Proof of homologous blood transfusion through quantification of blood group antigens

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Background and Objectives. Athletes may illegally enhance endurance performance by transfusing homologous red blood cells (RBCs) and thereby increasing the oxygen carrying capacity of their blood. Detecting this dangerous practice is difficult by currently used methods. The aim of this work was to develop tests capable of detecting a mixed red cell population by flow cytometry, utilizing the likelihood of differences in minor blood group antigens.

Design and Methods. Twelve antisera directed against blood group antigens, derived from donor plasma, were used in conjunction with a secondary antibody directly conjugated with fluorescein to label IgG-coated RBCs. Optimal concentrations of RBCs and antibodies were determined on panel cells used in blood banking for the identification of specific antibodies. Blood samples from 25 patients purportedly transfused with 1-3 units of RBCs were screened for evidence of transfusion, and the percentages of antigen-positive and antigen-negative red cells were automatically calculated by the software installed in the flow cytometer after setting gates around these populations on histograms of fluorescence.

Results. Mixed RBC populations were identified in 22 of 25 patients tested. The three patients with antigenically homogeneous populations of RBCs were subsequently found not to have received their scheduled transfusions.

Interpretation and Conclusions. This technique can detect small (<5%) populations of cells that are antigenically distinct from an individual's own RBCs. These results show the potential for flow cytometry to identify illicit homologous blood transfusion in athletes, and suggest the risk of false positives may be low.

Key words: transfusion, flow cytometry, blood-doping, blood group antigens.

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Blood doping is the scourge of endurance sports since it provides immoral athletes with an illegal performance advantage.[1] Athletes use blood doping to increase the absolute quantity of hemoglobin in their blood, and therefore the amount of oxygen that can be transported from the lungs to exercising muscle. Circulating hemoglobin can be increased by three methods: 1) using an exogenous substance such as recombinant human erythropoietin to stimulate the bone marrow to produce extra red blood cells (RBCs); 2) infusing hemoglobin-based oxygen carriers; or 3) transfusing RBCs.[1] Since recombinant human erythropoietin is detectable in urine[2] and hemoglobin-based oxygen carriers are demonstrably of non-human origin,[3] there is a commensurate risk of detection for athletes using either approach. However antidoping authorities do not have a sensitive test for blood transfusion, and anecdotal reports indicate that athletes have resorted to this technique in order to avoid sanction.

Every red cell of any individual has an identical and specific spectrum of blood group antigens which is under genetic control. Currently the only test available to antidoping authorities that is capable of detecting the consequences of blood transfusion is a partial agglutination or *mixed field* reaction.[4] This test relies on visual detection of the incomplete agglutination of RBCs in the presence of certain specific antisera. Whereas this technique may be adequate to identify a mixed field of agglutination when the RBC populations are nearly equal, it certainly cannot detect a mixture containing 10% antigen-negative RBCs, which perforce will fail to agglutinate with the antiserum. It should be noted that the infusion of one unit of blood (approximately 450 mL) will dilute the blood of the recipient (a volume of approximately 5000 mL)[5] by around 10%.

Many hematology and immunology laboratories throughout the world have established the feasibility of quantifying mixed RBC populations by flow cytometry through the use of standard blood bank antisera in combination with a fluorescent-labeled secondary antibody directed against human immunoglobulin. Analysis of red cell antigens labeled in this way is a simple and objective means to identify mixed cell populations resulting from transfusion through detection of antigens specific for either the donor or recipient.[6] Because so many antigens are involved, it would be very rare for a recipient and donor to be perfectly matched in their minor blood groupings. Provided there is at least one

antigen mismatched between donor and recipient, recognizing a subpopulation of 10% donor cells is well within the sensitivity of flow cytometric analysis (for a minor population of Rh(D)-positive RBCs within a population of Rh(D)-negative RBCs, this sensitivity has been estimated to be 0.07%).[7]

The principal objective of this research was to develop a sensitive test to detect the abuse of blood transfusion by athletes. We sought to validate the use of blood group antisera to identify mixed RBC populations in blood samples from patients receiving homologous transfusion, using the technique of flow cytometry. We also determined the optimal antibody concentrations for our panel of antisera by testing against a screening panel of RBCs used in blood banks for the identification of specific antibodies.

Design and Methods

Subjects

Blood samples were obtained from patients attending a nearby medical facility. Most patients were undergoing orthopedic surgery, which enabled evaluation of a pre-surgical blood sample (collected for routine cross-matching), together with a post-surgical sample taken within a few days of surgery (for routine blood tests). Medical records of transfusion candidates were evaluated to ensure no subjects had received a blood transfusion in the previous six months, and prior to transfusion each patient was phenotyped by a standard blood bank protocol using a tube method (in all cases patients were homogenous for antigen groups pre-transfusion). Positive and negative controls from a screening panel of RBCs were included, with antisera that were different from those used in the flow cytometric analysis of the post-transfusion sample.

In compliance with standard blood bank practice, all donor units were phenotyped only for ABO and Rh(D), normal practice except when a recipient is sensitized to a particular antigen, in which case the donor unit is then tested only for the relevant antigen(s). It was impractical to phenotype the donor blood transfused to the patients in this study because of the strictly limited access to donor unit(s) prior to transfusion.

Flow cytometer protocol

Blood samples were analyzed using a Coulter Elite XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA), with an air-cooled, software controlled, 15 mW argon ion laser operating at 488 nm. The instrument was checked daily using the standard fluorospheres available from Beckman Coulter. For RBC analysis, the voltage settings were chosen to select the appropriate cell population, the scatterplot of forward and side scatter using linear scaling, a discriminator setting on forward scatter of 100, and a bitmap drawn to exclude white cells, platelets and debris from the analysis (these settings are different for every flow cytometer).

Antigen-positive (labeled) and antigen-negative (unlabeled) red cells were evaluated using a single-parameter histogram to display fluorescence data, with the green fluorescence channel (FL1) represented on the horizontal axis on a logarithmic scale and the cell count (linear) displayed on the vertical axis. Fifty thousand events were counted in each analysis with the analyzer set to autoscale (the highest peak is displayed within the histogram and the y-axis scale then varied according to the height of that peak). The display is greatly simplified by the division of the horizontal axis into a series of discrete units or *channels*. Electrical impulses generated by the particles passing the laser light source for each of the channels are counted separately and as an absolute total, thereby generating a percentage figure as well as the individual numbers of events. Gates are set with reference to an antigen-negative population and then by eye according to the position of the peaks representing RBCs that are either positive or negative for the antigen in question. The percentages of antigen-positive and antigen-negative red cells are automatically calculated by the software installed in the flow cytometer once the gates have been set around these populations (the mean channel fluorescence or MCF values, representing the intensity of the fluorescence, are also given). The shape of the resulting histogram is greatly influenced by the type of amplification (log or linear) applied to the signals. In clinical flow cytometry, pulses which have undergone a log amplification prior to histogram display are typically utilized for the analysis of fluorescent-labeled antibody emissions.[8]

The commercially available buffer tablets normally used in preparation of wash buffers for flow cytometry have proved to be unsatisfactory because of high backgrounds in cytometric analysis of RBCs.[9] Therefore throughout the experiments described here, we used a phosphate-buffered saline (PBS) made from nine parts 0.9% sodium chloride and one part 0.15 M potassium hydrogen phosphate, adjusted to a pH of 7.2 with hydrochloric acid.

Antisera

Primary antisera to blood group antigens were obtained from a number of companies (Table 1). The source of the antibodies in every case was donor plasma, and all material had been tested and found to be negative for infectious agents according to methods licensed by the FDA. Each antibody batch, predominantly of class IgG, had been rigorously tested by the manufacturers against panels of RBCs positive and negative for the antigen of

Table 1. Antisera used in the quantification of blood group antigens by flow cytometry.

Antibody to	*Company*	*Cat. No.*	*N. batches tested*
C	Dominion Biologicals Ltd., Dartmouth, NS, Canada		1
	Immucor Inc., Norcross, GA, USA		1
c	Gamma Biologicals Inc. Houston, TX, USA	7231	1
	Dominion Biologicals Ltd., Nova Scotia, Canada		1
E	Immucor Inc., Norcross, GA, USA		1
	Dominion Biologicals Ltd., Dartmouth, NS, Canada		1
e	Gamma Biologicals Inc. Houston, TX, USA	7241	3
	CSL Ltd., Parkville, VIC, Australia	01791001	1
	Blood Bank Network, San Juan Capistrano, CA, USA		1
	Dominion Biologicals Ltd., Dartmouth, NS, Canada		1
K (KEL 1)	CSL Ltd., Parkville, VIC, Australia	02430501	1
	Biotest AG, Dreieich, Germany	808 080	1
	Dominion Biologicals Ltd., Dartmouth, NS, Canada		1
k (KEL 2)	Gamma Biologicals Inc. Houston, TX, USA	7537	2
M	Dominion Biologicals Ltd., Dartmouth, NS, Canada		1
	Blood Bank Network, San Juan Capistrano, CA, USA		1
Fy^a	Biotest AG, Dreieich, Germany	808 186	1
	Gamma Biologicals Inc. Houston, TX, USA	7591	1
	Dominion Biologicals Ltd., Dartmouth, NS, Canada		1
Fy^b	Biotest AG, Dreieich, Germany	808 191	2
	Gamma Biologicals Inc. Houston, TX, USA	7594	1
	Blood Bank Network, San Juan Capistrano, CA, USA		1
Jk^a	Biotest AG, Dreieich, Germany	808 176	1
	Gamma Biologicals Inc. Houston, TX, USA	7601	2
	Dominion Biologicals Ltd., Dartmouth, NS, Canada		1
Jk^b	Biotest AG, Dreieich, Germany	808 181	2
S	Gamma Biologicals Inc. Houston, TX, USA	7454	1
s	Gamma Biologicals Inc. Houston, TX, USA	7457	1
	Biotest AG, Dreieich, Germany	808 065	1
	Fluorescein-labeled secondary antibody fragment		
	Sheep anti-human Ig (H & L), F(ab')2, Chemicon Australia Pty. Ltd., Melbourne, Australia,	HDF	2

interest (all antibodies were marked *for in vitro diagnostic use by the indirect antiglobulin test*).

In normal blood banking practice these antisera would be used in conjunction with an anti-human immunoglobulin directed against IgG, with or without specificity also for C3d, to induce agglutination of the cells. For flow cytometric tests, agglutination of the RBCs causes blockages in the instrument and gives rise to erroneous results, so it was necessary to choose primary antibodies of class IgG and a labeled secondary antibody to introduce a marker on the antigen-positive cells. The conventional anti-human immunoglobulin was therefore replaced by a fluorescein-labeled, affinity-purified

polyclonal antibody $F(ab')_2$ fragment. We used sheep anti-human Ig (H & L), $F(ab')_2$ (Chemicon Australia Pty. Ltd., Melbourne, Australia, Cat. No. HDF) at a dilution of 1:50. Some primary antisera may require treatment with 0.01 M dithiothreitol (DTT)[10] to destroy any IgM component in the polyclonal mixture, since this can cause agglutination of the RBCs. The only antiserum in these experiments that needed this treatment was an anti-C from Dominion Biologicals Ltd.

Optimizing the antibody concentration

The antibody concentration for blood group quantification by flow cytometry is critical and serial dilutions of each batch of antiserum must be tested with a standardized volume of RBCs.

For this purpose, reference panels of RBCs (Abtectcell III) were obtained from CSL Ltd. (Parkville, Australia). These panels consist of three vials of RBCs in a 3% v/v suspension from three donors of representative Rh type R1R1, R2R2 or rr (fresh panels are issued every two weeks). The cells are provided by volunteer donors and are selected, collected and tested by the Red Cross NSW Blood Transfusion Service. For nearly all the antigens of interest, it was possible to test for homozygous, heterozygous and negative antigen expression through the selection of a combination of panels still within their expiry period. A larger panel of 11 screening cells, and thus a wider selection of antigens, is available from the same company (Phenocell) and similar extended cell panels are available from other companies such as DiaMed (Mt Waverley, Australia), Gamma Biologicals Inc. (Houston, USA) and Biotest AG (Dreieich, Germany).

From these panel cells, aliquots of 50 µL of a 3% RBC suspension (approximately 2.5×10^8 cells/mL), containing 100% positive cells for the relevant antigen, were washed once with 2 mL PBS and the supernatant removed. Doubling dilutions of the appropriate antibody were prepared in PBS and 50 µL of each dilution were added to an aliquot of RBCs and mixed by brief centrifugation followed by vortexing. The cells were incubated with the antibody for approximately 30 min at room temperature (RT) then washed once with 2 ml PBS. A 50 µL aliquot of the secondary fluorescein-labeled antibody fragment was then added to each tube and mixed as before (brief centrifugation followed by vortexing). The cells were incubated for 30 min at RT in the dark, after which they were washed once with 2 mL of PBS, resuspended in 2 mL PBS, then briskly vortexed before analysis. Tests on each antibody included the undiluted product as well as a series of doubling dilutions up to 1:32, and the chosen RBC panel included cells negative for the corresponding antigen as well as cells with homozygous and heterozygous expression of the antigen.

To allow for checks on the optimal dilution for any one antibody, cell mixtures were prepared using panel RBCs negative (nine volumes) and positive (one volume), or vice versa, for the antigen of interest. Aliquots of the mixture were incubated with three separate dilutions of the antiserum (one doubling dilution on either side of the perceived optimal dilution). The optimal dilution was defined as that which showed the widest difference in MCF between negative and positive RBCs (since the largest difference in MCF provides maximal separation along the x-axis between the two populations). Each fresh batch of antiserum was re-titrated (it was assumed that results from a chosen dilution of antisera from a single batch and single supplier remain valid at least until the expiry date).

Determination of the quantity of the secondary antibody needed for adequate labeling of the cells began with RBCs already coated with antibody at a suitable dilution of one of the primary antisera. The concentration of the fluorescent antibody was generally dictated by the manufacturer's recommendations and dilutions of 1:25, 1:50 and 1:100 in PBS were found to cover the normal working range. The specificity of the primary antiserum did not appear to influence the optimal concentration of the labeled secondary antibody.

Precision

Within-run precision studies were carried out in a mixed cell suspension, containing a 10% minority population of e-positive, K-negative RBCs. Two operators each aliquoted 10 separate vials for each antigen, and these vials were then mixed with the appropriate diluted antibody. Each vial was run three times on the flow cytometer to detect a mixed antigen population, with gates set independently by each operator. Mean values and coefficients of variation (CV) were calculated based on 60 determinations for each antigen.

Studies of imprecision due to the use of different batches of antisera (and preparation) were carried out in two mixed cell suspensions that contained a minority and a majority of RBCs positive for antigen e. Two different batches of anti-ε from Gamma Biologicals Inc (Houston, TX, USA) were used. Ten separate vials were prepared for each mixed cell population, and these vials were then mixed with the appropriate diluted antibody. Each vial was run three times on the flow cytometer to detect a mixed antigen population. The data were analyzed with nested analysis of variance, using Minitab (Minitab Inc., State College, PA, USA).

Simulation of mixed cell detection overtime

To demonstrate the ability of flow cytometry to detect mixed cell populations in an antidoping setting, panel RBCs were used to prepare a sample containing a minority population of c-positive and e-negative RBCs (to mimic a transfusion of a single

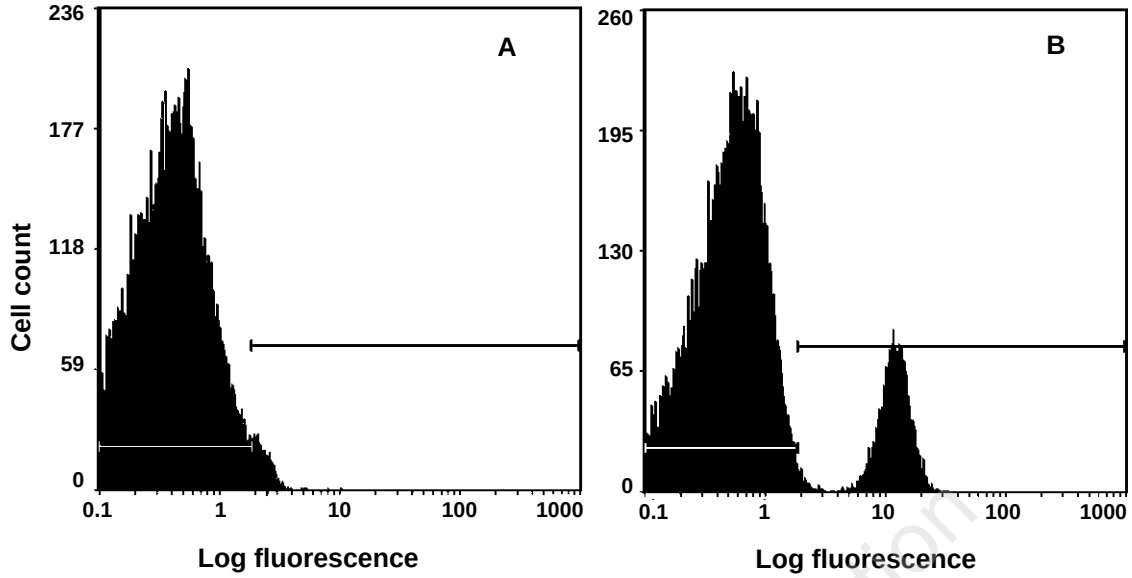

Figure 1. The effect of antibody concentration on the separation of RBCs positive or negative for the antigen KEL 1 (Kell) using flow cytometry. Antigen-negative RBCs are the highest peak, closest to the y-axis. In histogram 1A the antiserum was at a 1:2 dilution, and positive cells were barely detected as a shoulder on the right hand side of the main peak of negative cells. Histogram 1B shows the results of treatment of the same sample with a 1:8 dilution of antiserum, where the small peak representing the minority population of antigen-positive cells are clearly separated from the larger peak. The horizontal lines depict the gates set with reference to an antigen-negative and antigen-positive RBC population.

Table 2. Detection of the KEL 1 (Kell) antigen on RBC and the effect of antibody concentration on the mean channel fluorescence (MCF). Heterozygous positive and negative panel RBCs were treated with aliquots of two serially diluted antisera.

Anti-KEL 1 (CSL)			*Anti-KEL 1 (Biotest)*		
Dilution	*RBC⁺ MCF*	*RBC⁻ MCF*	*Dilution*	*RBC⁺ MCF*	*RBC⁻ MCF*
neat	0.60	0.19	neat	0.62	0.20
1:2	0.93	0.22	1:2	1.32	0.20
1:4	1.84	0.23	1:4	4.50	0.23
1:8	0.91	0.23	1:8	3.94	0.22
1:16	0.56	0.21	1:16	2.47	0.21
1:32	0.44	0.21	1:32	1.35	0.20

unit of homologous blood that differed from the recipient in being c-positive and e-negative). The mixtures were then diluted to simulate the gradual removal of the transfused cells over time by normal physiologic mechanisms. The dilution of the mixtures was calculated through the observation that following the infusion of two units of c-positive blood to a known c-negative transplant patient, the percentage of c-positive cells increased from 0.1% (equivalent to the *noise level* of the flow cytometer) to 20.6% immediately post-transfusion. The percentage of positive cells was still 9.3% after 50 days, 3.1% after 76 days and 0.4% after 111 days (*unpublished data*).

Analysis of patients' samples

Studies were carried out using blood samples collected from 25 post-surgical patients. Approximately 0.5 mL of well-mixed whole blood was washed once with 2 mL PBS and 30 µL of the packed cells were resuspended in 970 µL PBS to make a 3% suspension. Aliquots of 50 µL of the suspension were incubated with 50 µL of the appropriate dilution of each of the 12 antisera in the panel at room temperature for approximately 30 min. After one wash in 2 mL PBS, the tubes were incubated with 50 µL of the diluted secondary antibody and incubated in the dark at room temperature for approximately 30 min. The cells were washed once in 2 mL PBS and resuspended in 2 mL PBS. The cell suspensions were freshly vortexed before cytometric analysis.

Results

Optimal antibody concentrations

Many antisera exhibited a pronounced prozone effect at high concentration, but in such instances dilutions of 1:4 or 1:8 were found to give an increased MCF and good resolution between pos-

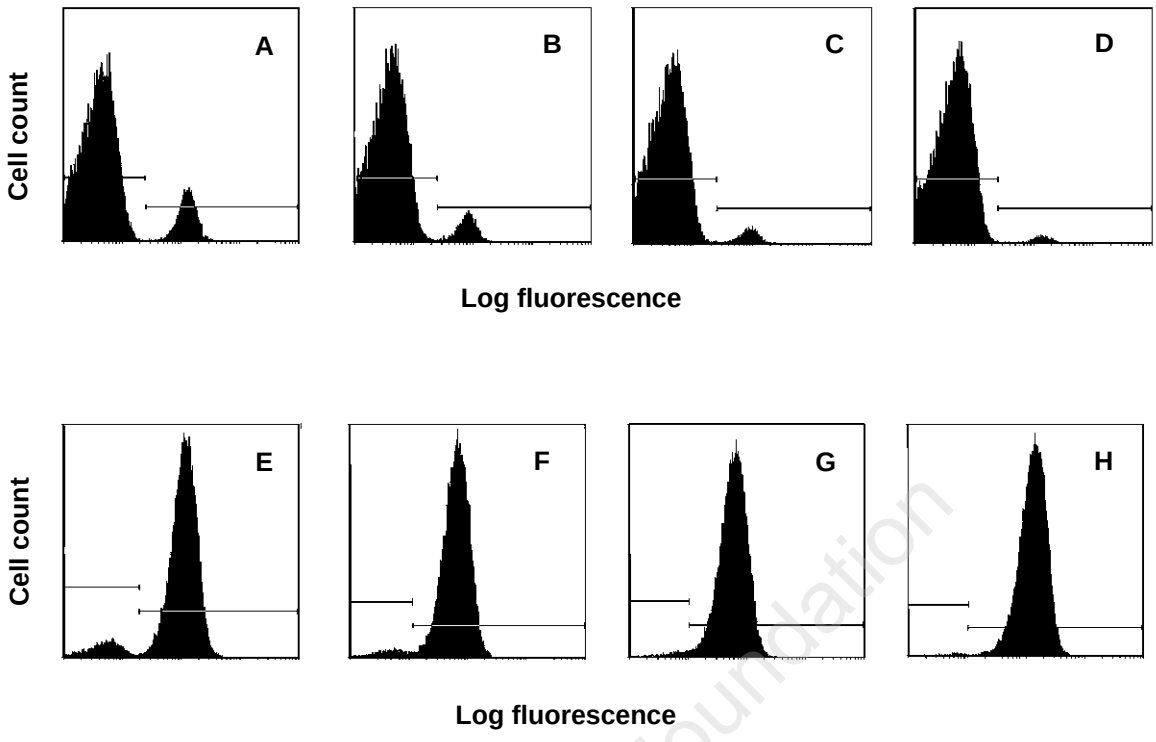

Figure 2. Flow cytometric analyses of a mixture of panel RBCs containing a majority population of c-negative, e-positive cells deliberately spiked with a minority population of c-positive, e-negative cells. Samples were subsequently diluted to demonstrate the gradual disappearance of the minor population over time. The top four histograms represent the blood sample labeled with anti-c, demonstrating a majority of c-negative cells (peak closest to the y-axis) spiked initially with 10.6 % c-positive cells simulating a transfusion with one unit of blood (2A). After 30 days, the percentage of c-positive cells would be expected to be approximately halved, as illustrated in 2B (5.5%); a further 30 days would give a histogram such as 2C (3.0%) and in another 30 days, a histogram like 2D (1.5%). The bottom four histograms represent the same cell mixtures but labeled with anti-e. The minor population was measured at 10.4% (2E), 5.1% (2F), 2.9% (2G) and 1.2% (2H).

itive and negative RBCs. It must be emphasized that because these reagents are derived from donor plasma, not only the optimal concentrations of antisera from different suppliers must be investigated, but also each batch of antiserum from any one supplier.

To demonstrate the effect of using different dilutions of antisera on the separation of mixed populations of RBCs, histograms representing two dilutions of a batch of Biotest anti-KEL 1 used in the

Table 3. Detection and quantification of a minority population of KEL 1 (Kell) antigen positive cells (heterozygous) using two different antisera at various dilutions. The MCF values for both populations are listed, together with the percentage of KEL 1-positive cells detected.

Anti-KEL 1 (CSL)				*Anti-KEL 1 (Biotest)*			
Dilution	*Positive RBC MCF*	*Negative RBC MCF*	*% Positive*	*Dilution*	*Positive RBC MCF*	*Negative RBC MCF*	*% Positive*
1:2	1.62	0.20	7.6	1:2	1.52	0.19	5.4
1:4	3.08	0.24	9.5	1:4	6.06	0.24	9.8
1:8	2.24	0.22	8.5	1:8	5.65	0.22	9.9
1:16	2.12	0.23	1.2	1:16	4.86	0.22	10.2

analysis of blood samples containing a minority population of antigen-positive cells are presented in Figure 1. Treatment of 50 µL aliquots of 3% RBC suspensions (approximately 10^7 RBCs) with a 1:2 dilution of the antiserum (Figure 1A) did not give an acceptable separation of antigen-positive and negative cells, whereas a further dilution of the antiserum to 1:4 (Figure 1B) showed a clear separation of the two populations. Dilutions of 1:8 and 1:16 resulted in histograms almost identical to those in Figure 1B. The gates were initially set using single KEL 1-negative and KEL 1–positive populations.

Table 2 lists the MCF of panel RBCs treated initially with serial doubling dilutions of anti-KEL 1 (Kell) from two different suppliers (CSL and Biotest). One of the panel RBCs tested was negative for KEL 1 and the other was a heterozygous positive. Despite the difference in the MCF values obtained with the individual antisera, the optimal dilution (chosen because it provided the highest MCF for the antigen-positive cells) was 1:4 for both. In a separate experiment, these same panel cells were used to prepare a sample containing 10% antigen-positive cells, and aliquots were treated with each of these diluted antisera. The results of the flow cytometric analysis of these mixtures appear in Table 3. The slight differences in MCF values between Tables 2 and 3 are a function of the incubation times with primary and secondary antibodies (normally each about 30 min), and to some extent the age of the diluted second antibody, which is light-sensitive.

Precision

For within-run precision studies, the mean (±SD) value (from 30 replicates from one operator) for the population of e-positive cells was 9.1±0.25% and 9.1±0.24% for the K-negative population. The coefficients of variation for the overall within-run precision from 60 determinations for the e and K antigens were 3.4% and 7.1% (respectively). Nested analysis of variance was used to identify the components of variation; CVs for *operators* were 2.1% and 6.7%, *samples within operators* 1.7% and 1.9%; and *replicates within samples* 2.0% and 1.6% (for e and K, respectively).

In addition to the overall CV, nested analysis of variance was used to identify three components of variation associated with different batches of antisera, namely *between batch*, *samples within batches* and *replicates within samples*. For the minority population of e-positive RBCs, overall CV was 2.9% and the components of variation were 0.0%, 2.5% and 1.4%, respectively. For the majority population of e-positive cells, overall CV (for e-negative cells) was 4.4%, with the three components of variation calculated to be 3.6%, 2.1% and 1.3%, respectively.

Simulation of mixed cell detection over time

The two antisera used in this test, anti-c and anti-e, provide clear evidence of two populations even when the minor one is at a very low level (Figure 2) . The top four histograms represent the cell mixtures labeled with anti-c. Figure 2A shows the majority of c-negative cells (the peak closest to the y-axis) spiked with 10.6% c-positive cells. After 30 days, the percentage of c-positive cells would be expected to be approximately halved, as illustrated in 2B (5.5% c-positive cells); a further 30 days would give a histogram such as that shown in Figure 2C (3.0%) and in another 30 days, a histogram like that in figure 2D (1.5%).

The bottom four histograms in Figure 2 represent the same cell mixtures but labeled with anti-ε (the recipient was e-positive and the transfused unit ε-negative). For this antigen, at the same (simulated) time intervals, the minor population was found to be 10.4% (Figure 2E), 5.1% (Figure 2F), 2.9% (Figure 2G) and 1.2% (Figure 2H). The explanation for why the peaks on the upper and lower histograms look different even though the percentages of minority cells were similar, is related to the log scale used on the x-axis rather than any difference in sensitivity. Specifically, because the x-axis is represented on a log scale, a minor peak close to the y-axis is spread out and the height of the peak much lower than is the case with a minor peak further along that axis. Therefore, since the peaks of e-negative cells (Figure 2G) are closer to the y-axis they will appear smaller than the peaks associated with the same percentage of c-positive cells (Figure 2C) which are further along the x-axis.

For the unmixed populations, positive events for a sample containing 100% c-negative red cells were measured at 0.2%, and positive events for 100% e-positive population were 99.5%. When the minor RBC population is positive, it is possible to be confident of a 1.5% result (Figure 2D), but when the minor population is negative, a histogram with just 2.9% (Figure 2G) still demonstrates a clearly identifiable second peak.

Not all antisera gave as clear-cut a separation between antigen-positive and negative cells as that illustrated in Figure 1B. Anti-M and anti-S frequently gave ambiguous results despite titration to optimize conditions. Such ambiguity was compounded by heterozygous expression of M or S on the donor or recipient RBC, and confidence was reduced further when the minor population was antigen-negative. However, histograms generated during the analysis of aliquots of 1, 2, 3 and 5 µL (approximately $5\text{-}25\times10^6$ RBCs) from a post-transfusion blood sample treated with an optimal dilution of anti-Fya were almost identical despite an approximately five-fold increase in the number of RBCs being tested, suggesting that the volume of

Table 4. Detection of mixed red RBC populations in 8 patients receiving 1-3 units of homologous blood matched for ABO and Rh(D) antigens and using antibody panel I. Patients 1-5 had undergone orthopedic surgery, patient 6 was an Rh(D) positive neonate transfused with Rh(D) negative blood after fetomaternal hemorrhage and patients 7-8 were transfused for correction of anemia following trauma. The hemoglobin values before transfusion are given at the bottom of the table. Rh(D) negative patients receiving Rh negative blood were not tested for other Rh antigens.

Antigen	*Patient*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
	% RBC positive							
C	99.5	88.3	8.0	99.8	99.8	21.1	70.7	
c	13.6	72.6	72.3	99.4	9.7	66.8	98.7	
E	16.6	0	86.6	10.7	0.1	0.2	19.5	
e	99.8	99.7	99.8	100	99.9	99.7	99.6	
K	81.7	68.6	69.2	0.1	0.1	0.4	10.1	0.1
k	99.8	100	99.7	99.9	99.8	99.8	99.7	99.8
Fy^a	82.4	67.0	67.2	11.5	100	37.6	10.5	25.0
Fy^b	99.8	99.8	9.8	88.7	99.7	99.8	99.5	87.7
Jk^a	100	99.7	99.8	99.9	89.2	99.8	98.4	99.9
Jk^b	99.8	99.8	100	0.3	99.4	30.2	99.6	13.2
S	0.2	29.5	29.9	11.7	89.7	0.2	0.2	24.1
s	99.9	99.9	99.8	99.9	99.9	100	99.5	88.0
Units issued	1	2	2	2	1	2 pedi	3	2
Units given	1	2	2	2	1	2 pedi	3	2
Hb g/L	113	73	80	88	108	103	68	94

Phenotype of the pretransfusion sample from each patient is as follows: 1. CCee, K+ k+, Fy^{a+} Fy^{b+}, Jk^{a+} Jk^{b+}, S- s+; 2. Ccee, K+ k+, Fy^{a+} Fy^{b+}, Jk^{a+} Jk^{b+}, S- s+; 3. ccEe, K+ k+, Fy^{a+} Fy^{b+}, Jk^{a+} Jk^{b+}, S- s+; 4. Ccee, K- k+, Fy^{a-} Fy^{b+}, Jk^{a+} Jk^{b-}, S- s+; 5. CCee, K- k+, Fy^{a+} Fy^{b+}, Jk^{a+} Jk^{b+}, S+ s+; 6. Ccee, K- k+, Fy^{a+} Fy^{b+}, Jk^{a+} Jk^{b-}, S- s+; 7. Ccee, K- k+, Fy^{a-} Fy^{b+}, Jk^{a+} Jk^{b+}, S- s+; 8. ccee, K- k+, Fy^{a-} Fy^{b+}, Jk^{a+} Jk^{b-}, S- s+.

RBCs is perhaps not as critical as is the antibody concentration.

Transfused patients

All patients received blood matched for ABO and Rh(D) except for patient 6, a baby affected by a fetomaternal hemorrhage, who received group O, Rh(D) negative blood. Patient 8 was Rh(D) negative, and it was assumed that the phenotype was most likely Rh(ccee); antisera against the Rh antigens were therefore not included in the test panel.

Table 4 lists eight patients whose post-transfusion blood sample was tested against a panel of 12 antisera (antibody panel I) including one against the antigen Cellano (k, KEL 2). This antiserum was included in the hope that the antigen expression of a heterozygote could be distinguished from that of a homozygote. The particular antiserum used in these experiments (a new batch from Gamma Biologicals Inc.) did not do this and an anti-M antibody was substituted in the subsequent test panel (antibody panel II).

In all eight patients there was clear evidence of mixed antigenic populations, and in every one there were at least two differences in antigenic repertoire. For example in patient 1, the appearance of minority populations positive for c and E, and a drop in the majority populations positive for K and Fy^a suggested that the single transfused unit differed from that of the recipient in having a CcEe, K-negative, Fy^a-negative phenotype. No transfused unit was fully phenotyped, but a mixed antigenic population was not detectable in the pre-transfusion sample. The percentage of RBCs in the minor antigen population bears a relationship to the degree of anemia as well as the number of units transfused. For example patient 7, with a hemoglobin value of 68 g/L before transfusion, received three units of homologous blood. The results of the flow cytometric analysis suggest all three were C-negative, two units were E-positive, one was K-positive and one was Fya-positive. These assumptions do not hold for the results from patient 6, a baby whose blood volume was therefore substan-

Table 5. Detection of mixed RBC populations in 7 patients undergoing orthopedic procedures and initially recorded as receiving 1-3 units of ABO and Rh(D) matched blood. Blood samples from these patients were tested using antibody panel II. The hemoglobin values before transfusion are given at the bottom of the table. After these tests were complete, patients 9, 10 and 12 were found not to have received their assigned units.

Antigen	*Patient* 9	10	11	12	13	14	15
				% RBC positive			
C	99.5	99.0	99.8	0.1	99.7	87.3	99.0
c	0.1	99.9	98.2	100	22.7	99.3	23.6
E	0.1	0.2	69.4	99.7	0.3	99.9	0.2
e	100	99.3	99.8	98.8	100	99.1	99.9
K	0.2	0.2	0.2	0.3	0.5	0.4	0.1
M	0.1	99.8	0.4	99.8	0.6	88.5	99.6
Fy^a	0.1	0.3	10.3	99.8	100	86.1	88.4
Fy^b	99.7	100	99.6	0.2	77.3	99.2	0.2
Jk^a	99.9	0.2	98.3	99.1	15.6	85.7	99.9
Jk^b	0.2	99.9	98.8	99.8	84.8	85.0	100
S	0.3	98.6	0.3	99.7	88.9	0.2	0.1
s	99.8	100	99.6	99.6	84.3	99.3	99.6
Units issued	2	2	2	2	2	2	2
Units given	0	0	2	0	2	2	2
Hb g/L	97	112	85	103	39	84	85

Phenotype of the pretransfusion sample from each patient is as follows: 9. CCee, K-, M-, Fy^a- Fy^b+, Jk^a+ Jk^b-, S- s+; 10. Ccee, K-, M+, Fy^a- Fy^b+, Jk^a- Jk^b+, S+ s+; 11. CcEe, K-, M-, Fy^a- Fy^b+, Jk^a+ Jk^b+, S- s+; 12. ccEe, K-, M+, Fy^a+ Fy^b-, Jk^a+ Jk^b+, S+ s+; 13. CCee, K-, M-, Fy^a+ Fy^b+, Jk^a- Jk^b+, S+ s+; 14. CcEe, K-, M+, ^{a}+ Fy^b+, Jk+ Jk^b+, S- s+; 15. CCee, K- M+, Fy^a+ Fy^b-, Jk^a+ Jk^b+, S- s+.

tially smaller (this patient received two 50 mL pediatric units or *pedipacks* of packed red cells).

The results from patients 9-25 tested with the antibody panel II are listed in Tables 5 and 6. There was no evidence of mixed antigenic RBC populations in patients 9, 10 and 12 despite computer records at the time the tests were done. It was subsequently found that the blood packs had been stored in the ward refrigerators (all six units were recovered).

The remaining patients were confirmed as having been transfused and there was flow cytometric evidence of transfusion with homologous blood as shown by the mixed antigenic populations. In all patients there was more than one antigen implicated in the demonstration of a mixed RBC population, except for the Rh(D)-negative patient 24.

Discussion

The technique of flow cytometry successfully identified mixed antigenic populations in patients who were transfused with at least one unit of homologous blood. The resolution of this technique is dependent on optimal concentrations of the primary antiserum and the secondary antibody fragment but less critically upon the volume of RBCs tested. The lifetime of an RBC is approximately 120 days[5] and transfused RBCs are known to remain in the circulation for at least 90 days. The capacity to detect small proportions of antigenically-distinct donor red cells, combined with the very low chance of being transfused with an identical homologous unit, recommends this as a potentially effective and sensitive method for identifying athletes who have transfused donor blood. False positive results do not appear to present a problem.

In a therapeutic setting it is essential that blood transfusions are compatible in terms of ABO blood grouping and generally also for the antigen Rh(D). Other antigenic groups present on the red blood cell surface are not considered to be of concern unless the patient has a known antibody directed against such an antigen, or has a history of adverse transfusion reactions. It is therefore unusual for patient and donor bloods to be matched for any of these minor antigens, and certainly not for all of them. Were a cynical athlete to select a donor whose red cells were lacking one antigen present on his or her own cells, it would be difficult for the

Table 6. Detection of mixed RBC populations in 10 patients transfused because of anemia caused by hemorrhage or, in the case of the neonatal patient 18, severe maternal pre-eclampsia. Samples were tested using antibody panel II. The hemoglobin values before transfusion are given at the bottom of the table. Rh(D) negative patients receiving Rh(D) negative blood were not tested for other Rh antigens.

Antigen	*Patient*									
	16	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*
	% RBC positive									
C	99.1		99.0			69.2	90.8			100
c	86.9		100			99.9	99.7			98.8
E	0.1		0.3			15.0	7.9			0
e	98.5		99.1			89.5	98.9			99.8
K	0.4	0.3	0.4	0.3	10.2	0.3	0.3	0.3	0.5	0.2
M	0.2	77.4	59.0	78.8	99.1	87.7	88.1	91.7	98.6	0.3
Fy^a	35.0	12.1	40.9	0.8	79.1	87.0	99.1	8.4	99.9	20.5
Fy^b	68.6	99.2	99.3	99.6	0.3	99.4	12.9	91.9	98.6	88.7
Jk^a	87.7	99.1	99.1	10.8	87.7	99.1	99.2	91.4	84.2	98.6
Jk^b	99.0	0.3	99.0	89.6	13.1	80.5	99.7	8.3	99.5	0.2
S	0.3	87.1	99.2	0.2	11.5	12.0	0.2	7.8	98.8	0.1
s	99.7	11.1	38.9	99.0	89.1	88.9	99.8	99.4	99.2	99.8
Units issued	2	2	1 pedi	2	2	2	2	2	2	2
Units given	2	2	1 pedi	2	2	2	2	2	2	2
Hb g/L	80	68	101	111	92	74	77	74	91	74

Phenotype of the pretransfusion sample from each patient is as follows: 16. Ccee, K-, M-, Fy^a- Fy^b+, Jka+ Jkb+, S- s+; 17. K-, M+, Fy^a- Fy^b+, Jk^a+ Jk^b-, S+ s-; 18. Ccee, K-, M+, Fy^a- Fy^b+, Jk^a+ Jk^b+, S+ s-; 19. K-, M+, Fy^a- Fy^b+, Jk^a- Jk^b+, S- s+; 20. K-, M+, Fy^a+ Fy^b-, Jk^a+ Jk^b-, S- s+; 21. Ccee, K-, M+, Fy^a+ Fy^b+, Jk^a+ Jk^b+, S- s+; 22. Ccee, K-, M+, Fy^a+ Fy^b-, Jk^a+ Jk^b+, S- s+; 23. K-, M+, Fy^a- Fy^b+, Jk^a+ Jk^b-, S- s+; 24. K-, M+, Fy^a+ Fy^b+, Jk^a+ Jk^b+, S+ s+; 25. Ccee, K-, M-, Fy^a- Fy^b+, Jk^a+ Jk^b-, S- s.

conventional partial agglutination reaction test to recognize that a minority of cells (10% donor) reacted differently to the larger population of agglutinated cells (90% recipient) during a test that targeted the missing antigen (the so-called *mixed field* scenario). In contrast, the technique of flow cytometry is able to quantify specific red cell antigens, and therefore unlike an agglutination-based approach, it can highlight small populations of cells that either express, or lack, a specific antigen. Flow cytometric phenotyping of recipient RBCs following transfusion has proved to have significant advantages over previous laborious techniques[11] and can be applied to mixed cell populations arising as a consequence of disease, or following bone marrow or peripheral blood stem cell transplantation, or through blood transfusion. These three scenarios are the only conditions under which an antigenically heterogenous RBC population could arise in any single individual, apart from special instances of hemorrhage between mother and fetus, intrauterine twin-twin transfusion, or in the rare tetragametic chimeras.[12]

The ability of flow cytometry to detect foreign cells has been extensively studied in the setting of fetomaternal haemorrhage, where a level of 0.25% represents a fetal bleed (approximately 12 mL of fetal blood). This is at the limit of efficacy of the normal maternal prophylactic dose of anti-D immunoglobulin; therefore, it is critical that a hemorrhage even slightly above this level should be accurately measured and appropriate treatment instigated.[13] Flow cytometry has been successfully applied in this specialized setting, and sensitivity at this level implies that the method has the capacity to detect antigenically-distinct RBCs for a considerable period post-infusion. Were an athlete to transfuse one or more units of homologous blood, the detection window would far exceed the 3-4 week period during which a meaningful perfor-

mance advantage could be obtained.

A limitation of this approach may be that athletes might seek to identify friends, colleagues or relatives who were antigen-matched for the specific panel of antibodies screened (candidates could be phenotyped by conventional blood banking techniques). The incidence of antigens corresponding to our panel II of antisera, as reported by Reid & Lomas-Francis,[12] means that the chance of an identical donor in a Caucasian population is 1.7 per thousand, in an Asian population (with fewer antigens surveyed) it is two per thousand and in a black American population it is three per million. However antidoping authorities are free to obtain still greater discrimination by introducing a more extensive panel of red cell antigens. Surface antigens, while the easiest to measure, are by no means the only way of identifying mixed RBC populations.

Fixing and permeabilizing the RBC allows cytosolic antigens and even internal membrane structures to be detected and quantified. It is estimated that there are over 250 discrete red cell antigens, but many of these represent very high or very low frequency polymorphisms and so are not particularly useful. We predict perhaps 20 antigens may be suitable for use in an antidoping setting.[12] Provided that the additional antigens are not publicly disclosed, it would be highly unlikely that donor blood would be specifically matched for the newly introduced test antigens.

This element of surprise would be reserved for use by antidoping authorities, and should become a significant deterrent to immoral athletes seeking to subvert the system.

The greatest problem concerning quantification of mixed RBC populations by flow cytometry is the lack of suitable IgG monoclonal antibodies directed against the minor blood group antigens. The IgG antibodies derived from donor plasma are specific in terms of their reaction with RBCs positive for the antigen in question, but there is no control over the avidity of the antibody and little control over titer. Not only are there differences in similar antibodies from different suppliers, but batch-to-batch variation from a single supplier can be even more startling. This has necessitated a close control over the antibody concentration used in the tests described here. Some antisera are considerably more satisfactory than others, a function not only of the antibody itself, but also of the number of antigenic sites expressed on each RBC. Antigen expression is not only a function of the zygosity, such that homozygous individuals theoretically express twice the number of antigens per cell than do heterozygous people, but there is also a large degree of variation between antigens. For example, Rh(D) has between 10,000 and 33,000 sites per RBC, whilst KEL 1 has only 3,000 per cell.[14] Should the flow cytometric method of detection of blood doping through transfusion become an accepted procedure, it is to be hoped that serological companies may be persuaded to develop and standardize their IgG monoclonal antibodies for use in flow cytometry rather than jettisoning them, which is their current fate. Because blood transfusion is an acute doping practice used at or immediately preceding competition, it is rational that athletes should be tested at the time of competition (post-race) rather than during out-of-competition periods. Analysis can be conducted on the same EDTA tubes (or indeed any anticoagulated blood) used for routine hematologic testing by antidoping authorities. The analytic technique is not dependent on cell characteristics prone to overt deterioration during limited storage, nor during temporary storage at room temperature, provided ambient conditions are not excessive (*unpublished observations*). Storage at 4°C is therefore ideal but not mandatory. Since donated blood is stable for five weeks in storage bags kept at 4°C, we predict antigens will be stable in an EDTA medium for at least several weeks. This provides ample opportunity for blood samples to be processed, packaged and transported to an appropriate testing laboratory.

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Pre-publication Report & Outcomes of Peer Review

Contributions

MN; analysis, protocols and interpretation of the data; MA: drafting of much of the Introduction and Discussion, particularly with regard to the current practices concerning blood doping; KS: establishment of the statistical validity of the test results; consensus was reached amongst the authors that a final decision on whether there was evidence for a mixed population should rest with the expertise of the technologist; HP: provided expert advice upon the red cell antigens that should be represented and the handling of the antisera. All four authors were involved in the revision of the manuscript. All four authors have agreed to the submission of the manuscript in the present form.

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In the following paragraphs, Professor Brugnara summarizes the peer-review process and its outcomes.

What is already known on this topic

Blood doping is a serious problem for endurance sports. While methods have been developed for the detection of recombinant human erythropoietin abuse and use of artificial blood substitutes, no reliable methods are available for the detection of blood doping via homologous blood transfusions.

What this study adds

A flow cytometric assay based on a panel of antisera against 12 blood groups antigens reliably detected the presence of homologous cells in the blood of patients who had previously received blood transfusions. The assay was capable of detecting transfused red cells with a lower limit of detection around 5% of the total red cell population. Since the antigens can be detected in blood stored at 4°C for a few weeks, confirmatory testing on duplicate samples is also a possiblity.

Caveats

In the absence of a monoclonal antibody-based panel of antisera, variations in the analytical performance of this test due to variability in potency or specificity of the polyclonal antibodies remain a major concern. Rigorous standardization of the assay will be essential to obtain reliable results in different laboratories.