Blood doping in athletes—Detection of allogeneic blood transfusions by flow cytofluorometry

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Athletes may undergo blood transfusion to increase their red cell mass and the oxygen carrying capacity of their blood in order to confer a competitive advantage. Allogeneic transfusions are normally mismatched at one or more minor blood group antigens. The most sensitive and accurate method known to detect this form of blood doping is flow cytometry. Low percentages of antigen-positive and antigen-negative red blood cells (RBCs) can be quantitated using suitable specific alloantibodies and careful analysis. By testing blood samples taken at various times, a reduction in the percentage of a minor population of RBCs will indicate transfusion has occurred. Am. J. Hematol. 83:657–667, 2008. **© 2008 Wiley-Liss, Inc.**

Introduction

Blood doping

The goal of blood doping in athletes is to increase circulating hemoglobin levels. This increases the oxygen concentration of arterial blood, and therefore the aerobic capacity of the athletes which can be useful for training and competitions [1,2]. The first alleged use of blood doping (i.e., transfusion) was in the 1960s, and this form of blood boosting became widespread after the 1968 Olympic games in Mexico City when it was realized that athletes from higher altitudes performed better mainly due to increased red blood cell (RBC) mass after high altitude training [3].

Three invasive methods that athletes have used to increase their circulating hemoglobin levels are administration of erythropoietic stimulants (e.g., recombinant human erythropoietin), hemoglobin-based oxygen carriers (also known as blood substitutes), and blood transfusions (autologous and allogeneic) [1,4]. To counteract blood doping, a urine test for the direct detection of recombinant human erythropoietin was implemented at the 2000 Olympic games in Sydney [5] and research is being performed on detection of the presence of hemoglobin-based oxygen carriers [6]. Because it is becoming more difficult to evade detection of erythropoietin abuse or blood substitutes, transfusion is again being utilized [5]. To detect autologous (self) transfusion, the only realistic way may be through an indirect method, such as a Hematologic Passport in which current hematology values are compared with historical values for each athlete [2,7,8]. In contrast, several tests have been developed to distinguish allogeneic blood (from another individual) after transfusion.

Detection of allogeneic transfusion

In practice, it is virtually impossible to transfuse completely matched blood from another person (except an identical twin) due to the large number of blood group antigens on RBCs and their variability among individuals. To detect allogeneic transfusions, currently available methods are based on the antigenic differences between the recipient's RBCs and the donor's RBCs. Originally, serological techniques were performed, such as detection of "mixed field" reactions when performing antigen typing and differential agglutination where percentages of agglutinated and unagglutinated RBCs were determined manually or by automated methods [9–13]. More sensitive quantitative techniques to characterize transfused RBCs are the enzyme-linked antiglobulin test [14] and flow cytofluorometry [15–23]. Applications of flow cytofluorometry (flow cytometry) have increased enormously over the last 20–25 years and this review will consider the merits of flow cytometry for detection and analysis of blood doping.

Advantages of analyzing RBCs for detection of blood doping

The main reason why RBCs, as opposed to other blood cells, are ideally suited to detection of allogeneic blood transfusion is because they express many well characterized blood group antigens that can be recognized by specific alloantibodies. They also have the longest survival of blood cells in vivo with a mean cell lifespan of ~110–120 days [24], so that after transfusion they should be detectable for up to about 3–4 months. This will depend somewhat on their time in storage before transfusion and the amount transfused, but would enable repeat samples to be taken for confirmation of transfusion and the percentage survival of transfused RBCs. Furthermore, RBCs can be stored frozen indefinitely and on reconstitution will retain their suitability for flow cytometry. Thus direct comparison could be made of samples collected from athletes at several times before and after competition, if necessary. The frozen specimens could also be retained for further testing if new antibodies or techniques became available or for settling legal disputes.

Use of Flow Cytometry for the Detection of Mixed RBC Populations

Transfusion of patients

Identification of the survival of transfused RBCs in patients has been determined by flow cytometry using alloantibodies directed against antigens on the transfused or recipient's RBCs. Typically this has been applied in the set-

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ting where one or more units of allogeneic blood (comprising about 200 mL packed cells per unit) were transfused [15–18,20–23]. Flow cytometry was also compared with ^{51}Cr-labeled RBCs to detect small aliquots of RBCs for survival studies [25–29]. It was found that transfusion of at least 10 mL of RBCs was needed in order to accurately follow the survival of the transfused RBCs by flow cytometry. In most cases, the transfused minor population was antigen positive, but in some studies a minor population of antigen-negative transfused RBCs in an antigen-positive recipient was followed [23,25,27,29]. Other applications of flow cytometry include phenotyping recipients' RBCs following transfusion [19] and determining red cell volume in transfused infants [30].

Feto-maternal hemorrhage

Flow cytometry has proven to be a very useful method to detect and quantify mismatched RBCs occurring after feto-maternal hemorrhage (FMH). In the early 1980s, very small numbers of fetal D+ RBCs in maternal D− blood (about 1 in 100,000 cells) were detected using cell sorting [31] or with labeled fluorescent microbeads [32]. Simpler flow cytometric methods using anti-D [33–36] or anti-hemoglobin F (HbF) [37,38] were later described to determine the size of a fetal bleed (FMH) in a pregnant woman so that the appropriate amount of anti-D immunoglobulin could be given. The sensitivity of these flow cytometric assays is about 0.1% (i.e., 2 mL RBCs) and the accuracy is good (CV $<$ 16% for samples with $>$0.1% fetal RBCs) if at least 50,000 events (i.e., cells) are analyzed; the accuracy is improved (CV $<$ 10%) if more events (e.g., 500,000) are analyzed [39]. Commercial kits are available and flow cytometry is used routinely in some clinical laboratories for the detection and quantitation of D-positive or HbF-positive FMH. Large D-compatible FMHs have been identified and quantitated using other blood group antibodies [40,41].

Chimerism and mosaicism

The analysis of mixed RBC populations occurring as a result of hematopoietic chimerism in twins [42–46] and in bone marrow transplant recipients [47–52] is easily performed by flow cytometry. This technique has also been used in studies of individuals with blood group mosaicism [26,53–55]. In practice, the most common applications are for RBC phenotyping after transplantation.

Blood doping (transfusion) in athletes

Flow cytometry was recently found suitable for the detection of allogeneic RBC transfusions in athletes [56–58] and is discussed in detail later. It was first used for this purpose at the 2004 Olympic games in Athens [2]. A gold medal winner in cycling at these Olympic games tested positive for allogeneic blood transfusion a month later at a different competition; this resulted in a 2 year suspension from international competition. Since then, at least two other cyclists have tested positive for this form of blood doping using flow cytometry.

Overview of Flow Cytometry

The flow cytometer

A flow cytometer is an instrument that analyzes light emitted from cells or particles individually as they pass in a fluid stream through a beam of excitation light. The reason why this technique is so effective at characterizing mixed cell populations, as in blood doping, is because each cell is analyzed separately. The most common excitation light source is an argon ion laser which emits light at a wavelength of 488 nm (in the blue region of visible light). Cells disrupt or scatter the incident light as they pass through the beam. Detectors measure light emitted from each cell at several wavelengths. Two detectors measure forward light scatter (FSC) which gives some information about cell size or volume and side light scatter (SSC) (also known as 90° or right angle scatter) which relates to the presence of intracellular structures such as granules and/or cell surface irregularities. If a fluorescent dye is attached to a cell, the dye will be excited to a higher energy state and will emit light of a greater wavelength. The intensity of fluorescence at the appropriate wavelength is measured by a third detector. The emitted light from several fluorochromes can be measured simultaneously with additional detectors. The power of the instrument lies in its capacity for acquisition and accurate analysis of many independent variables on each cell extremely rapidly.

Analysis

Each cell is analyzed individually by the flow cytometer with capture of its FSC, SSC, and fluorescence intensity data on the computer. Light and fluorescence signals are weak and are amplified by the flow cytometer's electronics on either a linear or logarithmic scale. The former is useful when there are small differences in signals from the cells being studied, but a logarithmic scale is more effective when these differences are large [59]. Some cells in whole blood can be distinguished from each other based on differences in how they scatter the incident light beam, when the FSC versus SSC data is displayed using linear amplification on a dot plot. By this means, the various white blood cell (WBC) types (granulocytes, monocytes, and lymphocytes) can be discriminated. However, for RBCs, it is best to display their FSC versus SSC data on a logarithmic scale when they form a discreet population (see Fig. 1). An electronic gate can then be set around this population to exclude background events such as small debris [39,60]. The fluorescence intensity of the selected (gated) cells is then normally plotted as a histogram of cell count versus channel number. The voltage of the fluorescence detector is adjusted so that antigen-negative RBCs fall within the first decade on a logarithmic scale. If two populations of RBCs are present, one without and one with a fluorescent antibody attached, this will be apparent and electronic markers can be placed over each group for the computer to calculate (a) the number and percentage and (b) the mean or median fluorescence intensity of the cells in each group. The accuracy increases with the number of cells analyzed; for rare event analysis the number analyzed is often 50,000 [33,37,38] to 500,000 [28,35,39,61]. For analysis of single cell populations, acquisition of 10,000 cells normally suffices. All the data for each individual sample is stored on the computer and can be reanalyzed later, which may be important for legal purposes. Some flow cytometers are semi-automatic enabling high-throughput sample processing and data acquisition.

Dyes and antibody conjugates

Fluorescent dyes, which are excited by a light source and then emit light of a higher wavelength, can be used to obtain additional information about cells. Some dyes label cells directly, e.g., thiazole orange is a blue-excited fluorochrome that labels RNA and DNA and is used for the detection and quantitation of reticulocytes by emission of green light. For most applications, including assessment of blood doping, other dyes are used to label primary antibodies (i.e., alloantibodies to blood group antigens) or secondary antibodies (i.e., antibodies that bind to the primary alloantibody). Commonly used fluorochromes for conjugation to primary or secondary antibodies are fluorescein isothiocyanate (FITC) and phycoerythrin (PE) which are

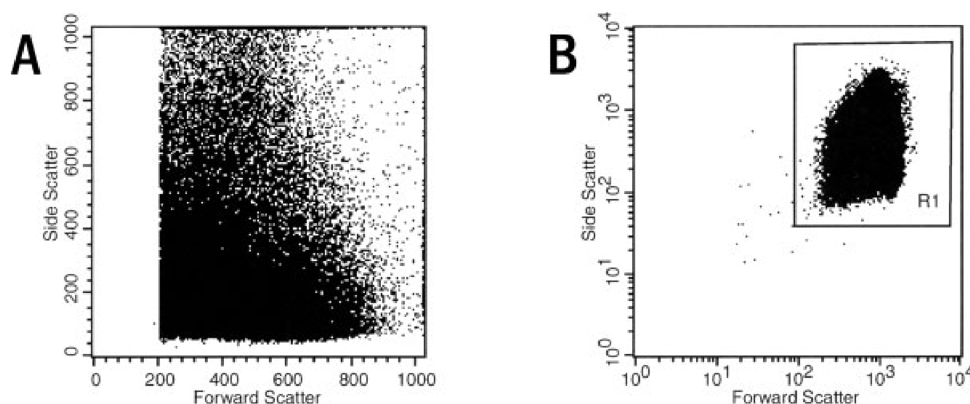

Figure 1. Gating. Comparison of FSC vs. SSC of 50,000 washed RBCs using linear amplification (A) or logarithmic amplification (B) of light scatter. Using linear amplification, the RBCs are widely distributed and it is not possible to set an electronic region to include all RBCs. With logarithmic amplification, the RBCs fall into a discrete group and it is easy to set a region (R1) around them to exclude debris but include all RBCs. In B, 99.96% of the events fall into Region 1.

excited by a blue laser and then emit green and yellow light, respectively. The fluorescence emitted by PE is greater than that of FITC. These fluorochrome-conjugated antibodies specifically label the antigens or bound alloantibodies on RBCs and the amount of fluorescence emitted by these labeled cells can be used to detect and quantify the antigens or alloantibodies on the cells. If there is a mixed population of cells (i.e., with antigenic differences) in the sample being analyzed, then these different groups can be analyzed individually via their different levels of fluorescence at the appropriate wavelength, without the need for physical separation.

Comparison of flow cytometry with other quantitative methods of analyzing RBCs

In contrast to flow cytometry, earlier manual methods to detect mixed cell populations such as differential agglutination [9] were tedious and subjective, with no electronic data storage. They were also less sensitive and less accurate. The immunofluorescence of individual cells can be determined microscopically, but again this is subjective as the fluorescence is not easily quantifiable, the fluorescence detection is not as sensitive as flow cytometry, and because relatively few cells can be examined manually this method is not satisfactory for rare event analysis.

Technical Considerations for Detection of Allogeneic Blood Transfusions by Flow Cytometry

Flow cytometry is widely used for the analysis of WBCs but much less commonly for the analysis of RBCs. Thus, most flow cytometry operators are unfamiliar with important aspects of testing RBCs (e.g., problems that can arise due to agglutination). Also, certain methods that pertain to WBCs (e.g., incubations at 4°C to prevent capping) are not relevant for RBCs. It is important that these differences are understood and that the appropriate methods are used when analyzing RBCs by flow cytometry.

Flow cytometric assays for the detection of allogeneic blood transfusion require RBCs (from the athlete and control non-transfused individuals), primary antibodies, and possibly secondary antisera. It is necessary to obtain a high fluorescent signal from antigen-positive RBCs while preventing agglutination in order to get reproducible, unequivocal, and sensitive data. The level of fluorescence of antigen-positive RBCs will depend upon several factors: (1) the number of antigen sites, (2) the strength of antibody, (3) the method used to label the antigen, and (4) the use of different fluorochromes. Important considerations of each component of the analyses will be discussed in detail.

Red blood cells

RBCs from individuals for flow cytometric testing are usually obtained as anticoagulated samples preferably in an anticoagulant-preservative such as acid citrate dextrose if the sample is not going to be tested immediately. Samples from athletes may need to travel from the training or competition sites to the testing sites. Although most blood group antigens are generally stable, it is important to minimize delays before testing to prevent sample deterioration that could affect results. When possible, the samples should be stored at 4°C prior to testing. Blood samples should be well mixed prior to removing subsamples for analysis. For quantitation of minor cell populations, several subsamples should be tested and a mean of the results determined. RBCs should be washed with saline prior to incubation with antisera to remove serum proteins, platelets and WBCs. For long-term storage (years), RBCs can be frozen in liquid nitrogen in a mixture of polyvinyl pyrollidone and bovine serum albumin [24; IBGRL modification] or sucrose and dextrose [62], or at −80°C using a glycerol solution [63]. They can be thawed for later testing.

Blood group antigens

As early as 1984, flow cytometry was applied to the study of blood group antigens [64,65]. It was shown that zygosity could be quantitated much more accurately by flow cytometry than by serology [66]. Currently, just over 300 blood group antigens have been described [67,68]. Most of these antigens are of either high incidence (found on greater than 99% of individuals' RBCs) or low incidence (found on less than 1% of individuals' RBCs) and thus mismatches of these antigens do not normally occur with allogeneic transfusions and they are not usually informative for the detection of mixed cell populations. For transfusion, patients are given ABO and Rh(D) identical or compatible blood and the same presumably applies to illegal blood transfusion in athletes. Group O blood can be transfused to group A or B recipients and D− blood can be transfused to D+ recipients. The blood group antigens that are the most useful are those found with a moderate frequency, e.g., those in the ABO (A, B), Rh (D, C, c, E), MNS (M, N, S, s), Kell (K), Duffy (Fy^a, Fy^b), and Kidd (Jk^a, Jk^b) blood group systems. Some investigators also include the Rh (e) and Kell (k) antigens in their investigations although these are of higher frequency (about 98 and 99.8% of individuals are positive for e and k, respectively). Blood group antigens vary in the number of sites on the RBC membrane with the amount of antibody bound being in proportion to the anti-

TABLE I. Ranges of Antigen Sites on Normal RBCs for Different Blood Group Antigens [69]

Blood group system	Antigen	Estimated number of antigen sites	
		Low end (phenotype)	High end (phenotype)
ABO	A	120,000 (A_2B adult)	1,170,000* (A_1 adult)
	B	610,000 (B adult)	830,000* (B adult)
Rh	D	10,000 (DCe/ce)†	33,500 (DcE/cE)‡
	C	21,500 (C+c+)	56,500 (C+c–)
	E	No data	25,500 (E+e–)
	c	37,000 (C+c+)	85,000 (C–c+)
	e	13,500 (E+e+)	24,500 (E–e+)
MNS	M and N	500,000 (heterozygote)	1,000,000 (homozygote)
	S	125,000 (S+s+)	250,000 (S+s–)
	s	85,000 (S+s+)	170,000 (S–s+)
Kell	K	2,300 (K+k+)	6,000 (K+k–)
	k	2,000 (K+k+)	5,000 (K–k+)
Duffy	Fy^a	7,000 [Fy(a+b+)]	~14,000 [Fy(a+b–)]
	Fy^b	No data	~14,000 [Fy(a–b+)]
Kidd	Jk^a	No data	14,000 [Jk(a+b–)]
	Jk^b	No data	No data

* Calculations indicate that this number may be twice as high, i.e., 2,000,000.
† "Weak D" phenotypes may have 300–9000 D sites per RBC.
‡ The rare –D– phenotype can have up to 200,000 D antigen sites.

gen-site density on RBCs (Table I). For example, there are many more A than D antigen sites and the antigen density of D is greater than K. For each antigen, variation can be seen in the number of sites on different individuals' RBCs based partly on natural variation but mainly on the genetic zygosity of the individual—homozygotes should theoretically have twice as much antigen as heterozygotes.

Primary antibodies

The antibody specificities that would be most useful to test would include anti-A, -B, -D, -C, -c, -E, -K, -M, -N, -S, -s, -Fy^a, -Fy^b, -Jk^a, and -Jk^b (and possibly anti-e). Monospecific antibodies (those that bind a single RBC antigen) are selected. Many antisera are available, both commercially and in immunohematology reference centers, to the common blood group antigens. Originally, antibodies to blood group antigens were human polyclonal antisera. In the last twenty years, monoclonal antibodies (mAbs) have been produced to some of the RBC antigens. Monoclonal anti-A and anti-B, recognizing sugar epitopes, are from mouse hybridomas and anti-M and anti-N are also murine. Antibodies to the Rh blood group antigens and some other minor blood group antigens are now generally obtained as mAbs produced from cell lines derived from B cells of immunized human donors as these protein antigens are not recognized by the murine immune system. Some mAbs to blood group antigens are now conjugated to FITC or PE, specifically for use by flow cytometry [35,52].

The immune system produces two main classes of blood group antibodies, IgM and IgG. In general, IgM antibodies are direct agglutinins as their large size allows them to bridge the distance between RBCs. IgG antibodies sensitize cells—they attach to RBC antigens, but due to their smaller size cannot usually agglutinate RBCs on their own (ABO antibodies are an exception). Many commercial mAbs used in routine immunohematology (e.g., for antigen typing) are agglutinins (IgM) and thus some precautions need to be taken when using these antisera by flow cytometry (see later section on agglutination). Anti-K, -S, -s, -Fy^a, -Fy^b, -Jk^a, and -Jk^b are still available from blood banking reagent manufacturers as IgG polyclonal human antibodies for use by the indirect antiglobulin test and thus can easily be applied to flow cytometry.

The strength (titer) of the antisera and the number of antigen sites on RBCs bound by antibody will vary for each antibody/antigen combination tested and will affect the fluorescent signal. Depending on the antigen-site density, an IgM antibody may bind to several antigens due to its high valency, in contrast to divalent IgG antibodies that can bind to no more than two antigens. Thus, the fluorescent signal from IgM may be weaker than that from IgG. It is important for detection and quantitation of minor cell populations that the antigen-positive population be clearly distinct from the antigen-negative population on the histogram display. Figure 2 shows results of RBC mixtures using three different IgG polyclonal antisera: anti-Fy^b, anti-K, and anti-s. The Fy(b+) RBCs are not clearly differentiated from the Fy(b−) RBCs, whereas the K+ and the s+ RBCs are distinguishable from the K− and s− RBCs, respectively. The difference in fluorescence of the K+ RBCs from K− RBCs is less than that of the s+ RBCs from s− RBCs. Because of variations like this, the electronic marker separating the antigen-positive subpopulation from the antigen-negative subpopulation should be analyzed, and reset if necessary, for each antiserum. Different electronic marker settings may also be needed when analyzing a minor population of antigen-positive RBCs vs. a minor population of antigen-negative RBCs with the same antiserum.

Titration studies can be performed with antisera to determine the appropriate dilution for use by flow cytometry. RBCs from individuals with known heterozygous gene expression should be chosen as positive controls (e.g., in artificially prepared RBC mixtures) as they will have fewer antigen sites than those from individuals with homozygous gene expression. Comparison should be made of several antibodies from different sources for each antigen in order to select an antibody giving a high signal-to-noise ratio, i.e., high fluorescence of antigen-positive cells relative to the background autofluorescence of antigen-negative cells. This is important as it will ensure that the antisera selected are suitable for the detection of low density antigens on RBCs. It will not be known what most of the blood group antigens of the donor and recipient (athlete) are.

Secondary antibodies

If the primary antibody is not directly conjugated to a fluorochrome, a conjugated secondary antibody of the appropriate specificity (anti-human or anti-mouse, anti-IgM or anti-IgG) must be used to fluorescently label the RBCs. Secondary conjugated antisera are available in several formats: whole molecule and $F(ab')_2$ and Fab fragments [70]. Whole molecule IgG antibodies will agglutinate RBCs coated with primary antibody and will also non-specifically bind to contaminating WBCs via IgG Fc receptors and thus should be avoided, if possible. $F(ab')_2$ antibodies are commonly used for WBC analyses because they lack the Fc portion and will not bind nonspecifically, but could cause agglutination of antibody-coated RBCs and should therefore be avoided for RBC analyses. Fab antibodies are best for RBC analyses by flow cytometry because they cannot crosslink IgG on RBCs to form agglutinates (see Fig. 3). However, if the antigen-site density on RBCs is low, agglutination by the secondary antibody may not be a problem. Fluorescence may be enhanced by the use of a biotinylated secondary antibody followed by avidin or streptavidin conjugated to FITC or PE [71]. In addition, a third antibody directed against the secondary immunoglobulin or fluorochrome can be added for enhancement of weak signals

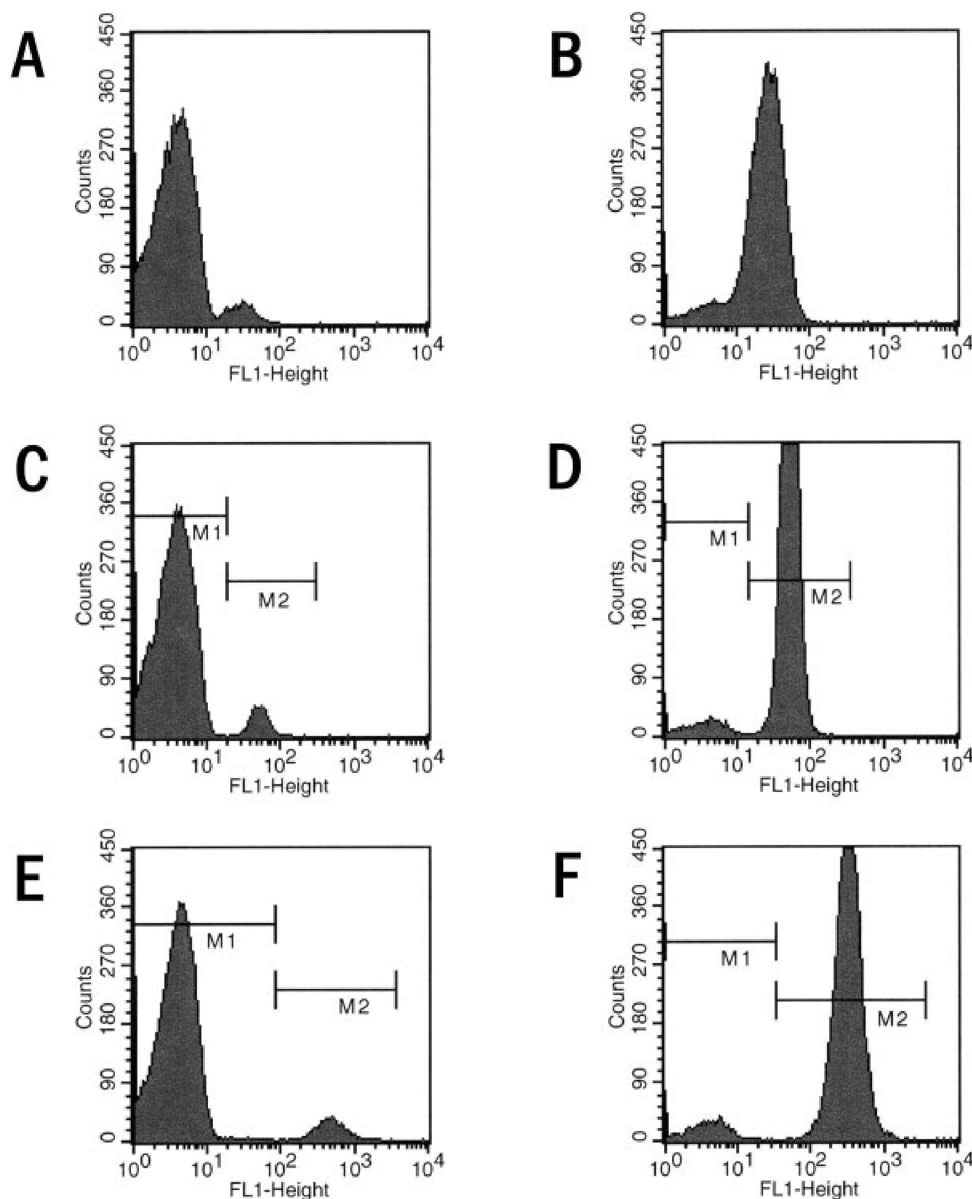

Figure 2. Minor populations of antigen-positive and antigen-negative RBCs. Fluorescence histograms showing results with 5% Fy(b+) in Fy(b-) RBCs (A), 5% Fy(b-) in Fy(b+) RBCs (B), 5% K+ in K− RBCs (C), 5% K− in K+ RBCs (D), 5% s+ in s− RBCs (E), and 5% s− RBCs in s+ RBCs (F). The RBC mixtures were incubated with polyclonal anti-Fy^b (A and B), polyclonal anti-K (C and D), or polyclonal anti-s (E and F). The secondary antibody for all tests was FITC Fab anti-human IgG and 50,000 events were analyzed. Note that the Fy(b+) and Fy(b−) RBCs are not clearly discriminated from each other. The K+ and s+ RBCs are separate from the K− and s− RBCs, but the s+ RBCs are clearly more fluorescent than the K+ RBCs and different markers (M1 and M2) are needed for quantitation with each different antisera. Marker settings are also different when the minor population is antigen negative vs. antigen positive.

[48,72]. Antibodies that have been used to label blood group antigens for analysis of mixed RBC populations are listed in Table II.

Agglutination and its avoidance

For flow cytometric analyses, agglutination should be avoided because each cell needs to be analyzed individually. Agglutination can be caused directly by a primary antibody or indirectly by a secondary antibody. Agglutinated cells can have increased light scatter (due to increased size and complexity; Fig. 3B) and, depending upon how the electronic gate on the FSC vs. SSC dot plot is set, could possibly be excluded from further analysis. If not excluded, the presence of an RBC agglutinate containing, for example, five to six RBCs, would be counted by the flow cytometer as one large "event" and if some or all of those RBCs were fluorescently labeled, then that event would be much more fluorescent than single cells (see Fig. 3D). This could give incorrect results of both the percentage of positive cells in a sample and of their mean or median fluorescence intensity, especially when the antigen-positive RBCs are the minor population. Agglutination may affect the final interpretation when following the survival of transfused RBCs over time, because the percentage of transfused cells must be accurately calculated on each occasion. In contrast, some agglutination could be tolerated in an antigen-positive population when determining whether transfused RBCs are present or not.

Different approaches to dealing with RBC agglutination for flow cytometric analyses, ranging from prevention to dispersal of agglutinates prior to analysis, are listed in Table III. The optimal choice would be to use nonagglutinating antibodies, but IgG nonagglutinating primary antibodies are

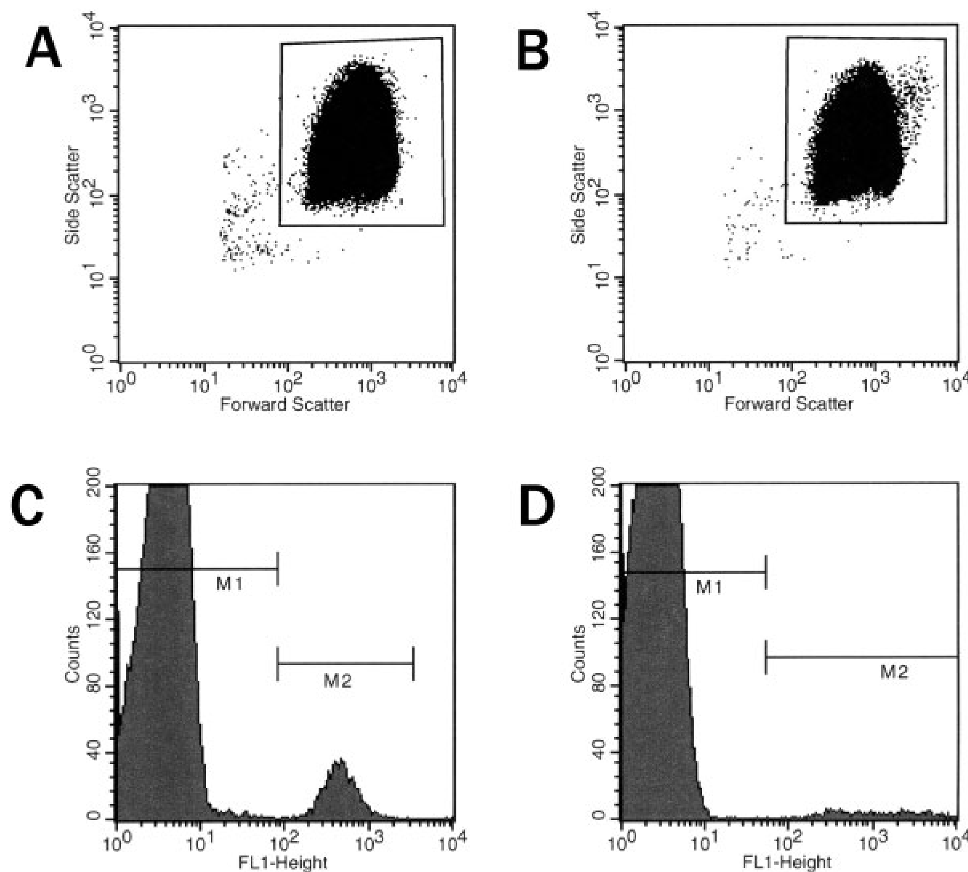

Figure 3. Secondary antibodies and agglutination. A mixture of 5% s+ in s− RBCs after incubation with polyclonal anti-s and either FITC Fab anti-IgG (A, C) or FITC $F(ab')_2$ anti-IgG (B, D) showing the FSC vs. SSC dot plots (A, B) and the fluorescence histograms (C, D). B and D show the presence of agglutinates with increased light scatter and increased fluorescence; no agglutinates are present in A and C. In C, 5.4% of the events fall in marker 2 (M2) representing the antigen-positive cells; in D, the antigen-positive cells make up only 1.1% of events due to many antigen-positive cells being in agglutinates. The electronic gate on the FSC vs. SSC dot plot has been set to include the agglutinated cells; if it had been set closer to the main RBC population, the agglutinates in B with increased scatter could have been excluded from further analysis which would have also resulted in an erroneously low percentage of labeled RBCs.

not always available. Commercial blood grouping reagents used for typing RBCs in immunohematology laboratories used to come from human sera and thus were polyclonal, often IgG, but over time these are being replaced by monoclonal reagents (usually IgM) which are chosen because of their ability to directly agglutinate cells. If human source antisera are available, they need to be strong enough (e.g., of sufficient titer) to differentiate antigen-positive from antigen-negative RBCs by flow cytometry.

For some antigens, e.g., A, B, M, and N, antibody mediated agglutination occurs due in part to the large number and topography of antigen sites on RBCs; this can be prevented by chemically fixing the RBCs prior to addition of antibody. Fixation prevents conformational changes of the

TABLE II. Reagents Used to Label RBCs in Mixed Cell Populations Using Blood Group Primary Antibodies With or Without Secondary Antisera

Primary antibodies	Secondary antisera	Blood group(s) studied
Human antisera (preferably high titer antibody)	FITC or PE anti-human IgG	ABO, Rh, Kell, Duffy, Kidd [40,48,49,50]
	Biotin anti-human IgG + (strept)avidin-FITC or -PE	ABO, Rh [27,48]
Commercial polyclonal antisera (from human sera)	FITC or PE anti-human IgG	ABO, Rh, MNS, Kell, Duffy, Kidd [21,41,45,57,58,72,73]
	Biotin anti-human IgG + (strept)avidin-PE	Rh, MNS, Kell, Duffy, Kidd [19,48,73]
Monoclonal antibodies (mAbs), mouse or human, IgG or IgM	FITC or PE anti-mouse or anti-human Ig	ABO, Rh, MNS, Kell, Duffy, Kidd [13,21,30,40,44,46,47,50,52,72,73]
	Biotin anti-Ig + (strept)avidin-PE	Rh, Kidd [73]
FITC- or PE-labeled mAbs, mouse or human	NA	ABO, Rh, MNS, Kell, Duffy, Kidd [30,35,40,47,52]
Biotin-labeled mAbs + (strept)avidin-PE	NA	MNS [30]

NA, not applicable.

Note that for signal enhancement some investigators have added a third antibody directed against the secondary antibody [48] or against the fluorochrome on the secondary antibody [72].

TABLE III. Ways to Avoid and/or Resolve RBC Agglutination for Flow Cytometric Analyses

To avoid agglutination caused by primary antibody:
Use nonagglutinating (IgG) antibodies
Chemically convert agglutinating IgM pentamers into nonagglutinating IgM monomers, e.g., using dithiothreitol [48,57] or 2-mercaptoethanol
Purify IgG fractions of sera on Protein G [49]
Dilute antisera past point of agglutination
Avoid use of potentiators
Chemically fix the RBCs prior to incubation with anti-A, -B, -M, or –N, e.g., with dimethylsuberimidate [74], formaldehyde [47,75], or glutaraldehyde [13,21,44,49]
To avoid agglutination caused by secondary antibody:
Use Fab fragments of secondary antibody
Use directly labeled nonagglutinating primary antibody and avoid use of secondary antibody
Dilute antisera past point of agglutination
To break up agglutination mechanically:
Vortexing (for weak agglutination)
Repeated agitation through a pipette microtip or small gauge needle [43,50]

red cell membrane required for RBCs to be crosslinked by multivalent antibodies [76]. Formaldehyde [47,75] is a satisfactory fixative for the A, B, M, and N antigens though rather tedious (a two-day, three-step process) and causes some hemolysis. Fixation with glutaraldehyde [13,21,44,49] is rapid and nonhemolytic, and although the A, B, and M blood groups are not destroyed, the N antigen is sensitive (A. Guest, Personal communication, IBGRL, Bristol). Fixation increases autofluoresence of RBCs and, more markedly, that of WBCs (see below). The increased RBC autofluorescence does not usually interfere with detection of highly fluorescent cells with large numbers of antigen sites (e.g., A or B) but it can mask weak fluorescence when few antibodies are bound to RBCs. Fluorescent labeled Fab fragments of some secondary antibodies are available, and should be used to avoid agglutination whenever possible when testing unfixed cells. Finally, if agglutination is unavoidable, mechanical disaggregation is an option although agglutination may not always be completely dispersed and strongly agglutinated RBCs (e.g., with anti-A or -B) can be hemolyzed by this process.

Problems with WBCs

Infrequently, the presence of leukocytes (WBCs) may interfere with accurate analysis if they are present in the labeled sample. WBCs can bind IgG through IgG Fc receptors, thus giving nonspecific fluorescence; $F(ab')_2$ or Fab fragments of secondary antibodies will avoid this problem but $F(ab')_2$ could agglutinate RBCs. Furthermore, autofluorescence of WBCs after fixation may be comparable to that of antibody-coated RBCs labeled with FITC or PE. In either case, WBCs can then appear as a minor population of labeled cells, or can increase the apparent number of fluorescently labeled RBCs. To prevent erroneous quantitation of a minor population of antigen-positive RBCs (generally when fixed samples or fluorochrome-labeled whole molecule antibodies are used), the WBCs must be gated out before analysis. This is best done on a FSC (or SSC) versus fluorescence dot plot—using the detector that is not measuring the fluorescent marker of interest—and excluding the minor group of cells with greater autofluorescence than the RBCs [37]. Unlabeled, unfixed WBCs could fall in the antigen-negative RBC area and an antibody specific to WBCs such as FITC-anti-CD45 could be used to identify them. Alternatively, a fluorochrome-conjugated antibody to glycophorin A (GPA) can be used to label RBCs in addition to the blood group antibodies under investigation to ensure only RBCs are analyzed, although care must be taken to avoid agglutination by anti-GPA [77]. Use of a fluorescent-labeled antibody to detect WBCs or of anti-GPA to label RBCs would mean that a different color fluorochrome would be needed to label the RBC blood group antigens in question. When two-color flow cytometric analyses are performed, electronic compensation is needed to subtract the overlap in emissions of the two fluorochromes [78].

Autofluorescence

Background fluorescence of RBCs consists of blue-excited green autofluorescence due to intracellular constituents such as flavins and pyridine nucleotides [78]. Autofluorescence increases after fixation of cells as discussed above, so to maintain antigen-negative fixed RBCs within the first decade on histograms, the voltage of the detector must be reduced. Instrument noise and nonspecific adherence of fluorochrome-labeled antibody to cells can also contribute to background fluorescence. All these factors limit the sensitivity of the test. It is important that the antigen-positive population is sufficiently labeled with fluorochrome so that it is clearly separated from the background fluorescence of the antigen-negative RBCs.

Background events

Background events are particles (not RBCs) that have fluorescence in the region used to detect antigen-positive or antigen-negative RBCs. Determination of background events is performed using labeled control blood samples either having or lacking the particular RBC antigen present in the test sample. For accurate quantitation of the number of labeled RBCs, the number of background events must be subtracted from the number of events of the test sample in the region of interest.

Selection of direct or indirect labeling method for each blood group antigen

For direct labeling, RBCs are incubated with a fluorochrome-labeled blood group antibody (e.g., FITC anti-Fy^a); for indirect labeling, RBCs are incubated with an unlabeled blood group antibody (the primary antibody, e.g., anti-Fy^a) and then with a fluorochrome-labeled anti-Ig reagent (the secondary antibody, e.g., FITC anti-IgG). The indirect method has been employed most often because of the lack of directly conjugated antibodies and also as a way to increase the fluorescent signal and discriminate labeled from unlabeled cells. Figure 4 shows the difference in fluorescence seen when an indirect method (anti-D + FITC anti-IgG) versus two direct methods (FITC anti-D and PE-anti-D) of labeling the D antigen are used. The advantage of the indirect method is that up to about six fluorochrome-labeled secondary antibodies can attach to each primary antibody thus increasing the signal per antigen up to six times [78]. Strongly fluorescent fluorochromes, such as PE consisting of up to 40 fluorescent bile pigments, amplify the signal (see Fig. 5). Furthermore, if PE is excited at a wavelength greater than 515 nm (e.g., by a green diode laser), then the blue-excited background autofluorescence is decreased [71,78]. A disadvantage of the indirect method can be increased nonspecific binding of the secondary antibody to antigen-negative cells. Another amplification method uses biotin-labeled antibody followed by (strept)avidin. Table II gives examples of approaches using secondary and/or primary antibodies that have been used by investigators when studying mixed RBC populations. A commercial kit is avail-

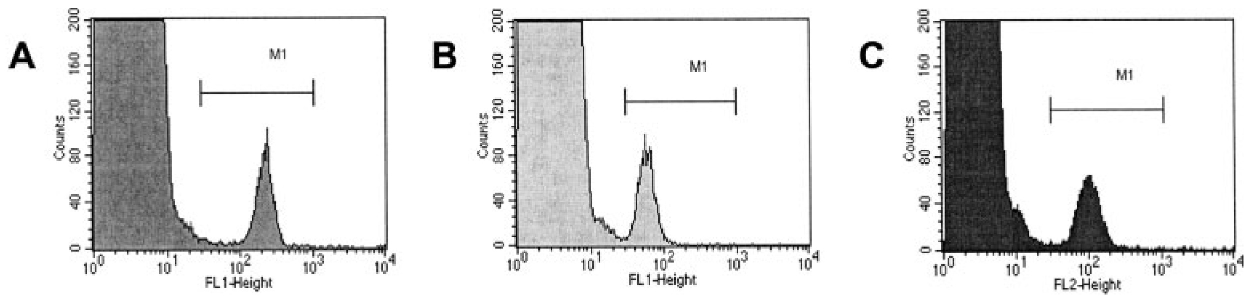

Figure 4. Direct vs. indirect labeling of RBC antigens. Fluorescence histogram results of a mixture of 1% D+ RBCs in D− RBCs after labeling by three different methods. RBCs were incubated with polyclonal anti-D and then FITC anti-human IgG (A), or FITC-labeled monoclonal anti-D (B), or PE-labeled monoclonal anti-D (C). The median fluorescence was greatest by the indirect method [210 (A)] than by the two direct methods [58 using FITC (B) and 102 using PE (C)]. B has the fewest background events.

able in some countries for quantification of mixed populations of RBCs.

Incubation time and temperature for primary and secondary antisera.
The optimal temperature of incubation for most polyclonal primary RBC antibodies is 37°C [79]; for monoclonal antibodies, room temperature can often be used (manufacturers' instructions should be consulted). For low-avidity antibodies to low-density antigens, incubation times of 60 min may enhance the number of bound antibodies and thus the fluorescence signal. Three to four washes of the RBCs with saline are required after incubation with the primary antisera if a secondary antibody is to be used; if unbound IgG is not completely removed, it could neutralize the secondary antibody [80]. For most flow cytometric methods, there is an incubation step for 30 min with the secondary antibody, often at room temperature and in the dark. At least one wash is performed after incubation with the secondary antibody prior to flow cytometric analysis.

Standardization
Commercial blood banking antibody reagents have been standardized for use with RBCs by methods that involve detection of agglutination but not for use by flow cytometry and thus this need to be performed. Standardization of primary antisera involves first determining the optimum dilution for use and then showing that antisera are specific for

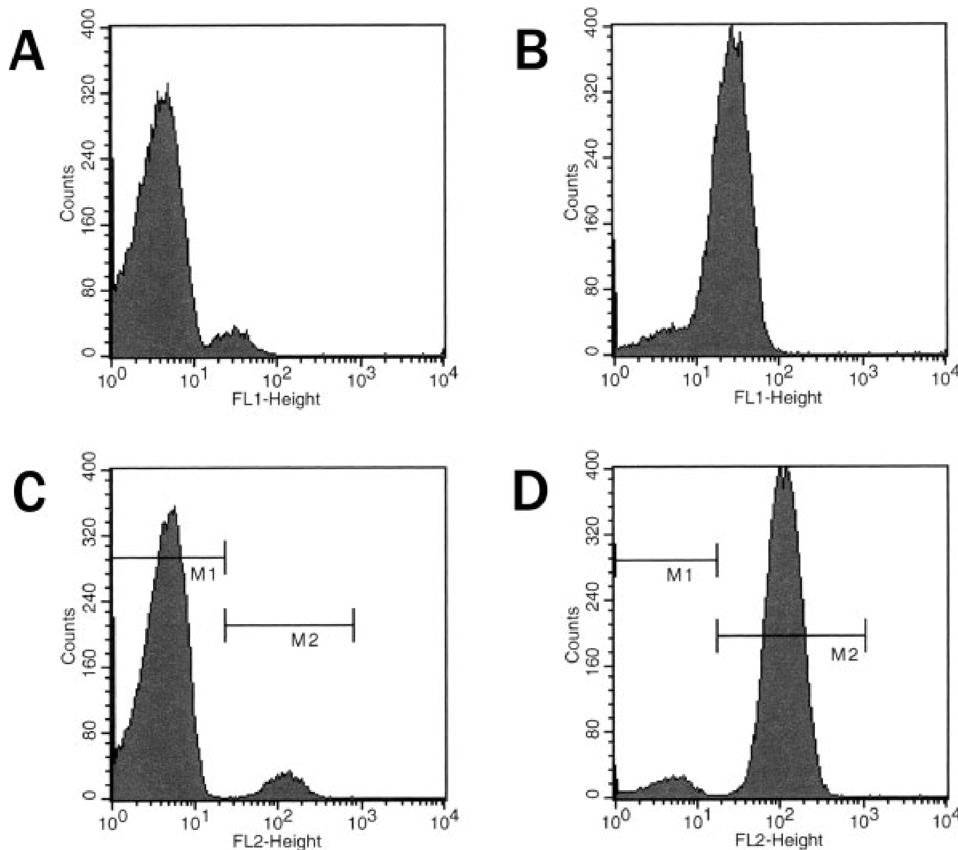

Figure 5. FITC- vs. PE-labeled secondary antibodies. Fluorescence histograms showing results of 5% Fy(b+) RBCs in Fy(b−) RBCs (A, C), and 5% Fy(b−) RBCs in Fy(b+) RBCs (B, D). The primary antibody was polyclonal anti-Fy^b; secondary antibodies were either FITC Fab anti-human IgG (A, B) or PE Fab' anti-human IgG (C, D). Use of the PE-labeled secondary antibody amplified the signal, resulting in clear separation between Fy(b−) and Fy(b+) RBCs, whereas use of the FITC-labeled secondary antibody did not.

the antigens they are directed against, by testing several antigen-positive and antigen-negative RBCs to rule out the presence of other common antibody specificities. Antibodies of animal origin (e.g., rabbit) obtained from non–blood-banking sources will need to be tested to show that they lack heterophile antibodies in addition to the above. This is done by testing fresh group O, A, and B RBCs with the antiserum; if reactivity occurs then the appropriate dilution when reactivity is no longer detectable needs to determined [81]. Fluorochrome-labeled secondary antibodies are available from immunology companies (not blood bank reagent manufacturers) and thus have not been standardized for use with RBCs and sometimes not even for use by flow cytometry. The appropriate dilution of secondary antibodies for labeling RBCs for flow cytometry must be determined. Assays must be standardized for each combination of primary antibody and secondary antibody (if used), determining the optimal fluorescence of antigen-positive RBCs and the signal-to-noise ratio, while avoiding agglutination.

Method Validation

The selected assays must be shown to reliably detect minor (e.g., 1–5%) RBC populations (antigen-positive and antigen-negative) in several samples, with low intra- and inter-assay CVs. This is to ensure that detection and survival of transfused RBCs can be followed over time in athletes before and after competition. The laboratory should participate in proficiency or external quality assurance testing for the assays. Accurate records of materials, methods, and results must be kept.

Flow Cytometry for Detection of Blood Doping

Three papers by Nelson et al. at the Royal Prince Alfred Hospital in Australia have been published specifically addressing the use of flow cytometry as a method to detect allogeneic blood transfusion in athletes [56–58]. Many of the issues discussed earlier were addressed: (a) using in vitro mixtures of RBCs, (b) testing samples obtained from patients after transfusion, and (c) performing validation studies with other laboratories. A more recent paper by Voss et al. at the Center for Blood Doping Research in Germany also investigated a flow cytometric method using commercial antibodies for detection of allogeneic blood transfusion [72].

In vitro RBC mixtures

Nelson et al. [56] first reported on studies with in vitro RBC mixtures. As the blood volume of an adult is 4–5 l, the authors assumed that transfusion of a single unit of blood would represent about 10% of the recipient athlete's RBCs. Thus, they spiked some RBC samples with 10% RBCs from donors matched for ABO and Rh(D) and then tested the samples blind by flow cytometry. In the first group of 10 samples, 10 antibodies were used (anti-c, -E, -e, -K, -k, -Fy^a, -Fy^b, -Jk^a, -Jk^b, and -s); minor populations were detected in six of the seven spiked samples and in none of the three unadulterated samples. In the second group of 10 samples, 12 antibodies were used (anti-C and anti-S were added); minor populations were detected in all of seven spiked samples and in none of three homogeneous samples. Thus, there were no false-positive results; the one false-negative in the first group would have been detected with the use of anti-S.

In vitro RBC mixtures and transfused patients

In Nelson's 2003 study, RBC phenotypes of 25 patients were determined serologically prior to transfusion of 1–3 units [57]. For flow cytometry, the primary antisera were polyclonal, mostly IgG (an IgM agglutinin in the anti-C reagent was destroyed by DTT treatment), then FITC-labeled secondary antibodies were used and 50,000 events were analyzed. When testing in vitro mixtures, not all primary antisera gave clear-cut separations between antigen-positive and antigen-negative RBCs. Anti-M and anti-S often gave ambiguous results. Using 12 antibodies, 22 of the 25 patients were determined to have mixed cell populations; 21 of these had mixed cell populations with more than one antisera (e.g., anti-c, -E, -K, and -Fy^a). Three patients did not have detectable mixed cell populations and it transpired that they were never transfused.

False-positive results were not a problem in this study [57]. With respect to false-negative results, Nelson et al. felt it would be unusual for a donor and a recipient to be matched for all antigens in the screen, and that more antibodies could be tested for further discrimination. They noted that transfused RBCs were detectable for some weeks post-transfusion, and that it was possible to detect a minor population of antigen-positive cells as well as a minor population of antigen-negative cells. Although antigen-positive (fluorescent) cells can be detected with a sensitivity of $<1\%$, it is more difficult to accurately measure very few antigen-negative cells unless care has been taken to eliminate debris when gating RBCs as these background events will not be fluorescent. The greatest problem associated with the use of flow cytometry for the detection and quantification of mixed RBC populations was found to be the lack of suitable nonagglutinating IgG monoclonal antibodies directed against appropriate RBC blood group antigens [57].

Inter-laboratory validation studies

In 2004, Nelson et al. reported on validation of their flow cytometric method [58]. They prepared and sent single or mixed RBC suspensions to six flow cytometry laboratories with reagents (e.g., diluted antisera) and detailed instructions, and received widely varying results in return. The authors examined each stage of the method and then asked four of the flow cytometry laboratories to progress stepwise through an extended procedure. In Step 1, the operators were supplied with pre-stained single populations of c+ and c− RBCs, and a 10% c+/90% c− mixture for analysis (with histograms to represent the expected results) so that they could optimize their flow cytometer settings; the results were in close agreement. In Step 2, the operators were sent single populations of K+ and K− RBCs and two K+/K− mixtures for staining and analysis. Two operators had difficulty obtaining separate peaks, but after some troubleshooting all four laboratories were able to correctly identify the samples with mixed cell populations and those with homogeneous populations. In Step 3, after resolving the sources of variation, six blinded samples were supplied to the operators for independent analysis; all agreed in their interpretation of evidence or lack of evidence for a mixed cell population. Background events for an antigen-positive minor population were found to be low ($\leq 0.4\%$) but were higher ($\leq 1.1\%$) for an antigen-negative minor population. Thus it was difficult to accurately quantitate a minor population of RBC lacking an antigen.

The issues that were identified in this study were: (1) the laboratories did not have a protocol for testing RBCs; (2) the operators learned to reset the electronic gates for each antiserum; (3) some instruments needed normal servicing or thorough cleaning; and (4) differences occurred in sample preparation that affected the results, e.g., centrifuge speed and decanting of supernatant during RBC washes. Nelson

et al. [58] felt that the use of directly conjugated IgG monoclonal antibodies might avoid some of these problems in the future.

Limitations of commercial IgM blood grouping antibodies

Voss et al. [72] reported their experiences when validating a flow cytometric procedure in their doping research laboratory. Using in vitro prepared mixtures of RBCs, they looked at linearity, specificity, recovery, precision, robustness, and inter-day precision for each of eight commercial primary antibodies. Six were monoclonal IgM antibodies (anti-C, -c, -E, -e, -Jk^a, and -Jk^b) and two were polyclonal IgG antibodies (anti-Fy^a and -Fy^b). PE-labeled anti-human IgM or IgG were used; a further amplification method of biotin-labeled anti-PE plus streptavidin-PE was used to intensify the fluorescence of weak signals and thus improve the separation of two overlapping populations. These investigators felt that their flow cytometric method was adequate for qualitative analyses, but not appropriately robust for quantitative purposes; they felt this may have been due to the use of IgM antibodies which cause agglutination and also bind to more than one antigen site resulting in low fluorescence of the labeled cells.

Troubleshooting for accurate blood doping analysis

False-positive and false-negative results must not occur. Antidoping test laboratories require repeat testing on a stored aliquot of the sample from the athlete when a positive result is obtained. False-positives could arise from poor laboratory techniques, e.g., the wrong sample being tested, contamination (carry-over) from another blood sample, or from the presence of WBCs that are highly autofluorescent or have bound IgG directly via Fc receptors. There should be at least two blood group antigens that show two populations and the percentages of the two (or more) minor populations should be similar. Clean pipette tips must be used and the flow cytometer should be adequately flushed between samples. If transfusion is suspected, a subsequent blood sample should be collected from the athlete to determine whether the percentage of transfused RBCs is reduced. It is unlikely that an athlete would have RBCs with different allogeneic blood groups without having being transfused. Although the rate of blood group chimerism has been reported to be relatively high in twins (8%) and triplets (21%) [82], DNA testing could be performed to determine if an athlete were a chimera.

False-negative results might occur if too few blood group antibodies were tested in order to detect a difference between the recipient's and donor's RBCs, as demonstrated by Nelson et al. [56]. Unavoidable false-negatives could occur if there are no antigen mismatches between donor and recipient RBCs, although this is unlikely as discussed above. However, poor techniques, such as incorrect gating, markers set improperly, too few washes of RBCs after incubation with primary antibody, instrument settings not optimized, or the presence of agglutination could all contribute to the wrong result. Insufficient labeling of antigen-positive RBCs can occur with IgM antibodies or if the antisera are too weak or not used at optimal dilution, so that the antigen-positive RBCs are not distinguishable from antigen-negative RBCs. Antigens of low copy number (e.g., Jk^a) may need amplification methods for their detection if strong antibodies are not available. High background events may camouflage an antigen-negative minor population; this would be more likely to occur in poor quality blood samples.

Conclusion

Flow cytometry has been shown by many investigators to be an excellent method for detection and quantitation of mixed RBC populations and transfused allogeneic RBCs. However, care must be taken in selecting appropriate antibodies and in designing robust methods that are necessary to achieve accurate results. This is essential for proof of blood doping. Since transfused RBCs can circulate for weeks after a transfusion, confirmation of a positive result could be accomplished by testing later samples from the athlete to show the disappearance of a minor population of cells over time.

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