Review: measuring red cell survival and determining the clinical significance of red cell antibodies

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Transfused red blood cells (RBCs) generally survive for long periods of time in the recipient's circulation. There are times, however, when transfused RBCs do not survive as well as expected. Compatibility problems involving RBC alloantibodies or autoantibodies may be the reason for shortened RBC survival. These cases may be studied by a variety of methods. Some methods focus on predicting RBC destruction or survival prior to transfusion, and some measure the percentage of RBCs surviving posttransfusion.

Survival studies may be performed when there are clinical difficulties, such as an unexplained hemolytic transfusion reaction or a serologic problem such as an antibody to a high-incidence antigen. Less frequently, studies are performed when a strongly reactive autoantibody may be masking the presence of an alloantibody, or when the presence of multiple antibodies do not allow clear identification of all specificities. In these situations, it is helpful to determine whether the blood that is available would be safe to transfuse, i.e., the RBCs would not be subject to immediate destruction.

Fortunately, over the years techniques have been developed to achieve this goal. Although the Ashby differential agglutination technique was the first method used to measure RBC survival,1 the method is tedious and can only be used with very powerful antisera (e.g., anti-A and anti-B). However, modifications that can be readily performed in any laboratory have been described.2 The "gold standard" measure of cell survival is radioisotope labeling (e.g., 51Cr) of RBCs. A newer method to measure RBC survival is flow cytometry.2,3 The latter two methodologies require reagents and instrumentation not always available to the routine transfusion service. These three ways to measure RBC survival require transfusion in the form of a small aliquot (radioisotope labeling),4 a larger aliquot (flow cytometry),5 or a full unit of RBCs (differential agglutination).1,2

Cellular assays, like the monocyte monolayer assay,6 mononuclear phagocyte assay,7,8 and antibody-dependent cellular cytotoxicity,9,10 are methods that can be used to prospectively predict RBC survival based on reactivity of antibody in an in vitro assay.

Ashby's Differential Agglutination Method

The first method described to determine the survival of transfused RBCs was Ashby's differential agglutination method.1 This method relies on differences in antigens present on the patient's RBCs and antigens present on transfused RBCs. The most useful antibodies for the test have optimal room temperature reactivity. As an example, when a recipient (usually group A) is transfused with group O donor RBCs, an aliquot of peripheral blood is studied by the differential agglutination technique. In this case, the recipient's group A cells are agglutinated with reagent anti-A, and the remaining number of unagglutinated group O cells are counted. Although this technique is valuable, it is quite tedious and somewhat limited, because potent direct agglutination reagents are needed, and a large volume of RBCs (usually one unit) has to be transfused in order to get a reliable count.

A modification of the Ashby technique by Molthan et al.11 was further modified by Postoway et al.,2 and it proved to be comparable to a flow cytometry procedure (to be described later). Although nearly all major blood group system antibodies could be used in this method,2 only full-unit transfusions were studied. With the use of a standard curve prepared with each test to mimic the transfusion circumstances, a high correlation between flow cytometry and the modified Ashby results was attained.

Red Cell Radiolabeling

Radioisotope methodologies have been used in transfusion science to study RBC life span, RBC survival during storage, and measurement of RBC survival after transfusion. Our focus is on the last application—mea-
suring the survival of transfused crossmatch-incompatible RBCs. The introduction of radioisotope labeling of RBCs provided precise measurements of incompatible RBC survival using small aliquots (1 mL). While many radioisotopes have been used to label RBCs (e.g., $^{14}$C glycine, $^{55}$Fe and $^{59}$Fe, and $^{32}$P), the use of radioisotopes in blood transfusion medicine has primarily focused on $^{51}$Cr. Others, including $^{99m}$Tc, $^{52}$Cr, and $^{111}$In, have been described recently. $^{13-17}$ $^{51}$Cr studies are valuable in determining the clinical significance of alloantibodies, and survival of greater than 94 percent of transfused RBCs after 60 minutes has shown good correlation with successful subsequent transfusion of larger volumes. $^{18,19}$ The $^{51}$Cr technique was first used by Gray and Sterling $^{20}$ to determine RBC volume, and the isotope was soon used to determine RBC survival. $^{21-25}$

$^{51}$Cr is the “gold standard” of the RBC labels, due mainly to a low stable rate of elution from the RBC, and a half-life of nearly 28 days. Other labels like $^{99m}$Tc, with a half-life of 6 hours, and $^{111}$In, with a half-life of nearly 3 days, are less useful in long-term survival studies. $^{16}$ $^{51}$Cr, however, is not suitable for imaging studies, whereas $^{99m}$Tc and $^{111}$In can be used to assess location of tagged cells in vivo. $^{16}$

The method for $^{51}$Cr studies to assess transfusion compatibility has been described by the International Committee for Standardization in Hematology (ICSH). $^{4}$ Mollison and others have reported on numerous RBC survival studies using $^{51}$Cr as well as $^{32}$P. $^{18,24}$

When compatibility for the purpose of transfusion is under study, it is important to have a transfusion medicine specialist and the nuclear medicine department involved in the selection of RBCs and interpretation of results.

Generally, studies are performed with a small volume of fresh donor RBCs of known phenotype that possess one single antigen with which the recipient’s serum is incompatible. The sampling times are important, and adequate time for mixing (usually 3 minutes) must occur. The shape of the survival curve showing percentage of survival versus time after injection is also important for the interpretation of the study.

According to Mollison, two types of curves are commonly seen. $^{24}$ The first can be described by a single exponential (one component curve). Mollison attributes this type of cell destruction as random, with no intervening factors. Antibodies that have shown this type of survival curve include IgG antibodies, e.g., anti-D, -C, -K1. $^{24}$ The second type of curve is illustrated by two or more components. The most common cause for these, according to Mollison, is acquired resistance to complement-mediated destruction. In this setting, destruction is observed to slow within a short time after injection of the incompatible cells (usually 10 minutes). $^{24}$ Mollison also notes that another reason could be heterogeneity of antigen distribution. Common antibodies involved in a two-component curve are those that are complement-fixing, e.g., anti-Jk$^a$. $^{24}$ A parallel between the RBCs of patients with cold agglutinin disease and cell survival curves with two or more components may exist. In cold agglutinin disease, the patient’s existing RBCs are destroyed at a slower rate than transfused cells. $^{25,26}$ Presumably the transfused cells acquire resistance to clearance (destruction) by having the active form of C3(C3b) converted on the RBC surface to the inactive form, C3d,g. Some cautions were raised by Mollison, particularly the time of the first sampling. Inactivation of C3 may occur within 10 minutes, so it is necessary to obtain the control 100 percent value with a sample drawn at 3 minutes. There are some clinical conditions that may delay mixing, such as splenomegaly or other conditions that may impede normal circulation. $^{12,27}$

Studies with $^{51}$Cr have shown excellent correlation with in vivo survival of RBCs. Silverglid et al. $^{19}$ reported studies on 38 patients. They found that all $^{51}$Cr survival studies with 60-minute survival above 70 percent yielded the appropriate rise in hematocrit and that there was no evidence of hemolytic transfusion when the patients were subsequently transfused with incompatible RBCs.

Pineda et al. $^{28}$, in their report of ten patients for whom compatible units were difficult to find, emphasized the importance of extending $^{51}$Cr studies to 24 hours if the 60-minute test gives normal results. By extending the studies to 24 hours, an anti-Jk$^a$ and an anti-Lan showed diminished survival. Bentley et al., $^{29}$ using 10 mL aliquots of autologous cells, showed that approximately 3.3 percent of 5-day-old cells were subject to rapid elimination in the first 24 hours after injection. There was no difference between autologous blood that was fresh (less than 1 day old) and 5-day-old autologous blood. $^{29}$ Bentley et al. $^{29}$ did not advocate use of blood older than 5 days for $^{51}$Cr studies, as storage studies for in vivo compatibility testing had not been performed. In studies of stored autologous blood, Myhre et al. $^{30}$ noted that there were differences between 24-hour survival rates in blood stored for 42 days.
Case reports of different antibodies have compared $^{51}$Cr survival with in vivo clinical evidence of survival or destruction.\textsuperscript{31-40} Although the referenced articles are examples from the literature, many more have been reported. Comparisons with other methods will be discussed later.

Much has been written about the analysis of long-term survival results.\textsuperscript{41-45} The ICSH recommends plotting the points on a graph or using an equation to determine RBC survival.\textsuperscript{4} It may be desirable (or necessary) to obtain a separate determination of RBC volume using autologous RBCs labeled with $^{99m}$Tc.\textsuperscript{12}

When the RBCs are compatible, ICSH states that the 60-minute sample counts will be 99 percent (range 94–105%) of the 3-minute sample. The committee also states that if the 60-minute survival is at least 70 percent and the plasma count is not more than 3 percent, then rapid destruction of large volumes of RBCs is unlikely. As Davey\textsuperscript{12} has recommended, the best way to ascertain if cell destruction of donor cells has occurred is to compare the survival of the incompatible cells to the survival of autologous cells or with compatible cells. Cases have been described where cell destruction was documented by $^{51}$Cr studies in the absence of serologically detected antibody.\textsuperscript{46,47} The expert panel of the ICSH who prepared the recommended method for radioisotope red cell survival studies described three methods that could be used.\textsuperscript{4} As this study was published in 1980, the committee did not address the use of units stored in additive preservative solutions (e.g., Adsol or CPDA-1). However, studies have been performed using RBCs stored in these storage systems as part of quality control requirements prior to public distribution of these collection systems. If fresh cells are used, the preservative used for collection makes little difference (R. Davey, personal communication, 1992).

While there are reports using $^{99m}$Tc and $^{111}$In for measuring the survival of transfused cells, $^{51}$Cr remains the “gold standard,” especially if long-term studies are desired.\textsuperscript{13-16}

A technique using nonradioactive chromium, $^{52}$Cr, has been described.\textsuperscript{17} $^{50}$Cr may also be a possibility for use in survival studies.\textsuperscript{12} Other isotopes, $^{57}$Co and $^{58}$Co, have been described that may gain popularity in the future.\textsuperscript{48,49} Animal studies using $^{59}$Fe and Rb (rubidium) have also been reported.\textsuperscript{50,51}

**Flow Cytometry**

Flow cytometry is a relative newcomer in the measurement of RBC survival. It relies on differences in the antigenic composition of the transfused cells under study. Most reports of this technique relied on larger volumes of RBCs to detect destruction (10 mL minimum),\textsuperscript{52-55} and some studies followed the survival of entire units.\textsuperscript{2,56-58} Other reports have also studied flow cytometry as a method to measure mixed populations of RBCs.\textsuperscript{59,60} Although this method is sensitive, it has not been reported to be capable of following the course of 0.5 mL of RBCs as is the $^{51}$Cr method.

Postoway et al.\textsuperscript{2} reported a series of patients transfused with blood that was not antigenically matched, so that survival could be studied by flow cytometry. Results of flow cytometry correlated well with a differential agglutination method and with results of known standard control mixtures. The use of control mixtures is important, as RBC survival experiments using the flow cytometer do not have established controls, as do radioisotope studies. Autoantibodies\textsuperscript{58} as well as alloantibodies\textsuperscript{53,54,56,57} have been studied. Some researchers have concentrated on differences in antigens of the ABO or Rh systems, while others have also studied antigens of the Kell\textsuperscript{2,57} and Kidd systems.\textsuperscript{2,56}

Read et al.\textsuperscript{61} evaluated the potential use of PKH2 (a green fluorescent lyophilic compound) as a binder to RBCs in order to detect minor RBC populations by flow cytometry. While this method has not been evaluated in vivo, it may be a promising method in the future.

The advantage of flow cytometry is that while incompatible RBCs must still be infused, the patient is not exposed to radioisotopes. The disadvantage is that flow cytometry methods thus far described require substantially more RBCs (10 mL) than do radioisotope studies (0.5 mL). Professor Mollison (personal communication, 1990) does not think the risk of larger infusion is prohibitive: however, the same caveats of RBC infusion in $^{51}$Cr studies apply.

Quantitation of minor RBC populations utilizes one of the design specifications of the flow cytometer. Using differences in cell membrane epitopes, populations of cells exhibiting and not exhibiting antigens can be ascertained and quantitated.

To fully comprehend the application of flow cytometry, a brief discussion of its mechanics follows. RBCs can be sensitized for analysis in one of two ways. The first uses a blood group antibody directly labeled with a fluorescent dye. This antibody is incubated with a mixture of antigen-positive and antigen-negative RBCs designed to represent the volume of RBCs transfused to
the patient under study (control), and RBCs obtained from the recipient after transfusion at timed intervals. After washing, the cells are ready for aspiration into the flow cytometer.

In the second method of RBC preparation for flow cytometric studies, RBCs (both control mixtures and timed-interval specimens) are incubated with the appropriate blood group antibody, then washed. These sensitized RBCs are then incubated with an anti-human globulin that has been tagged with the fluorescent label. After washing, these RBCs are ready to be aspirated by the flow cytometer.62

The RBCs are aspirated by the flow cytometer and manipulated so that they flow in single file (one at a time) through the sensing zone of a monochromatic source of light (usually a laser). It is in the sensing zone that fluorescent labels are excited to emit photons of light of lower energy. This different wavelength is selectively directed to a detector. Other light scattered by the interaction of laser light with the cell is also quantitated and gives information about cell size and complexity (internal structure granularity). In this way, only RBC-bound fluorescence is measured, nonfluorescent RBCs are easily differentiated from fluorescent cells, and both populations of cells can be quantitated.

Biological Crossmatch

Another method of evaluating individual response to transfusion is the biological crossmatch. In this setting, a small aliquot (10-50 mL) of the cells under study are transfused. The recipient is then closely monitored for signs of an immediate reaction by examining the posttransfusion plasma for free hemoglobin. This may be a way of detecting an immediate hemolytic reaction, but it is probably of no value in predicting the survival of transfused RBCs with the exception of ABO incompatibilities. This method may be very useful, however, in a critical situation, when time is of the essence. One benefit that has been noted is that patients involved in these studies are very closely monitored, so a reaction would be detected much sooner than in other transfusion cases.63

Invasive assays, such as the biological crossmatch, in vivo survival of radioisotope-labeled RBCs, and flow cytometry, have thus proven to be valuable tools to measure transfused RBC survival.

Cellular Assays

Generally, in patients with known antibodies, antigen-negative blood is provided for transfusion. That becomes difficult when the patient possesses an antibody to a high-incidence antigen. Situations like this are the usual cases for which red cell survival studies are requested. Until recently, the method most often regarded as the “gold standard” was the Cr survival study. However, there are now reports of a number of alloantibodies that have been studied by cellular assays in order to predict RBC survival. Correlation data are difficult to assess, however, as most patients are not transfused with crossmatch-incompatible blood and the alloantibodies occur rarely. Cellular assays such as the monocyte monolayer assay (MMA) are relatively noninvasive, requiring only a sample of peripheral blood from the patient.64

The antibody-dependent cell-mediated cytotoxicity test (ADCC)65,66 and chemiluminescence assays67 are also methods that may be used in the future in an attempt to predict cell survival.

Archibald68,69 was the first to use a cellular assay in the study of sensitized human red cells. One of the first correlations of a cellular assay with in vivo cell survival was reported by van der Meuten.9 This report focused on the study of RBCs from patients with a positive direct antiglobulin test. Later studies by Engelberts’s laboratory at the Netherlands Red Cross concentrated on the in vivo survival of RBCs of patients with hemolytic disease of the newborn (HDN).66,70 Four studies have reported the use of the MMA in the evaluation of autoimmune hemolytic anemia cases and HDN.71–74 Zupanska and her co-workers75–77 have also used cellular assays to correlate in vivo RBC survival in HDN and autoimmune hemolytic anemia. Other workers have concentrated on the correlation of cellular assays with alloantibodies and survival of transfused incompatible RBCs.6,8,78–82

The reactivity of sensitized RBCs with human monocytes was systematically studied by Abramson and Schur83 and Fiunt et al.,84 who evaluated the reactivity of monocytes with RBC antibodies of known IgG subclass. Correlation of the in vivo behavior of these antibodies was not reported. In the latter study,84 mononuclear cells were placed into a Lab-Tek tissue culture chamber/slide (Nunc Inc., Naperville, Illinois). Only the monocytes adhered to the slide, and sensitized RBCs were incubated on the monolayer of monocytes. The supernatant nonreactive RBCs were removed by washing the monolayer, then the slide was stained and evaluated for reactivity of the monocytes, either by adherence or phagocytosis. Other workers have estab-
lished clinical correlations with the Lab-Tek tissue cul-
ture chamber/slide.\textsuperscript{79,85}

Many factors have been reported as affecting MMAs.
It is important to keep these in mind when evaluating
the assay. Most of these factors would also have an effect
on ADCC.

\textbf{Macrophages versus monocytes}

Macrophages have been used in some of the studies
of alloantibodies.\textsuperscript{79,80} Although these cells are more
phagocytic than are monocytes against IgG-sensitized
RBCs, they are also more reactive with unsensitized RBCs.
Therefore, sensitivity may not be enhanced.

\textbf{Freshness of monocytes}

Monocytes, like other white cells, begin to lose activ-
ity as soon as they are removed from their native envi-
nronment. Therefore, separation and testing should
commence as soon as possible.

Garratty et al.\textsuperscript{85,86} reported data from storage of
monocytes for 24 and 48 hours at room temperature. It
was found that a dramatic decrease in reactivity
occurred with storage. It was recommended that if ship-
ment of autologous monocytes from the patient to the
laboratory is necessary, control monocytes should also
be drawn from a normal donor at the same time and
shipped with the patient’s monocytes. The two samples
of shipped monocytes should be tested in parallel with
freshly drawn monocytes to evaluate the amount of
dimination of monocyte reactivity in the shipped
samples.

\textbf{Monocyte reactivity differences among donors}

Garratty et al.\textsuperscript{85,86} also reported that the reactivity of
normal donor monocytes was variable. In one study,
eight donors were tested in parallel and a coefficient of
variation of 25 percent was seen when RBCs with a 3+
indirect antiglobulin test (IAT) were evaluated. A high-
er coefficient of variation, 41 percent, was observed
when less sensitized (1+) RBCs were tested. Variation
was seen even when the same donor’s monocytes (this
author’s) were tested over a 3-year time span using the
same source of control anti-D to sensitize Rh:1,2,3,4,5
RBCs. Schanfield et al.\textsuperscript{79,80} used frozen monocytes, and
expressed results as a percentage of the positive control
in an attempt to control this variable.

\textbf{RBC sensitization}

Optimal RBC sensitization in the preparation of in
vitro-sensitized RBCs is an important factor in the MMA.
It is as important in cellular assays as it is in the routine
procedures of a blood bank laboratory. All of the con-
siderations given to maintaining the optimal concen-
tration of cells and serum, suspending media, and the
temperature of incubation during the sensitization
phase should be given to RBC sensitization for cellular
assays.

Another consideration reported to be a factor in
the correlation of alloantibody significance is the pres-
ence of fresh complement in the RBC sensitization
phase.\textsuperscript{82,87,88} Results of studies with antibodies of speci-
ficities that have been associated with complement activ-
ation showed that 12 of 22 sera had increased reactivity
in the MMA when fresh normal serum was added to the
RBC sensitization milieu.\textsuperscript{87} The result changed from
negative (less than 3% total reactivity) to positive
greater than 3% total reactivity) in eight cases; the other
four became more reactive. Further studies indicated
that complement was the component of the fresh nor-
mal serum that caused the increased reactivity, as adding
fresh serum treated with EDTA did not result in
increased reactivity. It was also interesting that some
antibodies exhibited decreased reactivity when fresh
normal serum was added to the test system.\textsuperscript{87} Storage
of test sera has been reported to affect the outcome of
the assay.\textsuperscript{89}

\textbf{Assay specifications}

Incubation times and conditions are important in cel-
lar studies. Each assay has its own optimal incubation
time, which should be determined by correlation assays.

Incubation temperature is also important, as phago-
cytosis is most likely to occur at 37°C. This makes a
37°C incubation temperature vital for assays that con-
sider only phagocytosis to be a positive result.\textsuperscript{90}

Maintenance of neutral pH (7.2) is also mandatory for
optimal phagocytic activity. Therefore, in assays depen-
dent on the phagocytic activity of the monocytes, incu-
bation in a 5% CO\textsubscript{2} atmosphere may be necessary.\textsuperscript{90}
Nance et al.\textsuperscript{6} found that incubation in room air at 37°C
gave the best clinical correlations in their assay. As
always, correlation studies with antibodies of known
clinical significance are imperative to establish optimal
assay conditions.

Another variable in the assay specifications is the eval-
uation of the reactivity observed and the expression of
those results. Counting the number of reactive mono-
cytes in a specified total number by microscopic exam
is the method used by some groups, while others count the number of RBCs associated with a specified number of monocytes. Some groups count only phagocytosis, while others consider both adherence and phagocytosis of RBCs to be indicative of a reactive monocyte. Care must be exercised in any attempt to compare data obtained using different counting methods. It has also been reported that the total number of monocytes counted can drastically affect the coefficient of variation of the assay. It has been recommended that a minimum of 600 monocytes be evaluated if the percentage of reactivity is below 20 percent.

The expression of results also varies among reports in the literature. Some have reported percentage of reactive monocytes using adherence and/or phagocytosis. In some, results are expressed as total number of RBCs associated with monocytes. Still others report results as a percentage of a positive control value. Therefore, it is obvious that direct comparison of results may be difficult when reviewing the literature. It is essential that each group of researchers establishes its own normal range and does not rely on ranges reported in the literature.

Antibody-dependent cellular cytotoxicity (ADCC)

The ADCC technique has been reported in the evaluation of HDN. While not reported as a predicting tool for transfused RBC survival, it has been very useful in predicting significant cell destruction in neonates born to mothers known to have potentially clinically significant antibodies to RBCs.

Chromeluminescence

Chromeluminescence is a new assay that has also been used to study a mother's serum whose newborn is at risk for developing hemolytic disease. The value of this test for evaluating significance of alloantibodies in the transfusion setting has not been reported.

Discussion

The methods discussed in this paper may not be applicable to all laboratories.

While there is no reason why a reference laboratory (e.g., in a blood center) could not prepare cells and perform the radiolabeling studies, they are usually performed in the hospital setting. The flow cytometry method, however, is one for which transfusion of selected units could be easily performed at the hospital, and the flow cytometric assay of the timed samples performed in a reference laboratory.

The biological crossmatch, since it is used in critical situations, of necessity is performed at the bedside of the patient in the hospital. Performance of the laboratory tests (e.g., plasma hemoglobin) to evaluate cell destruction are performed in the hospital laboratory. Cellular assays with significant specifications are optimally performed in specialized laboratories and have no place in most routine blood bank laboratories.

References

Review: measuring RBC survival

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