ISOHEMOLYSINS IN BLOOD DONORS

Incidence in a Closed Panel of Donors

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MODERN workers in the field of blood transfusion therapy have a tendency to consider isoantibodies only in respect to their ability to produce agglutination. Too often the hemolytic component of isoantibodies is ignored, even though some of the earliest investigators stressed its importance.

There has been renewed interest in these antibodies with the realization that the problem of the dangerous universal donor is closely related to the presence of serum isohemolysins. The widely divergent reports in the literature concerning the frequency of such isohemolysins in the general population led to the studies that form the basis of the present report.

In the early part of the twentieth century numerous papers were published discussing isohemolysins and attempting unsuccessfully to correlate their presence with specific diseases. According to Landsteiner and Leiner,[1] Moragliano in 1892 reported the first observation of hemolysins in human sera against the red blood cells of other persons. His report was made before the concept of blood groups had evolved, but in all probability he did describe isohemolysins. This report and other early work were reviewed and were extended by Hesser[2] in 1924. In general, however, interest in isohemolysins remained dormant until recently, when the role of these antibodies in the dangerous universal blood donor problem became increasingly apparent. Most of the interest in isohemolysins has been centered around this aspect of the problem, as emphasized by Ervin, Christian, and Young[3] in 1950. This phase of the subject was critically reviewed by Dausset and Vidal[4] in 1951, and by A. D. Tovey[5] in 1958.

Isohemolysins occur in a significant percentage of sera that contain isoagglutinins. The isohemolysin titer is always low as compared to the isoagglutinin titer. In several studies the frequency of these antibodies in apparently normal persons has been reported. Landsteiner and Leiner[1] in 1905, reported that the blood of 50 per cent of children tested by them possessed isohemolysins. The first American series was reported in 1910 in a classic paper by Moss[6]; 25 per cent of the sera he studied contained hemolysins. This incidence is close to the 30 per cent reported in 1911 by Grafe and Graham.[7]

Several investigators reported a considerably higher incidence of these antibodies. In 1921, Jones[8] reported that the sera of 88 per cent of 144 adults demonstrated hemolytic activity against human blood cells of groups A and B. He stated that he read his reactions closely, and that this 88 per cent included sera that showed only a trace of hemolysis . Three years later, Hesser[2] reported on

results obtained with 52 hemolytic sera of 55 random ungrouped specimens. The three sera that showed no hemolysis, also were lacking in isoagglutinins, and presumably were group AB. However, these results were obtained using "kept corpuscles," described[2] as red blood cells that had been stored in the refrigerator until partial spontaneous hemolysis had occurred. These cells were then washed to remove the free hemoglobin, and the remaining cells were made into a saline suspension to be used as the antigen for the test. When the same sera were tested against a saline suspension of fresh cells, only 24 of the 52 specimens demonstrated hemolysis. The reaction with "kept corpuscles" is apparently a true antigen-antibody reaction, as the three sera from presumed group AB patients did not hemolyze these red cells. Subsequent surveys have reported the frequencies of isohemolysins in group O sera to be between 20 and 40 per cent. *Table 1* summarizes the data obtained from 14 surveys.[1,2,5-16] Not including Hesser's[2] data, only one report has placed the incidence of hemolysins as more than 50 per cent, and 9 of the 14 surveys reported incidences of less than 30 per cent.

Table 1.—*Reported incidence of hemolytic antibodies in normal human sera*

Investigators	Year of report	Incidence of hemolysins, per cent
Landsteiner and Leiner[1]	1905	50
Dienst[9]	1905	20
Moss[6]	1910	25
Grafe and Graham[7]	1911	30
Jones[8]	1921	88
Copher[10]	1923	25
Hesser[2] (kept corpuscles)	1924	100
Hesser[2] (fresh corpuscles)	1924	46
Jonsson[11]	1936	14.5
Wiener[12]	1943	30
Crawford, Falconer, Cutbush, and Mollison[13]	1952	20
Gardner and G. H. Tovey[14]	1954	20
Van Loghem and Reepmaker[15]	1955	28
A. D. Tovey[5]	1958	20
Lockyer and G. H. Tovey[16]	1960	18

By far the largest study was that reported by A. D. Tovey[5] in 1958, which was based on 90,000 blood samples; the incidence of alpha-isohemolysins in group O sera was 20 per cent, and of beta-isohemolysins, 2.4 per cent. The most recent

series, published in 1960, is that of Lockyer and G. H. Tovey;[16] they describe a screening test for hemolysins using papainized group A^P pig erythrocytes. This test is described as less time consuming than conventional screening against human group A cells, and is certainly less sensitive. Of 2,500 specimens of blood studied by these authors, 18 per cent showed some hemolysis, and 3.6 per cent were positive in dilutions of ⅛ or greater when tested against human red blood cells; only 4.2 per cent, of the total number tested, hemolyzed the papainized group A^P pig cell suspension. Because experience at the Cleveland Clinic seemed to indicate that the incidence of hemolytic sera was considerably higher than this and most other reported figures, the limited study reported here was undertaken.

Materials and Methods

More than 400 random sera, primarily obtained from our professional blood donor population, but also from outpatients of the Clinic, and from medical and nursing staff personnel, were tested for hemolytic activity by the following procedure: 0.1 ml. of human sera was pipetted into a 65 by 100 mm. serologic tube containing a like volume of diluted guinea pig complement. This tube was used as the first tube in a series of doubling dilutions that were usually carried out to the seventh tube (dilution of 1 to 128), using the diluted guinea pig complement as the diluent throughout. The complement dilution was prepared daily from freshly reconstituted, titered, commercial guinea pig serum. The complement titration was made in the serologic laboratory against sheep red blood cells sensitized with amboceptor in the same manner as for the Kolmer complement-fixation test for syphilis.[17] The dilution of complement used in the tests for hemolysins was that which supplied two full units of complement in 0.1-ml. volume, and in most instances this dilution was 1 to 30. To the mixtures of diluted test serum and complement, a volume of 2 per cent suspension of freshly prepared washed human group A_1 or group B red blood cells was added. After being thoroughly mixed by shaking, the mixtures were incubated in the water bath at 37 C. for one hour. At the end of that time, each tube was observed and the absence or degree of hemolysis was recorded (negative, one, two, three, or four plus). In several instances, the titrations were allowed to stand overnight, either at room temperature or in the refrigerator. No significant change in the hemolytic titers was evident after these procedures. Centrifugation of the tubes also did not alter the hemolytic titer. However, any of these modifications in technic resulted in a great increase in the agglutinating titer of all sera tested; but because our primary interest lay in the isohemolysin titer, secondary incubation or centrifugation was not performed routinely.

Results

The data collected from these studies are presented in *Table 2*. Of 289 speci-

Table 2.—*Incidence of isohemolysins in sera of group O blood*

Isohemolysin	Number of sera			
	Total tested	Hemolysins present	Hemolysins absent	Percentage positive
Anti-A	289	240	49	83.1
Anti-B	239	144	95	60.3
Anti-A or anti-B or both	239	211	28	88.3
Anti-A*	289	201	88	69.5

*Hemolytic activity present beyond the ½ dilution.

mens of group O sera which were tested for anti-A hemolytic activity, 83.1 per cent contained such activity. Of these specimens, 239 were tested for anti-B hemolytic activity, and 60.3 per cent evidenced such activity. Of all specimens of group O sera tested, 88.3 per cent had one or both hemolysins. Approximately 12 per cent of our tested samples of group O sera was totally lacking in hemolytic activity. When the sera that contained hemolysin in low dilution were eliminated from the series, the incidence of anti-A hemolytic activity was 69.5 per cent, or 201 of 289 specimens of group O sera tested. These frequencies are considerably higher than those that have generally been reported by others, with the exception of Jones's,[8] and of Hesser's.[2]

A small series of blood sera of groups A and B specimens was studied *(Table 3)*. Seventy-six specimens of sera from group A were included. They were not

Table 3.—*Incidence of isohemolysins in sera of group A and group B blood*

Blood group	Number of samples			
	Total tested	Hemolysins present, positive	Hemolysins absent, negative	Percentage positive
Group A				
Anti-B	76	47	29	61.9
Anti-B*	76	30	46	39.5
Group B				
Anti-A	56	40	16	71.5
Anti-A*	56	30	26	53.6

*Hemolytic activity present beyond the ½ dilution.

subgrouped because previous studies in our laboratory had shown that there is no correlation between titers of the anti-B agglutinin and the subgroup of the A cells. Of these 76 specimens, 61.9 per cent or 47 specimens hemolyzed group B

cells to some degree. When those specimens of sera containing only traces of hemolytic activity are eliminated from the series, then 39.5 per cent or 30 specimens had a significant hemolytic titer. Of 56 samples of group B sera, approximately 70 per cent or 40 specimens had anti-A hemolysin, or, again eliminating those with "trace" reactions, 54 per cent or 30 of 56 of these sera were positive for hemolytic activity.

Discussion

The explanation of these results that show a high incidence of hemolytic activity in our series, falls into two categories: selection, and experimental technic. The factors by which selection may have influenced the present results are somewhat conjectural. A large number of the specimens tested were from young adults, primarily college students, as well as from the resident and nursing staff of the hospital. A majority of these young people have had recent immunizations. It has been shown by Crawford, Falconer, Cutbush, and Mollison,[13] by Dausset and Vidal,[4] and by Powell[18] that many such immunizations substantially increase the incidence, as well as the titer, of hemolytic antibody in the serum. Therefore, the nature of the present test population would favor an increased incidence of serum showing hemolytic activity due to such heterospecific stimulation. This is especially true when statistics from such a group are compared with statistics for the general population, especially a population group in rural England, where the total immunologic experience is probably less than in the United States.

Secondly, there is the factor of age. As stated, sampling was primarily from young healthy adults. Mollison[19] showed that in the third decade of life the isoagglutinin titer rises to a maximum, and decreases slowly as a function of age. From this observation it can be inferred that isohemolysin titers have a similar relationship.

Finally, A. D. Tovey[5] observed increased hemolytic titers in the sera of persons who are regular donors. This is an empiric observation; the cause is not known. Since a majority of donors who are included in the present survey are drawn every two or three months, this factor may also play some role in our results.

The technical factors that may have influenced our results are as follows: the use of a 2 per cent red blood cell suspension throughout the study, the arbitrary definition of significant hemolysis, and the use of dilute guinea pig serum as the test serum diluent. In contrast to most investigators, who use a 5 per cent red blood cell suspension, we used a 2 per cent suspension throughout this study. Jones[8] noted that hemolysis in weak-reacting sera could be inhibited by raising the red blood cell concentration. This observation has been amply confirmed by others. Chaplin, Wallace, and Chang[20] showed that some hemolysin will lyse 2 per cent but not 5 per cent cell suspensions. The exact mechanism of this phenomenon is not known. However, by utilizing a 2 per cent cell suspension, we

believe that hemolytic activity is demonstrable with weakly reacting sera, which might otherwise have been missed.

There are various arbitrary definitions of significant hemolysis. Some investigators regard any degree of hemolysis less than 50 per cent as a negative result, others less than 75 per cent, and so on. Jones,[8] whose incidence of 88 per cent most closely agrees with that of our report, suggests that any trace of hemolysis is a positive result. However, even when those sera with traces of hemolysis are eliminated from our study, the percentages are higher than those usually reported.

The use of dilute guinea pig serum as the test serum diluent, may have influenced our results. It was not always possible to test the specimens immediately after collection. Titered, dilute, guinea pig serum was used as a test serum diluent to obviate the loss of natural complement activity in the test serum because of storage. This method gave more consistent results than the results from titrations with saline diluent, because there was absolute control of complement activity in each test.

Mollison and Thomas[21] showed that the hemolytic effect of the ABO hemolysin system is potentiated by the use of animal serum as the complement source. It seems possible that in our series the number of sera showing hemolytic activity was increased in this manner. In some sera, guinea pig complement consistently potentiated the reaction *(Table 4).*

Table 4.—*Comparison of complement activity of guinea pig and of human group AB sera in the anti-A hemolytic system*

Test sera group O, sample no.	Complement activity			
	Fresh sera, no added complement	Sera aged 7 days at room temperature	Aged sera plus ½ guinea pig sera	Aged sera plus ½ AB sera
1	1:8	0	1:8	1:8
2	1:8	0	1:8	1:8
3	1:8	Tr.*	1:16	1:16
4	1:4	0	1:8	1:4
5	1:2	0	1:2	1:2

*Signifies trace reaction.

The literature contains many contradictory statements about the relative efficacy of guinea pig complement in the study of isohemolysins. In addition to Mollison and Thomas,[21] Lockyer and G. H. Tovey[16] showed that guinea pig serum is a satisfactory source of complement for isohemolytic reactions. Both of these reports are contrary to the opinion of Boorman and Dodd[22] who believe that animal complement will not function in isohemolytic reactions. Because of these contradictory findings, it seemed necessary to determine the merits of var-

ious complement sources in our hemolytic system. *Table 4* shows the outline and results of a typical experiment. The test sera were first titered using saline solution as a diluent, establishing the maximum hemolysin titer in a system that utilized "natural" complement. The complement was then destroyed by storing the serum at room temperature for seven days. After this time, hemolytic activity was almost completely absent. The sera were then retitered, utilizing in one case dilute guinea pig complement, and in the other, fresh human AB serum as a complement source. As can be seen, hemolytic activity was restored in almost all instances.

Conclusions

As a result of this and similar experiments, we believe that the following statements may be made: (1) Properly titered lyophilized guinea pig serum is a satisfactory source of complement activity, utilized in the ABO isohemolytic system. Moreover, it is readily obtainable, is easily stored, and is easy to prepare. It can be standardized by well-accepted technics, and therefore standardized amounts of complement are present in each tube of the system; thus a further constant factor is supplied in the hemolytic system under investigation. (2) It has been stated that human AB serum is an unsatisfactory source of complement because of the presence of traces of AB substance. From our observations it would seem that AB serum is a good source of complement when used at optimum dilution as determined by a standard complement titration. In these dilutions, the traces of AB substance that may neutralize the hemolysin are probably diluted to an insignificant amount. An additional advantage of AB serum is the absence of isoagglutinin activity against group A and group B cells.

A. D. Tovey[23] reported the restoration of hemolytic activity to heat-inactivated group O serum by the addition of neat human or guinea pig serum, but not by the addition of diluted human or guinea pig serum. In the present study, it was impossible to restore hemolytic activity by using either fresh or lyophilized guinea pig serum, or with human complement in dilutions known to contain adequate complement activity as tested against the serum before heating. Nor could the hemolysin be reactivated by adding fresh, undiluted human serum of the same blood group as the heat-inactivated test serum. We believe, therefore, that the hemolysin is heat labile, and once inactivated cannot be reactivated. This is contradictory to the findings of Moss,[6] who studied this problem in 1910, and to those of Grafe and Graham,[7] who reported similar work in 1911.

In this same report, A. D. Tovey[23] mentions the presence of a hemolysin in the serum of an occasional guinea pig which lysed human A cells. This observation was investigated further, since we were using guinea pig serum so extensively. *Table 5* summarizes a typical experiment. Fresh neat guinea pig serum and neat lyophilized guinea pig serum were allowed to react for one hour at 37 C. with a panel of cells belonging to various blood groups. Samples from a total of six

guinea pigs from three different litters, as well as several lots of commercial serum, were tested in such fashion. The guinea pig serum contained an antibody that acts both as a hemolysin and as an agglutinin and that seems almost totally specific for human A cells. Lyophilization apparently inactivates the hemolytic component. When any of these sera were used in the usual dilutions to provide adequate complement activity, neither hemolysis nor agglutination of the test cells was noted.

Table 5.—*Hemolytic activity of undiluted guinea pig serum*

	Fresh guinea pig serum, undiluted		Undiluted, reconstituted, lyophilized guinea pig serum	
	Titers		Titers	
Antigen	Hemolytic	Agglutinating	Hemolytic	Agglutinating
Cells				
O	—	—	Tr.*	—
A_1	++++		— to tr.*	++++
A_2	++	++++	—	++++
B	Tr.*	—	—	—

*Signifies trace reaction.

A word about agglutinins in the human sera we studied seems in order. Our experiences are much the same as those reported by Witebsky[24] and other investigators who have studied extensively the relationships of isoagglutinins and isohemolysins. In general, the presence of a strong hemolysin usually is accompanied by a high agglutinin titer, but high agglutinin titers do not always imply a strong hemolysin; and secondly, immune-reacting agglutinins are found with relative frequency in low-titer hemolytic sera.

Summary

The problem of defining the dangerous universal donor is closely related to the isohemolysins in the donor serum.

There may be high incidence of isohemolysins in bloods from a certain selected donor population; 88.3 per cent of the sera from 289 group O donors in this study showed isohemolysins against either group A or group B erythrocytes, or against both antigens. Similar results were obtained with smaller numbers of sera from group A and group B persons; 61.9 per cent of group A donors had isohemolysins against group B cells, and 71.5 per cent of group B donors were able to hemolyze group A cells. These percentages are higher than most published

figures, and are explained on the basis of a closed panel donor population.

Thus, age distribution, recent immunologic experience, and frequency of donations can influence the incidence of hemolysins in a select donor population. These factors could be of some importance in defining the incidence of the dangerous universal donor in the blood bank that depends on its supply of blood from a local restricted population.

The technical factors that influence the incidence of demonstrable isohemolysis are: (1) concentration of red cell suspension, (2) arbitrary definition of significant hemolysis, (3) the use of dilute guinea pig serum as the test diluent.

Lyophilized, guinea pig serum is an accurate, reliable, and readily available source of the necessary complement in the demonstration of this hemolytic system, and its use is recommended for general work in the routine blood bank laboratory.

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