donors A,B,C - Erythrocytes washed, treated with complement.

TABLE I

TREATMENT OF WASHED HUMAN ERYTHROCYTES WITH COMPLEMENT
(DATA FROM FIG.1) A,B AND C ARE DONORS

No. Washings	OD540	O.D. 540 100% Lysis Calculated	% Lysis
		A	
0	0.52	10.0	5.2
1.	0.62	10.0	6.2
2	0.45	10.0	4.5
3	0.36	10.0	3.6
		B	
0	0.225	10.6	2.1
1	0.585	11.6	5.04
2	0.28	10.6	2.6
3	0.25	11.0	2.7
6	0.17	10.4	1.6
		C	
0	0.17	8.5	2.0
1	0.2	7.5	2.7
2	0.18	7.5	2.4
3	0.1	6.0	1.6

2. TREATMENT OF NORMAL ERYTHROCYTES WITH RHEUMATOID FACTOR

The presence of rheumatoid arthritis sera does not inhibit lysis by the destruction of the complement (Bowman, 1966) but rather seems to block the complement from combining with the sensitized cell. If the lysis occurring in experiment 1 is immune lysis, it may be inhibited by the presence of the rheumatoid serum. The following experiment was designed to investigate this possibility.

Procedure

The erythrocytes were obtained and washed as outlined in experiment 1. After the cells had been washed twice in DBS, the washed packed erythrocytes were made up to a 6% suspension in DBS. The 6% cells were then divided into two equal aliquots each containing 3.0 ml. of 6% cells. Set I was incubated with 1.0 ml. of DBS. These sera were obtained from the Rheumatoid Arthritis Clinic and were of high titre. The incubation was carried out at 37 C for twenty minutes. The C' was then added in 2.0 ml. amounts (containing 100 HD_{50}/ml.) to both Sets I and II and incubated at 37 C for sixty minutes.

After the incubation period, the tubes were centrifuged and the supernatants were read on the spectrophotometer. Blanks for reading Set I were made up of 4.0 ml. of DBS plus 2.0 ml. of C' and for Set II of 3.0 ml. of DBS, 1.0 ml. of 1:4 RAS plus 2.0 ml. of C'.

The above procedure does not determine whether the C' has been destroyed. A titration of the C' was therefore performed on

the supernatants from Sets I and II after the final incubation. The test system consisted of sheep erythrocytes sensitizied with guinea pig-produced amboceptor. Bowman has shown (1966) that RAS can cross react with rabbit amboceptor but little or no cross reaction takes place with guinea pig-produced amboceptor.

Results

The results of this experiment are shown in Fig. 2 and indicate that the RAS does inhibit the lysis of the washed normal erythrocytes. The titre of the C' before the experiment was 0.0125 ml. of undiluted C' necessary to cause 50% lysis of sensitized cells. After the experiment, the C' titre changed to 0.016 ml. of C' for the DBS control and 0.017 ml. of C' for the RAS inhibition. Hence rheumatoid factor can prevent this lysis from occurring. The results obtained, illustrate this conclusion. Titration of the C' demonstrated that there was no C' destruction when the cells were treated with rheumatoid serum, and that C' destruction cannot be invoked as an explanation of the decrease in lysis in the presence of rheumatoid factor.

3. OSMOTIC FRAGILITY OF NORMAL ERYTHROCYTES

The lysis which was observed as described in experiment 1 could have been due to changes in the osmotic fragility of the cells as the erythrocytes were being washed. The following experiment was carried out in order to answer this question.

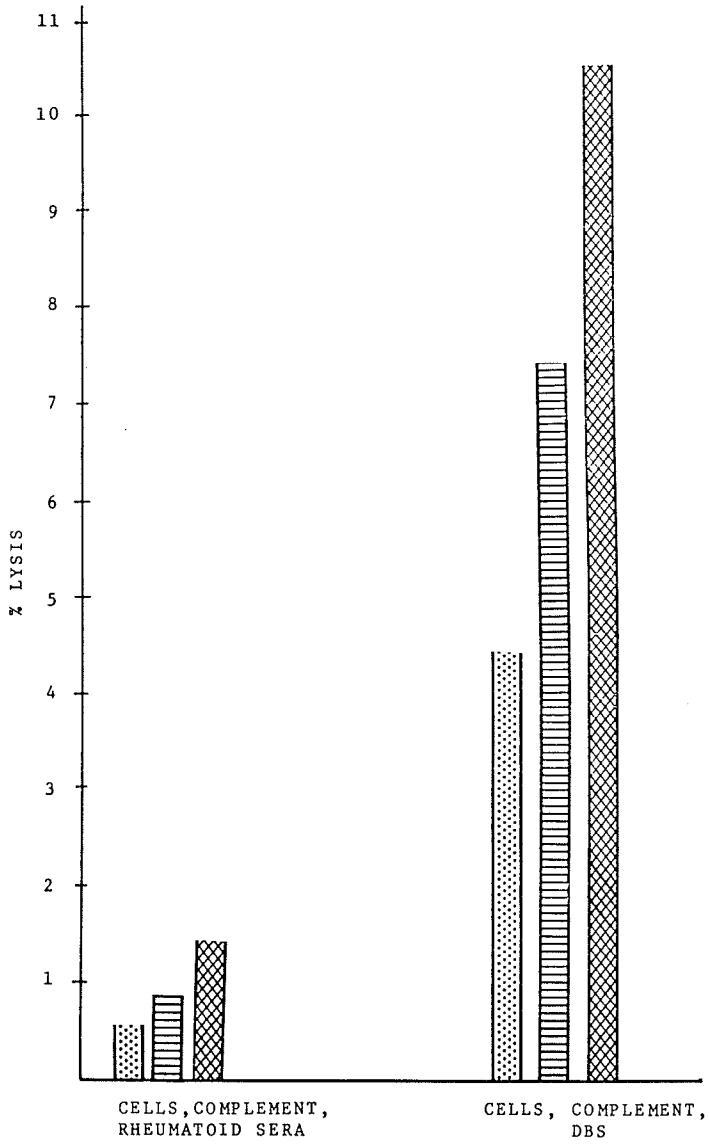

Fig. 2. The effect of rheumatoid sera on washed human erythrocytes in the presence of complement.

Three samples of cells are shown.

Procedure

If normal erythrocytes are suspended in a series of salt solutions of graded concentration from 0.5% to 0.28% NaCl, it is found that the first concentration in which any lysis at all is tested is usually about 0.46%, ("initial lysis") and that complete lysis occurs when the concentration is about 0.32%. Lysis is usually determined by simple inspection of the supernatants after centrifugation. Cells which are abnormally "fragile" show "initial" and "complete" lysis at higher salt concentrations.

NaCl solutions were prepared ranging from 0.5% to 0.28% NaCl. The difference between adjacent tubes was 0.02%. The solutions were distributed in 1.25 ml. amounts into each tube and rows were set up corresponding to the number of washings which had taken place. After each wash the supernatant was removed and an aliquot of packed cells was made up to a 50% suspension in DBS. One drop of this suspension was delivered through a Pasteur pipette to each tube in the corresponding row. This procedure was continued and after each wash, the 50% suspensions were distributed. The tubes were allowed to stand at room temperature for two hours. After centrifugation of the tubes, they were checked for any lysis occurring. The lysis was determined by means of visual interpretation. In salt concentrations decreasing from 0.85% (physiological), lysis of normal cells will first appear and is partial, at 0.40 to 0.46% NaCl. This is termed"initial" lysis. "Complete" lysis occurs between 0.3 and 0.36% NaCl.

Results

The cells were tested for their changes in osmotic fragility

as they were being washed. Results shown in Fig. 3 demonstrate that there was no change in their fragility as the washing occurred. When the procedure for osmotic fragility was accompanied by the procedure for the hemolytic susceptibility complement, the results were as observed. Fig. 4 illustrates that as the cells were being washed the amount of lysis followed the expected pattern, when the cells were treated with complement while the osmotic fragility of the cells remained unchanged. These results seemed to further suggest that this phenomenon which was occurring was not merely mechanical but rather an immune reaction. The osmotic fragility of the cells was tested three times and in each case, the figures recorded remained constant.

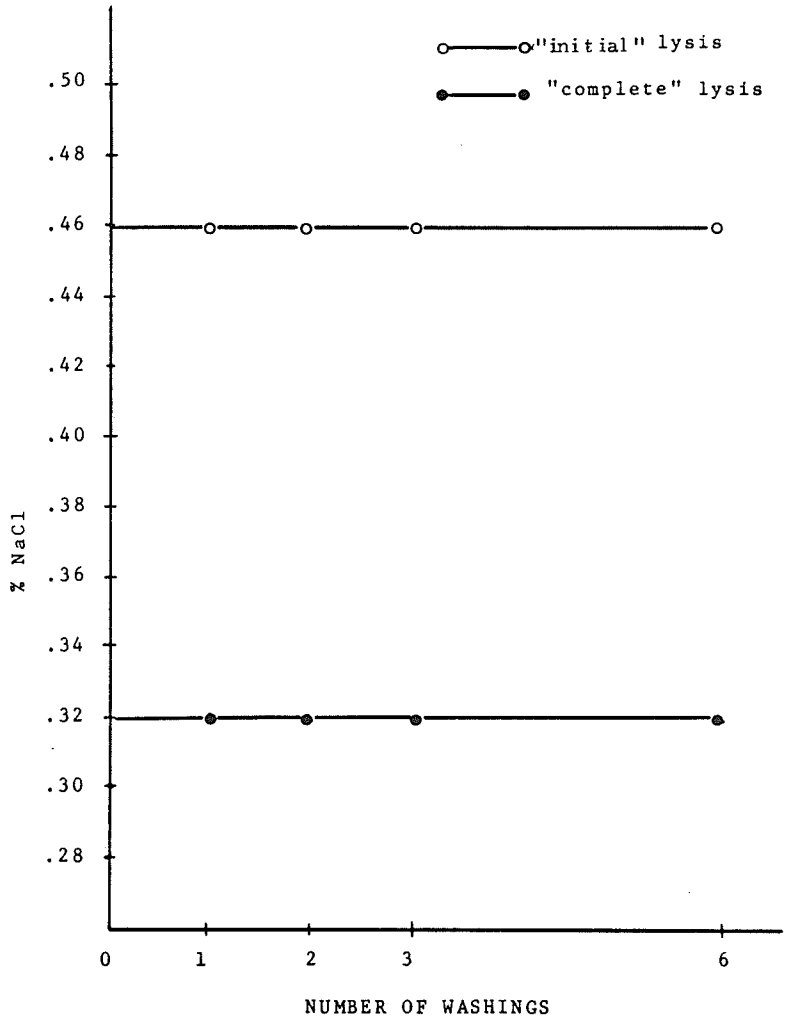

Fig. 3. Osmotic fragility of human erythrocytes repeatedly washed.

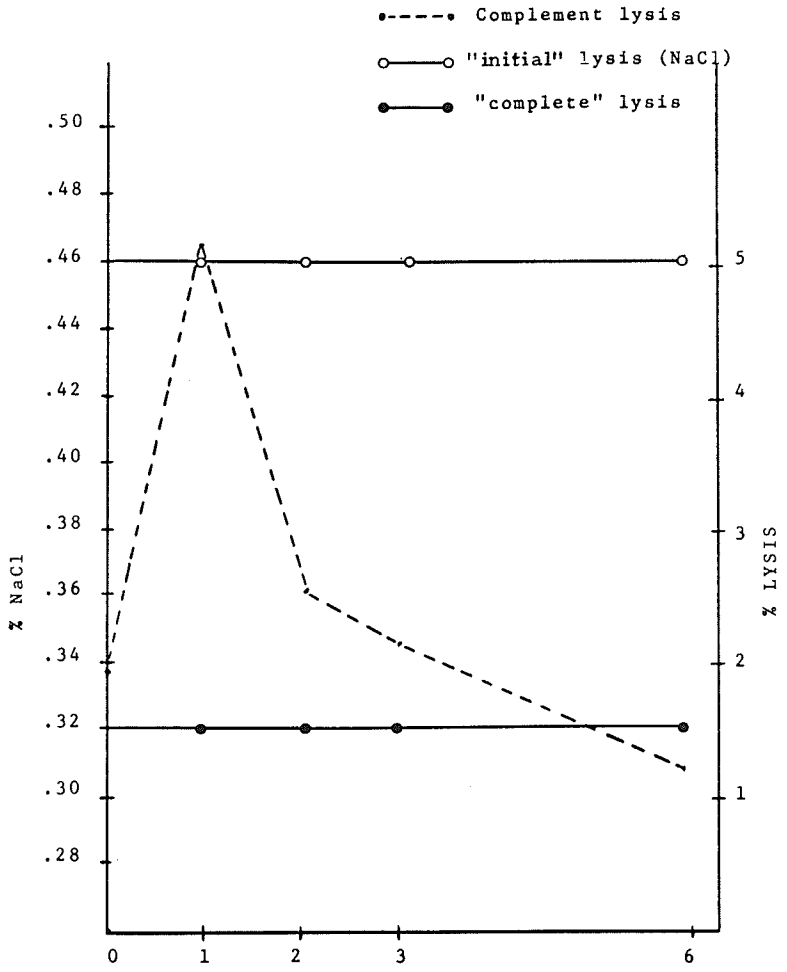

Fig. 4. Comparison of Osmotic fragility and haemolysis of erythrocytes treated with complement.

4. PRESENCE OF AN ANTIBODY IN THE COMPLEMENT

The lysis which occurred when normal washed erythrocytes were treated with C' could be due to the presence of an antibody in the C' which was directed against the erythrocyte.

4A. DOUBLE TREATMENT OF THE "UNSENSITIZED" HUMAN ERYTHROCYTES

If this lysis were dependant on an antibody in the complement then double treatments with the complement-containing guinea pig serum should give more lysis than single treatments. The following experiment was set up to test this.

Procedure

Group O, Rh positive erythrocytes were washed three times in DBS . A suspension containing 6.0 ml. of washed packed erythrocytes and 114.0 ml. of DBS was made, that is, a 5% cell suspension. The C' used in the test was heat inactivated at 58 C for thirty minutes. Eight test tubes were set up as shown in Table II.

TABLE II

EXPERIMENTAL SETUP FOR EXPERIMENT 4A

	1	2	3	4	5	6	7	8
5% cells	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
C' (inact)	1.1	1.1	1.1	1.1	-	-	-	-
DBS	-	-	-	-	1.1	1.1	1.1	1.1

The tubes were then left at room temperature for thirty minutes, with intermittent shaking. After centrifugation the supernatants were discarded and 4.2 ml. of DBS added to each tube. Tubes 1, 2, 5 and 6 received 1.1 ml. of fresh, active C'. Tubes 3, 4, 7 and 8 received 1.1 ml. of heated C'. Again the tubes were incubated at 37 C for twenty minutes and again centrifuged at 1000 x G for five minutes at 5 C.

Results

Results of this experiment are shown in Table III. The first two tubes received a treatment of heat inactivated complement followed by a treatment with fresh active complement. Tubes 5 and 6 received active fresh complement only. There was no difference between the two sets of tubes and if an antibody were present, the double treatment should have shown more lysis. This experiment was carried out twice with the same results each time. Hence, there appears to be no antibody in the complement unless it is a heat labile substance.

4B. ABSORPTION OF COMPLEMENT WITH HUMAN RBC

Absorption in the cold would not destroy such an antibody, and if it were present, it should be absorbed from the serum on the test erythrocytes.

An experiment was set up in order to decide whether there was a heat labile antibody in the C' which was directed against the human RBC. If such an antibody were present it would be destroyed

due to the heat inactivation process. Therefore, the following experiment was designed.

TABLE III

OPTICAL DENSITY OF TUBES FROM TABLE II WITH DOUBLE DOSES OF COMPLEMENT.

Complement Batch No.	1	2	3	4	5	6	7	8
A	0.8	0.76	0.02	0.02	1.2	0.9	0.01	0.01
B	1.4	1.5	0.04	0.03	1.4	1.4	0.02	0.01
C	1.0	1.2	0.015	0.015	1.5	1.4	0.015	0.015

Procedure

A pool of guinea pig serum was allowed to melt and was divided into two equal amounts. Fresh human erythrocytes were washed twice in DBS. Set I consisted of 2.5 ml. of two times washed packed cells plus 10.0 ml. of guinea pig serum. Set II consisted of guinea pig serum alone. Both sets were placed at 4 C for eighteen hours. The tubes were then centrifuged and the serum collected. A complement titration was performed on each of the sets and 100 HD_{50} calculated. Fresh two times washed human erythrocytes were then mixed with each of the sets of complement. The system was made up of 3.0 ml. of 7% cells, 1.0 ml. of DBS, and 2.0 ml. of the C'. The tubes were allowed to incubate at 37 C for sixty minutes.

Along with these tubes were also tubes which contained "cold complement treated cells" as well as "cold untreated cells". These cells were mixed with fresh active C' which had been titrated and adjusted to contain 100 HD_{50}. After the incubation, 0.5 ml. of each tube were removed and added to 4.5 ml. of distilled water. This provided a measure for one hundred per cent lysis. These tubes were read against distilled water. The test tubes from the first part of the experiment were read against blanks containing 4.0 ml. of DBS plus 2.0 ml. of the appropriate C'.

Results

Table IV illustrates the results. The absorbed C' when

TABLE IV

HEMOLYTIC ACTIVITY OF "ABSORBED" AND "UNABSORBED" COMPLEMENT

	O.D.540	O.D.540 100% Lysis Calculated	% Lysis
"Absorbed" and Fresh RBC	0.07	9.4	0.74
"Unabsorbed" and Fresh RBC	0.69	9.2	7.5
C' (Fresh) and "Absorbed" RBC	1.50	9.6	15.6
C' (Fresh) and "Unabsorbed" RBC	0.52	9.0	5.8

treated with fresh twice washed cells showed very little lysis. The unabsorbed C' when mixed with the same cells showed the expected amounts of lysis as seen in the prior experiments. When the cold complement treated cells were mixed with fresh C' the amount of lysis was much greater than when the same C' was mixed with the cold untreated cells.

4C. TREATMENT OF ERYTHROCYTES WITH "ABSORBED" C'

The results of the above experiment seem to indicate the presence of an antibody in the C' which is directed against the human erythrocyte. However, another possibility could explain these results. If during the adsorption procedure a substance were being added to the C' from the erythrocytes and this substance had an inhibitory effect on the lysis the results would be the same. This material might inhibit the lysis of the erythrocytes in the same manner as does rheumatoid factor. In order to investigate this possibility, the following experiment was designed.

Procedure

Human erythrocytes were washed twice in DBS and were made up to a 7% suspension, in the usual manner. The guinea pig serum was melted along with the melting of "absorbed" guinea pig serum. The experiment was set up as shown in Table V.

TABLE V

EXPERIMENTAL SETUP FOR EXPERIMENT 4C

	1	2	3	4	5
Fresh C'	2.0	-	-	3.0	2.0
"Absorbed" C'	-	2.0	-	-	1.0
DBS	1.0	1.0	3.0	-	-
Cells	3.0	3.0	3.0	3.0	3.0

After centrifugation of the tubes at 1000 x G for five minutes at 5 C, the supernatants were read and recorded.

Results

Table VI illustrates the results obtained. Tube 5 shows fifty per cent inhibition when compared to tube 4. These results indicate the inhibitory effect of the "absorbed" complement.

5. LABELLING OF ERYTHROCYTES

Fe^{59} is an isotope of iron which may be used to label the hemoglobin of newly formed erythrocytes. Cells were labelled in order to see if there was preferential lysis of new or old cells in the phenomenon described in experiment 1.

Procedure

A 10.0 ml. sample of blood was taken and added to sodium

citrate. After centrifugation, 5.0 ml. of plasma was removed and added to 15 uCi 59Fe-ferrous citrate under sterile conditions and incubated at 37 C for thirty minutes. The sample, with the label, was then reintroduced via venipuncture. Samples of blood were taken at 5, 10, 15, 30 and 90 minute intervals to determine the plasma clearance of the isotope. These samples were centrifuged and the plasma samples counted to determine the time required to remove one half of the isotope from the circulation. This was called $T\frac{1}{2}$ clearance. Samples were collected at 3, 5, 11 and 13 day intervals to determine the incorporation rate of the isotope with respect to the whole blood. After 13 days, the incorporation of the isotope was over 90%. All samples were counted in a scintillation well type counter and the counts recorded as counts per minute (CPM). The background counts were calculated on the basis of the counts obtained with distilled water in place of the samples.

At regular weekly intervals, samples of blood were taken and treated with complement, as described in experiment 1. The supernatants which showed lysis were then analysed for the radioactive counts and these results were compared with the results obtained when the erythrocytes were lysed by complement.

Results

In the calculations of experiment 1 the per cent lysis was calculated by the formula given in the materials and methods chapter.

TABLE VI

THE EFFECT OF "ABSORBED" COMPLEMENT ON THE LYSIS OF HUMAN ERYTHROCYTES.

	O.D.540	100% Lysis Calculated O.D.540	% Lysis
1	0.50	10.0	5.0
2	0.07	9.0	0.7
3	0.00	9.5	0.0
4	0.43	11.0	3.9
5	0.19	9.5	2.0

The calculated per cent lysis for this experiment was calculated as:

$$\text{per cent} = \frac{\text{CPM of the supernatant}}{\text{CPM of the 3.5\% suspension}} \times 100$$

In each case, the background CPM was subtracted from the total counts. If there were preferential lysis of newly formed erythrocytes then the CPM of the supernatants should reveal greater lysis than if merely random lysis was occurring. If a maximum level of 5% lysis is attained through complementary treatment, then approximately 5% of newly formed cells should also lyse if there is no preferential lysis.

Preferential lysis of newly formed cells was not observed. However, it should be noted that in order for significant readings to be obtained the counts should be at least the square root of the background above background, i.e., if the background were equal to 100 CPM, then readings must be above 110 CPM in order to be classified as statistically significant.

The results, with respect to experiments on two donors, indicate that no significant readings were obtained (see Table VII) and that there was no preferential lysis of newly formed cells. There could still be preferential lysis of old cells.

6. CONCENTRATING POOLED SUPERNATANTS

The initial rise in lysis and subsequent fall seems to indicate the presence of a protective substance which was being removed initially. Later washing brings about the removal of the

lytic agent. If the latter statement is true, then the concentrated supernatants from the descending portion of the curve should contain this factor. Therefore, procedures were designed to concentrate the pooled supernatants from the descending portion of the curve.

6A. CONCENTRATION BY POLYETHYLENE GLYCOL

Procedure

The supernatants from the descending portion of the curve were pooled. These pooled supernatants were placed in dialysis tubing and the ends were sealed off. Polyethylene glycol powder (Carbowax 20M Union Carbide Canada Ltd., Toronto, Ont.) was spread over the surface of the tubing and the apparatus was placed at 4 C for twenty-four hours. After this period of time the tubing was removed and the contents recovered.

TABLE VII

LYSIS OF ISOTOPE LABELLED HUMAN ERYTHROCYTES

Days after injection	Donor	Background	Counts above the background on Supernatants from cells washed				
			Zero	Once	Twice	Thrice	3.5% Cells
5	A	100	1	2	2	1	725
19	A	98	4	10.5	13	4	148
26	A	42	0	0	5	0	
6	B	98	5.5	4.5	7	5.5	189
13	B	39	4.1	5.5	6	4.5	246

6B. CONCENTRATION BY FREEZE DRYING

Procedure

The supernatants were obtained by the same method as described in experiment 6A. The supernatants were then placed in dialysis tubing and the ends were sealed off. The tubing was then placed into 10x volume distilled water. This was left at 4 C for forty-eight hours with changes of distilled water after twenty-four hours. Later, the supernatant was freeze-dried using the freeze drier, Thermovac Corp., N.Y. The remaining material was reconstituted with DBS to restore isotonicity.

7. AGAR GEL DIFFUSION

If the concentrated supernatants from experiment 4A and 4B do contain a lytic material, agar gel diffusion techniques should reveal a line of precipitation if the appropriate antisera were used. Therefore, various antisera were tested against the concentrated supernatants.

Procedure

The slides were poured using 1% Noble agar. After allowing them to cool, they were placed in a humid atmosphere for forty-eight hours at 5 C before use. A template was then used to cut holes in the agar. Two wells were filled with the concentrated supernatant

and two were filled with DBS. In the center wells, was placed, goat anti-human group O serum, rabbit anti-human globulin, anti-γ M or anti-γ G. These test slides were then left for forty-eight hours at 5 C. After twenty-four hours of washing with DBS, to remove the excess protein on the slides, the slides were placed in the incubator at 37 C for six to eight hours. Each of the slides was covered with strips of filter paper to prevent the cracking of the agar,while the drying was taking place. The slides were then placed in 10% glacial acetic acid in 90% ethanol for ten to fifteen minutes and into amido black (see Appendix) for the same period of time. To decolorize, the slides were placed in 10% glacial acetic acid for ten minutes. After decolorizations, they were washed with distilled water and dried.

Results

The antisera used were anti-human, anti-7S and anti-19S. The only antisera which did not give lines of precipitation were the anti-19S sera. There appears to be a globulin fraction present which is of the 7S variety (see Table VIII). This experiment has been carried out four times on the same supernatant, each time using a different antisera.

8. RESENSITIZATION OF ERYTHROCYTES

The concentrated supernatants from 4A and 4B were added to fresh cells in order to try to show the presence of a lytic agent which was being removed as the cells were being washed.

TABLE VIII

PRECIPITATION LINES OF ANTISERA AND CONCENTRATED SUPERNATANTS

	Anti-h	Anti-γ-globulin	Anti-γ-G	Anti-γ-M
Supernatant	+	+	+	-
DBS	-	-	-	-

Procedure

The erythrocytes were washed three times and 7% cells were made. The concentrated supernatants (from experiment 6A) were added to the suspension of cells. The system was 3.0 ml. of 7% cells, 1.0 ml. of concentrated supernatant and 2.0 ml. of C' (containing 100 HD_{50}). First, the concentrated supernatants and the cells were incubated at 37 C for twenty minutes, followed by the addition of the C' and a further incubation for thirty minutes. The tubes were then centrifuged at 1000 x G for five minutes at 5 C. The optical densities were read at an O.D. of 540 mμ. The blank to zero the machine contained 3.0 ml. of DBS, 1.0 ml. of the concentrated supernatant and 2.0 ml. of the C' used. One hundred per cent lysis was obtained using 0.5 ml. of cells plus 9.5 ml. of distilled water, blanked to distilled water.

Results

In each attempt to resensitize the erythrocytes with the concentrated supernatants (Table IX illustrates the results), the amount of lysis in the test, i.e., cells plus supernatant was less than the amounts of lysis which occurred in the controls. When the C' was titrated after the test it was found that the C' titre was comparable with the C' titre when it was kept in an ice bath.

9. ARTIFICIAL SENSITIZATION OF HUMAN ERYTHROCYTES

If an immune phenomenon is involved in the rise and fall,

TABLE IX

RESENSITIZATION OF WASHED ERYTHROCYTES

	O.D. 540	100% Lysis Calculated O.D.540	% Lysis
Cells + Concentrated Supernatant	0.16	8.4	1.9
Cells + DBS	0.21	8.6	2.4

with respect to the lysability of human erythrocytes which are repeatedly washed, we should be able to prepare cells artificially and duplicate the washing phenomenon. This was attempted.

Procedure

Group O Rh negative erythrocytes were washed three times in DBS. Anti-human group O amboceptor was used to sensitize the washed erythrocytes. The initial experiment used erythrocytes plus amboceptor. The amboceptor was diluted to 1.0 ml. of amboceptor to 7.0 ml. of DBS. Equal volumes of amboceptor were mixed with washed packed erythrocytes. The suspension was incubated at 37 C for thirty minutes. After this incubation period, the suspension was put through the mechanics of experiment 1.

Secondly, the sensitized cells were also mixed with rheumatoid sera, which acts as an anti-γ-globulin. The same procedure, as mentioned above, was followed, i.e., packed washed erythrocytes, amboceptor but in addition the rheumatoid sera. The rheumatoid sera was diluted to 1.0 ml. of RAS to 9.0 ml. of DBS. The sensitized cells were centrifuged at 1000 x G for five minutes at 5 C and the supernatant taken off. The RAS dilution was then added and after vortexing briefly, the mixture was incubated at 37 C for twenty minutes. Experiment 1 was then carried out on these cells which had been sensitized and also had had the addition of anti-γ-globulin.

Results

This experiment has only been carried out once and was a

preliminary test to see if an artificial system could be prepared. When amboceptor was used to sensitize the erythrocytes, followed by treatment with complement, there was a decrease in the amount of lysis as the number of washings increased, as seen in fig. 5.

With the addition of rheumatoid sera to the sensitized cells there was a slight initial rise in lysis followed by a decrease, as shown in fig. 6. This rise was not pronounced, as was the rise seen in fig. 1.

These results are suggestive that an artificial system could be prepared using red cells, amboceptor and rheumatoid sera which would behave in a manner similar to normal erythrocytes, but do not conclusively show this.

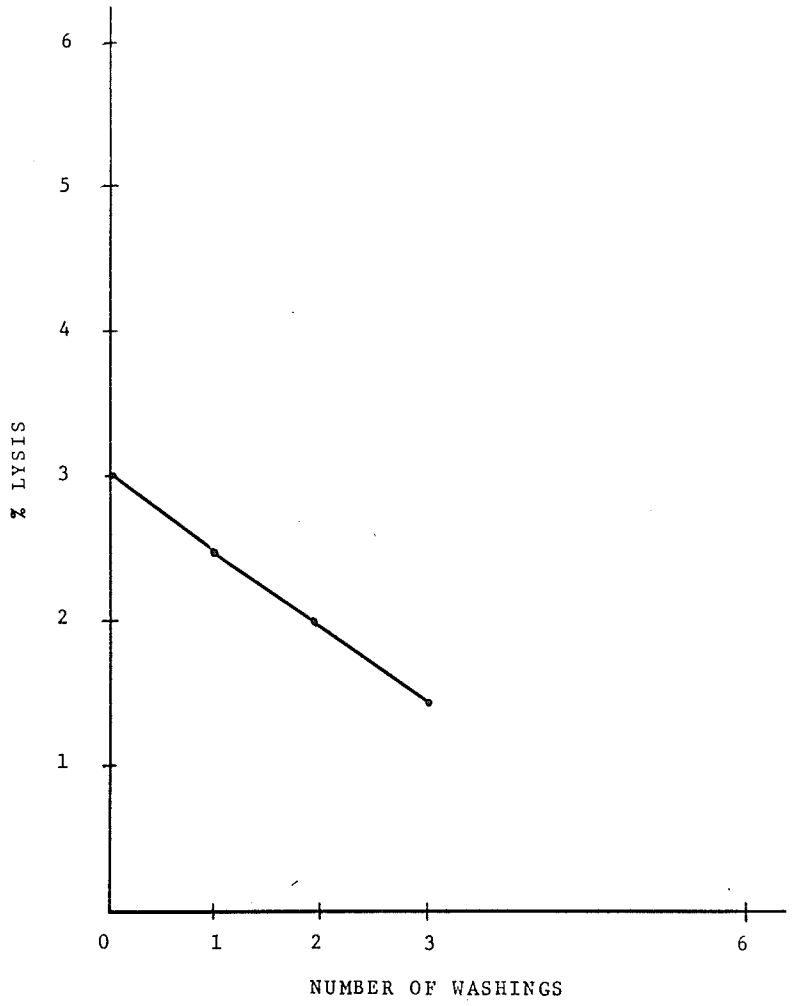

Fig. 5. Washed artificially sensitized Cells treated with complement.

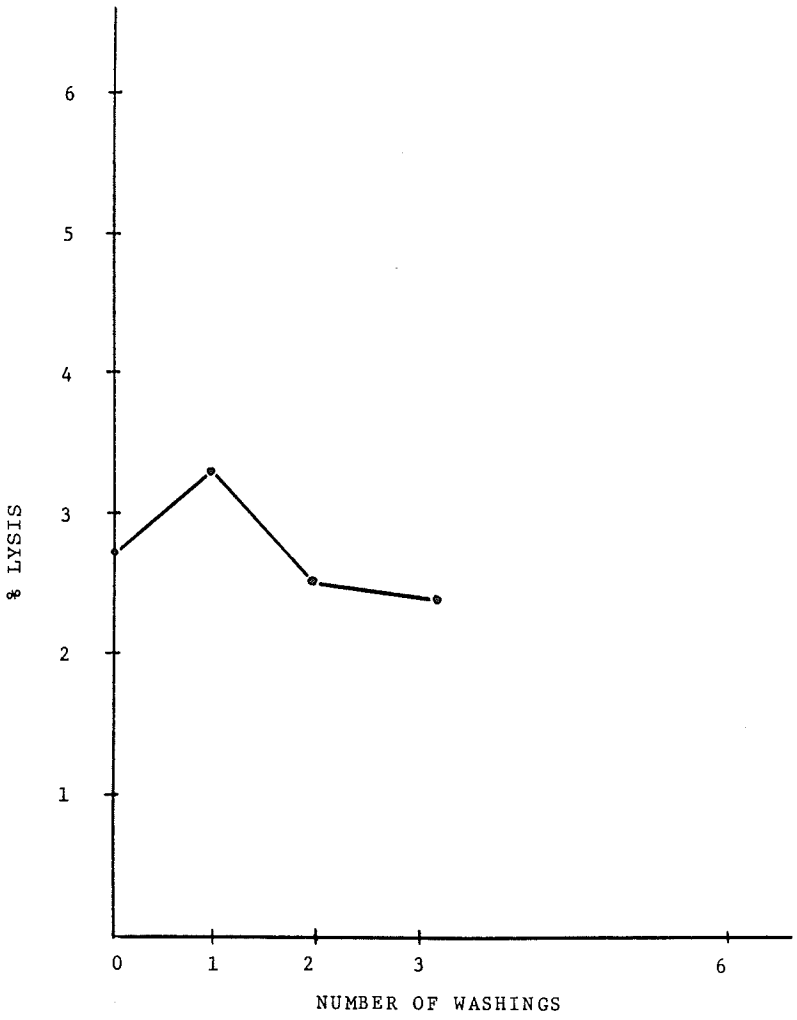

Fig. 6. Erythrocytes sensitized with amboceptor and anti-γ - globulin repeatedly washed and treated with complement.

10. LATEX FIXATION TESTS

The latex fixation test is a measure of the ability of sera to cause agglutination of inert latex particles of uniform size coated with a preparation of human gamma globulin. This test was performed in order to study the complement which had been absorbed with the washed human erythrocytes, to observe any difference between the erythrocyte treated and untreated complement. The experiment was set up as follows:

Procedure

Reagents were obtained from the Consolidated Laboratories (Canada) Ltd., Weston, Ontario, and the procedure suggested by the manufacturer was followed. The quantitative tube agglutination test was performed on the test sera.

The buffer was diluted to the suggested dilution and 0.5 ml. of buffer were added to each tube of each row. Serial doubling dilutions of the sera to be tested were made in 0.5 ml. amounts. The antigen was added, 0.5 ml., and the tubes were shaken for one minute. Incubation at 56 C for one hour followed. After the incubation the tubes were refrigerated for one hour. Centrifugation of the tubes followed at 1000 x G, for twenty minutes, at which time the tubes were read for agglutination.

Results

Both the "absorbed" and "unabsorbed" guinea pig sera produced some agglutination, to a dilution of 1:8. There was no

difference in the titres of the two. This observation suggests that the sera involved have a material which will cross react with the latex coated particles. The agglutination observed was only slight and could merely be due to non-specific agglutination of the latex particles.

CHAPTER IV

DISCUSSION OF RESULTS

DISCUSSION OF RESULTS

Normal human erythrocytes when treated with complement were found to be susceptible to lysis. The amount of lysis rose to a maximum level as the number of washes increased, and after the second or third wash, the lysability of the cells began to decrease. When the osmotic fragility of the cells was tested, it was found that as the number of washings increased, the fragility of the cells remained unchanged. Rheumatoid factor protected the erythrocytes from lysis, without the destruction of complement. Radioactive isotope labelling of the erythrocytes did not reveal any preferential lysis of newly formed cells.

In the field of immunology today there are several factors, which are hemolytic in the presence of complement and are found in normal human sera. The possibility of one of these factors causing this lysis requires discussion.

Dacie (1950a) has shown that cold agglutinins, present in normal human sera, are capable of causing lysis in the presence of complement. Certain requirements must be fulfilled before this can occur. Firstly, the medium involved must be of an acidic pH and secondly, the temperature of the medium must be below 35 C. In view of these facts, cold agglutinins seem improbable as the cause of the hemolysis which has been observed. The experimental temperature, when the cells are treated with complement is constant at 37 C and the pH of the environment is about 7.2.

Another possibility is the action of immunoconglutinins,which

are antibodies against fixed complement, (Coombs and Coombs, 1953). It is a substance which will agglutinate certain complexed of antigen, antibody and complement. Coombs et al (1961) suggest that immuno-conglutinins, which are present in normal human sera, are poorly hemolytic. Coombs goes on to say that conglutinin will only be absorbed by fixed complement, and since, in our system, complement is added after the cells are washed, conglutinin is unlikely to be responsible for the lysis.

The erythrocyte membrane can adsorb certain other substances. The substances include: I^{131} labelled fatty acids of the plasma lipids, which can exchange with red cell membrane lipids (Hughes-Jones and Gardner, 1962; Costea et al., 1962). It has also been shown that free I^{131} labelled inorganic iodine exchanges with the intra-cellular red cell anions (Costea et al., 1962).

If these substances were involved in the experimental test system, it seems unlikely that they would cause the lysis which is observed. If so the amount of lysis should not rise and later fall, but it should start at a maximum level and after successive washings reach a minimal level.

From the results previously described two explanations for the complementary hemolysis of washed normal erythrocytes are possible. First, there may be a hemolytic antibody on the surface of the normal cell itself, or second, there may be a hemolytic antibody present in the guinea pig complement which is directed against the erythrocyte.

If there was an antibody in the complement, then it would

sensitize the normal erythrocytes and bring about their complementary lysis. The results of the absorption experiment suggest that such an antibody was absorbed from the guinea pig complement during incubation of the serum with normal human erythrocytes. The absorbed complement showed little power to lyse fresh erythrocytes. However, the results of the washing experiment, which show the initial rise and subsequent fall in the amount of lysis as the number of washings increase, do not support the theory of there being an antibody in the complement. If an antibody were present, then it would be present in a a definite quantity, and since the complement was the same throughout a single experiment the quantity of antibody being added after each wash would also be constant, the only variable being the number of washings. Therefore, if an antibody were pre- the amount of lysis occurring would not rise and later fall, with increasing washings, but rather would remain constant.

The other explanation for this phenomenon assumes that a normal antibody is present on the surface of the erythrocyte. On the basis, the results of the absorption experiment could be explained by the release from the cells, during the absorption procedure, of a factor which would inhibit the lysis of the erythrocytes. This factor would inhibit the lysis of the fresh erythrocytes. The results of the test on the complement "absorbed" with red cells (experiment 4C) did, in fact, reveal that the "absorbed" complement did reduce the lysis of additional cells, by 50%, even when fresh "unabsorbed"

complement was present.

Rheumatoid factor can inhibit the lysis of sensitized erythrocytes in the presence of guinea pig complement, and does not bring about the destruction of the complement (Bowman, 1966). Heimer (1963) has shown that normal human sera as well as sera from rheumatoid arthritis patients, has the ability to inhibit the lysis of sensitized sheep erythrocytes. The inhibition of lysis produced by rheumatoid sera is consistent with either of the above interpretations and is suggestive, in itself, that the lysis is immune in nature.

The osmotic fragility of the erythrocytes, as the cells were being washed, was tested and remained constant throughout the procedure. Since the results of this test were constant the possibility of the lysis observed being due to mechanical effects is slight. The test is sensitive in distinguishing between normal erythrocytes and erythrocytes from patients with hematological disorders associated with reduced fragility.

Examination of the supernatants from the descending portion of the graph, when concentrated, also, suggests the presence of IgG globulin. Such globulin might come from the surface of the erythrocyte or may represent residual plasma IgG globulin.

The rise and subsequent fall in lysis with repeated washing has been partially reproduced by artificial sensitization of the erythrocytes, with amboceptor and rheumatoid factor. Only further investigation will reveal whether in fact the two agents, namely

antibody and rheumatoid factor, are involved in the lysis that occurs.

In the literature review it was noted that the indirect Coombs test would reveal the presence of an antibody in the serum. When there is none in the serum, that is, in less acute diseases, such an antibody is then detectable on the red cells using the direct Coombs test. However, in this case there must be a large enough proportion of red cells carrying the antibody to agglutinate fairly grossly in the test is to be positive. The present experiments suggest that amounts of such antibody too low to be revealed by the direct Coombs test might be observable using this technique.

In view of these findings, the following hypothesis is proposed to explain these results:

Normal human sera may possess a distinct if indeed muted spectrum of antibodies reacting with various autologous or isologous tissue components. In the norman human system, it is possible to have naturally occurring autoantibodies, which normally are not harmful. Also present, along with the autoantibodies, are naturally occurring anti-γ-globulins, which inhibit immune hemolysis. Erythrocytes combine with autoimmune antibody and these "sensitized cells" combine with anti-γ-globulin. The protective anti-γ-globulin prevents immune complementary lysis. The exact nature or purpose of these globulins is not as yet known, but in disease states of the autoimmune variety the delicate balance which has been set up is disrupted and the resultant disease occurs. In rheumatoid arthritis,

the production of high quantities of anti-γ-globulin is predominant, whereas in acquired hemolytic anemia, the opposite is true. It has been said that "A little autoantibody--unlike learning-- is not a dangerous thing" (Castanedo, 1967).

Recently, Frommel et al (1967) have shown that the IgG globulin is capable of being absorbed to normal human erythrocytes when the two mixtures are incubated together. Unaltered IgG did not bind as well as did denatured IgG but nevertheless the possibility exists of there being sites on the erythrocyte which have the ability to take up IgG. These sites might be free anti-γ-globulin valences.

Immunoglobulins bound to the surface of erythrocytes probably represent a readily available reserve which can be drawn upon in emergencies similar to the situation postulated for fibrinogen by Traber and Kolmen (1965). As the fibrinogen concentration increases, the percentage of the total content on the erythrocyte surface will correspondingly increase. Therefore, as the blood becomes hypofibrinogenic, the carrier fibrinogen dissociates from its binding sites on the red cell, thus increasing the concentration in the plasma until normal conditions have been reached. Similarly, immunoglobulin bound to the erythrocyte may leave the cell on occasion.

Chaplin (1960) demonstrated the presence of a substance on the surface of normal erythrocytes which behaved as a protein or protein-like material and was able to elicit an antibody response in rabbits. The response obtained with the eluate from normal red

cells was definite although considerably weaker than the responses to eluates from sensitized cell stroma.

Only further experimentation on these remaining problems will help to shed some light on the solutions. Several possible lines of investigation may be useful through eluate analysis. The methods of Ponder (1948) and Greenwalt (1956) have proven benificial in the work of Chaplin. The use of eluates prepared from the cells to stimulate an antibody response in rabbits would enable further exploration of the exact nature of the substance on the surface of the erythrocytes. Labelling of the anti-γ-globulin, that is the rheumatoid factor, would help to explain the mechanism involved when the rheumatoid serum demonstrated inhibition of lysis. The nature of the inhibition mechanism as well as its mode of action could be further studied. With a labelled anti-γ-globulin other methods of analysis could be employed to discover the exact mechanism involved. The use of immunoelectrophoresis or radio-immunodiffusion with the eluate and labelled anti-γ-globulin could confirm that the substance on the cell is a globulin. Studies with the Fab and Fc fragments would further reveal whether the adherent substance is bound specifically or nonspecifically, and to what fragment.

In conclusion, the most likely explanation for the complementary lysis of washed normal erythrocytes is that the cells involved are coated or carry on their surface an antibody. Under the artificial circumstances of these experiments, complementary lysis occurs, but <u>in vivo</u> it is prevented by the presence of normal anti-γ-globulin.

CHAPTER V

SUMMARY

CHAPTER V

SUMMARY

Experiments were performed on fresh human erythrocytes to observe their susceptibility to lysis by guinea pig complement. The following conclusions have been drawn:

1. As the erythrocytes are washed, the amount of lysis increases to peak, and further successive washings decrease this susceptibility.

2. Rheumatoid factor can inhibit this lysis from occurring.

3. The cells which are lysing do not appear to be preferentially newly formed cells.

4. This lysis phenomenon has been partially reproduced using cells treated with amboceptor and anti-γ-globulin.

5. The guinea pig serum (C^1) does not contain an antibody which is directed against the human erythrocytes.

6. It is proposed that normal human erythrocytes have on their surface a cytolytic antibody and an anti-γ-globulin.

Leaf 73 omitted in page numbering

BIBLIOGRAPHY

BIBLIOGRAPHY

Abderhalden, E. (1908). "Textbook of Physiological Chemistry in Thirty Lectures". (trans. Bell, W.T. and Defran, D.) Wiley, New York.

Abrahams, E.P. The nature of antigens and antibodies. In General Pathology, ed., H.W. Florey, Third Edition, London: Lloyd-Luke, p. 778, 1962.

Bishop, C., and Surgenar, D.M. (1964). "The Red Blood Cell". Acidemic Press. New York and London.

Blagorazumova, M.A. (1959). Sn. Nauchn. Rabrt. Stalingrad. Med. Inst. 12:27

Bowman, D. The Antiantibody character of rheumatoid factor. Med.B. Sc. Thesis. Department of Bacteriology and Immunology, University of Manitoba, August, 1966.

Boyer, M. (1962). Proc. Europ. Reg. Conf. Electron Misc. Delft.

Brun, G.C. (1939). "Cholesterol Content of the Red Cells in Man". (trans. Andersen, H.) Lewis, London.

Burnet, F.M. The Clonal Selection Theory of Acquired Immunity. Cambridge, England, Cambridge University Press, 1959.

Casey, T.P. Aspects of Autoimmunity, V. The Detection of anti-erythrocyte Antibody. New Zeal. Med. J. 65:300-303, 1966.

Castanedo, V.P., White, J.G., and Williams, R.C. Jr., (1967). Anti-nuclear antibodies in normal human subjects. Arth. and Rheum. 10:431.

Chaplin, H. Jr., and Cassell, M. (1960). Studies on anti-eluate sera I. The production of antiglobulin (Coombs) sera in rabbits by the use of antibodies eluted from sensitized red blood cells. Vox. Sang. (Basel) 5:32

Cook, G.M.W. (1962). Linkage of sialic acid in the human erythrocyte ultra-structure. Nature 195:159.

Coombs, A.M., and Coombs, R.R.A. (1953), The conglutination phenomenon. IX. The production of immuno-conglutinin in rabbits. J. Hyg. (London) 51:509

Coombs, R.R.A., Mourant, A.E., and Race, R.R. A New test for the detection of weak and "incomplete" Rh agglutinins, Brit. J. Exp. Path. 26:255, 1945.

Coombs, R.R.A., Coombs, A.M., and Ingram, D.G. (1961). "The Serology of Conglutination and its Relation to Disease". Blackwell, Oxford.

Corsini, F., Pintozzi, P., Cacciani, E., and Manfredi, G. (1959b). Distribution of adlolase activity in erythrocyte fractions with different osmotic resistance. Boll. Soc. Ital. Sper. 35:398

Costea, N., Schwartz, R., and Dameshek, W. (1962). The use of radioactive anti-globulin for the detection of erythrocyte sensitization. Blood 20:214

Dacie, J.V. Acquired haemolytic anaemia. In Lectures on the Scientific Basis of Medicine Vii. London: Athlone Press, p. 59, 1959.

Dacie, J.V. (1950a). The presence of cold hemolysins in sera containing cold hemagglutinins. J. Path. Bact. 62:241

_____. The Haemolytic Anaemias. Congenital and Acquired. Part I. - The Congenital Anaemias. Second Edition. London: J. and A. Churchill Ltd. 1960a.

_____. Acquired Haemolytic Anaemia. In Lectures of Haemotology, ed. F.G. Hayhor. Cambridge: University Press, p. 1. 1960b.

_____. The Haemolytic Anaemias. Congenital and Acquired. Part II. - The Autoimmune Anaemias. Second Edition. London: J. and A. Churchill Ltd. 1962.

_____. Demonstration of autoantibodies to erythrocytes. I. - Warm Autoantibodies. In Immunological Methods. A Symposium, ed., J.F. Ackroyd. London: Blackwell, p. 515, 1964.

Dameshek, W., Schwartz, R. and Oliver, H. Current Concepts of Autoimmunization: An interpretive review. Blood 17:775, 1961.

Ehrlich, P., and Morgenroth, J. (1901). Berl. Klin. Wschr. 38:251

Evans, R.S., and Weiser, R.S. The serology of autoimmune haemolytic disease. Observations on forty-one patients. Arch. Intern. Med. 100:371, 1957.

_____. Bingham, M. and Boehmi, P. Autoimmune haemolytic disease: Antibody dissociation and activity. Arch. Intern. Med. 108:338, 1961.

Eylar, E.H., Madoff, M.A., Brady, O.V., and Oucley, J.L. (1962). The contribution of sialic acid to the surface charge of the erythrocyte. J. Biol. Chem. 237:1992.

Eyster, M.E., Nachman, R.L., Christenson, W.L. and Engle, R.L. Structural Characteristics of red cell autoantibodies. J. Immunol. 96:107, 1966.

Frommel, D., Grob. P.L., Masouredis, S.P., and Isliker, H.C. (1967) Studies on the mechanism of immunoglobulin binding to red cells. Immunol. 13:501

Fudenberg, H.H., and Kunkel, H.G. Physical Properties of red cell agglutinins in AHA. J. Lab. Clin. Med. 106:689, 1957.

Greenwalt, T.J. (1956). Method of eluting antibodies from red cell stroma. J. Lab. Clin. Med. 48:634

Hack, M.H. (1947). Estimation of phospholipids in human blood. J. Biol. Chem. 169:137

Harboe, M., Muller-Eberhard, H.J., and Fudenberg, H. (1963). Identification of the componenets of complement participating in the antiglobulin reaction. Immunol. 6:412

Helyer, B.K., and Howie, J.B. Spontaneous autoimmune disease in NZB/B mice. Brit. J. Haemat. 9:119, 1963.

Heimer, R., Levin, F., and Kahn, M.F. Inhibition of complement fixation by Human Serum II. J. Immunol. 91:866, 1963.

Heremans, J.F. Vaerman, J.F., Carbonara, A.K. Rhodain, J.A., and Heremans, M.T. γ A-globulin: Its isolation, properties, functions and pathology, In Protides of the Biological Fluids. X Colloq. Peetevs, H. ed. New York, Elsevier, 1963, p. 108.

Holmes, M.C., and Burnet, F.M. The natural history of autoimmune disease in NZB mice. A comparison with the pattern of human autoimmune manifestations. Ann. Intern. Med. 59:265, 1963.

Hughes-Jones, N.C., and Gardner, B. (1962). The exchange of I^{131} labelled lipid and I^{131} labelled protein between red cells and serum. Biochem. J. 83:404

Ishisaka, K., Ishisaka, T., Lee, E.H. and Fudenberg, H.H. Immunochemical properties of human γ A isohemagglutinin. J. Immunol. 95:197, 1965.

Kabat, E.A. and Mayer, M.M. Experimental immunochemistry. 2nd ed. Charles C. Thomas, Springfield, Illinois, 1964.

Kunkel, H.G. and Rockey, J.H. β_2A and other immunoglobulins in isolated anti-γ-antibodies. Proc. Soc. Exp. Biol. Med. 113:278, 1963.

Leddy, J.P. Immunological Aspects of Red Cell Injury in Man. Sem. in Hemat. 3:48, 1966.

Marrack, J.R., Sensitivity and specificity of methods of detecting antibodies. Brit. Med. Bull. 19:178, 1963.

Merchant, D.J., Kahn, R.H. and Murphy, W.H. Handbook of Cell and Organ Culture. p. 162. Burgess Publishing Co., Minneapolis, Minnesota, 1961.

Mollison, P.L. Blood Transfusion in Clinical Medicine. 3rd Edit. Oxford, Blackwell, 1961.

Osler, A.G., Straues, J.H., and Mayer, M.M. Diagnostic Complement Fixation. I. A. Method. Amer. J. Syphil. 36:140, 1952.

Pickles, M.M. Haemolytic Disease of the Newborn. Oxford:Blackwell. 1949.

Pirofsky, B. The structure and specificity of erythrocyte auto-antibodies. Ann. N.Y. Acad. Sci. 124:448, 1965.

Ponder, E. (1948). "Hemolysis and Related Phenomenon". Grune and Stratton, New York.

_____, Present concepts of the structure of mammalian red cells. Blood 9:227.

Polley, M.J., Mollison, P.L. and Soothill, J.F. The role of 19S gamma globulin blood group antibodies in the antiglobulin reaction. Brit. J. Haemat. 8:149, 1962.

_____. Adinolfi, M., and Mollison, P.L. Serological characteristics of anti-A related to type of antibody protein (7S γ or 19S γ). Vox. Sang. 8:385, 1963.

Prager, M.D., and Bearden, J. Blood group antibody activity among the γ_1 A globulins. J. Immunol. 93:381, 1964.

Race, R.R., and Sanger, Ruth. Blood groups in Man, Fourth Edition. Oxford; Blackwell, 1962.

Rawson, A.J., and Abelson, N.M. Studies of blood group antibodies. VI. The blood group isoantibody activity of γ_1 A globulin. J. Immunol. 93:192, 1963.

Romeyn, J.A. and Onysko, E. The Effects of "anti-antibody" on immune hemolysis. Immunology 7:217, 1964.

Stratton, F., and Jones, A.R. (1955). Normal human red cells and Coombs (antiglobulin) serum. J. Immunol. 75:423.

Traber, D.L., and Kolmen, S.N. (1965). Absorption of fibrinogen onto erythrocytic surfaces. Tex. Rep. Biol. Med. 23:782.

APPENDIX

APPENDIX

Alsever's Solution (modified from Oslet et al, 1952)

10.25	gm. dextrose
4.00	gm. sodium citrate
2.10	gm. sodium chloride
0.275	gm. citric acid
500.00	ml. distilled water

Adjust to pH 6.1

Filter with millipore filter and store in sterile containers

Dulbecco Phosphate - Buffered Saline (DBS) (modified from Merchant et al, 1961, p. 162)

r1	In each of two liter flasks:	80.0	gm. NaCl
		2.0	gm. KCl
		11.5	gm. Na_2PO_4
		2.0	gm. KH_2PO_4
		1400.0	ml. distilled water
r2	In a 500 ml. flask:	10.0	gm. dextrose
		400.0	ml. distilled water
r3	In a 500 ml. flask:	0.34	gm. $CaCl_2$
		400.0	ml. distilled water
r4	In a 500 ml. flask:	2.0	gm. $MgCl_2$-$6H_2O$
		400.0	ml. distilled water

Autoclave all units separately at 10 lbs. for thirty minutes. To each of the two 2 liter flasks (r1) add: 200 ml. r2

200 ml. r3

with sterile precautions 200 ml. r4

Bottle with sterile precautions in sterile screwcapped bottles. Dilute 1:5 with distilled water prior to use.

Amido Black Stain: (for agar gel diffusion)

0.5 gms. amido black
5.0 gms. mercuric chloride
5.0 ml. glacial acetic acid

Mix in 100 ml. of distilled water.