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Caractérisation des microparticules dans les concentrés érythrocytaires : une analyse des antigènes de groupe sanguins par cytométrie de flux

THESE

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par

Giorgia CANELLINI

Médecin diplômé de la Confédération Suisse
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Caractérisation des microparticules dans les concentres érythrocytaires: une analyse des antigènes de groupes sanguins par cytométrie de flux

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Madame le Professeur Stephanie Clarke
Directrice de l'Ecole doctorale

RAPPORT DE SYNTHESE

Cette thèse a pour but de caractériser les microparticules isolées à partir des concentrés érythrocytaires et plus précisément de déterminer la présence d'antigènes de groupes sanguins à leur surface. Elle est divisée en trois parties, sous forme d'articles publiés à partir d'un travail de recherche mené au Centre de Transfusion Sanguine d'Epalinges.

Dans l'article « Microparticles in stored red blood cells : an approach using flow cytometry and proteomic tools » publié dans *Vox Sanguinis* en 2008, il est question de la lésion de stockage des globules rouges. Grâce à des techniques alliant la cytométrie de flux et la protéomique, il a été montré que la génération de microparticules augmente au cours du stockage des concentrés érythrocytaires et que leur composition se modifie au cours du temps.

L'article de revue « Analysis and clinical relevance of microparticles from red blood cells » publié dans *Current Opinion in Hematology* en 2010, explique les mécanismes de formation et d'élimination des microparticules de globules rouges. Il fait une revue des implications cliniques liées à la génération de microparticules et discute leur conséquences potentielles dans le domaine de la médecine transfusionnelle.

L'article « Red blood cell microparticles and blood group antigens : an analysis by flow cytometry » publié dans *Blood Transfusion* en 2012, décrit l'étude des antigènes de groupe sanguins à la surface des microparticules générées à partir de concentrés érythrocytaires après ajout de calcium ionophore. Les résultats de cette étude indiquent que les antigènes de groupes sanguins appartenant aux systèmes RH, KEL, JK, FY, MNS, LE et LU sont présents à la surface des microparticules. Ces antigènes pourraient potentiellement être source d'allo-immunisation après transfusion.

ORIGINAL PAPER

Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools

O. Rubin,[1] D. Crettaz,[1] G. Canellini,[1] J.-D. Tissot[1] & N. Lion[1,2]

[1]Service Régional Vaudois de Transfusion Sanguine, Lausanne, Switzerland
[2]Laboratoire d'Electrochimie Physique et Analytique, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Background and Objectives Microparticles (MPs) are small phospholipid vesicles of less than 1 μm, shed in blood flow by various cell types. These MPs are involved in several biological processes and diseases. MPs have also been detected in blood products; however, their role in transfused patients is unknown. The purpose of this study was to characterize those MPs in blood bank conditions.

Materials and Methods Qualitative and quantitative experiments using flow cytometry or proteomic techniques were performed on MPs derived from erythrocytes concentrates. In order to count MPs, they were either isolated by various centrifugation procedures or counted directly in erythrocyte concentrates.

Results A 20-fold increase after 50 days of storage at 4°C was observed (from 3370 ± 1180 MPs/μl at day 5 to 64 850 ± 37 800 MPs/μl at day 50). Proteomic analysis revealed changes of protein expression comparing MPs to erythrocyte membranes. Finally, the expression of Rh blood group antigens was shown on MPs generated during erythrocyte storage.

Conclusions Our work provides evidence that storage of red blood cell is associated with the generation of MPs characterized by particular proteomic profiles. These results contribute to fundamental knowledge of transfused blood products.

Key words: blood product storage, microparticles, proteomics, red blood cells, transfusion.

Introduction

Millions of blood products are transfused worldwide every year; many lives are thus directly concerned by transfusion. Since the beginning of blood transfusion, numerous efforts have been made to secure blood products and gain knowledge about their molecular structures. The progress of proteomics allows re-examining important issues in blood research [1] and transfusion science [2] with the tools of large-scale biology [3].

The three main labile blood products used in transfusion are erythrocyte concentrates (ECs), platelet concentrates and fresh-frozen plasma. Each of these products has to be stored according to its particular components. However, during storage, modification or degradation of those components may occur and are known as storage lesions. Among these lesions, the generation of microparticles (MPs) has been identified [4–6].

Ageing erythrocytes in blood bank conditions differ from *in vivo*, therefore, it has been suggested that erythrocyte physiological ageing process may be accelerated by storage conditions [7]. Indeed, during storage, several biochemical and physiological changes occur in ECs, including an increase in the concentration of free haemoglobin, lipids, MPs and a pH reduction. Concerning red blood cells (RBCs), they lose adenosine triphosphate, 2,3-diphosphoglycerate or potassium and their membrane undergoes various modifications, such as more rigidity, disruption of phospholipids asymmetry, protein clustering, lipid raft rearrangement, loss

Correspondence: Niels Lion, Service Régional Vaudois de Transfusion Sanguine, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland
E-mail: niels.lion@srtsvd.ch

of fragments or even release of MPs [8–10]. The exact effects of storage lesions on transfusion are still unknown.

Microparticles are small phospholipid vesicles of less than 1 μm in size, also known as microvesicles [11] or ectosomes [12]. They are released from a variety of cells, such as platelets, RBCs and white blood cells, or endothelial cells [13]. They contain a subset of proteins derived from their parent cells. However, MPs are heterogeneous and vary in size, phospholipid and protein composition. Release of MPs is a highly controlled process prompted by various stimuli, such as shear stress, complement attack, proapoptotic stimulation or damage [14].

Microparticles have long been considered as cell fragments or 'debris' without any biological function. Although their true biological function is still unknown, there are more and more indirect evidence that MPs are involved in a broad spectrum of biological activities, such as haemostasis [15], thrombosis [13], inflammation [15], transfer of surface proteins [16] or even angiogenesis [17].

An increase in the number of MPs in plasma has been demonstrated under various pathological conditions, such as heparin-induced thrombocytopenia [18], thrombotic thrombocytopenic purpura [19], diabetes [20,21], acute coronary syndromes [22], cardio vascular disease [23] or sepsis [24]. Despite their potential important activities, only few studies are available on MPs in blood products. It has been demonstrated that the number of MPs increases with the age of blood products [25] and that there is a link between the risk of transfusion complication and the age of the transfused blood products [26,27]. Thus, an appealing hypothesis is that a high number of MPs in ECs is linked to adverse transfusion reactions. In addition, Koch *et al.* recently demonstrated a link between the age of transfused RBCs and post-transfusion complications [28], indicating that progress in the knowledge of stored RBCs biology is urgently needed.

In order to have a better understanding of MPs in stored ECs, qualitative and quantitative experiments using flow cytometry or proteomic techniques were developed and performed. The goals of this study were: (i) to test centrifugation methods for the isolation of MPs from ECs; (ii) to count MPs directly in ECs and in their supernatant fraction; and (iii) to gain insight into the protein content of MPs as compared to RBC membranes, and (iv) to evaluate if erythrocyte-derived MPs also express Rhesus antigens on their surface.

Materials and methods

Erythrocyte concentrates

Whole blood was collected and prepared at the Lausanne or Bern blood banks, according to standardized procedures. Only ECs that did not satisfy quality criteria for transfusion were used for this study, notably those collected from donors presenting with elevated levels of alanine transaminases (ALAT). Briefly, 450 ± 50 ml of whole blood was drawn by venipuncture and collected in blood bags (Fenwal, Lake Zurich, IL, USA) containing the anticoagulant solution (citrate–phosphate–dextrose). Leucocytes and platelets were then removed by filtration. After separation of plasma from erythrocytes by centrifugation, RBCs were finally suspended in 100 ml of preservative solution sodium–adenine–glucose–mannitol. For the experimental purpose of this study, ECs were stored up to 50 days at 4°C instead of 42 days, the usual expiration date for concentrates in sodium–adenine–glucose–mannitol solution.

Flow cytometry analysis and microparticles counts in erythrocyte concentrates

Samples were analysed on a FACScalibur flow cytometer with CellQuest pro software (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometer was daily calibrated with CaliBRIT™ 3 kit (BD Biosciences) containing different fluorescent beads. Size events were defined using flow cytometry size beads of 1–1·4 μm (Spherotech, Lake Forest, IL, USA). For the different windows used, the flow cytometer was set on a logarithmic scale.

Fluorescein isothiocyanate (FITC) anti-human CD47 (BD Pharmingen, San Diego, CA, USA) and phycoerythrin (PE) anti-human CD235a (or glycophorin A) (BD Pharmingen) were the two antibodies used to tag erythrocyte MPs. Moreover, experiments have been done with FITC human annexin V (BD Pharmingen) that tags negative phospholipids present on MPs surface.

Microparticles counts were determined in the supernatant of a stored EC at 4°C for 38 days, after various centrifugation conditions (870 *g*, 1850 *g*, 2550 *g* and 3250 *g*). Four samples of 50 ml from the EC were spun down twice for 20 min, and 100 μl of supernatant was then mixed with 3 μl of FITC anti-human CD47 or 3 μl PE anti-human CD235a or both for double staining. After 20 min of incubation on an orbital shaker in the dark at 4°C, 400 μl of phosphate-buffered saline (PBS) was added and flow cytometry analysis was carried out within 1 h in a Trucount™ tube (BD Biosciences). Isotypic controls were performed with PE immunoglobulin G2b (IgG2b) or FITC IgG1 (both from Diaclone, Besançon, France). MPs were also determined in the supernatant of 13 different ECs stored from 2 to 50 days at 4°C after two centrifugations at 1850 *g* for 20 min. Measurements were done in triplicates. The flow cytometer settings for counting MPs in supernatants were as follows (detector: voltage): FSC: E00, SSC: 360, FL1: 500–600, FL2: 500–600 and FL3: 570.

Finally, to avoid pre-analytical variability due to centrifugation conditions, MPs counts were determined in ECs from seven different blood donors (without centrifugation). For each EC, six measurements were performed from day 2 to day

50 of storage at 4°C. Red blood cells were carefully mixed with the storage solution, and 5 µl of the RBC suspension was mixed with 4 µl of FITC anti-human CD47 for 5 seconds. The mixture was then incubated for 20 min on orbital shaker in the dark. Lastly, 4 µl of the solution was diluted to 1 ml with 0·9% NaCl in a Trucount™ tube, and was directly analysed by flow cytometry. Isotypic controls were performed with FITC IgG1 (Diaclone). The flow cytometer settings for counting MPs in concentrates were as follows (detector: voltage): FSC: E00, SSC: 300, FL1: 650, FL2: 520 and FL3: 600.

Proteomics

Proteomic analysis was done on MPs and erythrocyte membranes of the same 42-day stored ECs. MPs were obtained after three centrifugations (1850 *g* twice and 3200 *g* once, 20 min at 4°C) and the supernatant containing MPs was collected. Then, three ultracentrifugations at 120 000 *g* for 90 min at 4°C were done, each time pellets were suspended in PBS. To obtain erythrocyte membranes, RBCs were washed in PBS 10× and spun at 1850 *g* for 20 min at 4°C three times. Collected pellets were then washed in deionized water and after another centrifugation at 1850 *g* for 20 min at 4°C, pellets were collected and prepared for future analysis.

To determine the quantity of sample to load, protein concentration of each sample was measured according to the Bradford's method [29]. For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 30 µg of proteins was loaded onto a 4–12% NuPAGE Novex Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). The migration was carried out at constant voltage (200 V).

For mass spectrometry (MS) analysis, SDS-PAGE was run as previously described, but 300 µg of proteins was loaded. Upon electrophoresis completion, the gels were rinsed twice with deionized water and stained with colloidal Coomassie blue (National Diagnostics, Atlanta, GA, USA) overnight. The gels were destained with deionized water. Bands of interest were excised from the gels and transferred into an Eppendorf. In-gel proteolytic cleavage with sequencing-grade trypsin (Promega) was automatically performed in the robotic workstation Investigator ProGest (Perkin Elmer Life Sciences) according to the protocol of Shevchenko *et al.* [30]. Digests were evaporated to dryness and resuspended in 3 µl of α-cyano-hydroxycinnamic acid matrix [5 mg/ml in 60% (v/v) acetonitrile : water), of which 0·7 µl was deposited in duplicate on a target plate. Matrix-assisted laser desorption/ionization MS (MALDI-MS)/MS analysis was performed on a 4700 Proteomics Analyser (Applied Biosystems, Framingham, MA, USA). After MALDI – Time of Flight (TOF)/MS analysis, internal calibration on trypsin autolysis peaks and subtraction of matrix peaks, the 10 most intense ion signals were selected for MS/MS analysis. Non-interpreted peptide tandem mass spectra were used for direct interrogation

of the Uniprot (Swissprot + TrEMBL) database using Mascot 2·0 (http://www.matrixscience.com). The mass tolerance for database searches was 50 p.p.m. MASCOT was set up to only report peptide matches with a score above 14. With the parameters used, the threshold for statistical significance ($P < 0.05$) corresponded to a total (protein) MASCOT score of 33. Proteins scores above 80 were automatically considered valid, while all protein identifications with a total MASCOT score between 33 and 80 were manually validated. Validation included examination of the peptide root mean square mass error and of individual peptide matches. Peptide matches were validated only if at least an ion series of four consecutive y ions were matched, in addition to ions belonging to other series. Generally, only proteins matched by at least two peptides were accepted.

Western blotting

From 20 to 50 µg of proteins from the samples described before (MPs and erythrocyte membranes) were loaded onto a 4–12% NuPAGE Novex Bis-Tris polyacrylamide gel (Invitrogen). After migration, carried out at constant voltage (200 V), proteins were transferred to polyvinylidene fluoride membranes using a Novex blot module (Invitrogen) for 1 h 45 min at fixed voltage (30 V), according to the manufacturer's instruction. After transfer, blotted membranes were soaked overnight in blocking solution with PBS, 0·1% Tween-20 (v/v), 5% milk and 1% BSA (w/v). Four Western blots were done, each one with a different antibody, namely, anti-human CD235a (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human actin (Sigma, Saint Louis, MO, USA), anti-human stomatin (Proteintech Group, Chicago, IL, IL) and anti-human CD47 (from Santa Cruz or from AbCam, Cambridge, UK). All antibodies were used at a dilution of 1 : 500. The goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Dako, Baar, Switzerland) were both used at a dilution of 1 : 10 000. Subsequent visualization was performed using enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden). The signal was finally captured using X-ray film.

Expression of Rhesus antigens

Flow cytometry was used to determine the presence of Rh antigens on MPs generated during storage. MPs were first isolated from the supernatant of an EC after two centrifugations at 1850 *g* at 4°C for 20 min. The supernatant was then spun down at 18 000 *g* for 5 min to pellet MPs. Pellet was dissolved in 100 µl of PBS. One µl of the primary antibody (anti-D, anti-C, anti-c, anti-E and anti-e, respectively) was later added and mixed on orbital shaker for 90 min. IgM anti-D were obtained from Orthobiotech (Bridgewater, NJ, USA), whereas IgM anti-C, IgM anti-c, IgM anti-E and IgM anti-e

were obtained from Biotest (Dreichen, Germany). One µl of the secondary antibody was then added, and after 1 h on orbital shaker in the dark, 400 µl of PBS was added, and samples were analysed by flow cytometry within an hour. Secondary antibody directed against primary IgM was FITC anti-human IgF(ab) from Chemicon (Melbourn, Australia). To demonstrate the presence of various Rh antigens on MPs, blood samples expressing different Rh phenotypes, such as DCCee, DccEE or dccee, were selected. The antithetical phenotype was used with each antibody as a negative control.

Results

Microparticle counts

Using flow cytometry, MPs clearly were distinguished from RBCs by their size as well as by the negatively charged phospholipids on their outer membrane detected by annexin V. Indeed, the great majority of MPs were annexin V-positive, while merely a few percentage of erythrocytes were positive. MPs were also identified using either anti-CD47, anti-CD235a or both antibodies, without any differences in their numbers according to the choice of the antibody (Figs 1 and 2).

In both methods used, an increase in the number of MPs during storage of ECs was observed (Figs 2 and 3). The number of MPs was clearly related to centrifugation conditions (Fig. 2). In the measurements performed directly in the concentrate, the number of MPs increases about 20-fold after 50 days of storage at 4°C and considerably varies among different samples; it starts from 3370 + 1180/µl after 6 days, up to 64 850 + 37 800/µl after 50 days of storage (Fig. 3). The intra-assay coefficient of variation was evaluated. With both methods, the coefficient was less than 15%, even after 50 days of storage (data not shown). Nonetheless, and without evident explanation, we observed a huge individual variation of the MPs counted among different donors.
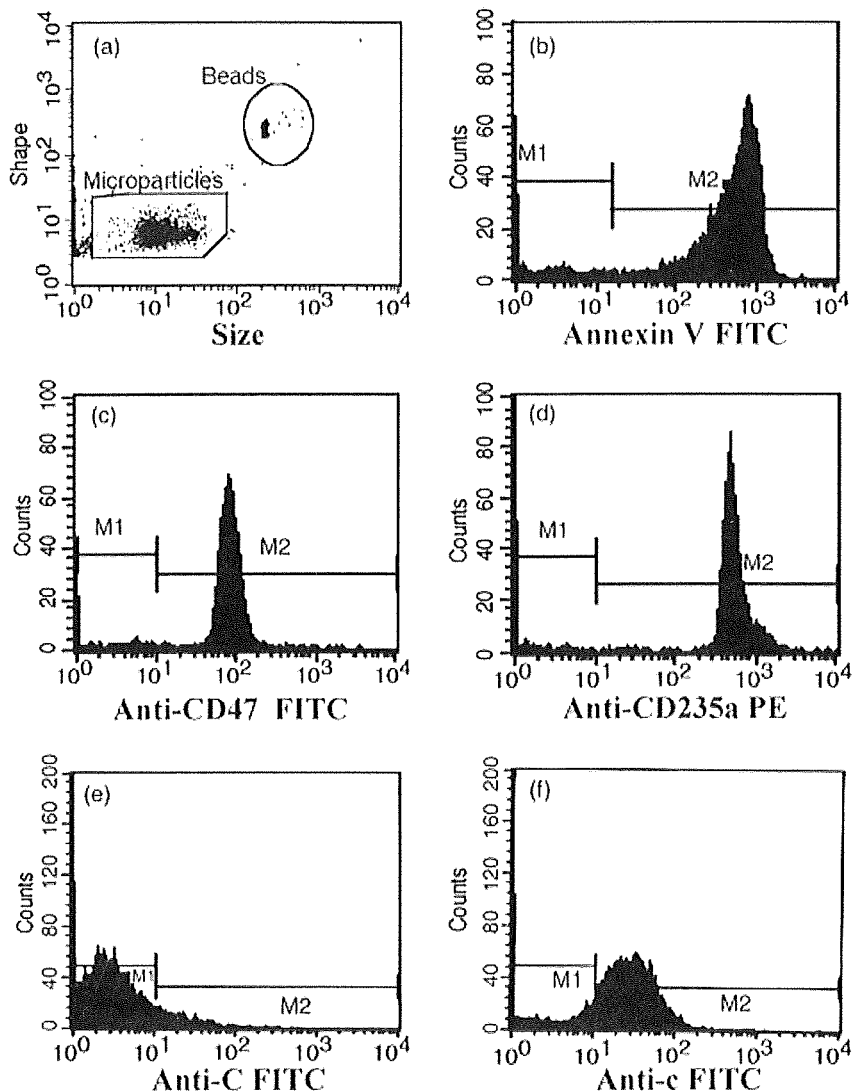

Fig. 1 Flow cytometry analysis of microparticles from a supernatant after centrifugation of erythrocyte concentrate. (a) Events that were sorted according to their size and shape. Two different regions were determined; microparticles and beads. A precise number of beads were used to determine the number of microparticles in each sample. Histograms represent events from the microparticle region (b–e) of (a) according to their fluorescence (due to an FITC- or PE-labelled antibody). M1 represents non-stained events whereas M2 shows stained event. (b) Microparticles stained with annexin V; (c) microparticles stained with anti-CD47 FITC; and (d) microparticles stained with anti-CD235a. FITC, fluorescein isothiocyanate; PE, phycoerythrin. In (e) and (f), microparticles were derived from an erythrocyte concentrate from a donor typing dccee. In this example, microparticles were negative for anti-C antibody (e), while they were positive for anti-c antibody (f).
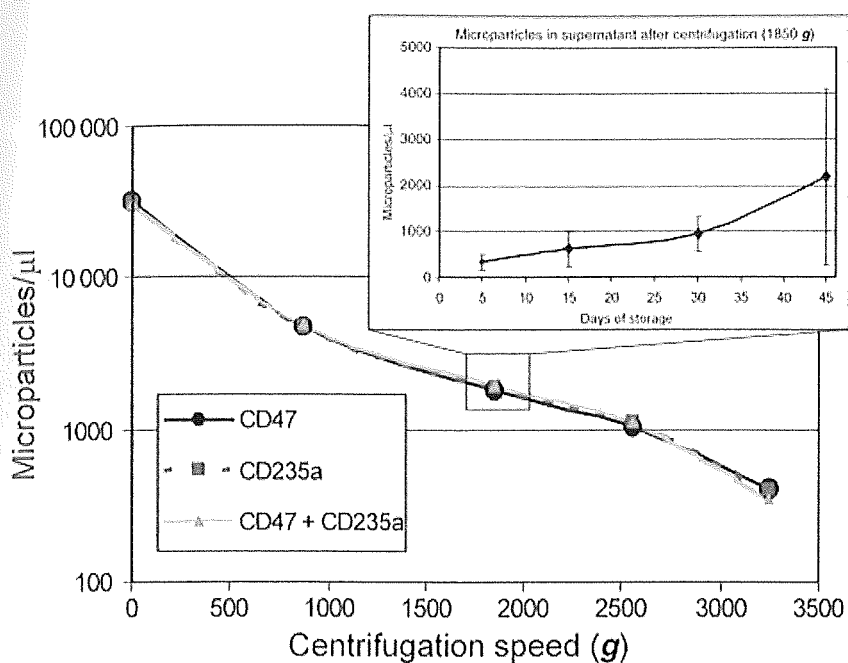
Fig. 2 Count of microparticles in supernatant of erythrocyte concentrates (ECs). The number of microparticles measured decreased with increasing centrifugation speed (test on one EC stored for 38 days). Note that the numbers measured were identical with the two antibodies used in this study (anti-human CD235a or CD47). The inset shows an increase in the number of microparticles in supernatant of ECs during storage (after centrifugation at 1850 *g*, here anti-human CD235a was used).
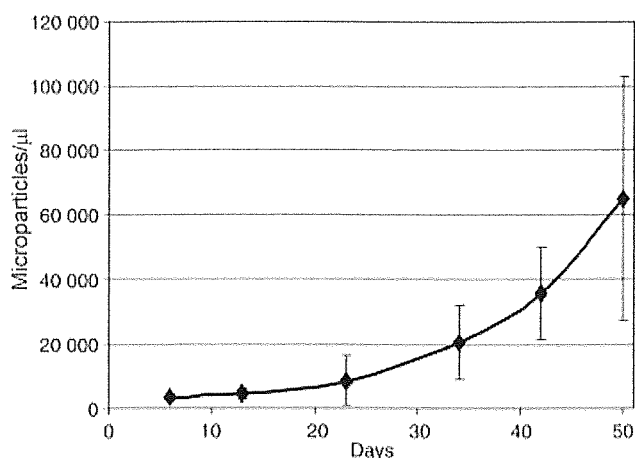

Fig. 3 Count of microparticles directly in erythrocyte concentrates during storage (without centrifugation). Data are expressed as the mean ± SD experiment (*n* = 7). At day 5, 3371 ± 1188 microparticles/μl were counted, whereas at day 50, their numbers were 64 858 ± 37 846 microparticles/μl. Anti-human CD47 was used to stain microparticles.

Proteomics

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis showed a number of large as well as discrete bands obtained after electrophoresis of RBC membranes (Fig. 4, lane A) and MPs (Fig. 4, lane B). A major difference was observed in the 25–35 kDa region; thus, in order to perform protein identification, bands of interest stained with Coomassie blue were excised and proteins were identified by MS. Table 1 lists the proteins identified in the three excised bands of lane A (erythrocyte membranes) and lane B (MPs). Not surprisingly,

abundant proteins such as carbonic anhydrases or peroxiredoxins were identified both in RBC membranes and MPs with good sequence coverage. A set of 14-3-3 proteins was also identified in both samples: 14-3-3 proteins are abundant and ubiquitous proteins [31] that act as regulators of a number of processes, such as modulation of protein kinase activities, signal transduction [32]. Remarkably, 14-3-3ζ has been shown to be implicated in GPIb-IX-V translocation to the cytoskeleton during platelet activation [33]. It was thus not surprising to find 14-3-3 proteins in RBC ghosts. The gel band that appeared to be quantitatively the most different between lanes A and B was identified as stomatin, identified in MPs with a score of 1131, a sequence coverage of 76·4% from 46 peptides, whereas it was identified in RBC membranes with a score of 156, a sequence coverage of 14·9% from four peptides, indicating that stomatin was largely enriched in MPs compared to erythrocyte membranes.

Interestingly, some of the identified proteins did not have molecular weights that corresponded to their respective position on the gel. For example, haemoglobin subunits α and β, which have a molecular weight of about 15–16 kDa, were observed in the region corresponding to 25–35 kDa on the gel. However, it has been documented that denatured and cross-linked haemoglobin strongly binds to the cytoskeleton during RBC storage in blood banking conditions [34]. It is thus highly probable that the haemoglobin subunits identified were present as homogeneous or heterogeneous dimers. Additionally, Band 3 (a major membrane protein) as well as Rhesus protein were identified in MPs only, from three and two sequenced peptides, respectively. For Band 3, the identified peptides correspond to the cytoplamic domain of the protein (spanning the region 117–180), which means that the Band 3
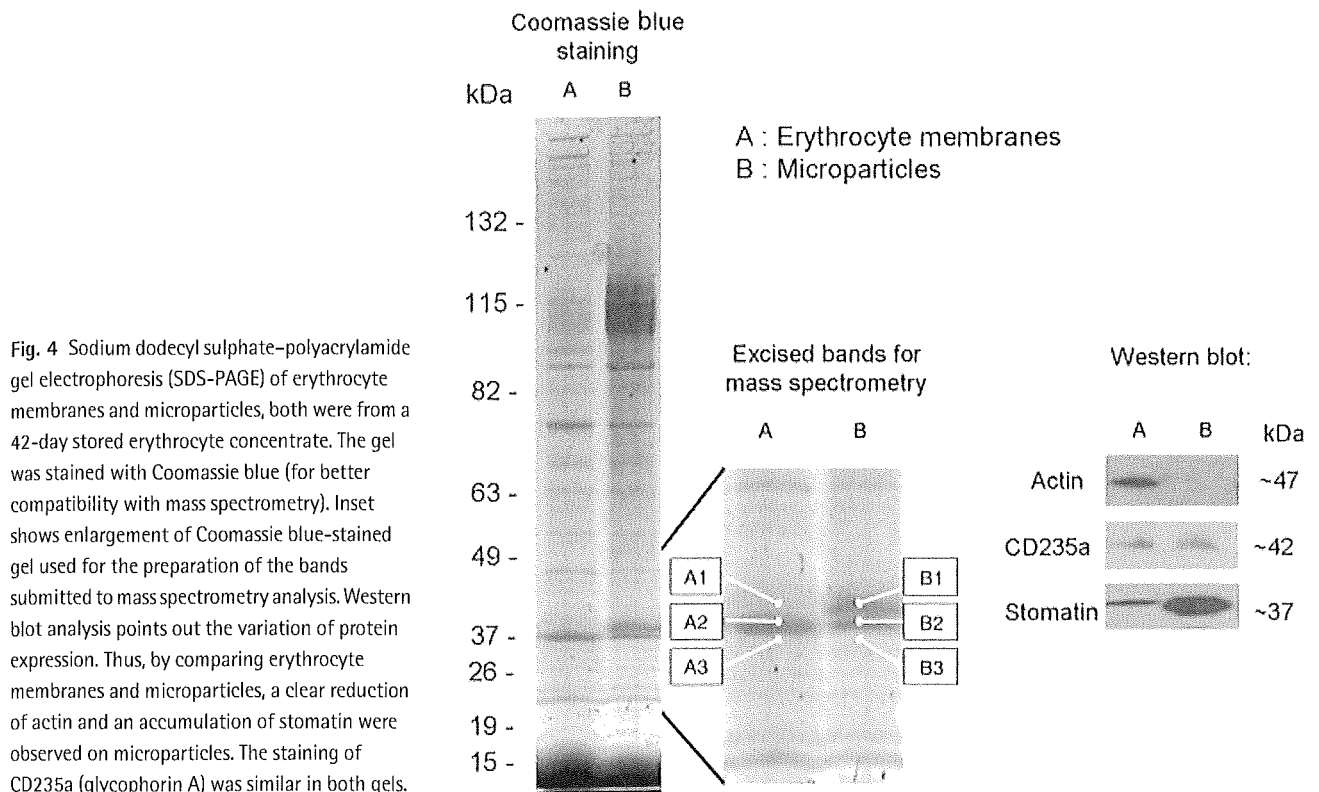
Fig. 4 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of erythrocyte membranes and microparticles, both were from a 42-day stored erythrocyte concentrate. The gel was stained with Coomassie blue (for better compatibility with mass spectrometry). Inset shows enlargement of Coomassie blue-stained gel used for the preparation of the bands submitted to mass spectrometry analysis. Western blot analysis points out the variation of protein expression. Thus, by comparing erythrocyte membranes and microparticles, a clear reduction of actin and an accumulation of stomatin were observed on microparticles. The staining of CD235a (glycophorin A) was similar in both gels.

fragment that appears on the gel in the 25–35 kDa region belongs to the cytoplasmic domain of the protein. The Rhesus protein was identified from two peptides covering the 17 last amino acids on the C terminus of the protein sequence. Of interest, Mascott allowed the identification of Rh peptides (Table 1; lane B of Fig. 4). However, it was not possible to discriminate between RhD and Rh(CE) proteins, a well-known problem in proteomics [35]. Nevertheless, the presence of various Rh antigens at the surface of MPs was confirmed by flow cytometry (see below).

Western blots

Western blotting confirmed the presence of CD235a, actin and stomatin on RBC membranes as well as on MPs, with a clear reduction of actin and an accumulation of stomatin on MPs (Fig. 4). However, using this technique and the antibodies available, neither CD47 nor Rh-proteins could be detected after SDP-PAGE. With blot techniques, no quantitative difference was observed between MPs samples from ECs stored for 5 or 42 days.

Rhesus systems

The presence of blood group antigens of Rhesus system on MPs surface was investigated by flow cytometry using specific antibodies. In each assay, positive and negative

samples were selected for the corresponding antigen (Fig. 1e,f). By this approach, the presence of C, c, D, E or e antigens was observed on MPs when they were present on the RBCs from which they derived. However, it was not possible to exclude that a population of MPs was Rh negative.

Discussion

There is no standardized method to count MPs. Several approaches have been proposed in the literature, the majority dealing with platelet MPs from whole blood or platelet concentrates. Centrifugation speeds, for MPs characterization, varied from 200 g to 13 000 g [36]. In our hands, complete elimination of RBCs from supernatant at low centrifugation was obtained after two centrifugation at 1850 g for 20 min. Annexin V is frequently used to detect phosphatidylserine, a negatively charged phospholipid known to be present on the outer leaflet of apoptotic cells as well as on MPs of various origins [18]. In this study, MPs were counted using flow cytometry with different antibodies, notably anti-CD235a and anti-CD47. These antibodies were chosen because both are reactive towards RBC membrane molecules known to be present on MPs [14,29].

Counting MPs directly within the homogenized ECs appeared as the simplest approach, avoiding handling, centrifugation and washing. The drawbacks were due to the presence of a great number of RBCs, the need of quite large

Table 1 Proteins identified in the region 25–35 kDa of RBC membranes (lane A of Fig. 4) and microparticles (lane B of Fig. 4). Reported are the sequence coverage (%) and the number of sequenced peptides

AC	Entry name	Protein name	MW (Da)	RBC (sequence coverage %)	RBC (identified peptide)	MPs (sequence coverage %)	MPs (identified peptide)
P31946	1433B_HUMAN	14-3-3 protein β/α	28 082	25·2	7	16·7	4
P62258	1433E_HUMAN	14-3-3 protein epsilon	29 174	34·9	11	NO	NO
P61981	1433G_HUMAN	14-3-3 protein γ	28 303	NO	NO	13·4	4
P27348	1433T_HUMAN	14-3-3 protein θ	27 764	14·3	5	NO	NO
P63104	1433Z_HUMAN	14-3-3 protein ζ/δ	27 745	44·5	11	8·2	2
P02730	B3AT_HUMAN	Band 3 anion transport protein	101 792	NO	NO	6·8	3
Q4TWB7	Q4TWB7_HUMAN	β-Globin chain (Fragment)	11 487	93·3	15	NO	NO
P07738	PMGE_HUMAN	Bisphosphoglycerate mutase	30 005	45·9	13	37·8	10
P00915	CAH1_HUMAN	Carbonic anhydrase 1	28 870	72·8	27	65·5	20
P00918	CAH2_HUMAN	Carbonic anhydrase 2	29 246	72·7	25	71·9	15
P07451	CAH3_HUMAN	Carbonic anhydrase 3	29 557	36·2	8	17·7	4
A0N071	A0N071_HUMAN	δ-Globin chain (haemoglobin δ)	16 055	55·1	10	55·1	9
P27105	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	31 731	14·9	4	76·4	43
P17931	LEG3_HUMAN	Galectin-3	26 188	11·6	3	NO	NO
P78417	GSTO1_HUMAN	Glutathione transferase ω-1	27 566	33·2	10	7·9	2
P69905	HBA_HUMAN	Haemoglobin subunit α	15 258	71·1	9	71·1	9
P68871	HBB_HUMAN	Haemoglobin subunit β	15 998	83·0	16	93·9	15
Q16775	GLO2_HUMAN	Hydroxyacylglutathione hydrolase	28 860	8·8	2	NO	NO
P30041	PRDX6_HUMAN	Peroxiredoxin-6	25 035	25·9	6	16·1	4
P18669	PGAM1_HUMAN	Phosphoglycerate mutase 1	28 804	8·3	2	NO	NO
Q06323	PSME1_HUMAN	Proteasome activator complex subunit 1	28 723	16·5	4	NO	NO
P25788	PSA3_HUMAN	Proteasome subunit α type 3	28 433	6·7	2	NO	NO
P25789	PSA4_HUMAN	Proteasome subunit α type 4	29 484	12·3	4	NO	NO
P60900	PSA6_HUMAN	Proteasome subunit α type 6	27 399	9·3	2	NO	NO
O14818	PSA7_HUMAN	Proteasome subunit α type 7	27 887	21·4	4	NO	NO
P00491	PNPH_HUMAN	Purine nucleoside phosphorylase	32 118	59·5	16	17·6	4
Q0KG01	Q0KG01_HUMAN	RhD protein	45 052	NO	NO	4·1	2

AC, accession number; MW, molecular weight in Da; NO, not observed.

amount of antibodies and an intra-sample variability in the number of MPs counted. In addition, samples cannot be stored, contrasting with supernatants containing MPs, which can be kept at 4°C or even be frozen before being evaluated by flow cytometry. Disadvantages of working with supernatants were related to the handling procedures and, more importantly, to the influence of centrifugation conditions. After centrifugation, residual RBCs were eliminated. However, a number of MPs appeared to be pelleted together with RBCs (Fig. 2). In any case, an increase in the number of MPs in ECs during storage was observed, even if the number of MPs counted differs according to the method. From our results, it is really clear that the number of MPs counted in EC was dependent on the centrifugation protocols.

Whereas it cannot be excluded that MPs from platelets, white blood cells or endothelial cells be present in the starting EC, this increase in total of MPs count can be attributed only to the shedding of MPs from RBCs present in the concentrate.

Noteworthy, the increase varied quite importantly from donor to donor. The reason of such a variation is unknown, but factors like ABO blood group, age, fasting or sex of blood donor may have a role and should be investigated. Finally, the most important parameter associated with the number of MPs in ECs was the duration of storage at 4°C.

A set of experiment with three ECs satisfying quality criteria for transfusion (normal ALAT level) was done and gave very similar, if not identical MPs count when compared to ECs with elevated ALAT level (data not shown).

Microparticle tend to aggregate at high concentration, either related to the methods used for their isolation or to their 'intrinsic' adhesion properties, which have been already evidenced with platelet-derived MPs [16]. We observed heaps of erythrocyte MPs by electron microscopy (data not shown), thus erythrocyte MPs may also have adhesion properties. This observation is important for the quantitative results, because MPs counts are evaluated according to their sizes

using flow cytometry. Thus, in samples with high concentrations of MPs, their number are probably underestimated due to the fact that MPs tend to form more heaps and flow cytometry does not distinguish between big MPs or aggregated MPs, even if the technique is the method of choice to study MPs [36]. So, the counting approach of MPs presented in this study (as well as in other published studies) should be considered as semiquantitative.

According to our proteomic and Western blot studies, MPs from stored RBCs appeared to be enriched with stomatin. Remarkably, the enrichment in stomatin, depletion in actin and stability of glycophorin A (as compared to erythrocyte membranes) were the same at day 7 and day 40. In this respect, MPs generated after a few days of storage or at the end of storage appear equivalent. Those results are well in line with previous reports [37,38]. Stomatin is a membrane protein involved in regulation of monovalent cation transport through lipid membranes [39]. Interestingly, stomatin (which has a structure similar to caveolin) is a major lipid-raft component of erythrocytes [40]. Precise reasons of stomatin enrichment in MPs are not well known and are still subject of investigation, but may have a role in membrane microdomains modulation leading to membrane budding and MPs release [38]. The cell membrane plays a key role in the formation of MPs. Indeed, following a stimulus, increase in intracellular Ca^{++} occurs and activates proteases that cleave cytoskeleton proteins (actin and spectrin). Membrane is thus less rigid and can bud until formation of MPs. Furthermore, the asymmetry between the neutral phospholipids on the outer membrane and the negatively charged phospholipids on the inner membrane held by translocases is broken [37]. Consequently, phosphatidylserine, a negatively charged phospholipid, is also located on the outer side of MP membrane. Using annexin V, flow cytometry confirmed that phosphatidylserine was present on MPs derived from erythrocytes but essentially lacked from fresh RBCs and was externalized in only a small fraction of old RBCs. Finally, as shown by Western blots, actin was not a dominant protein of MPs when compared to RBC membranes.

The precise reasons for the huge increase in MP counts during storage of EC observed in this study are unknown. Although controversial, it has been speculated that MPs could be a means for erythrocytes to prevent a premature removal from circulation when they are still functional or when lesions are reversible [41]. According to this hypothesis, MPs would allow erythrocytes to clear away non-functional molecules that would trigger an apoptosis-like pathway, or to get rid of autologous IgG binding senescent erythrocytes for removal by spleen macrophages [8,10,42]. Indeed, Willekens et al. have recently shown that MPs contain erythrocyte removal proteins such as bound IgG and altered Band 3, and thus concluded that microvesiculation serves as a removal pathway for damaged proteins [42]. Complementarily, a detailed proteomic investigation of RBCs and MPs generated during storage led Bosman et al. to hypothesize that there are two possible mechanisms at work in MPs generation: first, immunoglobulins could bind to senescent surface proteins, thereby triggering microvesiculation. Alternatively, oxidatively damaged proteins could bind to or disrupt normal interactions within the cytoskeleton, thereby altering the tight balance between the cytoskeleton pressure and the membrane bending stress [10]. These two passive mechanisms could well be only one part of the picture, because activation of protein kinases has been demonstrated to trigger phosphatidylserine exposure in erythrocytes [43], as well as tight concomitant regulation of microvesiculation and Band 3 phosphorylation/dephosphorylation [44]. Lastly, lysophosphatidic acid, an important lipid mediator, has been shown to be able to trigger phosphatidylserine exposure and microvesiculation in erythrocytes [45]. These studies show that MP generation can be triggered by various processes, including senescence or protein alteration, external or internal exposure to lipid mediators, and that phosphorylation plays a role in microvesiculation control.

In this study, we also showed evidence that Rhesus blood group antigens are located on erythrocyte MPs. The presence of theses proteins was evidenced by determination of Rh peptides by MS and by flow cytometry, indicating that the antigenic parts of the Rh proteins are located outside MPs membranes. The presence of the Rh complex is also reinforced by the expression of CD47 (shown by flow cytometry), which is a member of the complex within the RBC membrane. Those blood group antigens present on MPs are likely immunogenic, and thus may play a potential role in RBC alloimmunization after transfusion.

As recently shown by Koch et al., there is a link between duration of RBC storage and complications after cardiac surgery [28]. According to the result of this research, transfusion of erythrocytes that have been stored for more than 14 days in patients undergoing cardiac surgery significantly increases the risk of postoperative complications and reduces survival time. Reasons for such complications remain unclear; however, storage lesions may be a possible mechanism. Physicochemical changes occurring during storage of ECs are indeed known to affect RBCs function and viability. Our results confirm that important changes occur during storage of RBCs and that storage techniques allowing a better conservation of the integrity of the membrane should be thus developed in the future.

Acknowledgements

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Analysis and clinical relevance of microparticles from red blood cells

Jean-Daniel Tissot, Olivier Rubin and Giorgia Canellini

Service Régional Vaudois de Transfusion Sanguine, Lausanne, Switzerland

Correspondence to Jean-Daniel Tissot, MD, Service Régional Vaudois de Transfusion Sanguine, route de la Corniche 2, CH-1066 Epalinges, Switzerland
Tel: +41 21 314 65 89;
e-mail: Jean-Daniel.Tissot@mavietonsang.ch

Purpose of review

The mechanisms involved in the formation of red blood cell (RBC) microparticles *in vivo* as well as during erythrocyte storage are reviewed, and the potential role of microparticles in transfusion medicine is described.

Recent findings

Microparticles release is an integral part of the erythrocyte ageing process, preventing early removal of RBCs. Proteomics analyses have outlined the key role of band 3 – ankyrin anchoring complex and the occurrence of selective RBC membrane remodelling mechanisms in microparticles formation. The presence of several RBC antigens, expressed on microparticles, has been demonstrated. The potential deleterious effects of RBC microparticles in transfused recipients, including hypercoagulability, microcirculation impairment and immunosuppression, are discussed.

Summary

Formation and role of RBC microparticles are far from being completely understood. Combining various approaches to elucidate these mechanisms could improve blood product quality and transfusion safety. Implementation of RBC microparticles as biomarkers in the laboratory routine needs to overcome technical barriers involved in their analysis.

Keywords

microparticles, proteomics, red blood cells, transfusion

Introduction

Microparticles, also described as microvesicles [1] or ectosomes [2], are heterogeneous populations of phospholipid vesicles of less than 1 μm, released in circulating blood by erythrocytes, platelets, white blood cells or endothelial cells [3]. The production of microparticles is a highly controlled process, triggered by various stimuli, including cell stimulation and apoptosis [4]. If microparticles have been first described as cell dusts [5], they are now recognized as being involved in a broad spectrum of biological activities, such as thrombosis and haemostasis [3,6], inflammation [6], vascular and immune function [7*], apoptosis [4] or even intercellular communication by the transfer of surface proteins [8]. Microparticles are detected in healthy individuals and their increase has been observed in a variety of diseases with elevated thrombotic risk, vascular involvement or metastasis [9,10,11*].

Proteomics analysis has been recently applied to the study of red blood cell (RBC) microparticles (reviewed in [12]) and allows identification of numerous different proteins in biological samples. Proteomics has been successfully used in the field of transfusion medicine [13–15] and allowed the deciphering of various blood diseases [16]. Nevertheless, and whatever methods are employed to characterize microparticles, preanalytical issues remain of major importance to correctly assess microparticles in blood [17]. The lack of standardized methods has impaired microparticles analysis implementation in the clinical setting [18*].

Formation and clearance of red blood cell microparticles

The normal erythrocyte has a unique membrane and cytoskeleton organization with redundant surface area and sufficient flexibility to undergo extensive deformation during its transit through the spleen [19]. Under physiological conditions, the asymmetric distribution of RBC membrane phospholipids is maintained by the cooperative action of translocases (flippase, floppase and scramblase), concentrating negatively charged aminophospholipids, like phosphatidylserine on its inner leaflet [20].

Microvesiculation

Rapid externalization of phosphatidylserine after cell activation or apoptosis modifies the neutral membrane

charge into negative with loss of phospholipid asymmetry, leading to a cascade of events which disrupts the interactions between membrane and cytoskeleton proteins, specifically spectrin and protein 4.1R. The stability of the membrane is thus directly affected, becoming less rigid and allowing the formation and release of microparticles [21,22]. During their lifespan, RBCs lose approximately 20% of their haemoglobin (Hb) content, and similar surface area through vesicles emission, thus decreasing their favourable surface-to-volume ratio [23**]. Microvesiculation occurs throughout the erythrocyte lifespan and is triggered by different types of stimuli [23**], such as shear stress, complement, attack, oxidative stress and pro-apototic stimulations [4]. As shown during complement activation, microvesiculation prevents immediate killing of RBCs by eliminating the membrane attack complex C5b–9; this survival strategy is especially used when expression of complement regulatory proteins on the cell surface are decreased [24]. It remains obscure how the spleen facilitates microparticles formation during the second half of the erythrocyte lifespan; interestingly, improvement of RBC osmotic fragility due to increase of surface area has been described in patients with hereditary spherocytosis after splenectomy [25,26].

Erythrocyte ageing models

Microparticles formation has been described as an integral step of RBC senescence [23**]. Two major models of erythrocyte ageing have been proposed so far: the eryptosis and the band 3 clustering. The eryptosis model, which is similar to apoptosis of nucleated cells, could be regarded as the RBC response to various stresses, whereas the band 3 model may explain the physiology of RBC ageing [27*]. In the eryptosis model, intracellular flux of calcium through possibly altered nonspecific cation channels leads to activation of several enzymes, such as scramblases, calpains and transglutaminase 2. This results in phosphatidylserine externalization, degradation and cross-linking of cytoskeletal proteins, followed by modifications in the phosphorylation status of band 3 [28*,29,30]. The band 3 clustering model is characterized by protein oxidation [31]. The oxidation of Hb contributes to hemichrome formation, which is constituted Hb-derived products (likely met-Hb) linked to the inner leaflet, followed by the clustering and aggregation of band 3 multimers in the membrane [32]. Band 3 clustering forms or uncovers senescent neoantigens, probably because of relatively small structural modifications that are recognized by naturally occurring autologous IgG with subsequent complement activation [33–35].

Both models share the similar final outcome that leads to modifications of band 3 and induces perturbations of the inner leaflet microenvironment, which could alter the tight balance between the membrane and cytoskeleton forces, thus resulting in microvesiculation. It has been shown that modifications of the band 3–ankyrin anchoring complexes could enhance lateral compression forces of the cytoskeleton, making the RBC membrane prone to distortion. Thereby, band 3 anion exchanger should be considered as a key protein in the regulation of erythrocyte structure and function [23**], through binding cytoskeleton proteins, glycolytic enzymes and Hb [36].

Microparticles composition

Reviews of proteomic analysis of microparticles have been published elsewhere [17,37,38**,39]. The composition of RBC microparticles may vary according to the stimulus and differs from their parental cell by nearly complete absence of cytoskeletal-linked molecules, decrease of membrane proteins content, presence of more metabolic proteins and Hb and exposure of removal signal molecules such as phosphatidylserine and autologous IgG [23**,40]. Furthermore, enrichment of several erythrocyte membrane components has been described, especially band 3, glycophorins, complement receptors, glycosyl-phosphatidylinositol (GPI)-anchored proteins and lipid-raft markers, suggesting selective membrane lipids and protein sorting. In lipid-raft origin of RBC microparticles, the oxidation of cytoskeleton proteins may promote lateral movement of stomatin oligomers in the membrane, which serve as nuclei for lipid-raft aggregation and extension, leading to the budding of membrane lipid-patches detached from cytoskeleton and segregation of molecules, including stomatin and GPI-anchored proteins [39,41].

Clearance mechanisms

Once generated in circulating blood, phosphatidylserine-exposing RBC microparticles are rapidly and efficiently removed by binding to the macrophage's scavenger receptors of the organ in which they originate [40]. Alternatively, RBC microparticles carrying senescent neoantigen-specific autoantibodies are recognized by Fcγ-receptors and eliminated through the mononuclear phagocyte system [42].

According to the limited capacity of RBCs for self-repair, vesicles formation, which is an ATP-free mechanism even faster than translocation back of phosphatidylserine [40], may not only help to remove membrane-damaged molecules, postponing the phagocytosis of otherwise functional erythrocytes, but also prevents the exposure of potentially dangerous molecules [39]. Once microvesiculation capacity is exceeded, old erythrocytes are most likely phagocytosed.

Red blood cell microparticles and transfusion medicine

Under blood bank conditions, RBCs undergo progressive structural and biochemical changes commonly referred to
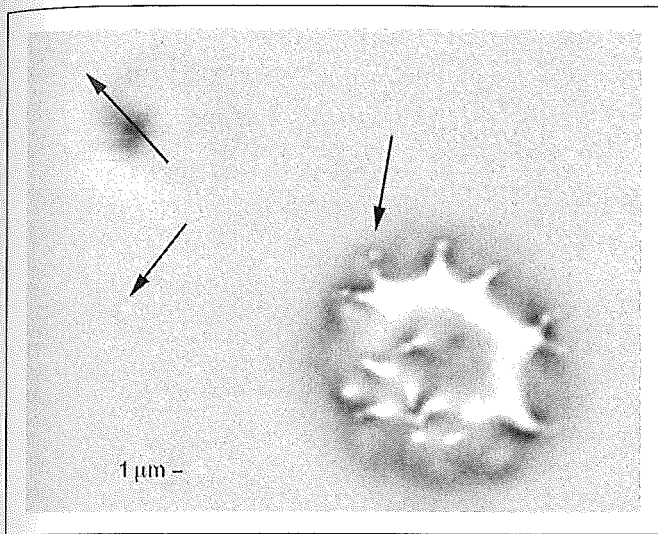
By confocal differential interference contrast microscopy, it is possible to observe forming microparticles (arrows) from spicules of echinocytes (magnification ×4000).

as 'the storage lesion' [39]. As shown in Fig. 1, erythrocytes show progressive cell shape transformation from biconcave disk to rigid spheroechinocyte [42], accompanied by the release of microparticles from the tips of spicules [6,43]. In addition, there is a depletion of ATP, pH acidification, haemolysis and microparticles accumulation observed in the medium [41]. Although differences have been reported between in-vitro and in-vivo RBC senescence such as denaturation of spectrin, changes in carbohydrate part of the membrane and increased mean cellular volume, the storage lesion shares similar features with the cell ageing process [38••,44•]. RBC membrane modification during storage is triggered by ATP depletion and oxidation and is centred on changes in band 3 leading to membrane detachment and disorganization that probably affect RBC deformability, osmotic resistance and survival after transfusion [45,46•].

Microparticles in erythrocyte concentrates
RBC microparticles formation represents a continuous process of membrane remodelling, which occurs early during blood banking [41,47•], and prevents the exposure of phosphatidylserine on RBC [48]. Almost all microparticles found in erythrocyte concentrates originate from RBCs and their number gradually increases with storage time [37,42,49••]. The level of vesiculation in erythrocyte concentrates may vary not only with the length of storage but also according to the product and the storage solution: RBC microparticles are increased in washed blood products [50,51], whereas they could be lowered with additive solutions that manage effectively the oxidative stress [44•]. The level of circulating microparticles could sig-

nificantly increase, as already observed in transfused patients presenting with paroxysmal nocturnal haemoglobinuria [50].

The composition of RBC microparticles in erythrocyte concentrates is nearly similar to those generated *in vivo*, except for the increased levels of stomatin, making it likely that a raft-based process is responsible for microvesiculation at low temperatures [41]. As those generated *in vivo*, the vesicles found in erythrocyte concentrates are devoid of most of the RBC integral membrane proteins or cytoskeletal components, with the exception of actin and band 3, found in aggregated or degraded forms [6,39,41]. Proteomics data confirm exposure of phosphatidylserine, binding of immunoglobulins and partial activation of complement on their surface. The significant difference observed in membrane composition between RBCs and microparticles outline that vesicles are generated by specific processes, which allow sorting of lipids and proteins [39]. Nonetheless, the pathways responsible for membrane lipids remodelling before microvesiculation remain mostly unknown. According to the absence of immunologic removal, microparticles in erythrocyte concentrates become more heterogeneous over time with a gradual increase of their size and of their content of proteasome components, and a decrease of phosphatidylserine exposure [38••,42], suggesting that either microparticles structure or nature of vesicle formation may vary with storage time.

Microparticles and blood group antigens
Half of the RBC transmembrane proteins carry various blood group antigen specificities [52]. Some of them, such as the rhesus (Rh) proteins, contribute to membrane stabilization through their link with protein 4.1R [53]. Blood storage is associated with the generation of soluble ABH antigens in the bag, parallel to the decrease of their expression on erythrocytes [54]. Various studies have shown that blood group antigens are located on microparticles [55,56]. The identification of Rh peptides by mass spectrometry and Rh antigens by flow cytometry (Fig. 2) indicates that the protein is present within the membrane and that the antigenic part is located at the outer side of the microparticles membrane [37]. Our flow cytometry analyses demonstrated the presence of several antigens such as RH2 on microparticles (Fig. 2) as well as RH1, RH3, RH4, RH5, FY1, FY2, JK1, JK2 and KEL1 (data not shown). Despite their cytoskeletal dissociation, Rh antigens on microparticles keep the property to react with the corresponding antibodies. However, it is not known whether they are able to elicit an immune response.

Another striking feature is the suppression of RBC antigens during autoimmune haemolytic anemia and during transfusion of incompatible blood [57,58]. It has been

Figure 2 General scheme presenting the most used analysis methods of microparticles
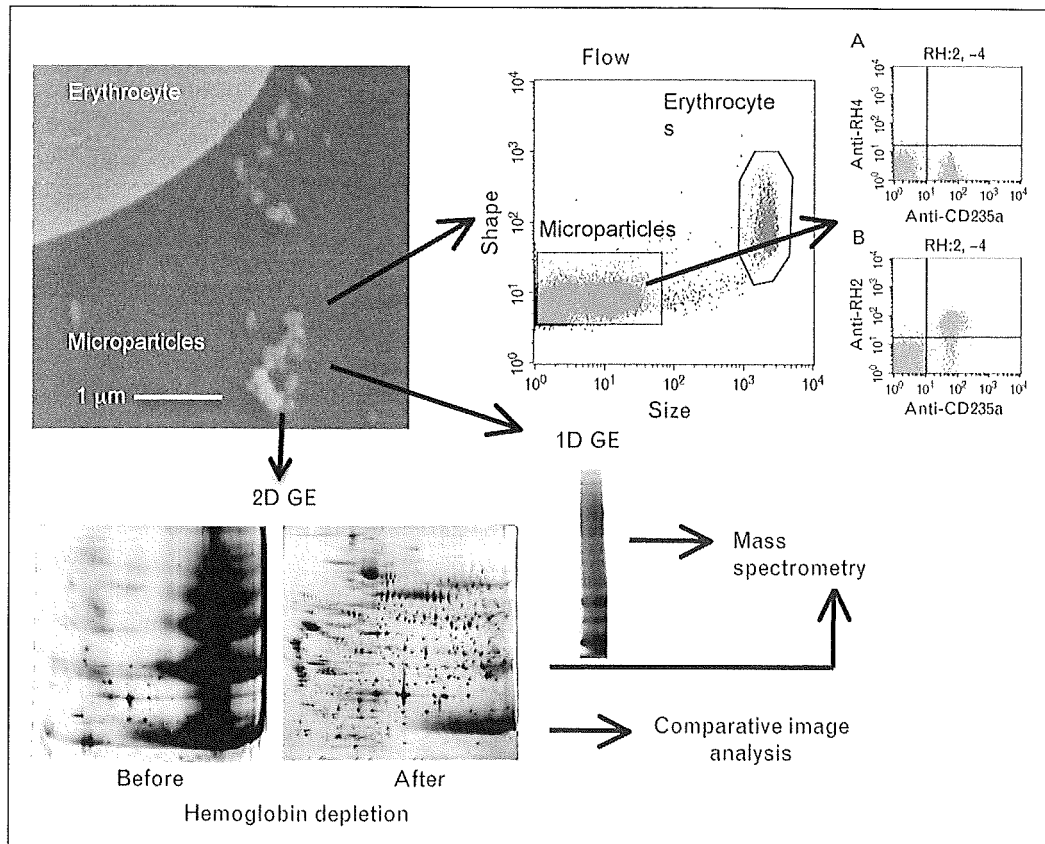
The upper left corner of the figure shows part of a red blood cell and microparticles (scanning electron microscopy; magnification, ×37 000). By flow cytometry technique, it is possible to sort microparticles from erythrocytes according to size and shape. Panels (A) and (B) show the region of microparticles sorted according to their fluorescence. Microparticles issued from a RH:2,-4 typed erythrocyte concentrate were tagged by RH2 antibodies (B), but not with RH4 antibodies used as negative control (A) (tagged microparticles are on right and nontagged on the left) (adapted from [37]). Microparticles were double labelled with anti-CD235a (glycophorin A) which binds to the vast majority of erythrocyte microparticles (histograms show 'MP region' according to their fluorescence; the tagged microparticles are on the right and nontagged on the left of panel A and B). Flow cytometry analysis also may reveal other red blood cell antigens on microparticles (such as Duffy or Kell). Microparticles proteins can be separated by different techniques such as SDS-PAGE (one-dimensional gel electrophoresis) or two-dimensional gel electrophoresis combined either with image analysis or mass spectrometry. Using this approach, various microparticles proteins were identified. Of note, two-dimensional gel electrophoresis of microparticles is hampered by the presence of large amounts of haemoglobin, which can be removed by off-gel electrophoresis, allowing a resolution sufficient for comparison image analysis. GE, gel electophoresis. Adapted with permission from Rubin et al. [12].

reported that antibody binding could induce disruption of membrane organization as well as vesicle formation with consequently the loss of the corresponding blood group antigens [59]. The exact mechanism of antibody-induced antigen suppression, which allows transfused RBC to escape haemolysis, is not known but it is tempting to speculate that selective loss of antigen might occur through RBC vesiculation.

Clinical relevance
A large proportion of Hb is enclosed in RBC microparticles [60*,61] and, with free Hb, participates in the increase of extracellular Hb during storage. Hb composition of microparticles resembles that of the oldest RBC and is enriched in denatured and high molecular weight fractions [39,42]. Due to their small size and the lack of most of the cytoskeletal proteins, it is expected that RBC

microparticles could localize near the endothelium and will probably scavenge nitric oxide as effectively as cell-free Hb [62,63*]. This reduction of nitric oxide bioavailability will affect adversely the microcirculation by promoting platelet aggregation and endothelial adhesion, impairing vasodilatation and generating reactive oxygen species [60*].

Moreover, phosphatidylserine exposure on RBC microparticles surface offers a binding site for prothrombinase and tenase enzyme complexes [12,64] and is associated with increased activity of factors VIII, IX and XI [65*], thus participating in thrombin generation and amplification. Hypercoagulable state associated with enhanced microparticles levels has been observed in chronic haemolytic anemia, including in sickle cell patients [65*,66]. Although the relationship between RBC microparticles

concentration and increased risk of arterial or venous thrombosis events after blood transfusion is not evident, this question has been raised recently in cancer patients [67].

The RBC microparticle potential role of cell-to-cell communication has not been investigated so far. RBC microparticles, as other cell-derived microparticles, could transfer molecules and modify cell phenotype such as illustrated by the transfer of GPI-anchored proteins CD55 and CD59 from vesicles found in erythrocyte concentrates to erythrocytes and granulocytes of paroxysmal nocturnal haemoglobinuria transfused recipients [50]. Moreover, the recruitment of RBC microparticles by nucleated cells allows the transfer of removal signals such as phosphatidylserine to innocent bystanders [68], labelling them with an apoptosis marker. These data offer a perspective of further investigations in the field of cell-to-cell communication capacity of RBC microparticles.

In addition to activating the classical complement pathway through IgG on their membrane, storage vesicles may affect the innate immune response by inhibiting the production of pro-inflammatory cytokines such as tumour necrosis factor-α and interleukin-8 [49$^{\bullet\bullet}$]. To what extent this anti-inflammatory signal may explain some of the immunosuppressive effects of blood transfusions remains to be clarified.

Although vesiculation contributes to erythrocyte homeostasis in eliminating sorted oxidized proteins [42], the impact of RBC microparticles on the microcirculation coupled with their immunomodulation and thrombogenic activities could potentiate adverse clinical outcomes observed in susceptible recipients transfused with older blood [69,70]. Undeniably, these observations raise important questions about the clinical impact of RBC storage lesions; however, this topic remains a matter of debate and needs strong epidemiologic data to clarify its role in transfusion medicine.

Conclusion

Extensive proteomic analyses of RBC microparticles have enabled a better comprehension of erythrocyte changes during ageing and blood storage and outlined the pivotal role of band 3–ankyrin anchoring complex. Indisputably, vesicle formation represents an integral part of the erythrocyte ageing process. RBC microparticles could be considered as the 'Dr Jekyll and Mr Hyde' in the field of transfusion: on one hand, improving RBC survival in transfused recipients by allowing the elimination of toxic molecules and removal signals; on the other hand, enhancing the deleterious events like microcirculation impairment, thrombosis and immunosuppression in susceptible patients.

Despite the currently available proteomics and immunochemical data, the exact and complete mechanisms of erythrocyte microvesiculation remain only partially elucidated. Combining comparative and qualitative proteomic analysis with other different approaches including immunochemical data or flow cytometry will help to discover the precise components involved in RBC microparticles formation and may identify the potential role of microvesicles in transfusion medicine. Therefore, proteomic analysis will provide a tool for the development of methods that could enhance RBC quality and survival after transfusion. Nevertheless, the use of microparticles as biomarkers in clinical routine or blood banking needs the technical barriers involved in their analysis to be overcome.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest
Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 608–609).

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Red blood cell microparticles and blood group antigens: an analysis by flow cytometry

Giorgia Canellini, Olivier Rubin, Julien Delobel, David Crettaz, Niels Lion, Jean-Daniel Tissot

Service Régional Vaudois de Transfusion Sanguine, Epalinges, Switzerland

Background. The storage of blood induces the formation of erythrocytes-derived microparticles. Their pathogenic role in blood transfusion is not known so far, especially the risk to trigger alloantibody production in the recipient. This work aims to study the expression of clinically significant blood group antigens on the surface of red blood cells microparticles.

Material and methods. Red blood cells contained in erythrocyte concentrates were stained with specific antibodies directed against blood group antigens and routinely used in immunohematology practice. After inducing erythrocytes vesiculation with calcium ionophore, the presence of blood group antigens was analysed by flow cytometry.

Results. The expression of several blood group antigens from the RH, KEL, JK, FY, MNS, LE and LU systems was detected on erythrocyte microparticles. The presence of M (MNS1), N (MNS2) and s (MNS4) antigens could not be demonstrated by flow cytometry, despite that glycophorin A and B were identified on microparticles using anti-CD235a and anti-MNS3.

Discussion. We conclude that blood group antigens are localized on erythrocytes-derived microparticles and probably keep their immunogenicity because of their capacity to bind specific antibody. Selective segregation process during vesiculation or their ability to elicit an immune response in vivo has to be tested by further studies.

Keywords: microparticles, erythrocytes, blood group antigens.

Introduction

Microparticles (MPs) are membrane vesicles released by various cells, including red blood cells (RBCs). They are defined by a size of less than 1 μm[1] and contain proteins derived from their parent cell. Microvesiculation represents a controlled process[2] which is triggered by various stimuli and initiates with externalization of negatively charged phospholipids. Erythrocyte-derived MPs (EMPs) represent the most abundant source of MPs in certain pathological states, such as sickle cell disease[3].

Cell activation or apoptosis promote calcium influx and lead to a cascade of events which breaks the links between the membrane and the cytoskeleton proteins, specifically spectrin and protein 4.1R. Therefore, the membrane becomes unstable, allowing the release of MPs[4,5]. During their lifespan, RBCs lose a certain amount of their haemoglobin content and surface area through this mechanism[6]. As RBCs are exposed to a constant oxidative stress and have a limited capacity for self-repair, vesicle formation certainly represents one of the solution to clear senescent antigens and prevent the exposure of dangerous molecules on their surface[7]. MPs formation has been considered as an integral step of RBC aging, as outlined by two recent models of erythrocyte senescence and is strongly correlated with spectrin oxidation[6,8-16]. Once vesiculation capacity is exceeded, old erythrocytes are removed by the reticuloendothelial system[17,18].

The composition of EMPs has been established through proteomic analysis. EMPs proteome differs from its parental cell by the decrease of cytoskeletal-linked molecules, the enrichment of several components (especially hemoglobin, band 3, glycophorins, complement receptors, glycosyl-phosphatidylinositol-anchored proteins and lipid-raft markers) and the exposure of removal signal molecules such as phosphatidylserin and autologous IgG[6,7,17,19,20]. These differences are explained by a process of selective sorting during microvesiculation process[7].

Under blood bank conditions, RBCs undergo structural and biochemical changes[21,22], described as the "storage lesion"[7,23-26]. This lesion shares similar features with RBC aging, with the peculiarity that oxidation of cytoskeleton proteins plays a central role[7,27-31]. It has been shown that the number of glycophorin A (GPA) positive EMPs increases in blood units over time and that their structure gradually change with accumulation of stomatin, compared to EMPs generated *in vivo*[6,18,20,32]. These observations make likely a raft-based process responsible for vesiculation under these conditions[7,17,22,33]. EMPs structure becomes also gradually more heterogeneous, according to the absence of their immunologic removal in the bag[18,28].

There are 31 RBC group systems identified so far[34], five of which hold antigens with carbohydrate structures (ABO, H, GLOB, P, LE) while the others consist of proteins[35]. Up to half of the RBC transmembrane proteins carry blood group antigens, including proteins that are major constituents of the RBC membrane, such as band 3 (ABO and DI), GPA and glycophorin B (MNS), glycophorins C and D (GE), Rhesus polypeptides (RH) and Aquaporin 1 (CO)[36,37]. These highly-expressed molecules are of two types: either transporters proteins with multiple membrane spanning domains (band 3, RH, CO) or sialylated glycoproteins with a single transmembrane domain (GPA, GPB). Due to their density, it would be expected that they play important structural or functional roles[37]. RBC cytoskeleton network is essential for membrane stability and consists of spectrin backbone anchored to the lipid membrane through interaction with actin, protein 4.1R and ankyrin[38]. Some of these erythrocyte proteins, such as the antigens of the RH and FY systems, belong to macromolecular complexes and contribute to membrane stabilization, through their link with protein 4.1R[39].

During storage, RBCs show a decrease of blood group antigens expression, concomitant to the progressive increase of soluble blood group concentration in the plasma[40,41]. Evidence of blood group antigens activity on MPs originated from erythrocyte concentrates (ECs) has been reported by some authors, using agglutination/inhibition tests, Western blotting, immunoelectron microscopy or radio-labeled anti-D antibodies[22,41-44]. In a previous study on EMPs, we identified RH peptides by mass spectrometry from SDS-PAGE of proteins isolated from EMPs[20]. Thus, our work aims to study the presence of clinically significant blood group antigens on MPs. We induced microvesiculation of RBCs stored in ECs after activation with calcium and performed the flow cytometry analysis with antibodies routinely used in immunohematology laboratories for agglutination tests.

Materials and methods
Whole blood collection, processing and storage

Whole blood was collected from normal volunteer donors, attending the Service Régional Vaudois de Transfusion Sanguine and held in bags containing an anticoagulant solution (citrate-phosphate-dextrose) for a maximum of 24 hours at room temperature, prior to processing. The bags were centrifuged, plasma was removed and the packed RBCs were suspended in 100 mL of SAG-M (sodium-adenine-glucose-mannitol) additive solution, afterwards leukocytes were removed by filtration. The ECs were finally stored at 4 °C.

Donor selection was based on homozygous expression of RH, JK, FY or MNS antigens except for KEL3 (Kp[a]) and LU1 (Lu[a]). Only ECs that did not satisfy quality criteria for transfusion were dedicated to this study (particularly those from donors with elevated liver enzymes).

Labeling

The experiment consists in an indirect staining of RBCs with specific blood group antibodies, followed by the generation of RBC-derived MPs via calcium ionophore activation and their analysis by flow cytometry. Samples with anti-RH antibodies undergo a double labelling with anti-CD235a (anti-GPA).

Samples of stored ECs were washed in phosphate-buffered saline solution (PBS) and centrifuged at $1,000 \times g$ for 30 seconds at 4 °C. The residual RBC pellets were incubated with antibodies against blood group antigens according to their phenotype (Human IgM Monoclonal anti-RH2 [anti-C], anti-RH4 [anti-c], anti-RH3 [anti-E], anti-RH5 [anti-e], anti-MNS3 [anti-S]; Human IgM Polyclonal anti-JK1 [anti-Jk[a]], anti-JK2 [anti-Jk[b]]; Human IgG Polyclonal anti-KEL3 [anti-Kp[a]] anti-KEL4 [anti-Kp[b]], anti-FY1 [anti-Fy[a]], anti-FY2 [anti-Fy[b]], anti-MNS4 [anti-s], anti-LU1 [anti-Lu[a]], anti-LU2 [anti-Lu[b]];

Mouse IgM Monoclonal anti-MNS1 [anti-M], anti-MNS2 [anti-N], anti-LE1 [anti-Le^a], anti-LE2 [anti-Le^b]; all from Biotest, Dreieich Germany), adjusted to 100 μL of PBS and mixed constantly for 90 minutes. Excess of antibody was removed by two washing steps in PBS.

As the primary antibodies are not fluorochrome-conjugated, the remaining RBC pellets were incubated with a secondary antibody (100 μl of diluted fluorescein isothiocyanate (FITC)-conjugated antibody), namely FITC-conjugated anti-human IgM (Caltag Laboratories, Burlingame, CA, USA), FITC-conjugated anti-human IgG (Chemicon, Temecula, CA, USA) or FITC-conjugated anti-mouse IgM (BD Biosciences, Franklin Lakes, NJ, USA). The samples were agitated continuously for 60 minutes in the dark. The excess of antibody was removed as described above with centrifugation and washing steps.

Generation of microparticles

Calcium inophore solution (2.6 μg of Ca ionophore A23187; Sigma-Aldrich, St-Louis, MO, USA) and 5 μL of CaCl$_2$ (Fluka, Buch, Switzerland) in 1 mL of PBS were added to the remaining pellet and agitated in the dark for 60 minutes, at 37 °C. Five μL of phycoerythrin (PE)-conjugated anti-human CD235a, diluted 1/10 (BD Pharmingen, San Diego, CA, USA) were added only to the samples labelled with anti-RH antibodies and these were agitated 20 minutes in the dark at room temperature. All the samples were then diluted with 50 μL of PBS and spun down at 1,000 × *g* for 30 seconds to remove RBCs and keep the supernatant containing EMPs. The supernatants were transferred to 5 mL plastic tubes (Falcon, BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometry

Supernatants were diluted in PBS. MPs size was determined using flow cytometric light scatter (FACScalibur, BD Biosciences, Franklin Lakes, NJ, USA). The flow cytometer was calibrated using beads (CaliBRIT™ 3 kit, BD Biosciences, Franklin Lakes, NJ, USA) to ensure standard instrument settings for each analysis. MPs were defined as particles less than 1 μm. Sizing beads of 1-1.4 μm (Spherotech, Lake Forest, IL, USA) were used to verify the appropriateness of the gate. EMPs were gated on the basis of their forward scatter and side scatter signals with logarithmic fluorescence scales (Figure 1). Results of blood group antigens expression were analysed on dot plots and histograms (Figure 2) with negative threshold defined by EMPs obtained from RBCs lacking the corresponding antithetic antigen.
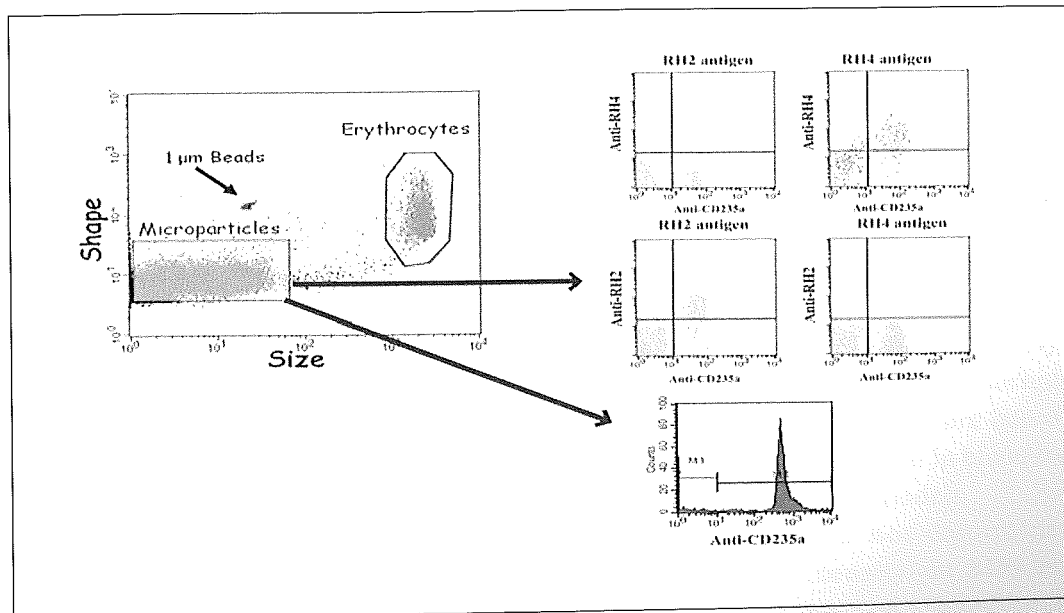

Figure 1 - Flow cytometry analysis of microparticles in erythrocyte concentrates. Size of events is defined by calibration beads of 1 μm. Two gating regions are represented: on the left, the events smaller than 1 μm which contains the microparticles and on the right, the erythrocytes. The expression of Rhesus antigens RH2 (C) and RH4 (c) (depending on the Rhesus phenotype of the donors) and glycophorin A (anti-CD235a) is illustrated.
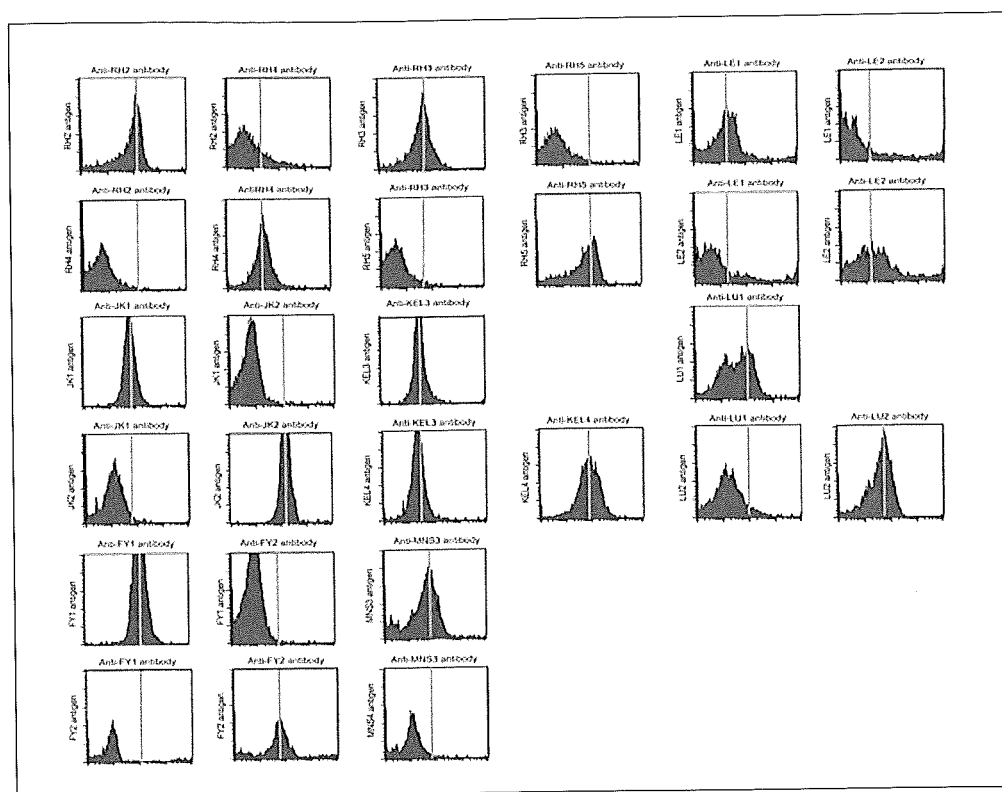
Figure 2 - Detection of clinically significant blood group antigens on microparticles derived from erythrocyte concentrates.
The red blood cells were selected according to their homozygous expression of blood group antigens (vertical axis), except for KEL3 (Kp^a) and LU1 (Lu^a). Flow cytometric histograms show the expression of RH2 (C), RH3 (E), RH4 (c), RH5 (e), KEL3 (Kp^a), KEL4 (Kp^b), JK1 (Jk^a), JK2 (Jk^b), FY1 (Fy^a), FY2 (Fy^b), MNS3 (S), LU1 (Lu^a) and LU2 (Lu^b) antigens on the erythrocyte-derived microparticles. The absence of the antithetic antigen on microparticles originated from homozygous erythrocytes served as negative control.

Results

Activation of RBCs with calcium allows the generation of MPs which are less than 1 μm (Figure 1). The erythrocyte origin of the MPs is confirmed by the anti-GPA staining. Intensities of detection of blood group antigens are shown on flow cytometric histograms (Figure 2). Using this method, the following antigens were detected on EMPs: RH2 (C), RH3 (E), RH4 (c) and RH5 (e) antigens, KEL3 (Kp^a) and KEL4 (Kp^b) antigens, JK1 (Jk^a) and JK2 (Jk^b) antigens, FY1 (Fy^a) and FY2 (Fy^b) antigens, MNS3 (S), Le^a (LE1) and Le^b (LE2), and finally LU1 (Lu^a) and LU2 (Lu^b) antigens. The presence of M (MNS1), N (MNS2), and s (MNS4) antigens could not be demonstrated because of agglutination of RBCs after the addition of the primary antibody, despite successive dilutions of the latter. However, glycophorin A was clearly identified on the surface of EMPs, either by flow cytometry or immunoblot analyses[20]. Gylcophorin B was detected with MNS3 antibodies (Figure 2).

Discussion

This study shows that MPs generated from ECs express clinically significant blood group antigens. Detecting their expression by flow cytometry indicates that most of them keep the property to react with the corresponding antibodies despite cytoskeletal dissociation during vesiculation. The activities of M (MNS1), N (MNS2), and s (MNS4) antigens have not been demonstrated owing to technical difficulties. For caution, these findings are based on artificially induced microvesiculation in blood bags and request verification in human models.

Our results did not determine whether the antigenic sites density on MPs corresponds to those of the parent cell. Thus, the question of the selective segregation (or even enrichment) of blood group antigens into MPs cannot be answered. Prior imunoelectron microscopy analysis of EMPs has ruled out the clustering of FY monomeric chains formation during vesiculation[42]. However, antigen

selection could arise in MPs, as suggested by the loss of specific RBC antigens during autoimmune hemolytic anemia or transfusion of incompatible blood[45, 46]. In these examples, antibody binding could induce disruption of membrane organization. Therefore it is reasonable to speculate that selective loss of antigen may occur through RBC vesiculation, although the exact mechanism involved has not been elucidated so far[47].

Duffy protein, also named DARC protein, is a receptor for chemokines. RBCs without DARC protein have lost the Duffy-dependent chemokine binding capacity. This may partly explain the tendency of FY:-1,-2 sickle cell patients to chronic inflammation and alloimmunization[37]. Some authors have shown maintenance of blood group antigens function on EMPs, especially the chemokine binding activity of DARC protein[42]. The hypothetic role of Duffy positive MPs in clearing local inflammation mediators has to be further investigated.

As their antigenic activity is oriented outwards and recognized by specific monoclonal antibodies, blood group antigens located on MPs may elicit an immune response *in vivo*. EMPs are easily phagocytised by antigen-presenting cells and despite their small size and low total volume, may represent a significant immunogenic load during transfusion[17, 18, 48]. Recently, Rhesus immunization originating from EMPs was suspected in an aphaeresis platelet recipient[49]. However, the capacity of EMPs to induce alloimmunization after transfusion needs to be demonstrated, especially whether they are more immunogenic and over which threshold they may trigger antibody formation.

The level of EMPs in blood of sickle cell patients is significantly elevated and strongly correlates with the degree of intravascular hemolysis and premature aging of RBCs[3, 50, 51]. Red cell antibody development is a well recognized complication of chronic transfusion in sickle cell patients, with an incidence of 10-40% in prior published reports[52-54]. Transfusion of RBCs with phenotype matching for C (RH2), c (RH4), E (RH3), e (RH5) and K (KEL1) antigens have reduced to six fold the immunization rate[55,56]. Several factors contribute to RBC alloimmunization in these patients. Therefore it will be important to define if EMPs contained in blood units will participate to the stimulation of the immune system and which strategies need to be developed by blood banks for its prevention.

Conclusion

Our work has brought further insights in the composition of EMPs generated during ECs storage upon calcium stimulation and raised questions about their potential immunogenicity. Nevertheless, the MPs capacity of immunization during transfusion needs animal models to investigate the immune effectors side. As well, determining antigen density on EMPs using antibody calibration kit will provide further information about the hypothesis of selective sorting during erythrocyte microvesiculation[57].

Although EMPs contributes to erythrocyte homeostasis in eliminating sorted oxidized proteins[18], they represent a "two-edged sword" with the capacity to induce adverse clinical outcomes in transfused recipients through their immune effects (immuno-modulation and alloimmunization), thrombogenic activities and negative impact on the micro-circulation[3,32, 58-60]. The analysis of EMPs generated during blood processing and aphaeresis techniques will permit to improve the quality of blood products[61,62]. Understanding the real impact of EMPs and minimizing their formation during blood processing represent a future challenge in the field of transfusion.

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Correspondence: Jean-Daniel Tissot
Route de la Corniche 2
CH-1066 Epalinges, Suisse
e-mail: jean-daniel.tissot@mavietonsang.ch