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Mononuclear cells from a rare blood donor, after freezing under good manufacturing practice conditions, generate red blood cells that recapitulate the rare blood phenotype

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Abstract

Background—Cultured red blood cells (cRBCs) from cord blood (CB) have been proposed as transfusion products. Whether buffy-coats discarded from blood donations (AB) may be used to generate cRBCs for transfusion has not been investigated.

Study Design and Methods—Erythroid progenitor cell content and numbers and blood group antigen profiles of erythroblasts (ERYs) and cRBCs generated in Human Erythroid Massive Amplification (HEMA) culture by CB (n=7) and AB (n=33, three females, three males, one AB with rare blood antigens cryopreserved using CB protocols) were compared.

Results—Variability was observed both in progenitor cell content (2-fold) and number of ERYs generated (1-log) by CB and AB in HEMA. The average progenitor cell contents of the subset of AB and CB analyzed were similar. AB generated numbers of ERYs 3-times lower ($p<0.01$) than CB in HEMA containing fetal bovine serum but similar to CB in HEMA containing human proteins. Female AB contained 2-times less ($p<0.05$) erythroid progenitor cells but generated numbers of ERYs similar to those generated by male AB. Cryopreserved AB with a rare blood group phenotype and shipped to another laboratory generated great numbers of ERYs, 90% of which matured into cRBCs. Blood group antigen expression was consistent with the donor genotype for ERYs generated both by CB and AB but concordant with that of native RBCs only for cells derived from AB.

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Conclusion—Buffy-coats from regular donors, including a donor with rare phenotypes stored under conditions established for CB, are not inferior to CB for the generation of cRBCs.

Keywords

Cultured red blood cells; Erythroblasts; HEMA culture; reagent red blood cells; rare blood phenotypes; alloimmunization; transfusion

Introduction

There are 34 human blood group systems which include >260 different antigens the prevalence of which varies with ethnicity and geographical distribution of the human population^{1,2}. Migrations of individuals to areas where their blood group antigens are rare increase the risk of alloimmunization and consequently the risk of transfusion reactions³. Management of alloimmunization has both clinical and diagnostic challenges. The clinical challenge of providing sufficient blood for patients with rare phenotypes is being met through the development of local and national registries of rare blood donors and establishment of repositories of cryopreserved blood. While these initiatives have increased overall availability of rare blood, such blood is often not available in the quantity needed^{4,5}.

The diagnostic challenge has been addressed by improving the sensitivity of alloantibody detection assays and by developing DNA-based techniques to identify patients and donors with rare red blood cell (RBC) phenotypes (confirmed by serology)⁴. These diagnostic tools require reagent RBCs (rRBCs) with rare and common phenotypes. Since donors with rare phenotypes may be temporarily/permanently deferred from donation, rRBCs with rare phenotypes may be unavailable⁶. Examples of rRBCs not available in US are Cartwright (Yta-/Ytb+); Colton (Coa-/Cob+), Dombrock (Doa+/Dob-; Jo(a+)/Jo(a-); Hy+/Hy-); and Landsteiner-Wiener (LWa+/LWb-)⁷. Donors with these phenotypes may be available in other countries^{8,9} but lack of standardized licensing criteria prevents sharing of these diagnostic tools.

The development of culture techniques, defined by us as massive human erythroid amplification cultures (HEMA), that produce large numbers of cultured RBCs (cRBCs)¹⁰⁻¹⁴ allowed studies which demonstrate that CD34^{pos} cells from various sources generate *ex-vivo* great numbers of erythroblasts (ERYs) that mature into functional RBCs¹⁵. A subsequent study demonstrated that autologous cRBCs generated by mobilized CD34^{pos} cells survive *in vivo* as long as their natural counterpart, providing the proof-of-concept that cRBCs may be used for transfusion¹⁶. However, reservations still exist whether it will be ever possible to generate cRBCs in numbers sufficient for transfusion. The conviction that the erythroid expansion potential of adult blood (AB) is inferior to that of cord blood (CB) and mobilized peripheral blood^{17,18} suggests however that buffy-coats discarded from AB with rare blood phenotypes are unsuitable to generate cRBCs in numbers sufficient for transfusion^{19,20}. However, whether these buffy-coats may generate rRBCs in numbers sufficient for antibody screening/identification tests which require only several mL of blood is yet to be established¹⁵.

The aim of this study was to assess whether currently discarded buffy-coats from blood donations are suitable to generate rRBCs. These experiments involved 1) comparison of the BFU-E content, a marker that may predict erythroid expansion potential, and numbers of ERYs generated in HEMA by AB and CB; 2) analyses of the effect on erythroid expansion of media based on bovine serum (HEMA^{ser}) or human proteins (HEMA^{def})^{21, 3)} comparison of female and male AB donors and 4) determination that processing, cryopreservation and shipment procedures established for CB transplantation are suitable to preserve and ship the buffy-coat from a donor expressing rare blood group antigens for generation of cRBCs. The results obtained indicate that the erythroid expansion potential of AB from regular donors is similar to that of CB in HEMA^{def} and that conditions established for banking CB are suitable for cryopreservation of AB buffy-coats that generate large numbers of cRBCs expressing normal levels of blood group antigens.

Materials and Methods

Human specimens and cell preparation

All specimens were collected and provided for this study as de-identified material according to guidelines established by the relevant institutional ethical committees. AB: Buffy-coats (approximately 50 mL/unit) from 31 whole blood donations (450 mL/unit) were collected and prepared by Unit of Immunoematology and Transfusion Medicine of “Sapienza” University of Rome (Italy). Buffy-coats were prepared by the hard spin centrifugation method (3,550 g for 10 min at 22°C using Sorvall RC 12BP centrifuge (Thermo Fischer Scientific Inc, Waltham, MA)) used to prepare platelets. Seven CB were obtained from full-term pregnancies at the time of delivery at the Public Hospital of Pescara (Italy). DNA genotyping of red blood cell antigens of one adult donor and three cord blood donors was performed (HEA-Bead Chip, Immunocor, Norcross, GA). Sex determination was performed by PCR genotyping for sequences present on the Y chromosome³¹. Mononuclear cells (MNC) from AB buffy-coats and CB were isolated by Ficoll-Paque (Sigma, St. Louis, MO) density centrifugation according to the manufacturer's protocol and cryopreserved in Iscove's modified Dulbecco's medium (40% vol/vol IMDM Gibco-Invitrogen, Carlsbad, CA) fetal bovine serum (FBS, 50% vol/vol, Sigma,) and dimethylsulphoxide (DMSO, 10% vol/vol, Sigma). Cryopreserved samples were placed in a Nalgene Cryobox (Thermo Fisher Scientific, Roskilde, Denmark) at -80 °C overnight and then stored in liquid nitrogen.

AB donor with a rare combination of blood antigens: A caucasian male A Rh negative (ccdee) Cw-, U+, Lub+, Coa+, Vel+, PP1Pk+ donor with the combination of rare blood group antigens K-k+, Fya+b-, Jka+b- and S-s+ was identified by solid phase serological technology (Galileo, Immucor, Norcross, GA). A one-time exception from the certified laboratory used to manufacture CB under Good Manufacturing Practice (GMP) conditions at the Centre of Blood Transfusion and Immunohematology in Milan, Italy was obtained to collect, process and store blood from this donor. The buffy-coat was obtained from a regular blood donation using the dual-phase centrifugation method developed to prepare platelets. The final volume of 60 mL contained 36.93×10^9 /L white blood cells (WBC); 0.24 % packed RBCs and 1.518×10^9 /L platelets. The buffy-coat was diluted to 120 mL with DMSO (20% v/v) and dextran (80% v/v) and divided into 5 bags designed for CB storage.

Each bag contained 24 mL and 443×10^6 WBC. The bags were placed into a controlled rate freezer (Nicool Plus PC, Air Liquide Sanita', Milan, Italy) and cryopreserved as previously described for CB²³.

Colony forming assay

MNC (10^5 cells/mL) were cultured in methylcellulose cultures (MethoCult, Stem Cell Technologies, Inc., Vancouver, BC) stimulated with human recombinant SCF (10 ng/mL, Amgen, Thousand Oaks, CA), IL-3 (10 ng/mL, Biosource, San Jose, CA), G-CSF (100 ng/mL, BOUTY SpA, Milan, Italy) and EPO (5 U/mL, Neorecormon, Auckland, New Zealand). The cultures contained either FBS (30% vol/vol, Hyclone, Logan, UT, USA) (HEMA^{ser}) or clinical grade proteins of human origin (HEMA^{def})²¹ and were maintained for 14 days at 37 °C and 5% pCO₂ in a fully humidified incubator.

HEMA culture

MNC (10^6 cells/mL) were cultured in IMDM plus FBS (HEMA^{ser}) or supplements of human origin (HEMA^{def})³⁰ and stimulated with SCF (50 ng/mL), IL-3 (1 ng/mL), EPO (5 U/mL), dexamethasone and estradiol (both 10^{-6} M, Sigma-Aldrich)¹³. HEMA^{def} is formulated with deionized human serum albumin (HSA, 10% v/v, Baxter International Inc., Deerfield, IL, USA), human iron saturated transferrin (h-TRF, Sigma-Aldrich), liposomes of HSA plus cholesterol (400 µg/mL, Cat. no. C3045, Sigma-Aldrich) and soybean lecithin (1.2 mg/mL, Cat. no. P3644, Sigma-Aldrich), recombinant human insulin (Merck KGaA, Darmstadt, Germany, endotoxin content <0.25), β-mercaptoethanol, sodium pyruvate, nucleosides, trace elements and L-glutamine (all from Sigma-Aldrich). Details on the preparation of the components of HEMA^{def} can be found in²¹. The cultures were kept for 7-17 days at 37 °C and 5% pCO₂ in fully humidified incubator. Day 7-ERYs (5×10^6 cells/vial) from the AB with a combination of rare blood phenotypes were cryopreserved as described²⁴.

Differentiation cultures

ERYs were induced to mature by 5 days of culture with EPO (3 U/mL) and human recombinant insulin (10 ng/mL, Calbiochem, Darmstadt, Germany)¹³. Cryopreserved day 7-ERYs were thawed and cultured for three additional days in HEMA^{ser}²⁵ and induced to mature by 7 days culture in StemSpan (Stem Cell Technologies) plus EPO (10 U/mL), thyroid hormone (1 µM), insulin (40 µg/mL), holotransferrin (100 ng/mL) and human serum (3%, vol/vol)¹⁴.

Determinations of cell numbers, viability and maturation state

Cell numbers and viability were determined by microscopic evaluation of cells stained with trypan blue (Boston Bioproducts, Ashland, MA, USA) in a Burker chamber. Viability and maturation was assessed by flow cytometry determinations of propidium iodide staining and expression of CD235a (Glycophorin A) plus CD36 or CD71, and CD117 (cKIT). Eucleated cells were recognized on the basis of size and absence of Hoechst staining^{14,25}. Fluorescence was measured either with Aria (Becton Dickinson, Franklin Lakes, NJ) or FACS LSRII (Becton Dickinson Biosciences, Breda, The Netherlands) cell sorters and data

analyzed with Flowjo Software (Tree Star, Inc., Ashland, OR). Maturation state was confirmed by visual examination of cytocentrifuged cell preparations (Cytospin 3, Shandon, Astmoor, England).

Blood group antigen profiling

ERYs and native RBCs were analyzed by flow cytometry using antibodies recognizing antigens present on proteins in the 4.1R [Duffy (Fy^a, Fy³), Kell (Kell prot, K/k, Kp^a/Kp^b, Js^b), glycophorin C (GYPC, Ge2)] and ankyrin R [glycophorin A (GYPA, CD235a, M, EnaFS), RhAG, band 3 (Wr^b)] complexes and on GYPB (s, U), Kidd (Jk3), CD35, CD55 and CD59. cRBCs were analyzed with antibodies for the Rh protein (BRIC69; IBGRL, Bristol, UK), RhAG (2D10; Sanquin, Amsterdam) and Band-3 (BRIC6; IBGRL, Bristol, UK).

Statistical analysis

Statistical analysis was performed by Anova, unpaired and paired t test, as appropriate, with Origin 5.0 for Windows (Microcal Software, Inc., Northampton, MA, USA).

Results

Erythroid progenitor cell content does not predict the numbers of ERYs generated in HEMA by AB and CB MNC

Variability in frequency of progenitor cells^{26,27} and ability to generate ERYs in liquid culture^{13,28} by MNC from different AB and CB has been reported. To establish whether the two variabilities are related, numbers of hematopoietic colonies generated in semisolid cultures and of ERYs generated in HEMA^{ser} by 26 AB and seven CB donors were compared.

CB MNC contained more CFU-GEMM (9 ± 6 vs 1 ± 1 per 10^5 MNC) and CFU-GM (65 ± 32 vs $19 \pm 13/10^5$ MNC) than AB MNC but the numbers of BFU-E (47 ± 32 vs $53 \pm 22/10^5$ MNC) and of total progenitor cells (123 ± 82 vs $73 \pm 28/10^5$ MNC) present in the MNC of these subsets of CB and AB donors was not significantly different (Table 1).

By contrast, CB MNC generated 4-times more ERYs than AB MNC (Figure 1). Fold increases (FI) ranged from 12-89 for CB and 1.7-47 for AB by day 14 and 8-178 for CB and 3-37 for AB by day 17. The mean FI observed at day 14 and 17 with CB and AB MNC were significantly different (day 14: 39 ± 27 vs 13 ± 11 , $p < 0.005$ and day 17: 51 ± 63 vs 12 ± 10 , $p < 0.05$).

To evaluate the reproducibility of ERY expansion from individual donors, numbers of ERYs generated in triplicate HEMA^{ser} cultures of three de-identified donors were determined. Consistently with previous observations¹³, FI observed in three replicate cultures from the same AB differed only by 10% (data not shown).

Using three donors, we reported that the use of humanized media (HEMA^{def}) increases the numbers of ERYs generated by AB MNC but not those generated by CB MNC²¹. To further assess the effects of media on ERY expansion, numbers of ERYs generated by day 14 and

17 by 7-19 AB and 3-6 CB in HEMA^{ser} and HEMA^{def} were compared (Figure 2). HEMA^{def} media did not reduce the variability in expansion observed in cultures from different donors which remained high. However, it significantly increased the numbers of ERYs generated by AB MNC (day 14: FI 14±11 vs 67±63, $p<0.01$; day17: FI 15±12 vs 136±200, $p<0.005$). In this limited (n=three) number of experiments, increases in numbers of ERYs generated by CB MNC in HEMA^{def} were not statistically significant (day 14: FI 38±27 vs 88±88; day 17: FI 51±63 vs 228±244).

Previous studies documented that gender affects the frequency of progenitor cells present in the circulation²⁹. To assess whether gender may affect the erythroid expansion potential of AB MNC, number of hematopoietic colonies and of ERY generated in HEMA^{def} and HEMA^{ser} by MNC from three male and three female AB donors were determined (Table 2, Figure 3). In HEMA^{ser}, MNC from female donors generated ~40% less BFU-E than those from males (65±16 vs 113±23 BFU-E/10⁵ MNC, $p<0.05$) (Table 2). This gender difference persisted when two female donors and two male donors were cultured under HEMA^{def} conditions. By contrast, MNC from female and male donors generated similar numbers of ERYs both in HEMA^{ser} and HEMA^{def} (Figure 3). By day 14, the FI in cultures from females and males were 11±3 vs 11±7 in HEMA^{ser} and 80±20 vs 82±10 in HEMA^{def}. Also in this set of experiments, AB MNC generated 10-times more ERYs in HEMA^{def} than in HEMA^{ser} ($p<0.005$).

These results confirm the existence of great donor-related variability in numbers of ERYs generated by AB MNC in vitro and indicate that components of the media (HEMA^{def}) play a greater role in determining the number of ERYs generated in culture than ontogenetic stage and gender.

Blood group antigen profiling of ERYs generated by CB

Proper expression of blood group antigens will play a major role in determining whether cRBCs will be considered suitable for transfusion³⁰. We previously reported that ERYs obtained from a donor of African-American descent express 19 blood group antigens at levels similar to those expressed by the native RBC from the same donor (³¹, Figure S1). Experiments were here performed to confirm these results and to assess expression of blood group antigens by ERYs generated by CB MNC.

First, the presence of nine blood group antigens on ERYs expanded from one AB donors and two CB donors was determined by molecular genotyping and by flow cytometry with specific antibodies (Table 3). Native RBCs from the same donors served as controls. By flow cytometry, concordance in antigen expression between ERYs expanded from the two CB and from AB and the corresponding native RBCs was 100%. The phenotype observed by flow cytometry for both ERYs and native RBCs was 95% concordant with that predicted by genotype.

The levels of 19 blood group antigens expressed by ERYs expanded *in-vitro* from three CB were then compared (Figure 4). These antigens included proteins present on the Ankyrin R and 4.1 R complexes as well as GYPB, the Urea transporter, the complement receptor CD35 and the proteins that mediate protection from complement CD55 and CD59. ERYs expanded

from CB expressed normal levels of antigens of the ankyrin R complex and of Duffy and GPYA on the 4.1R complex. However, although Kell protein and its k and Js^b epitopes were expressed at normal levels, the Kp^a epitope was expressed at levels 2-times greater than normal and K was not expressed. Normal levels of expression were also observed for U and s antigens on GYPB, Jk:3, CD35, CD55 and CD59 although a large cell-to-cell variability in expression levels was observed. In mature ERYs from CB, the expression of Ankyrin R associated antigens remained normal but antigens associated with complex 4.1R, JK:3 and CD35 were expressed at levels lower than normal (Figure 4).

Expansion and characterization of cRBCs from MNC from the donor with a combination of rare blood phenotypes

To clarify whether GMP conditions established for cryopreservation, storage and shipment of CB are suitable for buffy-coats, a feasibility study was performed. The buffy-coat from one donation from a Caucasian male with the ccdee/K-/Fyb-/Jkb-/S- phenotype was divided into 5 aliquots (450×10^6 WBC/each), and cryopreserved according to procedures standardized for collection of CB in Milan, Italy²³. One aliquot was shipped to Rome where it was thawed according to procedures established for CB³² and MNC prepared by Ficoll-fractionation. The separation provided 50×10^6 MNC that were cultured in HEMA^{ser}. By day 7, the cultures contained 50×10^6 ERYs (FI = 1 vs FI = 1.2 ± 0.6 observed at day 7 in HEMA^{ser} of 26 AB donors, Figure 1) with an immature CD36^{pos}CD235a^{neg} phenotype (not shown). Day 7-ERYs were cryopreserved (10 aliquots of 5 million each) according to procedures established for these cells²⁴ and one aliquot shipped to Dr. van den Akker for assessment of terminal maturation potential. Dr. van den Akker thawed the cells as described²⁴ and expanded them for three additional days in HEMA^{ser} and then induced their maturation by 7 days culture in differentiation media. At the end of this period, 93% of cells had the morphology of RBCs (not shown), were Hoechst 3342 negative and expressed robust levels of CD71, CD235a as well as of RhAg, Band 3 and Rh protein but did not express cKIT (Figure 5). The expression of RhAg and Band 3 was detected as a single narrow peak similar to that observed with native AB RBCs (Figure S1).

These results indicate that AB buffy-coats collected and stored under GMP conditions established for CB generate in HEMA^{ser} great numbers of ERYs capable of terminal maturation in vitro.

Discussion

Several investigators are exploring the use of low volume cord blood, a stem cell source currently discarded, to produce cRBCs for transfusion²⁸. Buffy-coats from donors expressing rare blood group phenotypes are currently discarded because they are thought unsuited to generate cRBCs for transfusion since their erythroid progenitor cell content is lower than that of CB²⁷. This study was conducted to clarify whether the erythroid expansion potential of buffy-coats from regular blood donations is inferior to that of CB and to assess whether conditions established to store CB for transplantation are suitable to store buffy-coats from donors with rare blood phenotypes.

First, numbers of ERYs generated in HEMA and erythroid progenitor cell content of MNC from 26 AB and seven CB donors were compared. Ten-fold differences in numbers of both BFU-E (Table 1) and ERYs (Figure 1) generated by MNC from different AB and CB donors were observed. However, the numbers of ERYs generated in replicate cultures of three AB donors varied only by 10% (data not shown), suggesting that the variability in numbers of ERYs generated in HEMA is an intrinsic property of the donor.

The erythroid progenitor cell content of the blood from the 26 regular donors was not statistically different from that of seven CB (Table 1). By contrast, previous studies demonstrated that blood from non-challenged adult individuals contains at least 10-times less progenitor cells than CB (<1 vs 40.88 ± 0.63 CFC per μL of blood, 30 vs 300 CFC per 10^5 MNC)³³. The greater CFC content of blood from regular donors observed in the present study may reflect either mobilization of stem/progenitor cells as a result of mild increases in erythropoietin levels^{34,35} and/or the presence in this population of genetic factors, still to be identified, that favor stem cell mobilization. While further studies will clarify the mechanism(s) underlying the greater progenitor cell content of blood from regular donors, this observation indicates that buffy-coats from regular donations are as suitable as CB to generate cRBCs.

The number of ERYs generated by AB MNC was lower than that generated by CB MNC in HEMA^{ser} (Figure 1) but AB and CB MNC generated similar numbers of ERYs in HEMA^{def} (Figure 2). As expected²⁹, MNC from female donors contained less BFU-E than those from males (Table 2) but generated numbers of ERYs similar to those generated by MNC from males both in HEMA^{ser} and HEMA^{def} (Figure 3). These results confirm that in HEMA^{def} AB MNC generate numbers of ERYs not inferior to those generated by CB MNC and indicate that BFU-E content may represent only one of the intrinsic properties of donor MNC that determine erythroid expansion potential in HEMA.

Blood group antigen expression is an important criterium to determine whether cRBCs are functional³⁰. In a subset of one AB and two CB, a good qualitative correlation between genotype and phenotype for 9 blood group antigens was observed both with native RBCs and with ERYs (Table 3). Similarly good qualitative correlations between blood group antigen genotype and phenotype of cRBCs obtained from CB and mobilized CD34^{pos} cells have been reported by other investigators¹⁶.

Quantitative data on blood group antigen expression on ERYs obtained in culture are scanty. We have previously reported that ERYs from AB express 19 blood group antigens at levels quantitatively comparable to those expressed by native RBCs³¹. Here we show that although expression of 8 antigens in ERYs obtained from three CB was qualitatively similar to that of native RBCs, expression of some of the 19 antigens previously analyzed for ERYs from AB was greatly reduced in ERYs from CB (Figure 4). Orthochromatic ERYs from CB express barely detectable levels of antigens present on proteins of the 4.1R complex. These complexes are partially assembled in the cytoplasm at early stages of erythroid maturation and then translocated to the membrane to be embedded into the lipid bilayer³⁶. Unbalanced expression of antigens of the 4.1R complex suggests that ERYs from CB may partially fail to complete the final stages of the assembly process. Orthochromatic ERYs and cRBCs from

CB also expressed reduced levels of CD35, Jk:3, CD55 and CD59 (Figure 4 and van den Akker, unpublished observations). CD35 recognizes an antigen on the erythrocyte complement receptor 1 the expression of which decreases in normal RBCs with aging³⁷. Jk:3, on the urea transporter, protects RBCs during the passage in the capillaries of the kidney³⁸. CD55 and CD59 protect RBCs from complement-mediated lysis³⁹ and their reduced expression is responsible for hemolysis in paroxysmal nocturnal hemoglobinuria⁴⁰. These considerations predict that reduced expression of CD35, Jk:3, CD55 and CD59 on cRBCs from CB makes them unsuited for both diagnostic and clinical use.

By contrast, cRBCs expanded from one AB donor with a rare blood phenotype expressed robust levels of three major blood group antigens (Figure 5), confirming previously published results on a larger number of blood group antigens³¹ suggesting that expression of these antigens on cRBCs from AB is quantitatively normal (Figure S1). Encouraging quantitative data on the numbers of cRBCs that may be generated by the buffy-coat of a rare blood donor were obtained. One-fifth of the buffy-coat from one donation stored under conditions established for CB generated in HEMA^{ser} 50×10^6 ERYs by day 7 which underwent further expansion in the van den Akker laboratory. Although FI in this second expansion was not determined, we estimate that one-fifth of buffy-coat would generate $\sim 6.8 \times 10^8$ ERYs by day 14 ($50 \times 10^6 \times 13$, the average FI for AB MNC in HEMA^{ser}, Figure 2). Since the efficiency of ERYs enucleation was $>90\%$ (Figure 5)¹⁹, one-fifth of buffy-coat can generate $\sim 6 \times 10^8$ cRBCs, a number of cells sufficient for >300 antibody detection/identification assays (2×10^8 rRBCs are sufficient for 100 assays). The normal levels of blood group antigen expression and these quantitative data suggest that currently discarded buffy-coats with rare blood phenotypes are suitable to generate cRBCs to be used as rRBC.

Blood centers have already established programs for cryopreservation and international sharing of rare blood products for clinical use⁴¹. The availability of molecular techniques for blood genotyping facilitates the establishment of national registries of donors with rare phenotypes and provides uniform reagents and methodology for genotype determinations. However, storage of buffy-coats from rare blood donations is not yet a standard procedure and international rules for sharing information and buffy-coats have not been established. As a result, if a donor with a rare phenotype becomes unavailable, there is no mechanism to obtain buffy-coats from donors with similar phenotypes in another registry. At least in Europe, buffy-coats are routinely produced from whole blood donations to harvest platelets for clinical use and then, after platelet recovery, are discarded⁴². We provide evidence that buffy-coats cryopreserved with procedures established for CB generate in other laboratories great numbers of ERYs and cRBCs expressing normal blood phenotypes. These experiments provide proof-of-principle that stored buffy-coats (and/or day 7-ERYs) may be distributed to generate rRBCs under conditions compliant with local GMPs. Since MNC from CB^{43,44} and ERYs from AB²⁴ remain functional for >8 -10 years after cryopreservation, this proof-of-principle suggests that establishment of buffy-coat (and/or day 7-ERY) repositories of donors with rare phenotypes represent a cost-effective approach to create a long-term resource for ex-vivo generation of rRBCs. Additional studies, including greater numbers of rare blood donors and comparative assessment of performance in diagnostic assays of cRBCs and RBCs from the same donor are necessary to validate the use of cRBCs as rRBCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparison of ex-vivo ERY expansion observed over time in HEMA^{ser} of MNC from different AB (n=26) and CB (n=7) donors

Results are presented as fold increase (FI) with respect to day 0. FI observed in cultures from individual donors are presented in A and B (each color a different donor) and mean ($\pm$ SD) FI values are presented in C and D. The mean FI observed at day 14 and 17 in cultures of CB (38.4 $\pm$ 26.6 and 50.6 $\pm$ 63.0) and AB (13.3 $\pm$ 11.3 and 12.0 $\pm$ 10.4) are statistically significant (p 0.005 and 0.05, respectively).

Figure 2. Comparison of ex-vivo ERY expansion observed at day 14 (panel A) and 17 (panel B) in HEMA^{ser} and HEMA^{def} cultures of AB and CB

Results are expressed as median (the line across the boxes) 25-75% inter-quartile range (the boxes) and maximum and minimum value (the end of the vertical line across the boxes) of FI obtained in cultures from different donors. The number (n) of individuals analyzed in each condition is indicated in parenthesis. Statistical analysis was performed by Anova and unpaired t test. Values obtained in HEMA^{ser} cultures of AB and CB are statistically significant (*, $p < 0.05$). Statistically different are also values observed in HEMA^{ser} and HEMA^{def} cultures of AB (##, $p < 0.01$; ### $p < 0.005$).

Figure 3. Comparison of ERY expansion observed over time in HEMA^{ser} (A) or HEMA^{def} (B) cultures of three separate female (red) and male (black) donors. MNC from each donor were cultured in parallel under the two conditions and results are expressed as mean ($\pm$ SD) FI of three separate experiments.

Figure 4. Quantitatively different blood group antigen expression by cord blood erythrocytes generated *In-vivo* and *Ex-vivo*

A) Flow cytometric (CD36/CD235a expression) and morphological characterization of the three cell populations analyzed: Native RBCs from the same CB used for expansion (top panels), erythroblasts generated at day 10 of HEMA^{ser} (Prol Day 10, middle panels) and orthochromatic normoblasts obtained after 5 days of culture under differentiation conditions (Diff Day 5). B-C) Flow cytometric analyses for the expression of blood group antigens presented on the Ankyrin R (B) and 4.1 R (C) complexes and of Glycophorin B, the urea transporter JK3, the complement receptor CD35 and the proteins that protect from complement CD55 and CD59 (D) of the same cells presented in A. Similar results were obtained in two additional experiments, each one with a different donor.

Figure 5. ERYs expanded *ex-vivo* from an adult donor expressing a combination of rare blood phenotypes generate numerous enucleated (93%) cRBCs expressing robust level of CD71, CD235a, RhAg, Band 3 and Rh but do not express cKit

A) Forward (FSC) and side (SSC) scatter analyses and Hoechst 3342 staining of ERYs at day 10 of expansion and of cRBCs obtained after 7 days of culture in differentiation media. B) Flow cytometric analyses for CD235a (GYPA) and CD71 (dot plot) and for antibodies against GYPA, RhAg, Band 3, the Rh protein and cKIT, the receptor for SCF, (histograms) of the cRBCs presented in A. Red histograms indicate isotype controls.

Table 1
Comparison of the progenitor cell content of MNC from different AB (n=26) and CB
(n=7) donors (the same donors analysed in Figure 1)

Results are presented as mean ($\pm$ SD) number of colonies per 10^5 MNC. The difference between the progenitor cell content of the AB and CB subset analyzed in these experiments is not statistically significant by t test.

	BFU-E	CFU-GM	CFU-GEMM	TOTAL
AB	53 $\pm$ 22	19 $\pm$ 13	1 $\pm$ 1	73 $\pm$ 28
	BFU-E	CFU-GM	CFU-GEMM	TOTAL
CB	47 $\pm$ 32	65 $\pm$ 46	9 $\pm$ 6	123 $\pm$ 82

Table 2
Comparison of progenitor cell content of MNC from three female and three male donors cultured under HEMA^{ser} conditions

Two male and two female donors were also cultured in HEMA^{def}.

HEMA ^{ser}	BFU-E	CFU-GM	CFU-GEMM	Total
Male (n=3)	113 ±23	32 ±17	2 ±1	147 ± 39
Female (n=3)	65 ±16	24 ±9	2 ±3	94 ±21
HEMA ^{def}	BFU-E	CFU-GM	CFU-GEMM	Total
Male (n=2)	121 ±21	37 ±18	4 ±2	164 ±36
Female (n=2)	47 ±12	24 ±17	7 ±5	79 ±35

p<0.05

not applicable

Table 3

Correlation between blood group antigen profiling at the DNA and protein levels in native and cultured ERYs from AB and CB.

Complex	Protein	Antigen	AB (African-American Donor)				CB A				CB C			
			DNA	Protein		DNA	Native	Protein		DNA	Native	Protein		Cultured
				Native	Cultured									
Ankyrin	Glycophorin A	M	n.d.	n.d.	n.d.	+	+	+	+	+	+	+	+	+
	Duffy	Fy ^a	+	+	+	+	+	+	+	+	+	+	+	+
		Fy ³	+	+	+	+	+	+	+	+	+	+	+	+
		K	-	-	-	-	-	-	-	-	-	-	-	-
4.1 Complex	Kell	k	+	+	+	+	+	+	+	+	+	+	+	+
		K ^{pa}	-	+	+	-	+	+	+	-	-	-	-	-
		K ^{pb}	+	+	+	+	+	+	+	+	+	+	+	+
		Jsb	+	+	+	+	+	+	+	+	+	+	+	+
Glycophorin B		s	+	+	+	+	+	+	+	+	+	+	+	+