EDITORIAL

On the Specificity and Reactivity of Coombs' Antiglobulin Sera

By Sol Haberman

The successful application of the Coombs', Mourant and Race antiglobulin test\(^1\) to a wide variety of antigen-antibody systems for the diagnosis of certain diseases\(^2\) has led to a heavy demand for Coombs' antiglobulin sera from commercial sources. This need has been met by (a) recognized biological and pharmaceutical houses, (b) research institutes, blood banks and laboratories producing this reagent for their own use and selling their surpluses, (c) small commercial laboratories producing this reagent for local sale only, (d) individual laboratories producing the antisera for their own uses, and (e) state, city or county health departments producing this reagent for their use and distribution. These producers of sera employ a variety of methods and animals in the production of this reagent. Because of known variations in the methods of preparation of this antiserum, as well as some previously observed differences between several such testing sera, it was thought desirable to compare the reactions of different sera on normal and antibody-coated erythrocytes from newborn children. The results were of such interest that the study was extended to include cases of hemolytic anemia as well as examples of erythroblastosis due to the ABO or Rh-Hr systems. In addition, selected Coombs' antiglobulin sera were used during a two-year survey on mothers and their infants born in the maternity division of Baylor University Hospital. During this period, 12,410 mothers and 12,525 infants were tested. The maternal sera were studied with the indirect Coombs' test, using a panel of selected erythrocytes containing known antigens. The erythrocytes of the infants were tested by the direct Coombs' reaction to detect the presence of adsorbed antibodies. Those infants showing positive Coombs' reactions were studied further using the heat elution\(^5\)\(^6\) technic to identify the adsorbed antibodies.

The six Combs' antiglobulin sera used in the report\(^7\) were obtained from four sources, and all met the "Minimum Requirements" of the National Institutes of Health.\(^8\) When these were tested with fresh bloods from the same patients, considerable variations were observed in their ability to detect adsorbed antibodies of the ABO system on the freshly collected erythrocytes of newborn infants; in addition, not all of the sera gave comparable reactions with the erythrocytes obtained from cases of acquired hemolytic anemia. Two of these sera failed consistently to show positive direct reactions with the red cells of infants having erythroblastosis due to the ABO groups. On the other hand, these two sera gave excellent reactions with the red cells of infants having erythroblastosis due to anti-D of the Rh system.

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Three different Coombs' sera were used in the indirect Coombs' technic to determine the titers of cryptagglutinoid (incomplete) anti-D and anti-A. When the antibody titration was performed on the anti-D serum, the same end-point was found for all three antiglobulin sera. However, when the same reagents were used to titrate an anti-A serum, a variation in end-points occurred with one serum giving a titer of 1/1,024, a second 1/512, and a third only 1/128. These titrations showed that while the antiglobulin reagents were relatively equal in sensitivity to anti-D, a significant variation in sensitivity to the immune anti-A of the cryptagglutinoid type could be detected.

The variations in specificity and sensitivity of the six antisera studied in this report indicated that the Coombs' antiglobulin sera being used by various laboratories might not give equivalent results. Although all were labeled "Coombs' Antiglobulin Serum," the failure of some of these to detect ABO iso-immunizations showed that these antisera varied in specificity. These differences could be due to factors such as the lack of desirable human globulins in the antigens used to stimulate the production of Coombs' reagents or the failure of the animals used to produce the desirable antiglobulin of the agglutinating variety. Such a production of agglutinoids (true blockers) or cryptagglutinoids (incomplete antibodies) against certain of the human globulins, as the beta or water soluble fractions, would result in reagents that varied in their ability to detect some human immune globulins in sera of patients. Thus, it would seem desirable to standardize the Coombs' antiglobulin sera against immune antibodies of the ABO and other systems as well as those of the Rh-Hr group.

When such standards were applied and the Coombs' antiglobulin serum was selected for its ability to detect ABO as well as Rh-Hr iso-immunization, an improved laboratory demonstration of the iso-immunization of pregnancy in mothers and infants of incompatible blood group pregnancies resulted. For example, in our earlier study of 33,823 mother-infant combinations in the maternity service, only 11 (0.032 per cent) cases were observed, 8 being due to anti-A in O mother-A infant pregnancies, and 3 to anti-B in O mother-B infants. However, in our later report, a total of 81 cases of ABO iso-immunization (0.653 per cent) out of 12,410 mother-infant combinations were detected when the selected Coombs' antiglobulin serum was used. This increase in the demonstration of adsorbed antibodies was further improved when the elution method was used to identify the specific antibody involved. The use of elution uncovered the fact that 22 of 81 cases were due to cross-reacting A + B (so-called "anti-C" of Wiener). This proof of the involved antibody would have been quite difficult to make without this elution method.

The data acquired with selected sera and the heat elution test seemed to indicate that there was a difference in the mother-infant blood group combinations which allowed for a greater incidence of iso-immunization to the ABO factors. For example, in the 1,372 O mother-A infant pregnancies, 53 or 3.86 per cent iso-immunizations with anti-A occurred and 22 or 1.60 per cent cases with cross-reacting anti-A + B were found. Among 337 O mother-B infants only 5 instances or 1.48 per cent were found that immunized to the B antigen. An even smaller percentage (0.72 per cent) was found among A
mothers-B infants in which only 1 case of immunization to the B antigen occurred. The 134 B mother-A infant combinations yielded no instances of iso-immunization. This decreasing incidence seemed to show that the group O mother was more likely to be immunized than group A or group B mothers. In addition, iso-immunization was more frequent when the O mother had A children rather than B children. The A mothers only rarely produced an affected B child, and the B mothers failed to do so in this study. Such results might not have been found in studies made with Coombs' testing sera of lesser specificity.

It would seem that the specificity of the antiglobulin determines whether or not the reagent can be applied to antigen-antibody systems other than Rh-Hr. It is of primary importance that antiglobulin sera should be composed of antibodies that are capable of producing the agglutination phenomenon when mixed with cells that have been adsorbed with immune globulins. The presence of "blocking" (agglutinoid) or other incomplete antibodies in the antiglobulins could render such a product of little value as a testing reagent because of the possible prozones that might result. Van Loghem et al., known for their Coombs' antiglobulin sera, found such prozones in some Coombs' antiglobulin sera produced in rabbits. These prozones, however, were attributed to an excess of antibody such as have been observed with the precipitin reaction in other antigen-antibody systems.

The fact that the antiglobulin sera might contain more than one reactive fraction was observed by Dacie who found that red cells sensitized with "incomplete cold antibodies" could remove the fraction effective for that antibody. Later, Dacie observed that when small amounts of human gamma globulins were added to antiglobulin sera, the fraction effective for the "warm antibody" was neutralized, while those effective for the "cold antibody" remained intact. These observations offer further evidence that the "incomplete cold antibody" is present in a fraction other than the gamma globulin. Such tests offer a method for differentiation between the "cold" and "warm" incomplete antibodies from cases of acquired hemolytic anemia. In addition, it was found that the "incomplete cold antibody" fixed both the heat-stable and the heat-labile portions of complement in the presence of the erythrocytes resulting in a firm and irreversible bond which resisted elution by washing with warm saline. On the other hand, the "incomplete warm antibody" observed in acquired hemolytic anemia did not need the thermolabile components (C'1 and C'2) of complement.

Other studies using selected adsorption of antiglobulins have shown that each globulin fraction removes its specific component from the antiglobulin serum. Further studies by Cutbush et al. reported that all examples (with one exception) of "warm type" antibodies of the anti-Rh were gamma globulins of the gamma-2 variety. The sole exception to this finding was attributed to a quantitative rather than a qualitative difference. They further observed that the "incomplete" anti-Le", the normal "incomplete cold antibody" and the adsorbed "cold antibody" of acquired hemolytic anemia were not gamma globulins. These were classified as alpha or beta globulins because of their reactions with their anti-alpha and anti-beta globulin prepared in rab-
bits. An immune anti-A and examples of anti-Fy* and one anti-Le' were found to be a mixture of mostly gamma with some non-gamma globulins. Such an observation had been made by Dacie17 who found an example of anti-Fy* that contained immune globulins other than the gamma type. The studies on the non-gamma globulin antibodies showed their need for factors in fresh serum to cause sensitization, thus confirming the observations of Dacie. These authors emphasized that antiglobulin sera used in blood group work should be carefully selected for their ability to react with non-gamma globulin and gamma globulin antibodies.

When such data on antibody globulins are examined in the light of peaks in the electrophoretic pattern, a narrow view with its attendant limitations are the result. This important tool of protein chemistry helps to classify serum globulins into groups that migrate in an electrical field at a similar rate of speed under given ionic strengths. The peaks thus formed represent complex groups of serum proteins with similar mobilities. Alterations of the ionic strength of the buffers or changes in the strength of the electrical field often result in differences in the visualized peaks. For example, preliminary fractionation of an anti-Rh-containing serum by ion-exchange resins18 yielded two fractions that migrated to the gamma position in the electrophoretic field, one being a salt soluble gamma, and the other a water soluble gamma globulin. These fractions possessed different antibody activity in that the salt soluble proteins (0.05-0.15 M NaCl) contained the saline agglutinin, while the water soluble fraction showed cryptagglutinoid and agglutinoid activities. The approach of Witebsky and Mohn19-21 utilizing dialysis resulted in the separation of water soluble from salt soluble proteins, each fraction of which possessed a distinct “spectrum” of antibody activity such as the separation of the “blocking” antibody from the saline agglutinins. These observations were confirmed by a third method, electrophoresis-convection.22 The combination of various methods of fractionating serum proteins, such as ion-exchange removal of salts, sodium sulfate precipitation, differential salt solubility and the location of the fractions by Tiselius electrophoresis patterns,23 has shown that the immune globulins may be separated by various methods. In addition, the actions of reagents such as tannic acid23 and 5M urea24 on sera containing mixtures of antibody reactivities gives evidence that the globulins possessing antibody activity are complex. Consequently, the use of limited criteria for the study of antibody globulins is to take a myopic view of immuno-chemistry. For example, the work of Sober et al.25 has shown that column chromatography using cellulose anion exchange resins could result in more sharp separations of serum proteins. Speer et al.26 applied this method to sera containing anti-D that had been mixed with labelled albumin-iodine-131 and transferrin-iron-59. They found that the proteins thus fractionated and compared to the electrophoresis patterns resulted in a scattering of anti-D activity over the alpha, beta and gamma fractions in a fashion similar to the findings of Cann with electrophoresis-convection.22 The position of the gamma globulin fraction of serum as an entity is summarized by the report of Tiselius et al.27 using calcium phosphate gels in column chromatography. He showed that the gamma globulin of electrophoresis may be separated into multiple com-
ponents. Thus, it seems necessary to abandon electrophoresis patterns as the sole criterion for labelling immune serum proteins.

In our experience\textsuperscript{28} it was found that an antiglobulin serum may give good reactions with red cells sensitized with one anti-D antiserum, yet give doubtful reactions with the same erythrocytes sensitized with another anti-D serum. Such results imply that some antiglobulin sera may fail to have antibodies specific for the immune globulins present in the patients.

It has long been recognized that some nonagglutinating immune anti-A can be detected by the indirect antiglobulin test\textsuperscript{29}; in general, however, the antibodies of the ABO system, both natural and immune, often do not react well with the Coombs’ reagent. Similar findings have been reported for the “Lewis” blood groups\textsuperscript{30} while other workers have found that their Coombs’ antiglobulin worked quite well with this antigen system. Such reported variations could well be attributed to differences in the Coombs’ reagents or to the need of complement in this system.

In the light of the above discussion, it would seem desirable to have reference standards for Coombs’ testing sera. Until such a time as standardization of this reagent can be accomplished, it is important for those individuals who use the Coombs’ test to understand that there are differences in sensitivity and reactivity of the various products bearing this label. This may indicate that in doing a Coombs’ test, reliance should not be placed on reactions obtained with a single antiglobulin serum. The broadened use of the antiglobulin serum from the diagnosis of hemolytic disease of the newborn to the investigations and diagnosis of auto- and iso-immune diseases, as in the acquired hemolytic anemias, seems to necessitate a re-evaluation of the criteria for an adequate Coombs’ antiglobulin serum.

REFERENCES

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