The Effects of Concentrated Eluted Anti-Red Blood Cell Antibodies on the in Vivo Survival of Normal Red Blood Cells

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Previous studies of in vivo survival of sensitized red blood cells have utilized either cells from patients with immune hemolytic anemia or cells coated in vitro by antibody present in whole serum. The present study reports in vivo survival studies using normal red cells sensitized by relatively “pure” antibodies, obtained by elution from well washed, sensitized red cells.

Materials and Methods

Patients.—Eluates were prepared from the red cells of two patients hospitalized with acquired hemolytic anemia; in one the Coombs test was consistently negative, in the other it was consistently strongly positive.

Sensitization of red cells by (a) normal cold incomplete antibody and (b) incomplete anti-D. (a) Four hundred and fifty ml. of defibrinated blood were obtained from a group A healthy normal laboratory technician whose serum was known to contain potent cold incomplete antibody. The fresh serum was incubated with 100 ml. of fresh normal group O red cells for 1 hour at 0 C. in a crushed ice bath. (b) One hundred ml. of serum were obtained from a group A Rh-negative mother whose serum was known to contain no complete anti-D antibody but a high titer (1/1024) of incomplete anti-D demonstrated by the indirect antiglobulin technic. The serum was incubated for 1 hour at 37 C. with 100 ml. of fresh normal group O Rh-positive cells.

Preparation of eluates.—Stroma was prepared approximately as described by Ponder. One hundred ml. of sensitized red cells were washed 5 times with 7 volumes of chilled normal saline, with the use of a refrigerated centrifuge for sedimentation of the red cells. The washed cells were then delivered into 6000 ml. of distilled water which had been acidified to pH 5.0 by bubbling through CO₂ gas. The stroma was allowed to settle for 1 hour in a cold room at 5 C. After aspiration of the clear supernatant, the residual stroma was washed in the cold 10 times, employing CO₂-acidified 0.1 per cent sodium chloride solution. Approximately 25 to 35 ml. of greyish-white stroma remained after the washing and was almost entirely free from hemoglobin as judged by the pale pink color of the final supernatant wash solution. The washed stroma was resuspended in 2 volumes of 0.85 per cent saline and heated at 56 C. with frequent mixing for 10 minutes, followed by rapid centrifugation for 20 minutes at 2500 rpm in heated centrifuge cups in a centrifuge of radius 22 cm. If necessary, the supernatant eluate was cleared by recentrifugation at 2,500 rpm for 30 minutes. A 2 ml. aliquot of the final eluate was set aside for in vitro testing for antibody activity.

Concentration of eluates.—Approximately 80 ml. of the clear eluate was dialyzed overnight against cold running tap water, whereupon it was lyophilized to dryness. The residue was resuspended in 4 ml. of sterile, pyrogen-free 0.85 per cent saline and the undissolved sediment separated by centrifugation. The concentrated eluate had a more or less brown color, depending upon how much hemoglobin was present in the original eluate.

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The eluate was either used immediately to sensitize normal cells for in vivo studies or was stored frozen at -22 C. until used. Storage never exceeded 1 week.

**Sensitization of normal red cells for in vivo survival studies.**—Fifteen ml. of blood from a normal subject of appropriate blood group were defibrinated in a sterile pyrogen-free flask. The defibrinated blood was transferred to a sterile centrifuge tube and centrifuged for 30 minutes at 2,500 rpm. Sufficient plasma was removed to raise the hematocrit of the residual contents to 67 per cent. One and one-half ml. of the 67 per cent red cell suspension (i.e., 1.0 ml of packed cells, 0.5 ml. fresh normal serum) was transferred to a sterile centrifuge tube. The concentrated eluate was sterilized by Seitz filtration employing a Swinny filter adaptor* on a 5 ml. Luer-Lok syringe. The filter pad was first saturated by passing through 1 to 2 ml. of the sterile serum previously removed from the defibrinated blood. The concentrated eluate was then filtered and 1.2 ml. added directly to the 1.5 ml. of 67 per cent cell suspension. The mixture was then incubated for 2 hours in a water bath at 37 C., with occasional gentle agitation. The remainder of the filtered eluate was retained for in vitro testing.

**Chromium-51 tagging.**—Two-tenths ml. sterile ACD (U. S. P. Sol. A) was added to the sensitization mixture on completion of the 2 hour incubation period. Fifty μc. Cr51 was added and the mixture allowed to stand for 30 minutes at room temperature. The sensitized tagged cells were then rapidly washed 3 times with 9 volumes of chilled saline and made up to a final volume of approximately 7.5 ml. Six ml. of the cell suspension were immediately injected into the recipient, with the use of a calibrated syringe. The time elapsed from drawing the cells from the normal donor to injection into the recipient was 3 and one-half hours. Chromium-51 activity of the injected red cell suspension and of all subsequent blood samples was measured in a well-type scintillation counter.

**Blood volume measurement.**—Blood volume was calculated from measurement of the plasma volume by the conventional Evans Blue dye method, employing suitable corrections of the observed hematocrit for plasma trapping² and for the body-venous hematocrit ratio.²

**Red cell survival.**—Estimation of expected 100 per cent red cell survival was based on knowledge of the exact number of counts injected and of the recipient’s blood volume. For reasons elaborated in the Discussion, it was decided that destruction of >25 per cent of the red cells within 24 hours of injection would be required to indicate a significant effect of the eluate-sensitization procedure. Radioactivity over the hepatic and splenic areas was measured with a collimated directional scintillation counter positioned vertically at skin level over carefully predetermined sites in the right and left upper quadrants.

**Ashby technique.**—A single red cell survival was performed by Dacie’s⁵ modification of the Ashby method, employing a potent powdered anti-M globulin to agglutinate the patient’s own type MN cells, leaving the transfused NN cells free to be counted.

**Direct Coombs tests.**—All direct Coombs tests were performed with the use of 10 serial saline dilutions of a single potent broad spectrum antiglobulin serum prepared in the authors’ laboratory.¹ The red cells to be tested were washed 5 times in 9 volumes of saline and made up to a 10 per cent suspension in saline. Equal volumes of each serum dilution and the 10 per cent cell suspensions were well mixed on a glass plate and gently rocked for exactly 3 minutes. The reactions were graded from 1 to 4 plus on gross visual inspection.

**Indirect Coombs test.**—Serial dilutions of the original eluates and the concentrated eluates were mixed with an equal volume of 2 per cent suspension of fresh normal red cells and incubated for 1 hour in a water bath at 37 C. The red cells were washed 3 times and incubated for 5 minutes at room temperature with one volume of a 1/10 dilution of the broad spectrum antiglobulin serum, followed by centrifugation for 2 minutes at 1000 rpm. Readings were microscopic and graded from 1 to 4 plus.

**Gamma globulin neutralization test.**—To determine whether the sensitization of red cells appeared to be gamma globulin or nongamma globulin in type, the undiluted broad

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*Becton, Dickinson and Company, Rutherford, N. J.*
spectrum antiglobulin serum was diluted with an equal volume of 0.1 per cent gamma globulin solution, thoroughly mixed, and after 5 minutes' incubation at room temperature was serially diluted in saline and compared in the direct antiglobulin test with the unneutralized antiglobulin serum. When the reaction with the unneutralized antiglobulin serum was completely suppressed by the addition of gamma globulin, the red cell sensitization was characterized as "gamma globulin" in type. "Mixed" and "nongamma globulin" sensitizations were characterized by partial and complete lack, respectively, of suppression by the gamma globulin neutralization procedure.

**Results**

**Patients with Acquired Hemolytic Anemia**

M. F. was a 36 year old white female with an established diagnosis of Hodgkin's disease, who entered the hospital with chronic anemia requiring supportive transfusion therapy; the hematocrit was 30 per cent, and there were 14.5 reticulocytes per 100 red cells. The direct Coombs test was repeatedly negative. A. O. was a 55 year old white male alcoholic with one year's history of intermittent idiopathic acquired hemolytic anemia ("warm" antibody type) who entered the hospital this time with hematocrit 15 per cent, reticulocytes 50 per 100 red cells. The direct Coombs test was repeatedly strongly positive.

**In Vivo Red Cell Survival Studies**

*Normal control.*—When 1 ml. of normal red cells was sensitized with concentrated eluate from 100 ml. of normal red cells, the results shown in figure 1 on the left were obtained. The original cells were direct Coombs-negative, as were the indirect Coombs reactions employing the eluates and the final direct Coombs on the injected cells. One hundred per cent immediate survival of the injected cells was obtained, and a normal red cell survival was observed (Cr T½, 28 days) without any evidence of preferential sequestration of the destroyed cells in either liver or spleen.

*Normal cells sensitized with concentrated eluate from cells coated by normal cold incomplete antibody.*—Although it is evident from figure 1 on right that the original cells were strongly sensitized by normal cold incomplete antibody, no antibody activity was demonstrable in the eluate even after concentration, nor was the direct Coombs reaction positive on the cells injected for the survival study. Normal initial and subsequent survival of the injected cells was observed (Cr T½, 26 days), without preferential hepatic or splenic sequestration of the destroyed cells.

*Normal cells sensitized with concentrated eluate from cells of a patient with Coombs-negative acquired hemolytic anemia.*—It is evident from the antibody studies in figure 2 (top on left) that autoantibody was not demonstrable on the patient's cells, in the eluate nor on the normal cells sensitized with the concentrated eluate. The "sensitized" normal cells were injected into the original patient, rather than into a normal recipient, because of fear of transmission of serum hepatitis (see Discussion). One hundred per cent immediate survival of the injected cells was observed, with a more rapid than
normal linear disappearance thereafter \( \text{Cr}^5 \overline{T}, 14 \text{ days} \). There was no preferential hepatic or splenic sequestration of the destroyed cells. The shortened red cell survival was attributed to the patient's underlying extracorporeal hemolytic process and not to any damaging effects of sensitization by the concentrated eluate, since a simultaneous Ashby survival study of normal donor cells revealed an entirely comparable shortening of red cell life span.

Normal cells sensitized with concentrated eluate from cells of a patient with Coombs-positive acquired hemolytic anemia.—Strong sensitization of the patient's cells is evident from the antibody studies in figure 2 (top on right), with striking antibody activity in the eluate and strong sensitization of the normal cells by the concentrated eluate. Once again the sensitized normal cells were injected into the original patient, rather than into a normal recipient, because of fear of transmission of serum hepatitis. One hundred percent immediate survival of the injected cells was observed, with a rapid linear disappearance yielding a \( \text{Cr}^{51} \overline{T}, 2 \) of 7 days. Surface scintillation counting did not reveal splenic sequestration of the destroyed cells. Although it was not possible to conduct simultaneous Ashby survival studies on normal cells in this patient, it was known that a \( \text{Cr}^{51} \) red cell survival conducted several months previously, when the patient was in a comparable clinical hemolytic state, had shown a \( \text{Cr}^{51} \overline{T}, 2 \) of 7 days.

Normal cells sensitized with the concentrated eluate from anti-D sensitized
cells.—The antibody studies recorded in figure 3 (top on left) indicate strong sensitization of the original cells, strong antibody activity in the eluate and strong sensitization of the normal group O Rh-positive cells by the concentrated eluate. When the sensitized normal cells were injected into an Rh-positive patient without evidence of hemolytic disease, only 88 per cent of the expected survival was observed 15 minutes after injection. The survival had declined to 78 per cent by 27 minutes after injection, yielding a Cr51 T½ of 1.3 hours. During this period of observation there was progressive accumulation of radioactivity of the recipient’s spleen. Twenty-four hours after injection none of the sensitized cells remained in the recipient’s circulation, and the ratio of splenic to hepatic radioactivity was 9 to 1. The in vivo survival of normal group O Rh-positive cells sensitized by the whole serum containing incomplete anti-D is shown in figure 3 on the right. Less than 50 per cent of the injected cells remained in the recipient’s circulation after 1 hour, and comparable splenic sequestration of the destroyed cells was observed.

Discussion

The present study was undertaken to explore the effects of eluted antibody on the in vivo survival of red cells. It was hoped that the lyophilization procedure employed for concentration of the eluted antibody would not alter...
Fig. 3.—See legend for figure 1. The graph on the left concerns studies with the eluate, the graph on the right describes results with the native serum. Liver to spleen ratios were obtained 24 hours after injection of the tagged cells. Note marked increase in in vitro activity of eluate following concentration (on left). The same anti-D serum was employed in both studies; the recipients were two patients not suffering from hemolytic disease. Red cell destruction was rapid, with predominant splenic accumulation of radioactivity in both instances.

the antibody’s physiologic properties and that by concentration it might be possible to elicit in vitro and/or in vivo evidence of antibody elutable from the red cells of patients with Coombs-negative acquired hemolytic anemia. Fresh normal serum was included in the final “sensitization” mixture in case complement should be required for the attachment of the antibody to the red cell.

It has been the authors’ experience that when D-positive red cells are relatively weakly sensitized in vitro by anti-D and then transfused, a proportion of the red cells is rapidly removed from the circulation (within a few hours), predominantly by splenic sequestration. However, the injected red cells which are not removed promptly will survive normally in the recipient, suggesting that in vivo elution of antibody has occurred and that the cells have suffered no intrinsic damage from their original sensitization. Mollison has encountered similar findings. With this concept in mind, it was decided that a positive in vivo result in the present studies would require significant destruction of the eluate-sensitized cells (i.e. > 25 per cent) within 24 hours of injection and that no attempt would be made to attribute shortened life span of red cells surviving after 24 hours to the effects of eluate-sensitization.
ANTIBODY EFFECTS ON NORMAL RED BLOOD CELL SURVIVAL

The entirely negative results of the control study (fig. 1, on left) were gratifying. There were no symptoms of any kind in the recipient, and the immediate and subsequent survival of the eluate-sensitized red cells were normal. By confirming that there was nothing intrinsically noxious in the concentrated eluate nor in the sensitization and chromium-tagging procedures, the control study cleared the way for studies of eluates prepared from red cells with known or suspected sensitization.

Sensitization by cold incomplete antibody (shown by Crawford et al. to have anti-H specificity) is known to be of the "nongamma globulin" type. Efforts by previous workers to demonstrate that "nongamma globulin" antibodies are elutable have been uniformly unsuccessful, a finding confirmed and strengthened by the negative in vitro results (fig. 1, on right) with both the unconcentrated and concentrated eluates from the cells strongly sensitized by cold incomplete antibody. Too little is known about the in vivo survival of cells sensitized with normal cold incomplete antibody to draw any firm conclusions from the survival studies illustrated in figure 1; however, the normal in vivo results tend to support the interpretation that physiologically active antibody was not present in the eluate.

Whenever a patient with unequivocal acquired hemolytic anemia is encountered who has a negative Coombs test, the question arises: is there an autoantibody present which is not demonstrable by the antiglobulin reaction or is the hemolytic process not mediated by an autoantibody mechanism? The study on the patient with Coombs-negative acquired hemolytic anemia (fig. 2, on left) supports the concept that the hemolysis in this patient was not on a conventional autoimmune basis. The patient herself was selected as the recipient of the normal cells sensitized by her concentrated eluate, since she had received numerous blood transfusions and had been jaundiced previously, and since there was no reason to believe that the preparation of the concentrated eluate would inactivate hepatitis virus if it were present in the blood from which the eluate was prepared. Ninety-five per cent of the injected cells were present in the patient's circulation 24 hours after injection. Although the Cr T/2 was shortened to 14 days, this was confirmed to be unrelated to the sensitization by concentrated eluate, since a simultaneous Ashby survival employing fresh normal donor cells revealed a comparable shortening of life span.

It is particularly interesting that there was no immediate loss of red cells sensitized by the eluate from the patient with strongly Coombs-positive hemolytic anemia (91 per cent survival at 24 hours; fig. 2, on right). The autoantibody responsible for sensitization of this patient's cells was of the gamma globulin variety and was easily eluted. It is evident from the in vitro studies that considerable concentration of antibody activity was achieved by the concentration of the eluate. The normal cells injected for the in vivo study were very strongly sensitized. The patient was again used as the recipient, for the same reasons enumerated above. While parallel Ashby studies were not carried out, it was known that the patient was hemolyzing actively during the course of the observations, and the Cr T/2 of 7 days was attributed to the patient's underlying extracorporeal hemolytic disease and not to the effects of sensitization by the eluate. The in vitro similarities between cells
Table 1.—In Vitro and In Vivo Comparison of Normal Red Cells Sensitized by Concentrated Eluates from Two Different Sources

<table>
<thead>
<tr>
<th>Source of cells for preparation of eluate</th>
<th>Direct Coombs on eluate-sensitized cells</th>
<th>Characterization of sensitization</th>
<th>Cr51 half-life</th>
<th>Splenic sequestration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient with acq. hemolytic anemia</td>
<td>1:1 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256 1:512</td>
<td>Gamma globulin 7 days 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitized in vitro by Anti-D</td>
<td>4 4 4 4 4 4 3 3 2</td>
<td>Gamma globulin 1.3 hours 4+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

sensitized by the Coombs-positive acquired hemolytic anemia eluate and by the anti-D eluate are summarized in table 1. Both sensitizations were of the gamma globulin type, and the apparent "strength" of sensitization of the normal cells used for both in vivo survival studies was entirely comparable. However, the contrast in the in vivo behavior is striking: the acquired hemolytic anemia eluate-sensitized cells had a Cr51 T½ of 7 days and were not preferentially sequestered by the spleen, whereas the anti-D eluate-sensitized red cells had a Cr51 T½ of 80 minutes and were almost exclusively sequestered in the spleen. The results emphasize that in vitro behavior in the antiglobulin test gives limited insight into the physiologic alterations brought about on the red cell surface by different antibodies.

The studies with eluted anti-D (fig. 3) are of value in establishing that the elution procedure and the subsequent concentration of the eluate did not alter either the in vitro or in vivo effects of the antibody. Rapid red cell destruction associated with splenic sequestration were observed both with sensitization by the original serum (graph on right) and with sensitization by the same antibody separated from its original serum by the elution procedure (graph on left). These findings make it less likely that the negative results obtained with the previously described concentrated eluates resulted from damage to the antibodies by the elution and concentration procedures.

Additional studies with eluates from a number of patients with acquired hemolytic anemia will be required before it can be assessed whether the in vivo method described in the present report will be a useful tool in investigation of acquired hemolytic anemia. Certainly the technical procedures have proved simple and safe. There is no reason why the same general protocol could not be applied to an expanded program of basic investigation of hemolytic syndromes, substituting in place of eluates extracts from known antibody-producing tissues and from pathologic tissues known to be associated with acquired hemolytic anemia.

**SUMMARY**

1. A method has been described for the preparation and sterilization of a concentrated eluate from human red cell stroma.
2. Red cells sensitized by such an eluate prepared from normal control red cells showed entirely normal in vivo survival, as did cells sensitized by eluate from anti-H coated cells.
3. Sensitization of red cells by concentrated eluates from a patient with Coombs-negative acquired hemolytic anemia and from a patient with Coombs-positive acquired hemolytic anemia did not cause significant alteration in the in vivo survival of the red cells.

4. Red cells sensitized by the concentrated eluate from anti-D sensitized cells disappeared from the recipient’s circulation very rapidly and were sequestered in the spleen, indicating preservation of the physiologic properties of the antibody throughout the elution, concentration and sterilization procedures.

**SUMMARY IN INTERLINGUA**

1. Es describite un methodo pro le preparation e sterilisation de un concentrato eluito a stroma eritrocitico human.

2. Erythrocytos sensibilisate per un tal eluito que habeva essite preparate ab normal erythrocytos de controlo exhibiva in vivo un superviventia completely normal. Les mesmo valeva pro cellulas sensibilisate per eluito ab cellulas revestite de anticorpore anti-H.

3. Sensibilisation de erythrocytos per concentrato eluitos ab un patiente con acquiritae anemia hemolytic Coombs-negative e ab un patiente con acquiritae anemia hemolytic Coombs-positive non causava un alteration significative del supervivientia in vivo del erythrocytos.

4. Erythrocytos sensibilisate per un concentrato eluito ab cellulas sensibilisate per anti-D dispareva rapidissimemente ab le circulation del recipient e esseva sequestrate n le splen. Isto indicava que le proprietates physiologic del anticorpore esseba preservate durante le processos de elution, concentration, e sterilisation.

**REFERENCES**


The Effects of Concentrated Eluted Anti-Red Blood Cell Antibodies on the in Vivo Survival of Normal Red Blood Cells

NEIL W. CULP and HUGH CHAPLIN, JR.