DIFFERENTIATION OF IgG AND IgM ERYTHROCYTE ANTIBODIES USING THE REDUCING AGENT, DITHIOTHREITOL

by

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INTRODUCTION

A. STATEMENT OF THE PROBLEM

The blood bank is often called upon to characterize the immunoglobulin nature of erythrocyte antibodies. The present investigation was primarily concerned with the differentiation of antibodies in the antenatal studies of obstetrical patients. Such information is important in this group of individuals since IgG-type antibodies may cross the placenta and cause hemolytic disease of the newborn. In contrast, IgM antibodies cannot cross the placental barrier and therefore pose no potential threat to the newborn.

Currently a number of techniques to determine the immunoglobulin nature of erythrocyte antibodies are available. However, they are either time-consuming and expensive, or insensitive and generally impractical for routine use in the blood bank. This study was designed to attempt to develop a rapid, practical and inexpensive method that would discriminate between immunoglobulin M (IgM) and immunoglobulin G (IgG) erythrocyte antibodies.

B. HISTORY OF HEMOLYTIC DISEASE OF THE NEWBORN (HDN)

The first complete description of what is now known as hemolytic disease of the newborn was reported in 1908 in Germany by Pfannenstiel (1). In 1917 the disease was described in America by Abt (2). At that time the name, "familial icterus gravis" was used, denoting the presence of severe jaundice in newborns of succeeding pregnancies. Hart in 1925 (3) first successfully treated a severely affected infant by repeated replacement of its blood. The exchange transfusion is still the

standard method of treatment for the severely affected infant, although other types of treatment are available for infants with mild jaundice.

In 1939, Levine and Stetson (4) described a case in which a group O woman had recently delivered a dead fetus. Upon transfusion with group O blood from her husband, the woman developed a severe reaction. Subsequent studies showed that her blood agglutinated most group O blood. These investigators postulated that the mother had been sensitized by the erythrocytes of the fetus which contained a factor inherited from the father but absent in the mother. They concluded that specific immunization could occur during pregnancy.

The following year, Landsteiner and Weiner described an immune blood group system produced by injection of Rhesus monkey blood into rabbits (5) and later into guinea pigs (6). The sera obtained from these animals after immunization contained a substance which was capable of reacting with certain human group O bloods, but not with others. The involved blood group was named the Rhesus (Rh) blood group although it is now thought that the antibody produced was not identical to the currently described anti-Rh, but rather another antibody, called anti-LW for Landsteiner and Weiner (7). Weiner and Peters (8) subsequently implicated the role of the "Rh factor" in causing hemolytic episodes developing after administration of blood to patients previously sensitized by transfusion or pregnancy. Levine, Katzin, and Burnham (9) in 1941 suggested that isoimmunization occurring during pregnancy was the cause of HDN.

Only a few years later Weiner (10) and Race (11) independently reported the existence of two types of anti-Rh antibodies. One was a "complete" antibody which could agglutinate erythrocytes suspended in a saline medium. The other was called an "incomplete" or blocking antibody. The latter was found to attach to the red blood cell (rbc) membrane but was not capable of causing direct agglutination. The term blocking antibody was used because it occupied the antigenic sites of the rbc so that agglutination did not occur when the cell was exposed to the "complete" antibody. Development of albumin (12), enzyme (13), and antiglobulin (14) techniques now promote agglutination of the coated cells and thus facilitate identification of this type of antibody.

In 1948, Weiner (15) made the important observation that "incomplete" anti-Rh antibody could traverse the placenta while the "complete" type antibody could not. He postulated that differences in molecular weight might account for this phenomenon. Ultracentrifugation studies enabled Fudenberg and Kunkel (16) to demonstrate that the "incomplete" antibodies did, in fact, have a lower molecular weight than the "complete" type. They obtained sedimentation coefficients (S_{20}) of 7S and 19S, respectively, showing that the 19S material had a much greater density than that of the 7S. The fact that blood group antibodies exist in more than one form was now established. The identical antigen could induce production of at least two forms of the same antibody.

The fact that the placenta was the site of antibody transfer was confirmed by Gitlin and associates (17) employing radioactive tagging of

injected antibodies. Amniotic fluid was found to contain very little antibody. In cord blood serum, almost no high molecular weight antibody was present, although it contained a higher concentration of the antibody of low molecular weight than the maternal serum. This observation allowed Kohler and Farr (18) to speculate that the "incomplete" antibody was transferred across the placenta by an active transport mechanism, rather than by passive diffusion. They postulated that this transport was due to the conformation of the molecule rather than its molecular weight.

The above observations suggest that the existence of an erythrocyte antibody does not necessarily imply a danger of HDN. The presence of an "incomplete" (IgG) antibody in a pregnant woman would be cause for concern. Although these antibody types are frequently demonstrated by albumin, enzyme, or antiglobulin techniques, some may also agglutinate rbc's in a saline medium. Conversely, the antibodies formerly called "complete" are now known to belong to the IgM class of immunoglobulins and have been shown to occasionally behave as incomplete antibodies (19). Therefore, agglutination in saline does not prove the presence of an IgM antibody, and agglutination by enhancing techniques is not sufficient evidence that the type of antibody present is an IgG.

Since discovery of the Rh blood group, specific typing for the Rho (D) antigen has been performed and patients receiving transfusions are given blood compatible for this group. Therefore, immunization to this antigen by transfusions has been markedly reduced. Increased use of transfusions has resulted in immunization to various other blood

groups, however. In fact, most other blood groups were discovered by demonstration of the presence of immune antibodies in either the blood of patients who had received multiple transfusions or mothers who had delivered erythroblastotic infants. Administration of blood specific for all the known blood group antigens is impossible. Identical twins would be required for this purpose. However, identification of any blood group antibody present in an obstetrical patient and determination of its immunoglobulin nature would be a valuable part of an antenatal study. Identification of the antibody is frequently carried out, as recommended by Pirofsky in 1965 (20), but its nature is rarely investigated due to the expensive and time-consuming techniques presently available. A simple technique useful for routine work is desirable and will be reported in this paper.

C. THE STRUCTURE OF ANTIBODIES

Proteins in human sera may be classified by their ability to migrate in an electrical field. In order of decreasing mobility they are identified as albumin, alpha$_1$, alpha$_2$, beta, and gamma globulin. Antibody activity resides in the gamma and beta portions. These immunologically active proteins are called immunoglobulins. Currently, five classes of immunoglobulins have been studied. They differ in activity and structure. The five classes are known as IgG, IgA, IgM, IgE, and IgD. Blood group antibodies have been found to consist mainly of the IgG and IgM varieties. Some anti-A and anti-B erythrocyte antibodies have been shown to be of the IgA variety. Rarely, other erythrocyte antibodies also occur in the IgA form (21).

The first major step in the delineation of antibody structure was made by Porter in 1959 (22). He hydrolyzed rabbit gamma-globulins into three fractions using the enzyme papain. The three fractions combined were found to account for more than 90% of the original molecule. Most of its biological activity was still present. Fraction I and II were later shown to be identical, and contained the antibody combining sites. This part of the molecule was later named the Fab portion. Fraction III, now known as the Fc fragment, contained the features necessary for placental transfer (23) and complement fixation.

Edelman and Poulik (24) approached the problem of antibody structure in another system. They employed mercaptoethanol in a strong urea solution to reduce the molecule. They obtained two major components differing in molecular weight. This led Porter (25) to suggest that the IgG molecule consisted of two pair of polypeptide chains connected by disulfide bonds.

The polypeptide chains obtained by Edelman and Poulik are now classified as light (L) chains and heavy (H) chains. The light chains exist in two forms, the kappa and the lambda type, differing in amino acid sequence and antigenic specificity. Only one type occurs in a particular immunoglobulin but both types are shared by all classes of immunoglobulins. They have a molecular weight of 25,000 and do not contain carbohydrate.

Immunoglobulins are classified by the type of heavy chains they contain. Thus, the heavy chains of IgG, IgA, IgM, IgD, and IgE are denoted as gamma, alpha, mu, delta, and epsilon, respectively. These

differ in amino acid sequence and immunologic specificity. They vary in MW from 50,000 to 70,000.

Both the light and heavy chains have a constant and a variable region (26). Light chains consist of approximately 214 amino acid residues. The carboxy-terminal half has a constant amino acid sequence while the amino-terminal portion is variable. The heavy chains differ in the number of amino acids present, but also contain a variable region of about 110 amino acid residues. The heavy and light chains are connected by a single interchain disulfide bond. Regularly spaced intrachain disulfide bonds connect portions of each polypeptide chain, allowing folding, or looping of the chain into its tertiary structure. Two of these bonds occur in each light chain and at least four are present in each gamma chain. The light chains are exterior to the heavy chains in Porter's model, and the two heavy chains are also attached by at least one interchain disulfide bond.

The above description outlines a working model of an IgG molecule. This structure has a MW of 160,000, and a sedimentation coefficient of 7S. The carbohydrate, which is attached to the heavy chains, accounts for about 3% of the molecule. The amount of IgG present in the serum varies from 8.0 - 16.0 mg/ml. Table I summarizes these characteristic properties.

Four distinct classes of human IgG, differing in amino acid sequence and biological activity have been identified (27). They have been denoted IgG1, IgG2, IgG3, and IgG4, and account for 75%, 11%, 10%, and 4% of the total serum IgG concentration, respectively. The number

TABLE I

CHARACTERISTIC PROPERTIES OF THE IMMUNOGLOBULINS

Immunoglobulin Class	IgG	IgA	IgM	IgD	IgE
Molecular Weight	160,000	170,000	900,000	180,000	200,000
Sedimentation Coefficient	7	7, 9, 11, 13	19	7	8
Carbohydrate Content	2.9%	7.5%	11.8%	12.0%	10.7%
Complement Fixation	+*	0	+	0	0
Placental Passage	+	0	0	0	0
Serum Concentration (mg/ml)	8.0-16.0	1.4-4.2	0.5-1.9	0.03	0.005
Number of Subclasses	4	2	2	—	—
Heavy Chain Specificity	gamma	alpha	mu	delta	epsilon

*The subclass IgG_4 does not fix complement.

of interheavy chain disulfide bonds differs with the particular subclass. Thus, IgG1 is thought to possess two interheavy chain bonds, while IgG2, IgG3 and IgG4 contain three, four and one, respectively. All four subclasses have been shown to be capable of placental passage (28). All except IgG4 are capable of fixing complement.

IgA antibodies usually exist in a dimeric form in which the basic monomers are held together by disulfide bonds. They have an S_{20} of 11 in the dimeric form which occurs mainly in secretions. In serum, IgA, at a concentration of 1.4 - 4.2 mg/ml, is mainly present in the monomeric form, which has an S_{20} of 7. The molecule contains 7.5% carbohydrate. These immunoglobulins do not traverse the placenta or fix complement. In secretions they contain another polypeptide chain called the secretory piece. This piece is thought to aid in membrane transport of the immunoglobulin.

Two subclasses of IgA, IgA1 and IgA2, are also known to exist. About 90% of the IgA in serum is IgA1 whereas 50% of salivary IgA is in the IgA2 form. IgA2 is an unusual immunoglobulin in that it lacks the light-heavy chain disulfide bond (29), while the light chains are connected by one disulfide bond. The light and heavy chains are weakly held intact by non-covalent interactions.

IgM antibodies rarely exist as monomers, although they may occur in this form. They usually are polymers of five basic structures similar to the IgG molecule, which are attached by disulfide bonds and arranged in a cyclic form. They have a MW of 900,000, and contain about 12% carbohydrate. Their sedimentation coefficient is 19S. They are not

capable of crossing the placenta but are able to fix complement. The immunoglobulin is present at a 0.5 - 1.9 mg/ml concentration in normal serum. Two subclasses have been identified which are very similar (30).

The amounts of IgD and IgE present in the serum are not certain, but combined they account for less than 1% of the total immunoglobulin in the body. They are present as monomers having molecular weights of 180,000 and 200,000, respectively. Neither one is capable of fixing complement or of crossing the placental barrier.

Recent evidence indicates that the IgA and IgM polymeric forms contain an additional polypeptide chain which is thought to aid in joining the monomers. For this reason it has been called the J, or joining chain (31, 32). It has not been shown to be present in IgA and IgM monomers. Synthesis of the J chain occurs in the plasma cell, where immunoglobulins are produced (33).

D. METHODS OF IMMUNOGLOBULIN DIFFERENTIATION

1. Ion-exchange chromatography

The separation of proteins by ion-exchange chromatography was first successfully accomplished by Sober and Peterson (34). They were able to develop an exchanger made of cellulose that afforded separation under conditions mild enough to preserve the protein. Fahey and Horbett (35) demonstrated that the anion exchangers separated 6.6S and 18S gamma globulins.

Anion or cation exchangers are available, although the former are more widely used for separation of immunoglobulins. The affinity of a protein for an exchanger depends on its size, charge distribution and

polymeric structure. The exchangers are packed in a column whose size depends on the amount of specimen to be studied and the degree of separation desired. Proteins containing an opposite charge from that of the exchanger are adsorbed onto its surface. Buffers of varying pH and/or ionic strength are then allowed to flow through the column. Variation in pH alters the charge on either the protein or the exchanger, while variations in salt concentration promote dissociation of electrostatic linkages between the protein and the adsorbent. Either gradient or stepwise elutions may be employed. Continuous gradients afford a greater resolving power but also dilute the specimen. Stepwise elutions are simple and adequate when there is a wide variation in elution conditions.

Separation of erythrocyte isoagglutinins by ion-exchange chromatography has been employed by Abelson and Rawson (36, 37). A stepwise elution using buffers of increasing ionic strength and decreasing pH affords separation of pure IgG and moderately pure IgM and IgA fractions.

2. Gel chromatography

In this procedure immunoglobulins are separated by their differences in molecular weight (38). Chemically inert solids, the most common of which are sold under the commercial name of Sephadex, are used to pack the column. Sephadex is a material consisting of dextrans in a cross linked arrangement. Aqueous solvents mixed with the dextran particles cause them to swell. Pores present in the swollen gel allow low molecular weight substances to penetrate it while excluding the high

molecular weight substances. Thus, the latter substances come off the column rapidly in the first phase of the elution while those of low molecular weight are eluted slowly.

Sephadex is sold according to the possible water regain per gram of dry gel. The G200 forms have a regain of twenty grams of water per gram of dry gel. Substances of molecular weight greater than 150,000 are excluded by the pores. Three major peaks consisting of 19S, 7S, and 4S proteins are obtained when human serum is applied.

This procedure is useful for separation of IgG and IgM antibodies due to the great difference in their molecular weights. IgA may be found in either fraction, however, due to its variable molecular weight. This method has also been applied to further purification of proteins previously incompletely separated by other means.

3. Density gradient ultracentrifugation

Another method for the characterization of antibodies is based on the principle that particles suspended in a liquid behave in a particular manner according to their density. Particles lighter than the liquid will float, while heavier ones will settle out. Molecules having the same density as the medium will remain suspended when a gravitational force is applied. Therefore, a density gradient of a particular solute such as sucrose, may be utilized. Components to be studied will sediment in bands or zones. The sedimentation coefficients may then be measured. Isohemmagglutinins were first studied by this method by Pederson (39). Since then it has been a useful tool in characterization of warm and cold autoantibodies, complete and incomplete anti-D, and other erythrocyte antibodies.

4. Reduction of disulfide bonds

The dissociation of 18S, 25S, and 32S macroglobulins after treatment with the reducing agent, 2-mercaptoethanol ($HSCH_2CH_2OH$) was first described by Deutsch and Morton (40). This led to the belief that macroglobulins were composed of monomers held together by disulfide bonds. These were intersubunit bonds susceptible to cleavage by low concentrations of reducing agents. Intrasubunit bonds remained intact. Higher concentrations of the reducing agents were later found to break the intrasubunit bonds and to yield individual polypeptide chains with no antibody activity. This difference in susceptibility to reduction was thought to be due to the three dimensional configuration of the molecule which rendered the intersubunit bonds more accessible (41, 42).

The 6.5S fractions obtained by mild reduction were seen to reaggregate upon removal of the sulphydryl compounds by dialysis. The components obtained were found to be different from the original molecules. Those possessing antibody activity were thought to have reaggregated with non-antibody macroglobulins, thus losing their activity although recovering their polymeric state. The presence of a very high concentration of a particular antibody could possibly result in specific reaggregation with a return of biologic activity.

The reduced 6.5S fractions could be maintained by dialysis against an agent such as iodoacetamide (ICH_2CONH_2), which blocked the free SH groups, and thus inhibited recombination. IgM antibodies active in serological tests were shown not to regain their erythrocyte

agglutinating activity even when reaggregation was allowed to occur (43). Therefore most investigators eliminated the blocking step when working with hemagglutination systems. IgG, or 7S immunoglobulins retained their activity even after treatment with 2-mercaptoethanol (2-ME). IgA antibodies were found to vary in their susceptibility to this reagent, possibly due to their polymeric state. Since isoagglutinins are only rarely IgA antibodies, the reduction procedure is very useful in differentiation of IgG and IgM antibodies.

Removal of the sulphydryl compounds by dialysis was continued when the procedure was applied to blood group antibodies, although the reason for this was not clear. The technique generally adopted by blood banks was described by Mollison (44). However, the cumbersome dialysis step was shown to be unnecessary by Reesink and associates (45) and by Decary and associates (46).

Agents capable of disulfide bond reduction have the general formula, RSH. These ionize and split groups having the general formula, R'SSR" into R'SH and R"SH portions. The R' and R" may represent any molecule or polypeptide chain held together by disulfide bridges. Besides 2-ME, other agents which have the same general formula are dithiothreitol (DTT) or Cleland's reagent, cysteine, glutathione and many others. Some of these have been employed for differentiation of immunoglobulins produced by some types of antigenic exposures, but have not been used for determination of the nature of erythrocyte antibodies.

DTT, $HS\text{-}CH_2(CHOH)_2CH_2\text{-}SH$ or Cleland's reagent (47), is a cyclic structure with OH groups on the middle carbons. These render the

compound water soluble and reduce the offensive odor of the thiol groups. The other reagents mentioned have an extremely repugnent odor and require use under a hood. This characteristic of 2-ME has made its routine use in the blood bank objectionable. In addition, dialysis of the treated samples is time consuming and requires sizable amounts of serum. Since methods such as column chromatography and ultracentrifugation require expensive equipment, time, and technical skill, the disulfide reduction method is the only practical approach to antibody characterization in the routine laboratory. Other shorter methods are available, but often lack accuracy. One of these is neutralization of specific immunoglobulins by precipitation with antibodies directed against them. In another method the serum is heated to 70°C for 20 minutes (48). IgM antibodies are inactivated by heating while most of the IgG types remain active.

Elimination of dialysis, shorter incubation periods, and use of a less offensive reagent would make the disulfide reduction procedure more feasible for routine work. Therefore, the action of DTT on erythrocyte antibodies was investigated. It was hoped that a procedure could be developed that would meet the above criteria, and be used routinely for characterization of antibodies in obstetrical patients. Other applications should be possible, such as the elimination of a potent IgM antibody in order to identify a more weakly reacting IgG antibody in the same serum.

MATERIALS AND METHODS

A. TEST SERA AND CELLS

1. Blood samples to be studied for anti-A and anti-B erythrocyte antibody activity were obtained from blood group O, A, and B individuals. The Portland Red Cross and the University of Oregon Medical School blood banking service found these sera to be free of additional erythrocyte antibodies by standard saline, enzyme, and antiglobulin tests. A pool of group O erythrocytes containing most of the known erythrocyte antigens were employed for this purpose. Such cell pools were obtained from Ortho and Dade Laboratories under the commercial names of Selectogen and Search-Cyte, respectively.

Blood samples containing other antibodies were obtained from various laboratories in the city. The sera were frozen until used in this study.

2. Test cells employed for determination of anti-A, anti-B and anti-Rho (D) titers were obtained from donors of blood type A, B, and O RHo (D) positive, respectively. The blood was collected in vacutainer tubes containing EDTA as an anticoagulant. Tests using these cells were performed within 48 hours after the sample was collected. Typing was performed by the University of Oregon Medical School blood bank service. Either Selectogen or Search-Cyte was employed for titration of the other antibodies studied.

B. 2-MERCAPTOETHANOL (2-ME) TREATMENT OF ANTIBODIES

2-Mercaptoethanol (2-ME) was purchased from Matheson, Coleman, and Bell. Ten milliliters (ml) of a 1.0M stock solution was prepared in

phosphate buffered saline (PBS), pH 7.2 - 7.4. A 1:10 dilution of the stock reagent was made in PBS to obtain a 0.1M working solution. The standard procedure using this reagent for discrimination of erythrocyte antibodies has been described by Mollison (44). Equal volumes of the 0.1M 2-ME and undilute test serum were mixed and incubated for two hours at 37°C. Controls consisting of equal parts of PBS and the test serum were simultaneously mixed and incubated. After incubation, aliquot samples of each were separated. One set of samples was dialyzed against PBS overnight at 4°C. The dialysis was performed in cellophane dialysis bags with a pore radius permeability of 24 Å. These are manufactured by Union Carbide. Dialysis was carried out in a beaker containing a PBS volume at least ten times the volume of the sample. A magnetic stirrer was used for continuous mixing. The remaining portions of test and control sera were stored at 4°C overnight without dialysis.

Antibody titers were determined after a refrigeration period of 16-24 hours. One drop of a 3-5% saline suspension of appropriate cells was added to 0.1 ml of a series of two-fold isotonic (0.85%) saline dilutions of each of the above treated and control sera. The dilutions were made in 10 x 100 millimeter test tubes. Anti-A and anti-B titers were determined by incubating the mixtures at room temperature for one hour, centrifuging in a Serofuge for fifteen seconds and quantitating the degree of agglutination by macroscopic readings. Incomplete antibody titers were obtained after incubation for one hour at 37°C. These tubes were also centrifuged fifteen seconds and agglutination was read macroscopically. In addition, samples not demonstrating agglutination

were washed three times with saline, drained, and centrifuged for fifteen seconds after addition of antiglobulin serum. The degree of agglutination was graded macroscopically over a range of trace to 4+.

Antibody titers obtained with the dialyzed and undialyzed samples previously treated with 2-ME were compared to their respective controls not treated with the reducing agent. A two tube (four-fold) or greater reduction in titer of the 2-ME treated sample from the control sample was considered significant, indicating the presence of an IgM antibody. Lesser reduction or an identical titer signified the presence of an antibody of the IgG variety.

C. DEVELOPMENT OF A STANDARIZED REDUCTION PROCEDURE USING DITHIOTHREITOL

1. Determination of an optimum dithiothreitol concentration

Dithiothreitol (DTT), commercially known as Cleland's reagent, was purchased in one gram quantities from Calbiochem. Concentrations of 0.002M, 0.004M, 0.005M, 0.006M, 0.008M, 0.01M, 0.05M and 0.1M dithiothreitol (DTT) diluted in isotonic saline were prepared in 10 ml volumes. Anti-A erythrocyte antibodies sensitive to 2-ME were tested after treatment with the above concentrations of DTT. Equal volumes of the test serum and the DTT were incubated together at a number of different temperatures for varying periods of time. Titers were then performed. These titers were compared to ones obtained by incubation of the serum with isotonic saline. The titration procedure was slightly modified from that described above. A 2% erythrocyte suspension, in a quantity of 0.1 ml, was employed rather than the single drop of a 5% suspension previously used.

A control to rule out inadvertant denaturation of antibodies was always employed. A serum containing anti-D unaffected by 2-ME was tested simultaneously with the DTT studies.

2. Determination of an optimum incubation temperature

Serum containing an anti-A erythrocyte antibody was treated with DTT and incubated at $4^{o}C$, $37^{o}C$, and $24^{o}C$ at several different intervals of time. Additional anti-A antibodies were tested after $24^{o}C$ and $37^{o}C$ incubation temperatures. Untreated controls consisting of equal volumes of serum and isotonic saline were incubated for identical times and at identical temperatures as the treated sera. Antibody agglutination titers were then determined.

3. Determination of optimum length of incubation

The length of incubation of the treated anti-A serum was also varied. In the first series, 0.1M, 0.01M, and 0.001M, DTT were prepared and each was incubated with a serum containing an anti-A antibody for two hours at $37^{o}C$, as in the 2-ME procedure. Antibody titers were then performed on one aliquot of each sample. Another was stored at $4^{o}C$ overnight and titers were performed the following day. Subsequently the antibody was tested after 15 minute, 30 minute, one and two hour periods of incubation with 0.01M DTT. Finally, the serum was treated with 0.05M and 0.005M DTT and incubated for fifteen and thirty minutes. Titers were then determined.

A control consisting of an anti-D antibody unaffected by 2-ME was simultaneously studied.

4. Determination of optimum pH

Isotonic saline solutions of Sorenson's phosphate buffer were prepared in a standard fashion. Buffers of 6.0, 7.0, 7.4, 7.8, and 8.2 were made. To 100 ml of each buffer 0.8 g of saline was added. A 0.01M solution of DTT was made in solutions of each pH. These were then tested by treatment with an anti-A antibody. Results were confirmed by identical treatment of one anti-D and seven anti-A antibodies.

5. Confirmation of the values

Seven anti-A antibodies in type O sera were then treated with 2-ME and titers were determined. Similarly, each antiserum was treated with 0.01M DTT in PBS, pH 7.2 - 7.4, and incubated for thirty minutes at room temperature. Other aliquots were treated with 0.005M and 0.01M DTT in PBS of the same pH. These were incubated for fifteen minutes at 37°C and titers were performed. Controls consisting of equal volumes of the serum and PBS were incubated simultaneously and titers determined.

6. Comparison of the activity of DTT when prepared in isotonic saline and in phosphate buffered saline, pH 7.2 - 7.4

Eight anti-A antibodies were treated with 0.01M DTT made in both isotonic saline and PBS, pH 7.4. Controls consisting of serum and PBS were simultaneously tested. These were incubated for fifteen minutes at 37°C. Titers of each were compared with titers obtained from a 2-ME treated sample of the same serum. One anti-D antibody was treated similarly as a control against denaturation.

7. Comparison of the activity of 2-mercaptoethanol and DTT on a variety of antibodies

The susceptibility of a variety of antibodies to treatment with a 0.01M solution of DTT was then determined. Each antibody was also tested after treatment with 2-ME. The same indicator cells were used for both methods. Titrations were carried out at room temperature or 37°C, depending upon the optimum activity of the particular antibody. Titers were compared to their own individual untreated control titers.

D. DETERMINATION OF THE STABILITY OF THE DTT

The stability of the DTT in both isotonic saline and in 7.2 - 7.4 phosphate buffered saline was studied. DTT concentrations of 0.01M were prepared in each of these solutions. One half of each was placed in 10 x 100 millimeter glass test tubes in 0.5 ml portions. These were covered with parafilm and frozen. The other half of each was stored at 4°C in a flask. Approximately once a week the activity was tested by treatment of an anti-A antibody with each of the frozen and refrigerated solutions of DTT. This antibody was simultaneously treated with 2-ME. Titers were performed as previously described.

The stability of the reagent when stored in both glass and plastic was also determined. A solution of 0.01M DTT was prepared in both normal saline and phosphate buffered saline. One half of each was stored in glass containers and the other half was kept in plastic screw top bottles. All were stored at 4°C. The contents of each were then tested weekly by treatment of an anti-A antibody.

E. SEPARATION OF IMMUNOGLOBULINS BY USE OF ANION-EXCHANGE CHROMATOGRAPHY

1. Preparation of buffers

Concentrations of 0.4M solutions of both dibasic sodium phosphate (Na_2HPO_4) and monobasic sodium phosphate (NaH_2PO_4) were prepared in six liter volumes. 0.1M and 0.0175M concentrations of each were prepared by appropriate dilutions of the 0.4M solutions. At each concentration, the desired pH was obtained by mixture of the dibasic and monobasic solutions. The final working solutions were: 0.4M sodium phosphate, pH 5.2; 0.1M sodium phosphate, pH 5.8; and 0.0175M sodium phosphate, pH 6.3.

2. Preparation of the exchanger

a. The cellulose ion exchanger, DE-52 was purchased in the microgranular form from Whatman suppliers. This product is a preswollen cellulose having diethyaminoethyl functional groups. The regain is 2.3 - 2.8 grams of water per gram of dry exchanger.

b. Equilibration

The preswollen DE-52 exchanger was weighed and dispersed in 0.4M sodium phosphate buffer, pH 5.2. A ratio of fifteen to thirty milliliters of buffer per dry gram of exchanger was used. The pH was adjusted to 6.3 with 10N NaOH. The mixture was stirred for ten minutes and then filtered through a Buchner funnel with Whatman #42 filter paper. After the excess 0.4M buffer was removed in this manner, the exchanger was washed at least three times with 0.0175M sodium phosphate buffer, pH 6.3, until this pH was maintained.

c. Removal of fine particles

The filtered exchanger was then dispersed in the 0.0175M buffer in a ratio of thirty milliliters of buffer for each gram of dry

exchanger. This mixture was transferred to a graduated cylinder. The suspension was allowed to settle for (t) minutes according to the formula, $t=nh$,* where h is equal to the height in centimeters of the suspension in the cylinder and n is a factor between 1.3 and 2.4. A value of n was arbitrarily chosen as 2.0 for the wet DE-52 exchanger. The supernatant was then removed by suction and fresh buffer added, making a final solution consisting of 150% of the wet settled volume of exchanger.

d. Degassing

The equilibrated exchanger was then transferred to a stoppered Buchner funnel connected to an aspirator. A vacuum was applied along with gentle swirling of the funnel until all excess bubbles were removed.

3. Preparation of the sample

The serum to be applied to the column was dialyzed overnight against two changes of the 0.0175M sodium phosphate buffer. The volume of buffer used was at least ten times that of the sample. Before application of the sample to the column, the precipitated protein was removed from the dialysate by centrifugation.

4. Column packing

The size of the column to be used was determined by the amount of sample to be applied. Both 1 x 40 centimeter and $1\frac{1}{2}$ x 30 centimeter columns were employed. The column was filled with the prepared exchanger, or slurry. Air pressure was applied for packing and the excess buffer was collected in a beaker. When the desired packed column

*Instruction manual, Ion-exchange chromatography, Whatman suppliers.

height was obtained, the remainder of the column was filled with buffer. The top end unit was then connected to the column and attached to a peristaltic pump (Sigmamotor, Inc.). Buffer had been allowed to pump through the tubing for several minutes previously in order to remove any excess air. The Sigmamotor was set at 60 and buffer pumped through the column for at least thirty minutes. The flow rate was checked by measuring the eluate collected in a graduated cylinder for fifteen minutes. If necessary, the Sigmamotor was then readjusted to give a flow rate of sixty ml/hour.

5. Addition of the sample and elution of protein fractions

The pump was then disconnected, the flow from the bottom of the column clamped off, and excess buffer above the packed exchanger removed. Glass beads were added to the top of the bed in order to minimize disturbance of the bed when the sample was applied. The dialyzed sample was then added with a Pasteur pipette. The remaining portion of the column was filled with buffer, the bottom end unit unclamped, and the top unit again connected to the peristaltic pump. 0.0175M sodium phosphate buffer was pumped through the column until the first protein fraction was removed. Sodium phosphate buffers having concentrations of 0.1M and 0.4M were then sequentially pumped through the column in order to elute the more firmly bound proteins.

Fractions were collected in volumes equal to the volume of the sample applied, by means of an automatic, refrigerated fraction collector (Buchler Instruments, Inc. #3R-4002). The protein content of each fraction was monitored at 280nm by an LKB 8301A Uvicord II and

recorded by an LKB 6520A chopper bar recorder. Location of the peaks was confirmed by reading the peak fractions with a Beckman DB spectrophotometer at 280mm.

6. Identification of the types of immunoglobulins present in the column fractions

The fractions were then tested for the presence of immunoglobulins by use of anti-IgG, anti-IgA and anti-IgM antiserum in an Ouchterlony diffusion reaction (49). Agar was prepared from Ionager #2 (Colab Laboratories, Inc.), and pipetted onto glass slides. Wells were made in the agar by means of an agar puncher. The antiserum was placed in a central well. Fractions were added to six wells evenly spaced around the central well. After overnight incubation at $4^{o}C$, the plates were observed for lines of precipitation between the antisera and the fractions.

Detection of the immunoglobulin by use of a ring test was also employed on several occasions. Antiserum was pipetted into 4 x 50 millimeter test tubes. A small amount of the fraction was layered over the antiserum by slow pipetting down the side of the test tube. A ring of precipitation at the interface within two hours was considered a positive test.

7. Examination of the column fractions

Eluates containing the maximum optical density and antibody activity with the 0.0175M and 0.4M buffers were then tested for agglutination or hemolysis of erythrocytes containing appropriate antigens. Tests were carried out at $24^{o}C$ or $37^{o}C$, depending on the antibody. The samples

showing no reaction in saline were tested with antiglobulin serum. If both phases were negative, the eluates demonstrating a particular peak were combined and concentrated with Lyphogel. (Lyphogel, manufactured by the Gelman Instrument Company, is a polyacrylamide gel for concentration of macromolecules.) These concentrated samples were retested. The eluates and concentrates that demonstrated reactivity were then dialyzed overnight at $4^{\circ}C$ against two changes of physiological saline.

The dialyzed samples were then treated with 2-ME and DTT prepared in both normal saline and phosphate buffered saline, pH 7.2 - 7.4 and antibody titers determined. Untreated portions of the eluates were simultaneously tested. A modification of the DTT procedure was found to be necessary for eluate samples. Equal volumes of the eluate and serum from a normal blood group AB individual (not containing anti-A or anti-B), were mixed previous to testing. This mixture was treated with an equal volume of DTT, and incubated for fifteen minutes at $37^{\circ}C$. The antibody titer was then performed. Anti-A and anti-B antibody testing was carried out at room temperature with erythrocytes containing A_1 and B antigens, respectively. Anti-D and anti-Kell titers were performed at $37^{\circ}C$. Saline activity was observed as described in Section B. Mixtures demonstrating negative reactions were tested with antiglobulin serum.

Two of the columns were not eluted with the 0.1M buffer. The 0.4M eluates were found to contain both IgA and IgM when tested by Ouchterlony agar diffusion. Each of these immunoglobulins was neutralized by precipitation with its specific antiserum. After precipitation, titers were again determined and compared with a titer performed on an untreated sample.

In the remaining columns, the 0.1M fractions were tested for erythrocyte antibody activity. Those giving positive results were also tested after precipitation with both anti-IgA and anti-IgM sera.

8. Rechromatography of 0.4M sodium phosphate eluates

The 0.4M sodium phosphate eluates of several columns were rechromatographed on DE-52 for further purification of the IgM fraction. The fractions demonstrating protein content from elution with this buffer were concentrated with Lyphogel. The sample and the ion exchanger were equilibrated with 0.1M buffer. The column was packed as indicated in Section D4. A continuous 0.1M to 0.4M buffer system was obtained by gravity flow from two beakers connected by a U-tube. One hundred milliliters of 0.1M sodium phosphate buffer, pH 5.8 was placed in one beaker and one hundred milliliters of 0.4M sodium phosphate buffer, pH 5.2 was added to the other. The concentrated sample was applied to the packed column and the gradient allowed to flow. Eluates obtained were concentrated and tested as previously described.

RESULTS

A. THE INFLUENCE OF DIALYSIS ON ERYTHROCYTE ANTIBODY AGGLUTINATION TITERS AFTER TREATMENT WITH 0.1M 2-ME

Anti-A and anti-B antibodies obtained from 103 blood group O, A, and B individuals were tested in dialyzed and undialyzed 2-ME reduction procedures. Significant reduction of activity was demonstrated in both dialyzed and undialyzed samples in 96 of 103 antibodies tested. One antibody was unaffected by either 2-ME reduction procedure and was classified as an IgG antibody. Accordingly, 94% of the study group gave identical results in both reduction methods. Six sera had differing effects in the undialyzed and dialyzed techniques. All demonstrated a significant two tube reduction of antibody titer in the undialyzed system, and only a single tube reduction in the standard dialyzed 2-ME technique. These differences are of questionable significance as outlined in the discussion section. Table II summarizes this data.

The 31 sera containing known incomplete erythrocyte antibodies were similarly studied. A significant reduction in antibody titer was not observed in either the dialyzed or undialyzed 2-ME procedure. Accordingly, removal of 2-ME in order to prevent denaturation of the IgG antibodies did not appear to be necessary. These results are presented in Table II.

The comparative sensitivity of the dialyzed and undialyzed reduction techniques to inhibit IgM erythrocyte antibody agglutinating capacity was evaluated in 36 blood samples. Table III summarizes these

TABLE II

THE INFLUENCE OF DIALYSIS ON ERYTHROCYTE ANTIBODY AGGLUTINATING TITERS AFTER TREATMENT WITH 0.1M 2-MERCAPTOETHANOL

Saline Active Antibody Source		Number Tested	Significant Reduction* of Antibody Agglutinating Titer			
Antibody	Blood Type		Both Procedures	Only in Undialyzed	Only in Dialyzed	Neither Procedure
Anti-A	O	43	39	4	0	0
Anti-B	O	26	23	2	0	1
Anti-A	B	20	20	0	0	0
Anti-B	A	14	14	0	0	0
Total		103	96	6	0	1
Antiglobulin Active Antibody Specificity						
Anti-D		21	0	0	0	21
Anti-Kell		7	0	0	0	7
Immune Anti-A		3	0	0	0	3
Total		31	0	0	0	31

*Four-fold or greater.

TABLE III

THE COMPARATIVE SENSITIVITY OF DIALYZED AND UNDIALYZED 2-ME REDUCTION TECHNIQUES IN INHIBITION OF IgM ERYTHROCYTE ANTIBODY AGGLUTINATION TITERS

Change	No difference between the two methods	Greater reduction in dialyzed than undialyzed procedure	Greater reduction in undialyzed than dialyzed procedure	Total
Number of specimens	16	12	8	36
Percentage of specimens	44.5	33.3	22.2	100

results. The two techniques do not appear to differ in the amount of reduction obtained. Therefore, the undialyzed procedure was utilized for comparative purposes in the following studies.

B. DEVELOPMENT OF AN ERYTHROCYTE ANTIBODY REDUCTION PROCEDURE UTILIZING DITHIOTHREITOL (DTT)

1. Results of varying incubation schedules and DTT concentrations on antibody agglutination titers

A preliminary investigation of the activity of DTT under conditions similar to those of the 2-ME technique was carried out. DTT, in concentrations of 0.1M and 0.01M denatured the protein after a two hour incubation period at 37°C. A concentration of 0.001M DTT did not result in reduction of antibody activity when tested after two hours of incubation at 37°C. Further incubation of this mixture at 4°C overnight did not induce reduction of antibody activity.

Table IV illustrates the effect of incubation time and temperature on anti-A antibody agglutination after treatment with 0.01M DTT. Incubation schedules of 60 and 120 minutes at 37°C resulted in protein denaturation. A significant reduction was obtained with all of the other incubation schedules. Varying degrees of reduction were seen with each of these schedules.

The results of treatment of an anti-A antibody with three concentrations of DTT are presented in Table V. It was again noted that the 0.01M DTT solution induced significant reduction. The test serum was denatured in all of the incubation schedules when 0.05M DTT was used. The antibody titer was significantly reduced in all of the

TABLE IV

EFFECT OF INCUBATION TIME AND TEMPERATURE ON ANTI-A ANTIBODY AGGLUTINATION AFTER TREATMENT WITH 0.01M DITHIOTHREITOL

Incubation Temperature	Reduction of Anti-A* Agglutination Titer			
	Incubation time, minutes			
	15	30	60	120
37°C	4-fold	4-fold	Denatured	Denatured
Room Temperature	4-fold	8-fold	8-fold	16-fold
4°C	4-fold	4-fold	4-fold	8-fold

* Reduced 8-fold by 0.1M 2-ME.

TABLE V

REDUCTION OF ANTI-A AGGLUTINATION AFTER TREATMENT WITH 0.05M, 0.01M, AND 0.005M DTT AT DIFFERING INCUBATION SCHEDULES

Incubation Temperature	Concentration of DTT	Reduction of Anti-A* Agglutination Titer	
		Time, in minutes	
		15	30
37°C	0.05M	denatured	denatured
	0.01M	4-fold	4-fold
	0.005M	8-fold	16-fold
Room Temperature	0.05M	denatured	denatured
	0.01M	4-fold	8-fold
	0.005M	2-fold	16-fold

* Reduced 8-fold by 2-ME.

other schedules except one in which a 15 minute, room temperature (RT) incubation using 0.005M DTT was employed. This is not consistent with the other results and probably represents an error in dilution.

Anti-D antibody control activity was not affected by 0.005M or 0.01M solution of DTT with incubation schedules of 15 and 30 minutes at room temperature and 37°C.

The influence of different concentrations of the DTT was further studied. Anti-A activity was reduced in most cases. This study was carried out using 15 and 30 minute periods of incubation at 37°C. The most effective reduction was obtained with 0.01M and 0.008M DTT concentrations. Table VI summarizes these results.

On the basis of these preliminary studies, a tentative standard procedure was established for detailed evaluation. Several combinations of concentration, time, and temperature were found to be effective. The most efficient appeared to be a system using 0.01M DTT incubated with the antibody for 15 minutes at 37°C.

2. The effect of pH on DTT reducing ability

Table VII illustrates the effect of pH on anti-A and anti-D agglutination titers after treatment with a 0.01M concentration of DTT prepared in buffers of varying pH values. Incubation schedules of 15 and 30 minutes at 37°C were tested and found to be similar. Anti-A antibody was reduced when treated with DTT prepared in buffers of pH 7.0, 7.4, and 7.8. None of these DTT solutions significantly reduced anti-D activity.

TABLE VI

EFFECT OF CONCENTRATION OF DTT ON ANTI-A AGGUTINATION TITER

Concentration Of DTT	Reduction of Anti-A* Agglutination Titer	
	Length of Incubation**	
	15 minutes	30 minutes
0.01M	16-fold	16-fold
0.008M	16-fold	16-fold
0.006M	8-fold	8-fold
0.004M	8-fold	8-fold
0.002M	8-fold	2-fold

* Reduced 8-fold by 2-ME.

** Incubation temperature 37°C.

TABLE VII

EFFECT OF DTT TREATMENT OF ANTI-A AND ANTI-D ANTIBODIES;

DTT SOLUTIONS OF 0.01M ARE TESTED IN BUFFERS OF DIFFERENT pH VALUES.

Antibody Specificity	pH of Buffer	pH After Addition of DTT	Reduction* of Agglutination Titers	
			Length of Incubation**	
			15 Minutes	30 Minutes
Anti-A	5.0	4.9	2-fold	None
	6.0	5.9	None	2-fold
	7.0	7.0	4-fold	8-fold
	7.4	7.3	4-fold	4-fold
	7.8	7.7	8-fold	4-fold
	8.2	7.9	4-fold	None
Anti-D	5.0	4.9	2-fold	2-fold
	6.0	5.9	2-fold	2-fold
	7.0	7.0	None	2-fold
	7.4	7.3	2-fold	None
	7.8	7.7	2-fold	None
	8.2	7.9	2-fold	None

* 4-fold or greater significant.

** Incubation temperature of 37°C.

Seven different anti-A antibodies were then tested with DTT prepared in the same buffers. The incubation schedule was kept at 37°C for 15 minutes. Table VIII illustrates these results. These studies indicate that the same three buffers as seen in the preceding study are equally effective. In addition, the DTT prepared in a buffer of pH 8.2 was also capable of significant titer reduction. One specimen did not demonstrate a significant titer reduction with any of the DTT solutions, or with 2-ME, and was considered to represent an IgG antibody. Anti-D activity was not reduced by DTT solutions prepared in these buffers. Of the possible buffers, that of pH 7.4 was chosen for further work, since it is often available in the routine laboratory.

Since the preceding studies had been carried out using DTT prepared in isotonic (0.85%) saline, a comparison of the reducing ability of DTT in isotonic saline, DTT in PBS, pH 7.4, and 2-ME was made. These results are presented in Table IX. Only one of nine specimens demonstrated any difference with the two DTT reagents, and the amount of difference was within the experimental error of the technique.

3. Confirmation of optimum time, temperature, and concentration using DTT prepared in PBS, pH 7.4

Tables IV, VI, and VII indicate that incubation of the DTT-serum mixture for 15 minutes at 37°C results in an equivalent reduction in titer as when the time is extended to 30 minutes. Table IV suggests that an incubation schedule of 30 minutes at room temperature may be more effective than the 15 minute, 37°C incubation schedule. Therefore, further studies were performed to evaluate anti-A antibody activity

TABLE VIII

RESULTS OF TREATMENT OF ANTI-A AND ANTI-D ANTIBODIES WITH 0.01M DTT IN BUFFERS OF VARYING pH VALUES*

Specimen Number		1	2	3	4	5	6	7	8
Antibody Specificity		Anti-A	Anti-A	Anti-A	Anti-A	Anti-A	Anti-A	Anti-A	Anti-D
pH of Buffer	REDUCTION OF AGGLUTINATION TITERS								
5.0		2-fold	2-fold	2-fold	2-fold	4-fold	2-fold	None	Not Tested
6.0		2-fold	4-fold	4-fold	2-fold	4-fold	8-fold	2-fold	Not Tested
7.0		8-fold	32-fold	32-fold	2-fold	16-fold	16-fold	16-fold	2-fold
7.4		16-fold	32-fold	32-fold	None	16-fold	16-fold	16-fold	2-fold
7.8		16-fold	32-fold	64-fold	2-fold	8-fold	16-fold	16-fold	2-fold
8.2		32-fold	16-fold	32-fold	None	16-fold	32-fold	16-fold	2-fold
2-ME Control		16-fold	16-fold	64-fold	2-fold	16-fold	16-fold	32-fold	2-fold

* DTT incubation schedule: 15 minutes at 37°C.

TABLE IX

COMPARATIVE REDUCTION OF ANTIBODY AGGLUTINATING TITERS AFTER TREATMENT WITH 2-ME AND TWO TYPES OF SOLUTIONS OF DTT, 0.01M*

Specimen Number	Antibody Specificity	Reduction of Agglutination Titers		
		DTT in isotonic saline	DTT in PBS, pH 7.4	2-ME
1	Anti-A	32-fold	16-fold	16-fold
2	Anti-A	8-fold	8-fold	8-fold
3	Anti-A	32-fold	32-fold	16-fold
4	Anti-A	64-fold	64-fold	32-fold
5	Anti-A	64-fold	64-fold	32-fold
6	Anti-A	32-fold	32-fold	8-fold
7	Anti-A	8-fold	8-fold	16-fold
8	Anti-A	32-fold	32-fold	64-fold
9	Anti-D	None	None	None

* DTT incubation schedule: 15 minutes, 37°C.

using these two incubation schedules. A 0.01M DTT solution prepared in PBS, pH 7.4 was now employed. Table X demonstrates these results. With three of the antibodies, activity was reduced to a greater degree using a 15 minute, 37°C incubation schedule. Three others were identical with both methods. One was not significantly reduced when either of these schedules was employed and was considered to be an IgG antibody. Therefore, the 15 minute incubation schedule was found to be the most effective in the buffered system.

In Table V, the 0.005M concentration of DTT appears to be more effective in antibody titer reduction than the 0.01M solution. Results obtained with 0.004M and 0.006M solutions, as seen in Table VI, were not as impressive as those obtained with 0.008M and 0.01M solutions. In order to evaluate the possibility that the 0.005M DTT solution in PBS was more effective than the 0.01M solution, seven anti-A antibodies were tested with these two solutions. An incubation schedule of 15 minutes at 37°C was employed. The results are summarized in Table XI. It can be seen that the 0.005M solution is not adequate for maximum reduction with most specimens when a buffered DTT solution is utilized.

The results outlined in this section indicate that DTT is most effective when used at a concentration of 0.01M, as previously determined. The solution may be prepared in either isotonic saline or PBS, pH 7.4. The most efficient incubation schedule was confirmed to be one in which the DTT is incubated with the test serum for a period of 15 minutes at 37°C.

TABLE X

COMPARATIVE REDUCTION OF ANTI-A AGGLUTINATION AFTER TREATMENT WITH 2-ME AND 0.01M DTT* AT TWO DIFFERENT INCUBATION SCHEDULES

Specimen Number	Reduction of Agglutination Titer		
	Incubation Schedule with DTT		0.1M 2-ME
	30 Minutes, RT	15 Minutes, 37°C	
1	4-fold	4-fold	2-fold
2	16-fold	16-fold	16-fold
3	2-fold	None	2-fold
4	2-fold	8-fold	8-fold
5	8-fold	16-fold	16-fold
6	8-fold	16-fold	16-fold
7	4-fold	4-fold	8-fold

* DTT prepared in PBS, pH 7.4.

TABLE XI

THE COMPARATIVE ABILITY OF 0.01M AND 0.005M DTT SOLUTIONS* AND 0.1M 2-ME TO REDUCE ANTI-A** AGGLUTINATION

Specimen Number	Reduction of Agglutination Titer		
	Test Solution		
	0.01M DTT	0.005M	0.1M 2-ME
1	4-fold	2-fold	2-fold
2	16-fold	2-fold	16-fold
3	None	None	2-fold
4	8-fold	2-fold	8-fold
5	16-fold	4-fold	16-fold
6	16-fold	4-fold	16-fold
7	4-fold	4-fold	8-fold

* Incubation schedule: 15 minutes, 37°C.

** Same specimens as tested in Table X.

C. COMPARISON OF THE REDUCING ABILITY OF 0.1M 2-ME AND 0.01M DTT ON A VARIETY OF ERYTHROCYTE ANTIBODIES

1. Comparison of the activity of DTT and 2-ME on erythrocyte antibodies which occur predominately as those of the IgM variety

A total of 223 anti-A and anti-B antibodies obtained from individuals of blood group O, A, and B were tested for activity after treatment with 2-ME and DTT reduction procedures. The results may be seen in Table XII. A significant reduction in titer from an untreated saline control was obtained with 213 of the 223 antibodies tested with both methods. Three sera exhibited no reduction or less than a four-fold decrease in titer, and were considered to contain antibodies of the IgG variety. Accordingly, 216 of 223, or 96.8% of the sera gave identical results with the two methods. Of the seven antibodies differing in the two procedures, five were reduced by DTT but not by 2-ME, while the converse was observed in the remaining two. These seven were retested with the two procedures. Table XIII summarizes the results. Identical titer reduction from the first series was obtained with only one specimen. One serum demonstrated a marked difference when treated with 2-ME on two different occasions. A technical error during one series of tests was probably responsible for this discrepancy. The remaining five were found to differ by one tube in a series of two-fold serial dilutions of the antibodies. This is considered to be within the range of technical error for this type of serological procedure.

2. Comparison of the activity of DTT and 2-ME on antibodies which are usually present in the IgG form

TABLE XII

COMPARATIVE ABILITY OF 0.1M 2-ME AND 0.01M DTT SOLUTIONS TO SIGNIFICANTLY REDUCE SALINE ACTIVE ERYTHROCYTE ANTIBODY TITERS

Saline Active Antibody Source		Number Tested	Significant* Reduction of Antibody Agglutinating Titer			
Antibody	Blood Type		Both Procedures	Only with 2-ME	Only with DTT	Neither
Anti-A	O	80	73	2	4	1
Anti-A	B	27	26	0	0	1
Anti-B	O	58	56	0	1	1
Anti-B	A	58	58	0	0	0
TOTAL		223	213	2	5	3

* Four-fold or greater.

TABLE XIII

ORIGINAL AND REPEAT RESULTS OF SEVEN SALINE ACTIVE ANTIBODIES DEMONSTRATING DIFFERING EFFECTS WHEN TREATED WITH STANDARD DTT AND 2-ME REDUCTION PROCEDURES

Specimen			Test	Amount of Reduction of Antibody Agglutinating Titer	
No.	Antibody	Blood Group		Original Test	Repeat Test
1	Anti-A	O	2-ME	2-fold	4-fold
			DTT	4-fold	4-fold
2	Anti-A	O	2-ME	2-fold	4-fold
			DTT	4-fold	2-fold
3	Anti-A	O	2-ME	4-fold	4-fold
			DTT	2-fold	2-fold
4	Anti-A	O	2-ME	4-fold	4-fold
			DTT	No reduction	2-fold
5	Anti-A	O	2-ME	2-fold	16-fold
			DTT	4-fold	4-fold
6	Anti-A	O	2-ME	2-fold	2-fold
			DTT	4-fold	2-fold
7	Anti-B	O	2-ME	2-fold	4-fold
			DTT	16-fold	32-fold

Table XIV illustrates the results of antiglobulin active erythrocyte antibody titers after treatment with DTT and 2-ME. Since they mainly occur in the IgG form, known antibodies belonging to the Rh blood group system and immune anti-A antibodies were studied. A total of 46 of 48, or 95.8% had no significant titer reduction by either method. One anti-D antibody contained an antiglobulin titer of 1:32 which was reduced by 2-ME but not by DTT. An anti-D saline titer of 1:16 was also present in this specimen. The anti-C which differed in the two procedures demonstrated a two-fold titer reduction with 2-ME and a four-fold reduction with DTT.

3. Comparison of 2-ME and DTT reduction procedures on erythrocyte antibodies whose immunoglobulin nature was unknown

Of the antibodies whose immunoglobulin nature was unknown, 30 of 31, or 96.8% demonstrated identical results with both reduction procedures. The one exception was a very weakly active anti-Le^a antibody whose titer was reduced by 2-ME but not by DTT. The end-point of the titer of this antibody was so difficult to determine that the significance of this result is questionable. This data is summarized in Table XV.

4. Comparative sensitivity of the 0.01M DTT and 0.1M 2-ME reduction procedures

The comparative sensitivity of the two methods in causing reduction of the agglutination titer was determined statistically. The Wilcoxon matched-pairs signed-ranks test was utilized for this purpose. The method of calculation of this test is described in Appendix I. The results may be seen in Table XVI. The known IgG control antibodies did

TABLE XIV

EFFECT OF 0.1M 2-ME AND 0.01M DTT ON INCOMPLETE ERYTROCYTE ANTIBODY AGGLUTINATION TITERS

Antiglobulin Active Antibody Specificity	Number Tested	Significant* Reduction of Antibody Agglutinating Titer			
		Both procedures	Only with 2-ME	Only with DTT	Neither
Anti-D	38	0	1	0	37
Anti-E	1	0	0	0	1
Anti-C	2	0	0	1	1
Anti-e	1	0	0	0	1
Anti-$\bar{c}$	1	0	0	0	1
Immune Anti-A	5	0	0	0	0
TOTAL	48	0	1	1	41

* Four-fold or greater.

TABLE XV

EFFECT OF 0.1M 2-ME AND 0.01M DTT ON AGGLUTINATION TITERS OF ERYTHROCYTE ANTIBODIES WHOSE IMMUNOGLOBULIN NATURE IS UNKNOWN

Saline Active Antibodies Antibody Specificity	Number Tested	Significant* Reduction of Antibody Agglutinating Titer			
		Both procedures	Only with 2-ME	Only with DTT	Neither
Anti-M	2	0	0	0	2
Anti-Le^a	3	2	1	0	0
Anti-I	3	3	0	0	0
Non-Specific Cold Agglutinin	3	3	0	0	0
Anti-P_1	1	1	0	0	0
Antiglobulin Active Antibodies Antibody Specificity					
Anti-Kell	13	0	0	0	13
Anti-M	1	0	0	0	1
Anti-Le^a	1	0	0	0	1
Anti-JK^a	1	0	0	0	1
Anti-JK^b	1	0	0	0	1
Anti-F_y^a	2	0	0	0	2

* Four-fold or greater.

TABLE XVI

STATISTICAL EVALUATION OF THE COMPARATIVE REDUCING ABILITY OF 0.1M 2-ME AND 0.01M DTT USING THE WILCOXON MATCHED-PAIRS SIGNED-RANKS TEST

Antibody Specificity	Blood Group	Number Tested	Z Score	Level of Significance	Test Demonstrating Greatest Degree of Titer Reduction
Anti-D	—	38	0.11	N.S.	Neither
Anti-B	O	58	0.38	N.S.	Neither
Anti-B	A	58	-2.43	$p<0.05$	2-ME
Anti-A	B	27	-2.40	$p<0.05$	2-ME
Anti-A	O	80	-3.31	$p<0.001$	2-ME

N.S. = not significant

not demonstrate more than a one tube (two-fold) decrease in titer by either method, which is within the range of error for the serologic tests. The effect of the two reagents on anti-D titers was calculated, however, in order to determine whether the strength of the reaction was reduced more in one method than the other. It was found that the two tests did not differ significantly in their effects on anti-D agglutination titers, as may be seen in Table XVI.

Of the predominately IgM antibodies, anti-A in blood group O, anti-A in group B, anti-B in blood group O, and anti-B in group A were studied independently. All except anti-B in group O demonstrated a highly significant difference in the degree of reduction of antibody titer obtained by the two methods. The amount of reduction with the 2-ME procedure was greater than that of the DTT in each case. The anti-B titers in blood group O were reduced to the same degree by both methods. It should again be emphasized that although statistically significant differences in magnitude of reduction were found, both procedures were equally capable of demonstrating the presence of the IgM erythrocyte antibody.

D. DETERMINATION OF THE STABILITY OF DITHIOTHREITOL IN A VARIETY OF CONDITIONS

1. A comparison of DTT prepared in isotonic saline and DTT prepared in phosphate buffered saline, pH 7.4

The reducing ability of the 0.01M DTT was determined after preparation of the reagent in solutions of isotonic saline and phosphate buffered saline, pH 7.4. The buffered reagent was found to lose its

reducing ability after a period of 14-21 days when stored at refrigerator temperatures. The DTT solution prepared in isotonic saline was capable of anti-A titer reduction for a period of six months of similar storage at which time the study was terminated. This data is presented in Table XVII.

2. Stability of the two reagents when stored at -20°C

The stability of the buffered reagent was found to be greatly enhanced if aliquots were frozen. The frozen solution demonstrated optimum reducing ability for at least six months. Optimum reducing ability was also maintained for this period of time with the DTT in isotonic saline, when stored at -20°C. Table XVII summarizes these results.

3. Stability of the two reagents when stored in glass and plastic containers

As may be seen in Table XVIII, solutions of DTT in phosphate buffered saline were found to lose antibody inhibitory activity more rapidly when stored in plastic containers. The same reagent was active for the usual period of 14-21 days when stored in glass containers. The stability of an isotonic saline solution of DTT did not appear to be influenced by storage in plastic containers when tested for a period of three months.

E. COMPARISON OF THE REDUCING ABILITY OF 0.1M 2-ME AND 0.01M DTT ON IMMUNOGLOBULINS SEPARATED BY ION-EXCHANGE CHROMATOGRAPHY

1. Separation of immunoglobulins

TABLE XVII

STABILITY OF TWO SOLUTIONS OF DTT WHEN STORED AT 4°C AND -20°C

Solution	Anti-A Antibody Titer After Reduction Treatment						
	Storage						
0.01M DTT in PBS, pH 7.4	Temperature	Time, in Months					
		1	2	3	4	5	6
	4°C	1:256	—	—	—	—	—
	-20°C	1:16	1:2	1:16	1:8	1:8	1:16
0.01M DTT in Isotonic Saline	4°C	1:32	1:2	1:16	1:4	1:8	1:8
	-20°C	1:16	1:2	1:16	1:4	1:8	1:16
Untreated Saline Control Titer		1:256	1:64	1:512	1:128	1:128	1:128

TABLE XVIII

STABILITY OF TWO SOLUTIONS OF DTT WHEN STORED IN EITHER GLASS OR PLASTIC CONTAINERS AT 4°C

0.01M DTT in PBS, pH 7.4		
Container	Anti-A Antibody Titer	
	Storage Time	
	10 Days	17 Days
Glass	1:16	1:4
Plastic	1:16	1:32
Untreated Saline Control Titer	1:256	1:32
0.01M DTT in Isotonic Saline		
Container	Anti-A Antibody Titer	
	Storage Time	
	8 Days	3 Months
Glass	1:16	1:8
Plastic	1:16	1:8
Untreated Saline Control Titer	1:512	1:128

Immunoglobulins were separated into more purified forms by means of ion-exchange chromatography in order to test the activity of these forms after treatment with 0.1M 2-ME and 0.01M DTT. Four peaks were obtained from the three sodium phosphate buffers used for elution. Ouchterlony double diffusion tests revealed that only IgG was eluted with a 0.0175M buffer of pH 6.3. Two peaks were obtained with a 0.1M buffer of pH 5.8. The first was a narrow peak which contained predominately IgA. Some IgG was also present in this fraction. The second was a broad peak containing albumin. The fraction eluted with a 0.4M buffer of pH 5.2, contained the IgM portion. This was always contaminated with some IgG and on some occasions this fraction also contained IgA. The original titers of the antibodies were not recovered in the column fractions.

2. Further purification of the IgM fraction

An attempt to obtain a more pure IgM component by rechromatography was unsuccessful. The antibody was diluted to such an extent that it was unable to induce agglutination of erythrocytes even when eluates were pooled and concentrated. Therefore, rechromatography was discontinued after it was employed on the 0.4M fractions from columns of the first two sera.

3. Results of 2-ME and DTT treatment of erythrocyte antibodies found in the 0.0175M fraction

Table XIX describes the antibodies obtained from the 0.0175M fractions of the various columns, and their susceptibility to reduction. Four of the anti-A antibodies demonstrated some agglutinating ability

TABLE XIX

SUSCEPTIBILITY OF 0.0175M ELUATES FROM DEAE-CELLULOSE COLUMNS TO REDUCTION WITH 0.1M 2-ME AND 0.01M DTT

Specimen Number	Antibody Specificity	Reciprocal of Eluate Titer*		Significant Reduction of Agglutination Titer					
				2-ME		DTT in Isotonic Saline		DTT in PBS pH 7.4	
		Saline	Antiglobulin	Saline	Antiglobulin	Saline	Antiglobulin	Saline	Antiglobulin
1	Anti-A	4	16	No	No	No	No	No	No
2	Anti-A	8	512	No	No	No	No	Yes	No
3	Anti-A	0	16	—	No	—	No	—	No
4	Anti-A	16	64	No	No	No	No	No	No
5	Anti-A	0	32	—	No	—	No	—	No
6	Anti-A	16	64	No	No	No	No	No	No
7	Anti-B	0	4	—	No	——— Too weak to test ———			
8	Anti-B	0	16	—	No	—	No	—	No
9	Anti-B	0	16	—	No	—	No	—	No
10	Anti-D	0	128	—	No	—	No	—	No
11	Anti-D	0	8	—	No	—	No	—	No
12	Anti-D	0	8	—	No	—	No	—	No
13	Anti-Kell	0	8	—	No	—	No	—	No

* Average titer of saline controls of the two test procedures.

in a saline medium. A higher titer was obtained using an antiglobulin technique. The remainder of the antibodies were only demonstrated by means of antiglobulin tests.

Because of the necessity of adding normal serum to the eluates before testing them with DTT, antibodies having titers less than 1:8 were too weak to give definitive results unless all treated and control sera demonstrated positive reactions in the 1:4 dilution. For this reason, the concentration of anti-B eluted from one of the columns was too weak for adequate testing with DTT.

Only one antibody obtained by this fraction procedure and assumed to be IgG showed significant reduction of antibody agglutinating capacity employing the disulfide reduction methods. The saline agglutination titer of this anti-A antibody was reduced by DTT in PBS, but not by DTT in isotonic saline or by 2-ME. In fact, some of the saline agglutination titers were increased after treatment with 2-ME and DTT. None of the antiglobulin agglutination titers were significantly reduced by either of the reduction procedures.

4. Results of 2-ME and DTT treatment of erythrocyte antibodies found in the 0.4M fraction

Five anti-B, four anti-A, one anti-D, and one anti-I erythrocyte antibodies were recovered in eluates of this buffer. All of these were saline active erythrocyte antibodies whose agglutination titers were significantly decreased by 2-ME and DTT reduction procedures. These results are summarized in Table XX. One of the anti-A antibody titers

TABLE XX

SUSCEPTIBILITY OF 0.4M ELUATES FROM DEAE-CELLULOSE COLUMNS TO REDUCTION WITH 0.1M 2-ME AND 0.01M DTT

Specimen Number	Antibody Specificity	Reciprocal of Eluate Saline Titer*	Significant Reduction of Saline Agglutination Titers		
			2-ME	DTT in Isotonic Saline	DTT in PBS, pH 7.4
1	Anti-A	32	Yes**	Yes	Yes
2	Anti-A	8	Yes	Yes	Yes
3	Anti-A	8	Yes	Too weak to test	
4	Anti-A	32	Yes	Yes	Yes
5	Anti-B	32	Yes	Yes	Yes
6	Anti-B	8	Yes	Yes	Yes
7	Anti-B	16	Yes	Yes	Yes
8	Anti-B	16	Yes	Yes	Yes
9	Anti-B	32	Yes	Yes	Yes
10	Anti-I	256	Yes	Yes	Yes
11	Anti-D	32	Yes	Yes	Yes

* Average titer of saline controls of the two test procedures.

** 1:16 antiglobulin titer after reduction of saline titer.

was reduced with 2-ME, but this antibody was not successfully recovered subsequently when DTT testing was performed.

All dilutions found to be negative for agglutinating capacity after reduction were tested with antiglobulin serum. Although some IgG was present in the eluates obtained with this buffer, only one antibody demonstrated reactivity with antiglobulin serum. Since a higher saline agglutination titer was present, it was not possible to determine antiglobulin agglutination titers prior to the reduction procedure.

5. Determination of immunoglobulin type of erythrocyte antibodies eluted with 0.1M sodium phosphate

It was of interest to test the possibility that some of these antibodies were primarily of the IgA variety. Therefore, titers were performed on the 0.1M fractions of all of the columns before and after precipitation with anti-IgA serum. All retained the same titer except low titered saline anti-A and anti-B antibodies from one column. The agglutination titers of these antibodies were reduced 8-fold and 4-fold, respectively, after neutralization with anti-IgA, indicating that this fraction contained an erythrocyte antibody of the IgA variety. Higher titered anti-A and anti-B antiglobulin antibodies present in this fraction were not neutralized by anti-A, however, indicating that the main variety was IgG rather than IgA. This fraction, in fact, did contain a significant amount of IgG. Some titers of the 0.1M fraction were decreased after IgM neutralization, although IgM was not demonstrated by Ouchterlony analysis.

DISCUSSION

It is clinically important to determine the immunoglobulin nature of erythrocyte antibodies discovered in obstetrical patients. IgG antibodies are capable of crossing the placenta and causing hemolytic disease of the newborn. IgM antibodies do not traverse the placental barrier and, therefore, are harmless in this respect. Documentation of the IgG nature of an erythrocyte antibody prior to delivery will alert the physician to the possibility of erythroblastosis. On the other hand, determination of the IgM nature of the antibody may avoid unnecessary anxiety.

A hemmagglutination system was employed for differentiation of these immunoglobulin types. Since IgG and IgM erythrocyte antibodies differ in their ability to cause agglutination of red blood cells, a brief discussion of this system is outlined below.

Agglutination of erythrocytes is a visual result of antigen-antibody interactions under appropriate conditions. Antibody binding sites and antigenic determinants on the erythrocyte surface become closely associated in a "lock-and-key" arrangement. Weak intermolecular bonds, as well as the three dimensional configuration of the complementary molecular structures, hold the reactants together. Covalent bonds are not involved. The reaction is reversible, and depends on the "goodness of fit," permitting close proximity of the antigen and antibody. The ionic strength and pH of the surrounding media, as well as the temperature of incubation, may affect the strength of the bonds or the rate at which combination occurs (50).

After antibody-antigen union, bivalent antibody molecules may form bridges between the cells, resulting in a lattice arrangement. Visual agglutination occurs when a sufficient number of cells are involved. Red cells repel each other due to the presence of negatively charged carboxyl groups of sialic acid on the cell membrane (51). In solution, cations are attracted to the negative cell surface and form a positive cloud of ions around the cell. Some of these cations travel with the cell and account for the total charge that keeps erythrocytes apart. The potential measured at the edge of the cation cloud is known as the zeta potential (52).

Several factors are thought to be involved in formation of the lattice leading to agglutination. Pollack (52) claims that the length of the antibody molecule is important. IgM antibodies, having a length of 750 Å, are long enough to bridge the distance between two cells separated by repulsive forces. Agglutination can therefore occur in ordinary saline media. IgG antibodies attach to the cell, but are too short (250 Å) to overcome these repulsive forces and form molecular bridges. Three types of substances are used to induce agglutination of erythrocytes coated with IgG antibodies. Antiglobulin serum acts as a bivalent antibody bridge between antibodies attached to different cells; proteolytic enzymes remove sialic acid from the cell membrane and reduce the negative charge; albumin and other colloidal materials absorb the cations surrounding the cell reducing the zeta potential. Erythrocytes coated with antibodies are then able to more closely approximate each other and agglutination may occur.

Besides the surface charge of the erythrocytes, other factors must be involved since some IgG antibodies induce agglutination in saline (53). Hoyer and Trabold (54) demonstrated that the number of antigenic sites may be important. A much greater number of antigen sites was found to be necessary for saline agglutination with IgG than with IgM antibodies. Pollack (55) has suggested that the position of the antigen in relation to the depth of the cell membrane may also be of importance.

Many blood bank services assume that saline active erythrocyte antibodies are IgM while those requiring enhancing media are of the IgG variety. While this is often true, many exceptions may occur, as noted above. Therefore, more definitive tests are required for discrimination of these immunoglobulin types.

This investigation was designed in order to establish a rapid, inexpensive method which would differentiate IgG and IgM erythrocyte antibodies. In order to accomplish this, it was necessary to evaluate the new test procedure against a technique known to be capable of distinguishing these two immunoglobulin types. A procedure for reduction of IgM erythrocyte antibodies, with a resultant loss of serological activity, has been described by Mollison (44). This method utilized 2-mercaptoethanol as a reducing agent. The procedure was reliable, but time consuming, since it required removal of 2-ME by dialysis after reduction. The necessity of dialysis has been questioned by several investigators since the start of the present investigation (45, 46). Elimination of this step would greatly reduce both the amount of serum needed for study, and the time spent performing the test.

Accordingly, the effect of dialysis on erythrocyte antibody agglutinating titers after 2-ME treatment was studied.

Antibodies known to occur most frequently as either IgM or IgG varieties were tested in dialyzed and undialyzed 2-ME reduction procedures. Identical results were obtained with 97 of 103 saline active anti-A and anti-B antibodies tested (Table II). The remaining six sera demonstrated a significant (four-fold) drop in titer employing the undialyzed but not the dialyzed procedure. However, a two-fold reduction in titer was obtained in these six, using the dialyzed method. Accordingly, the differences in titer represent only a variation of one tube in a set of serial dilutions. The most likely explanation is that this is within the range of technical error. Other explanations may be considered. The discrepancy between the two methods may reflect reaggregation of IgM monomers after removal of the sulphydryl compound. Dialysis, accordingly, would make the test less sensitive. Such significant reaggregation is unlikely. The large number of reduced IgM monomers of varying antibody specificities combine at random, and therefore, those specific for the same antibody are unlikely to recombine (44).

The lack of dialysis may introduce another complication. Denaturation of either the antibody or antigen could occur by permitting 2-ME to remain in the mixture. This would lead to titer reduction of IgG antibodies when dialysis is omitted. Mollison (44) claims to obtain false positive values with undiluted sera when dialysis is not employed, but offers no data to confirm this statement. The studies of Reesink et al.

(45) do not support this view. However, the only IgG antibodies studied were those of anti-A specificity. The current study evaluated the effect of dialysis on IgG antibodies having anti-D, anti-Kell, and anti-A specificities. Table II demonstrates that all incomplete antibodies evaluated preserved their agglutinating capacity without removing 2-ME. The comparative sensitivity of titer reduction induced by either removing or leaving 2-ME in the reacting mixture further supports this observation. It was found that the majority of antibodies had an identical degree of titer reduction with dialyzed and undialyzed procedures. In addition, similar numbers of antibodies demonstrated either more or less sensitivity in titer reduction when the two methods were compared (Table III).

The original procedure that Deutsch and Morton used to reduce macroglobulins (40) was applied to blood group antibodies and dialysis was incorporated as part of the method. The current study indicates that dialysis may be eliminated without adversely affecting the sensitivity of the test. Therefore, an undialyzed 2-ME procedure was employed as a standard reference test for evaluation of a new reduction procedure.

Dithiothreitol (DTT), has been utilized for reduction of disulfide bonds since it was first described for this purpose in 1964 by Cleland (47). Miller and Metzger (42) and Beale and Feinstein (41) employed this reagent for disulfide bond location in macroglobulins. The former investigators obtained IgM subunits in concentrations ranging from 0.001M to 0.027M DTT and an incubation period of one hour at 25°C.

Beale and Feinstein were able to produce 7S subunits using DTT in a final concentration of 0.000125M. Additional reduction with 0.005M DTT resulted in polypeptide chain formation. This study was also performed at room temperature for one hour. Pure 1% solutions of IgM in Tris-HCl buffered saline, pH 8, were treated with the different concentrations of DTT. Selective reduction was then used in conjunction with alkylation of the free SH groups to locate the number and position of the disulfide bonds in IgM molecules. Only one bond was found to connect any two 7S monomers. This bond was susceptible to mild reduction while intrasubunit bonds remained intact.

Following the work of Deutsch and Morton (40), it was shown that 2-ME reduction of 19S macroglobulins into 7S monomers resulted in a loss of most biological activity, including the ability to agglutinate red blood cells (43). The monomers have been shown to retain their ability to combine with specific antigens, however (56-58). Accordingly, mild reduction of IgM antibodies with DTT should also be accompanied by a loss of red blood cell agglutinating ability. One reference (59) mentions the use of DTT in detection of erythrocyte immunoglobulin types, but a formal procedure is not presented. No previous studies using DTT for this purpose have been reported. Usage of this reagent in the routine blood bank seemed to offer some advantages. Primarily, the offensive odor of 2-ME would be eliminated. This is a major reason for the reluctance of blood bank personnel to incorporate this procedure in the routine laboratory. Also, a more rapid test could possibly be developed by varying concentrations of DTT and incubation schedules.

The present study was initiated in order to investigate the effect of DTT on erythrocyte antibodies. Saline active anti-A antibodies demonstrating titer reduction after 2-ME treatment were treated with concentrations of DTT at different incubation schedules. A loss of activity indicated reduction of 19S IgM antibodies into 7S monomers. Anti-D antibodies not reduced by 2-ME were studied simultaneously as controls against further reduction of 7S molecules into polypeptide chains. Several combinations were capable of reducing the degree of anti-A antibody agglutination. A 15 minute incubation period at 37°C using a 0.01M solution of DTT was found to be the most efficient. Since equal volumes of serum and the DTT were utilized, the final concentration of DTT was 0.005M. This did not affect the activity of the IgG antibodies. This is somewhat surprising in view of the fact that the investigators mentioned above obtained separation of polypeptide chains using this concentration of DTT. They were working with a different system however, so the present studies and test systems are not comparable.

Anti-A antibody titers appeared to be reduced to a comparable extent when the 0.01M DTT solution was prepared in buffers of pH 7.0, 7.4, 7.8, and 8.2 (Tables VII and VIII). According to Cleland's original description of the reagent (47), the ability of DTT to reduce an S-S bond is enhanced by increasing pH values. This effect was not as striking in these studies, although pH values between 7.0 and 8.0 were more effective than values of 5.0 and 6.0 in a buffered test system. DTT in isotonic saline was found to reduce anti-A agglutination to an equal degree as DTT prepared in PBS, pH 7.4. This was

surprising since the pH of DTT in isotonic saline in our laboratory was 5.42, while the pH of the DTT in the PBS solution was 7.3. Solutions of DTT prepared in buffers of pH 5.0 and 6.0 were less effective in antibody reduction. The ionic strength at pH values of 5.5 in the buffered system is about 2.2 times that of the non-buffered system, so the salt concentration is the major difference in the two solutions. The higher salt concentration may interfere with the reaction, but this is doubtful. The ionic strength of the PBS solution at pH 7.4 is more than twice that of the PBS of pH 5.5. Accordingly, if any interference is involved it is overcome by the increase in pH.

Solutions of 0.01M DTT in PBS, pH 7.4, were repeatedly found to lose activity after storage for 14-21 days at refrigerator temperatures. Freezing the solution in aliquots increased its stability to a period of six months, at which time the studies were terminated. Solutions of DTT in isotonic saline were stable for at least six months at refrigerator or freezing temperatures (Table XVII).

The refrigerated solutions of DTT in PBS were found to lose activity even more rapidly when stored in plastic containers (Table XVIII). The same effect was not observed with the isotonic saline solution of DTT after three months of study. The reason for this observation was not further investigated. The buffer may induce increased leaching of the plastic causing interference of DTT activity. This is highly speculative, however.

In spite of the storage difficulties inherent in using DTT in PBS, this solution is preferable to isotonic saline solutions of the reagent,

since the latter varies from one laboratory to another. On the other hand, PBS solutions of DTT at a particular pH value should be consistent between laboratories. Freezing the solution in small aliquots takes very little time, and greatly prolongs the stability. Plastic containers should be avoided, when working with the buffered solution. Possibly, high grade plastics would not inactivate the reagent, but the effect of various types of plastics was not investigated.

After the standard DTT procedure was established, it was tested against a variety of antibodies. Since they exist predominately in the IgM form, saline active anti-A and anti-B antibodies were studied. At the same time, these antibodies were tested with the standard undialyzed 2-ME procedure. A total of 213 of 223 antibodies tested demonstrated significant titer reduction with both procedures (Table XII). Three were not reduced with either method and were assumed to represent IgG antibody forms. Two were reduced by 2-ME but not by DTT, while the converse was true of the remaining five. These seven were retested at a later time (Table XIII). The two that were significantly reduced with 2-ME but not with DTT gave essentially the same results when the tests were repeated. Three of the five that were reduced by DTT only in the first series, were reduced by both upon retesting. The opposite results were obtained with one antibody in the second study, while the remaining one was not reduced by either method when repeat tests were performed.

With the exception of one specimen, all of the antibodies differing in the two procedures demonstrated a change of only one tube in a set of serial dilutions. This is within the range of error of serologic

testing. Mixture of the reducing reagent with the serum, addition of the saline to the tubes to be used for the dilution, and transferrence of one dilution to the next in the series, are all possible sources of error in this technique. Since a two-fold and four-fold titer reduction each represent different interpretations of the type of immunoglobulin present, it would be advisable to repeat any tests giving these two reduction results. If the amount of serum is sufficient, the test should be performed in duplicate. Also, the use of master dilutions may reduce the amount of error. It should be emphasized that these difficulties are not solely the problem of employing DTT. Rather, they are inherent in any serologic procedure dependent upon quantitation by serial dilution.

The degree of titer reduction of IgM antibodies appeared to be greater when induced by 2-ME than DTT (Table XVI). The titer was often decreased to the same absolute level with both methods, but the strength of the agglutination in the positive dilutions was found to be greater with DTT than with 2-ME. In other words, a 1:2 dilution of the serum might exhibit a 3+ reaction after treatment with DTT and a 1+ reaction after treatment with 2-ME. The strength of the 1:4 dilution may be 1+ with both reagents, and the 1:8 dilution negative. This effect was observed repeatedly and accounts for the statistical difference in the two procedures. The DTT solution was at least as effective as 2-ME in distinguishing IgG and IgM antibodies. The degree of reduction is not critical for this important point. This observation could assume significance, however, if other applications of the procedure are

attempted. For example, the DTT procedure recommended in this paper may not be as useful as 2-ME for elimination of high titered IgM erythrocyte antibodies in order to test for more weakly reactive IgG antibodies.

Of the IgG antibodies tested, one anti-D titer was reduced by 2-ME only, and one anti-C titer was susceptible to reduction only by DTT (Table XIV). None of the other 41 antibodies of the Rh blood group demonstrated titer reduction with either of these two procedures. A statistical study of the anti-D antibodies revealed that the degree of reduction was the same with both methods. This demonstrates that neither one of the procedures induced additional reduction of the 7S monomers.

Thirty-one antibodies of unknown immunoglobulin nature were also studied with these two procedures (Table XV). Identical results were obtained with all except one saline active Le^a antibody which was reduced with 2-ME but not by DTT. The significance of this result is questionable since the antibody was very weak and the end-point was extremely difficult to determine. Neither of the two saline active anti-M antibodies studied were susceptible to reduction by 2-ME or DTT. IgG anti-M antibodies may be active in saline (44). Accordingly, these two antibodies would be cause for concern in an obstetrical patient, even though they demonstrate saline activity in classical agglutination techniques. The titer of these antibodies was not increased by use of antiglobulin serum. If contained in the serum, antiglobulin active antibodies were present at titer ranges the same or lower than the saline titer. Anti-M

antibodies usually occur in the IgM form, but have been shown to exist in IgG forms and cause hemolytic disease of the newborn (19, 60).

Two reduction procedures were compared and both appeared to be equally effective in the differentiation of immunoglobulin types. Accordingly, it was not possible to determine which one was correct if discrepancies were obtained. Unfortunately, the quantity of serum present after the tests were performed was insufficient for further testing of these samples. Therefore, a number of antibodies of various types were studied after purification of DEAE-Cellulose ion-exchange columns, in order to confirm that the two reducing agents act in an identical fashion on the specific immunoglobulins.

The purified immunoglobulins obtained from the column fractions confirmed the effectiveness of the 2-ME and DTT procedures in distinguishing immunoglobulin types. The fractions obtained with 0.0175M sodium phosphate buffer were found to contain IgG antibodies. Six anti-A, three anti-B, one anti-Kell, and three anti-D antibodies were eluted from ten different columns using this buffer (Table XIX). All were antiglobulin active antibodies. In addition, four anti-A antibodies had some saline activity. One of the saline titers was reduced significantly with DTT in PBS, but not by DTT in saline or by 2-ME. Since a higher antiglobulin titer was present and not reduced by any of the solutions, the significance of this result is questionable.

Titers of antibodies obtained from the columns were much lower than the original serum titers. This was especially true of the anti-D and anti-Kell varieties. The reason for the low recovery of these IgG

antibodies is not clear. As much as 25% of the IgM fraction may be lost during equilibration of the sample with this buffer (37). It has been shown that anti-D saline active antibodies represent IgM-type antibodies. This may account for the fact that the saline active portion of one anti-D antibody was lost.

A possibility that some of the antibodies were of the IgA variety was considered, since the existence of these forms of erythrocyte antibodies has been reported (21). The IgA portion was recovered in the 0.1M fraction when elution with the buffer of this concentration was included. This step was omitted when the anti-I antibody was tested. As a result, IgA and IgM were both eluted with the 0.4M buffer. If the antibody was IgA, this could explain why it was not reduced by DTT in the original tests. However, precipitation with anti-IgA and anti-IgM revealed that the antibody was IgM.

An antibody which occurred primarily in the IgA form could also account for the failure to recover it in the 0.0175M and 0.04M fractions. Accordingly, the 0.1M fractions were tested for antibody activity. Those demonstrating positive reactions were tested after precipitation with anti-IgA. Two low titered saline active antibodies (anti-A and anti-B) from a group O individual were reduced after this treatment. The more strongly active antiglobulin titers of the same antibodies were not affected. This could indicate that a portion of the saline active antibodies in this fraction were in the IgA form. However, the results are not completely conclusive.

SUMMARY AND CONCLUSIONS

In this study, two disulfide reduction procedures for differentiation of IgG and IgM erythrocyte antibodies were investigated. These tests are simple and may be utilized for routine work in the blood bank. The procedure previously available employing 2-mercaptoethanol (2-ME) required dialysis for removal of sulphydryl compounds prior to titration of the antibody. The necessity of dialysis was investigated. Identical results were obtained with 97/103 saline active anti-A and anti-B antibodies using dialyzed and undialyzed 2-ME procedures. The remaining six were reduced by the undialyzed technique only, and the difference probably represents a procedural variation. None of 31 antiglobulin active antibodies were reduced by either method.

A reduction procedure utilizing 0.01M dithiothreitol was established. It was found to be more efficient than the 0.1M 2-ME method in that maximum reduction of titer was achieved after treatment of the serum for 15 minutes at 37°C. In addition, the repugnant odor of 2-ME was avoided, so this compound did not require use of a hood. Dialysis was found to be unnecessary and a stable reagent is available when stored at appropriate temperatures.

Only ten of 302 antibodies of varying specificities studied in undialyzed 2-ME and DTT reduction procedures demonstrated differing results. Titers were reduced only by 2-ME with four specimens while six demonstrated DTT reduction only. Most of these differences appeared to be within the range of technical error of serological testing.

Purified immunoglobulins from DEAE-Cellulose column chromatography confirmed the role of DTT and 2-ME in specific reduction of IgM antibodies. None of the 12 antiglobulin antibodies obtained from the IgG fractions showed titer reduction with 2-ME or DTT, while all ten antibodies in the IgM fractions were reduced by both methods.

The DTT reagent may be prepared in isotonic (0.85%) saline or PBS, pH 7.4. The saline solution may be stored at either refrigerator or freezing temperatures. The PBS is preferable, but should be frozen in aliquots. Storage in plastic containers should be avoided.

It is well known that IgG erythrocyte antibodies may cross the placental barrier and cause Hemolytic Disease of the Newborn, while IgM antibodies are incapable of placental transfer, and therefore harmless. DTT may be utilized for routine differentiation of antibodies found in obstetrical patients. The procedure is rapid, inexpensive, and requires minimal amounts of serum. The successful employment of DTT in an erythrocyte antibody system suggests that this reagent may have general applicability in other antibody detection systems.

APPENDIX I

STATISTICAL CALCULATIONS

In order to determine whether there was a significant difference in the degree of reduction between the 2-ME and DTT procedures, the Wilcoxon matched-pairs signed-ranks test (61) was performed. Each titer was given a numerical value by summing the amount of agglutination obtained in each of the set of serial dilutions. A trace reading was assigned a value of one. Different base line values were required for each method. Separate untreated control titer values were obtained because the time of incubation was different with each technique. The total amount of agglutination in the treated titer was subtracted from the value of the titer of the untreated serum. In this manner a value relating to the degree of reduction with both 2-ME and DTT was obtained for the specific serum. The difference between the two methods was then determined by subtraction. These differences, eliminating values of 0, were ranked. Ties were given the value of the average of the ranks. After ranking, the sign of the difference was affixed to the number and the smaller sum of like-signed ranks was determined.

It was possible to use the formula, $Z = \frac{T-\mu^T}{\sigma^T}$, since the sum of the ranks, T, is practically normally distributed when the value of N is greater than 25. The value of T is the sum of the ranks with the less frequent sign; μ^T is the mean, where $\mu^T = \frac{N(N+1)}{4}$; and σ^T is the standard deviation with the formula: $\sigma^T = \sqrt{\frac{N(N+1)(2N+1)}{24}}$. N is equivalent to the total number of matched pairs minus the number demonstrating no difference. Values of p were extrapolated from a table of z scores.

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