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,