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On the association of lipid rafts to the spectrin skeleton in human erythrocytes ☆

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ABSTRACT

Lipid rafts are local inhomogeneities in the composition of the plasma membrane of living cells, that are enriched in sphingolipids and cholesterol in a liquid-ordered state, and proteins involved in receptor-mediated signalling. Interactions between lipid rafts and the cytoskeleton have been observed in various cell types. They are isolated as a fraction of the plasma membrane that resists solubilization by nonionic detergents at 4 °C (detergent-resistant membranes, DRMs). We have previously described that DRMs are anchored to the spectrin-based membrane skeleton in human erythrocytes and can be released by increasing the pH and ionic strength of the solubilization medium with sodium carbonate. It was unexplained why this carbonate treatment was necessary and why this requirement was not reported by other workers in this area. We show here that when contaminating leukocytes are present in erythrocyte preparations that are subjected to detergent treatment, the isolation of DRMs can occur without the requirement for carbonate treatment. This is due to the uncontrolled breakdown of erythrocyte membrane components by hydrolases that are released from contaminating neutrophils that lead to proteolytic disruption of the supramolecular assembly of the membrane skeleton. Results presented here corroborate the concept that DRMs are anchored to the membrane skeleton through electrostatic interactions that most likely involve the spectrin molecule.

1. Introduction

The study of lipid rafts has attracted considerable interest in recent years. The partition of glycosylphosphatidylinositol (GPI)-linked proteins in a low-density fraction of the membrane of epithelial cells that resisted solubilization at 4 °C by the nonionic detergent Triton X-100 was the original observation that advanced the concept of lipid rafts and also defined the methodological approach for most of the subsequent studies [1]. Thus, the biochemical characterization of these membrane microdomains has been traditionally based on fractions of the plasma membrane that resist solubilization by nonionic detergents at 4 °C (detergent-resistant membranes, DRMs) that can be isolated by centrifugation as a band of low buoyant density in sucrose gradients. The DRMs are believed to originate from the coalescence of larger aggregates of lipid rafts that exist as dynamic entities in the membrane of living cells, although the assumption that DRMs correspond to the lipid rafts has been questioned [2,3]. The lipid phase in the DRMs is in a liquid-ordered state, due to its enrichment in sphingolipids (that preferentially contain saturated acyl chains) and cholesterol. DRMs also contain characteristic subsets of integral and peripheral membrane proteins, such as GPI-linked and other acylated proteins [4]. It has been observed that lipid rafts interact with the cell cytoskeleton in various cell types [5–7].

The erythrocyte membrane is a complex entity, with unique properties, that comprises a sub-membranous lattice of proteins in the form of a network, composed mainly of spectrin tetramers, actin protofilaments, tropomyosin and protein 4.1. This lattice is linked, directly or indirectly with other peripheral proteins, such as ankyrin, to the lipid bilayer through "vertical interactions" with integral membrane proteins, mainly band 3, Rh-associated glycoprotein (RhAG) and glycophorin C (GPC). The sub-membrane spectrin network is defined as a membrane skeleton to distinguish it from the cytoskeleton found in all other nucleated cells, and confers mechanical robustness to this long-lived circulating cell while still maintaining the characteristic flexibility and deformability that the cell needs to traverse the circulatory system for approximately 170,000 times during its life [8,9].

Despite the wealth of data existing on lipid rafts and DRMs obtained from a variety of cell types, relatively little information is available concerning erythrocytes, particularly with respect to the interaction between the DRMs and the membrane skeleton, and the effective procedures for DRM isolation (Ref. [10] and citations therein, [11]). The erythrocyte can be obtained with ease and it is devoid of

☆ This work is dedicated to retired Professor Augusta Brovelli.

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subcellular structures, making it the model of choice for the study of biological membranes. The topology and topography of its lipid and protein components have been investigated in great detail. Nonionic detergents have been of value for determining the topological aspects of the integral and peripheral protein species and their disposition in the membrane [12,13], and the early studies identified that a significant portion of the membrane is resistant to solubilization by such detergents. Because the detergent-resistant membrane contains the bulk of the membrane skeleton, it has a high buoyant density and is therefore commonly named a "triton shell". Remarkably, this detergent-resistant portion was found to be enriched in sphingolipids [12–14]. In retrospect, it is natural to think of those early-described detergent-resistant membranes as containing the entities that later became known as lipid rafts. We have recently described that DRMs in human erythrocyte ghosts [10] and also in whole erythrocytes [15,16] are connected to the membrane skeleton through what appear to be electrostatic interactions. This conclusion was based on the fact that DRMs can be separated from detergent-treated ghosts or whole erythrocytes as a fraction in the low-density region of a sucrose gradient, after the ionic strength and the pH of the detergent-containing medium were increased. In most of the published work, however, lipid rafts were easily obtained from erythrocytes as a fraction of low buoyant density in flotation experiments, apparently without the need for any additional procedures to detach them from the spectrin skeleton. We show here that the purity of the erythrocyte preparations, particularly with respect to the level of contamination by neutrophils, greatly affects the experimental procedures needed to isolate DRMs.

2. Materials and methods

2.1. Antibodies and reagents

The following were used: Mouse monoclonal (29) anti-flotillin-2 (code 610384, Becton Dickinson Italia, Milan, Italy); goat polyclonal (M-14) anti-human stomatin (code sc-48308), mouse monoclonal (BRIC 10) anti-human GPC (code sc-59183), goat polyclonal (N-19) anti-human p55 (code sc-13490), mouse monoclonal (8C3) anti-human ankyrin-1 (code sc-12733), HRP-conjugated mouse anti-goat IgGs (code sc-2354) (Santa Cruz Biotechnology, Tebu-bio, Italy); mouse monoclonal (BIII-136) anti-human band 3 (code B9277, Sigma-Aldrich, Milan, Italy); mouse polyclonal anti-human protein 4.1 (code H00002035-A01, Abnova, Taipei, Taiwan); HRP-conjugated goat anti-mouse IgGs (code 170-6516, Bio-Rad Laboratories, Milan, Italy). *N*-Acetyl-eglin-C (recombinant) was from Enzo Life Sciences, USA. Di-isopropylfluorophosphate (DFP) was from Sigma-Aldrich, Milan, Italy.

2.2. Blood processing

Blood was freshly drawn from healthy human donors, after informed consent was obtained, in 0.1 volumes of 130 mM citric acid, 152 mM tri-sodium citrate, 112 mM glucose, or in 0.1 volumes of 3.8% (w/v) tri-sodium citrate, as anticoagulants. To remove leukocytes and platelets [17,18], whole blood or a 50% haematocrit (Ht) suspension of cells in PBS (5 mM Na-phosphate, 154.5 mM NaCl, 4.5 mM KCl, pH 7.4) were layered on a mixture made of equal parts, by weight, of α-cellulose and microcrystalline cellulose equilibrated with PBS. Filtered erythrocytes were washed three times in PBS or in one of the following buffers: HN (5 mM HEPES, 150 mM NaCl, 4.5 mM KCl) or HNM (10 mM HEPES, 150 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$). In some instances (see figure legends and text), the last wash was performed with HKM (10 mM HEPES, 150 mM KCl, 4.5 mM NaCl, 1 mM $MgCl_2$). The pH of all the buffers was adjusted to 7.4 and their tonicity to 300–315 mOsm/kg H_2O, as determined by a freezing point depression osmometer (Hermann Roebling, Berlin, Germany). When filtration was omitted, whole blood was centrifuged at 25 °C in a fixed-angle rotor centrifuge at 1000×*g* for 5 min. After the first sedimentation, the plasma, "buffy coat" and a thin layer of erythrocytes were aspirated and discarded. Approximately 4 volumes of PBS were added to the packed cells that were resuspended and subjected to a second centrifugation step under the same conditions. The supernatant, "buffy coat" and a thin layer of erythrocytes were again aspirated and discarded. The cells were resuspended in PBS, HN or HNM and subjected to two more washes, as described above.

Filtered or washed erythrocytes were resuspended at 20% Ht and aliquots of suspensions used for DRMs preparation, gelatin zymography, SDS–PAGE and Western blotting. For some experiments, the cells (20% Ht) were pre-incubated with 1–5 mM (final concentration) DFP at 37 °C for 10 min and washed three times before DRMs isolation.

2.3. Preparation of DRMs

DRMs isolation from filtered erythrocytes was carried out essentially as previously described [16], with modifications. Briefly, the cells (1.25×10^9) were incubated at 4 °C in HN or HKM containing Triton X-100, so that the final volume was 0.625 ml and the final detergent concentration 1% (w/v) (16 mM). After 30 min incubation, the samples were mixed with an equal volume of 80% (w/v) sucrose in 0.3 M Na_2CO_3 (or in 0.3 M K_2CO_3) and transferred to ultracentrifuge tubes (5 ml, Ultra-clear™, code N. 344057, Beckman Coulter, Milan, Italy). Sucrose solutions in the same buffer as cell lysates [2.5 ml of 30% (w/v) sucrose followed by 1.25 ml of 5% (w/v) sucrose] were layered on the samples and the tubes spun for variable times (see text) at 225,000 ×g_{max} at 4 °C in a bench-top ultracentrifuge (Optima-Max, equipped with a swinging-arm MLS50 rotor, Beckman Coulter, Milan, Italy). After ultracentrifugation, one fraction of 0.8 ml, one fraction of 1.0 ml, usually containing all the DRMs, and four fractions of 0.8 ml were collected from the top of the tube and saved for subsequent characterization. DRMs from washed erythrocytes were prepared by following the same procedure as for filtered cells, except for the omission of carbonate. Cell lysates were mixed with 80% sucrose in the same buffer as the lysate, before transferring to the ultracentrifuge tube.

2.4. Protein detection in sucrose gradient fractions

Aliquots of filtered or washed erythrocytes, and of sucrose gradient fractions, were subjected to SDS–PAGE [19] in 10% (w/v) isocratic or 5%-15% (w/v) gradient polyacrylamide gels, for Western blotting or Coomassie blue staining, respectively. Sucrose-gradient fractions (1–4 from above) were mixed with 0.5 volumes of reducing SDS–PAGE sample buffer [50 mM Tris–HCl pH 6.8, 5% (w/v) SDS, 5 mM EDTA, 200 mM dithiotreitol (DTT), 35% (w/v) sucrose, 0.01% bromophenol blue], while the haemoglobin-rich fractions (5 and 6 from above) and the total erythrocyte suspensions (20% Ht) were treated with 9 volumes of diluted SDS–PAGE sample buffer [1 volume of reducing SDS–PAGE sample buffer plus 1.7 volumes of 5% (w/v) SDS in water], heated at 60 °C for 15 min and stored at −20 °C until analysis. All the samples to be subjected to electrophoresis (except for samples shown in Fig. 1) were pre-incubated with 5 mM DFP (final concentration) for 5 min at 25 °C before addition of the SDS–PAGE sample buffer, to avoid proteolysis during heating in SDS [20].

For Coomassie blue staining, samples were processed as described above, except for fractions 5 and 6 that were mixed with three volumes of diluted sample buffer containing 100 mM DTT. After SDS–PAGE, the gels were stained with freshly prepared 0.2% (w/v) Coomassie blue R250 in 20% (w/v) trichloroacetic acid (TCA).

For Western blotting analysis, proteins separated by SDS–PAGE were electro-transferred to PVDF membranes [21] that were then incubated for 1 h at 25 °C in blocking buffer [20 mM Tris–HCl pH 7.4,

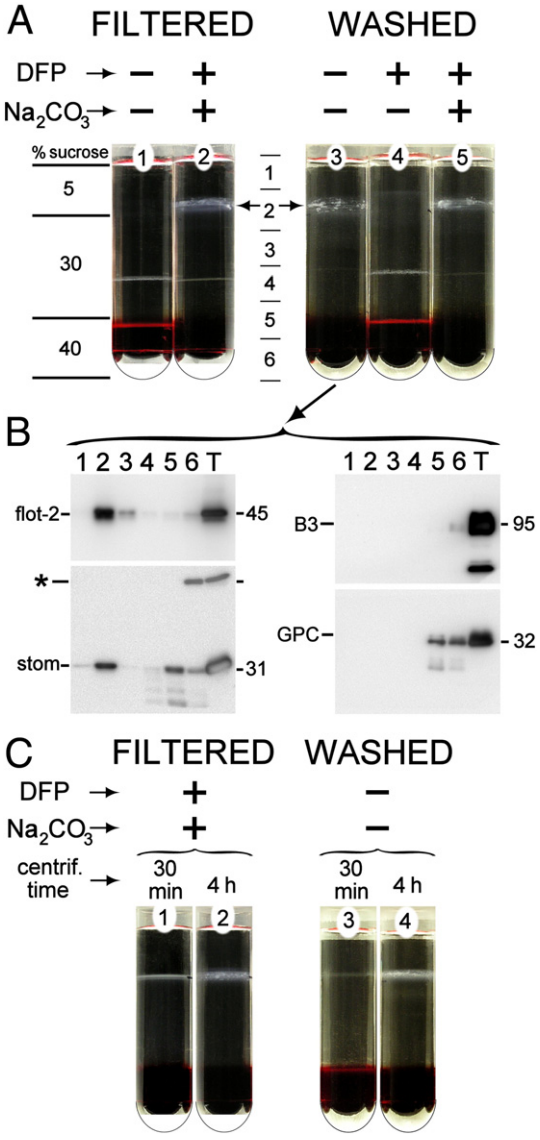

Fig. 1. Isolation of DRMs from filtered or washed erythrocytes. (A) Erythrocytes were treated with Triton X-100 in HN, mixed with an equal volume of 80% sucrose in HN (tubes 1, 3 and 4) or 80% sucrose in 0.3 M Na_2CO_3 (tubes 2 and 5), and subjected to ultracentrifugation for 16 h. Carbonate was required to obtain a band of light-density material (arrow) from filtered (tube 2) but not from washed (tube 3) erythrocytes. When DFP (5 mM) was added to the washed erythrocytes, the low-density band was not obtained (tube 4), unless carbonate was added (tube 5). (B) Six fractions collected from tube 3 were analyzed by Western blotting of flotillin-2 (flot-2), stomatin (stom), Band 3 (B3) and GPC. Samples of the total erythrocyte population (T) before lysis with Triton X-100 were loaded for reference. The asterisk in the stomatin blot indicates an aggregated form of the protein. The image is representative of results obtained in several similar experiments. (C) Filtered or washed erythrocytes were treated as described in (A), tubes 2 and 3, respectively. After only 30 min centrifugation, a DRM band was visible in the filtered, carbonate treated sample (tube 1) but not in the sample of washed erythrocytes treated with Triton X-100 in the absence of carbonate (tube 3). Tubes 1 and 3 were centrifuged again for 3.5 h. Only after this period a band of DRM-like material was visible in the washed erythrocytes sample (tube 4). The DRM band of tube 1 broadened during 4 h of centrifugation, due to diffusion at the interface between 5% and 30% sucrose (tube 2).

150 mM NaCl, 0.05% (v/v) Tween-20, 5% (w/v) skimmed milk], then overnight at 4 °C in the primary antibody at the specified dilutions (see below) in Washing buffer [50 mM Tris–HCl pH 7.5, 0.2 M NaCl, 1% (w/v) polyethylene glycol 20000, 1% (w/v) BSA, 0.5% (v/v) Tween 20], and finally with the secondary antibody diluted in Washing buffer as specified below, after which, the proteins were revealed by chemiluminescence detection (Amersham ECL Western blotting kit, GE Healthcare, Milan, Italy):

Primary antibody, dilution	HRP-conjugated secondary antibody, dilution
anti-stomatin, 1:1000	mouse anti-goat, 1:4000
anti-flotillin-2, 1:8000	goat anti-mouse, 1:4000
anti-band 3, 1:30000	goat anti-mouse, 1:30000
anti-GPC, 1:10000	goat anti-mouse, 1:10000
anti-p55, 1:200	mouse anti-goat, 1:4000
anti-protein 4.1, 1:2000	goat anti-mouse, 1:4000
anti-ankyrin, 1:6000	goat anti-mouse, 1:6000

2.5. Purification of granulocytes

Granulocytes were obtained by Dextran-70 sedimentation of blood followed by Ficoll-Hypaque gradient centrifugation, essentially as described by Böyum [22], with minor modifications [23], and washed in PBS supplemented with 2 mM EDTA and 0.5% (w/v) BSA. The residual erythrocytes in the granulocyte-rich fraction were eliminated by differential hypotonic lysis. The cells were resuspended in PBS and counted. Finally, cells were sedimented and resuspended in the appropriate HEPES buffer (see above) and used for the subsequent experiments. Aliquots of cells were also stored at −20 °C and then analyzed by gelatin zymography.

2.6. Gelatin zymography

Analysis of MMP-9 activity was performed by SDS–PAGE in 7.5 or 10% (w/v) polyacrylamide gels impregnated with 0.1% (w/v) type A-gelatin from porcine skin as described [24,25] with modifications. Briefly, blood cells, fresh or stored at −20 °C, were resuspended in PBS containing 5 mM DFP at the concentration of 5.3×10^5 cells/μl (erythrocytes) or 2×10^4 cells/μl (granulocytes) and then mixed with 0.5 volumes of non-reducing SDS–PAGE sample buffer (same as the reducing buffer, less DTT) without heating. After electrophoresis, gels were incubated for 1 h under agitation in 2.5% (v/v) Triton X-100, washed three times with deionised water and then incubated overnight at 37 °C in 50 mM Tris–HCl pH 7.6, 150 mM NaCl and 10 mM $CaCl_2$. After digestion, gels were stained for 30 min with 0.03% Coomassie brilliant blue R-250 in 50% (v/v) methanol, 9.2% (v/v) acetic acid, and then destained with 10% (v/v) isopropanol, 10% (v/v) acetic acid.

3. Results

3.1. Leukodepletion of blood and its effect on isolation of DRMs from whole erythrocytes

Following on from our previous work [10,15,16], we investigated why DRMs obtained from erythrocytes required an alteration in the ionic strength and the pH of the solubilization medium with Na_2CO_3. All our previous experiments were carried out with purified erythrocytes obtained by filtering the blood through cellulose. However, when the starting material consisted of washed, non-filtered erythrocytes, a low-density band of DRM-like material could be obtained in the absence of carbonate, at the 5%–30% sucrose interface in the gradients (Fig. 1A, tube 3). The isolated material was similar, in terms of both quality and quantity, to the DRMs prepared following the addition of carbonate when filtered erythrocytes were used as the starting material (Fig. 1A, tube 2): it contained approximately 30% of the total cell cholesterol (not shown), was enriched in flotillin-2 and stomatin and devoid of band 3 and GPC (Fig. 1B). The fractions obtained from the gradients of washed erythrocyte samples were examined and their protein content compared with that of untreated cells. A strong decrease in protein content was observed, particularly for band 3 and GPC, which are

normally depleted in the DRMs and which concentrate in the high-density region of the gradients [15] (Fig. 1B, compare fraction 5 and 6 with the sample of untreated cells, T). This suggested that extensive proteolytic degradation had occurred during the isolation of DRMs from the washed erythrocytes but not from the filtered erythrocytes.

When DRMs were obtained from washed erythrocytes, inclusion of DFP during treatment with Triton X-100 prevented the generation of low buoyant density-DRMs (Fig. 1A, tube 4), while treatment with Na_2CO_3 overcame this inhibitory effect (Fig. 1A, tube 5).

As previously observed, no DRMs could be obtained from filtered erythrocytes if carbonate was omitted (Fig. 1A, tube 1), while inclusion of carbonate allowed the subsequent isolation of DRMs after only 30 min centrifugation (Fig. 1C, tube 1). Conversely, no carbonate was required to isolate DRMs from washed erythrocytes, provided that the samples were centrifuged for at least 4 h (Fig. 1C, tube 4).

3.2. Effect of washing or filtration through cellulose of whole blood on the level of contamination by leukocytes

The fact that DFP as well as carbonate (implicating serine proteases and pH) strongly affected DRM generation suggested that at least part of the effect may have been due to the "buffy coat", consisting primarily of polymorphonuclear neutrophils (PMNs), the most abundant leukocytes in human blood. PMNs contain granules with a potent proteolytic activity and are therefore the likely source of proteases responsible for the observed breakdown of erythrocyte proteins. We used gelatin zymography for the quantification of

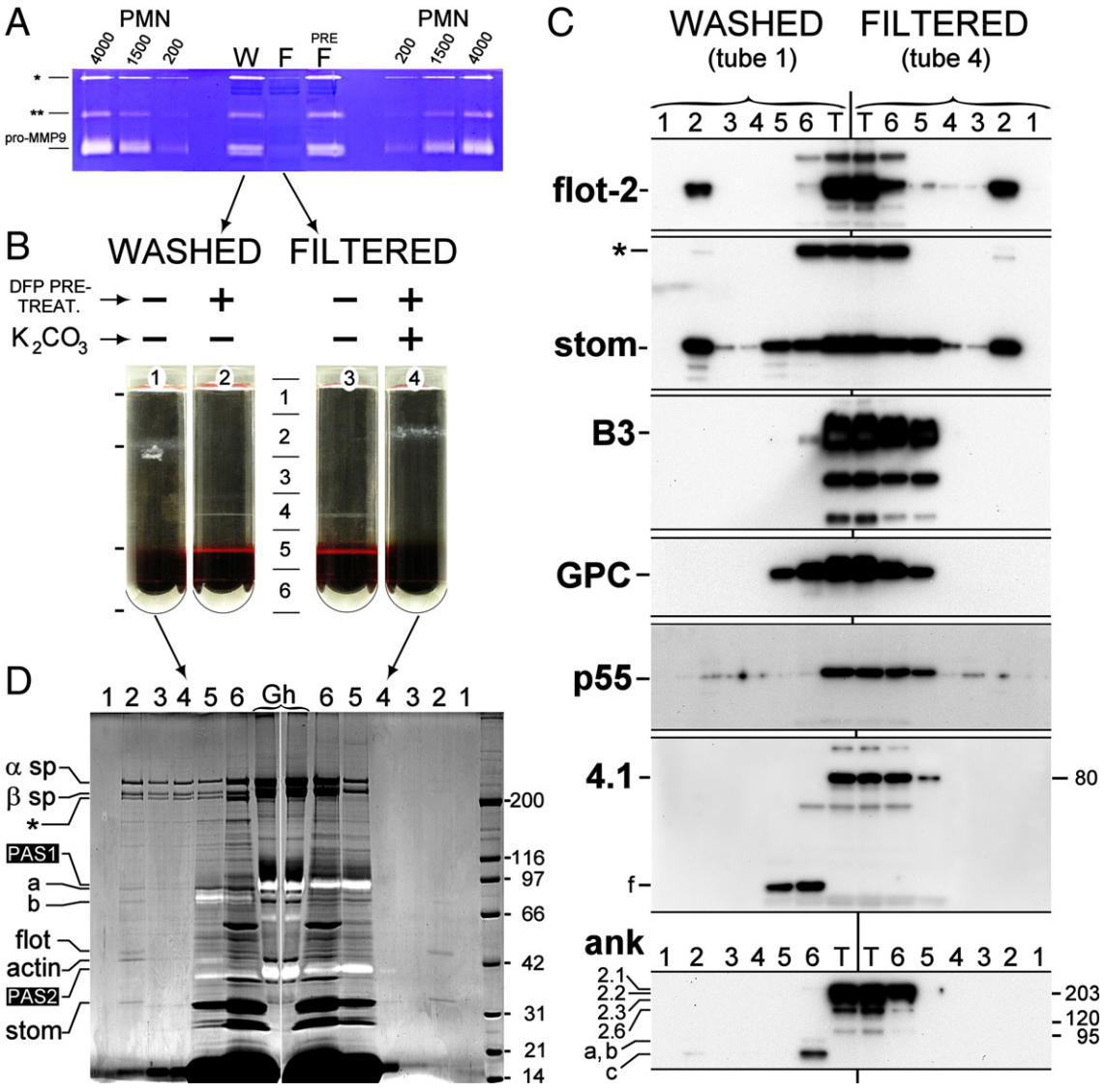

Fig. 2. Isolation of DRMs, in HKM buffer, from washed or filtered erythrocytes. (A) Gelatin zymography of total blood cells (preF), washed (W) or filtered (F) erythrocyte suspensions. The equivalent of 20 µl of packed erythrocytes (approximately 2×10^8 cells) was treated for gelatin zymography. In each lane, 10^7 erythrocytes were loaded. For calibration of gelatinase activity, purified PMNs were loaded in the numbers indicated. Three major bands are visible, corresponding to the gelatinase activity of pro-MMP-9 (92 kDa), of a 120-kDa band (**) corresponding to a complex of MMP-9 and the 25 kDa neutrophil-gelatinase-associated-lipocalin (NGAL), plus a minor band of MMP complex at 240 kDa (*), of unclear origin. In the lanes where erythrocytes were present, erythrocyte spectrins that stain with the Coomassie blue are visible above the lighter background of stained gelatin. (B) Washed or filtered erythrocytes, pre-treated or not with DFP (5 mM), were treated with Triton X-100 in HKM, mixed an equal volume of 80% sucrose in HKM (tubes 1, 2 and 3) or 80% sucrose in 0.3 M K_2CO_3 (tube 4), and subjected to ultracentrifugation for 16 h. (C) Six fractions from tubes 1 and 4 were collected, subjected to treatment with DFP before addition of SDS–PAGE sample buffer and analyzed by Western blotting of flotillin-2, stomatin, Band 3, GPC, p55, protein 4.1 (4.1) and ankyrin (ank). For samples "T", 0.24 µl equivalent packed erythrocytes (approximately 2.4×10^6 cells) prepared as described in Section 2.4 were loaded for reference. The asterisk in the stomatin blot indicates an aggregated form of the protein. Band "f" in the protein 4.1 blot and bands "a, b " and "c" in the ankyrin blot are some relevant degradation products of these proteins. (D) Coomassie blue-stained gel of gradient fractions from tubes 1 and 4. The PAS1 and PAS2 labels indicate the corresponding negatively stained bands in the samples of purified ghost membranes (Gh) and in fractions 5 and 6 from filtered cells.

PMNs in suspensions of washed or filtered erythrocytes. This very sensitive technique is based on the in-gel detection of the activity of metalloproteinases [26,27], which PMNs (but not erythrocytes) express in high amounts, in particular MMP-9 (neutrophil gelatinase B). Thus, detection of gelatinase activity in erythrocyte suspensions is a clear sign of contamination by PMNs.

Fig. 2A summarizes the results obtained by gelatin zymography and illustrates that washed erythrocytes (W) contain similar numbers of PMNs (150–400 PMNs per 10^6 erythrocytes) as found in unfiltered erythrocyte preparations (preF). However, filtration of whole blood (F) significantly lowered contamination by PMNs to less than 20 PMNs per 10^6 erythrocytes. In a number of experiments, DRMs were prepared from suspensions of filtered erythrocytes to which known numbers of purified PMNs were added. This revealed that the level of contamination by PMNs above which DRMs could be obtained without the addition of carbonate was approximately 100 PMNs per 10^6 erythrocytes (data not shown).

3.3. Membrane proteins in DRM preparations from washed erythrocytes or filtered and DFP-pre-treated erythrocytes

In order to determine the effects of DRM isolation procedures on erythrocyte membrane proteins, we prepared samples using (i) washed erythrocytes processed without the addition of DFP and (ii) filtered erythrocytes that were also pre-incubated at 37 °C with DFP. Both preparations were purified in HKM, with the purpose of reproducing more physiological conditions with respect to intracellular cations content [14], and providing a supply of Mg^{2+} ions for maintaining the stability of the membrane skeleton [28]. For the same reason, potassium carbonate was used, when appropriate, instead of the sodium salt. The samples were subjected to Triton X-100 treatment in HKM buffer, and fractions from the sucrose gradients were examined by Western blotting of seven different membrane proteins, and by Coomassie blue staining for evaluating the membrane-skeletal proteins. As expected, a fraction of low-density material was obtained, in the absence of carbonate, from washed erythrocytes (Fig. 2B, tube 1), while filtered, DFP-pre-treated erythrocytes released DRMs only when carbonate was added (Fig. 2B, tube 4). The low-density material from washed erythrocytes (Fig. 2B, tube 1, fraction 2) was examined on Western blots (deliberately overexposed to evaluate possible proteolytic degradation), and it was found to be relatively enriched in the typical lipid raft markers, flotillin-2 and stomatin, although both proteins were to some extent proteolyzed (Fig. 2C, "washed", tube 1 samples). Proteolysis of these proteins was especially evident as a decrease in their content in the high-density fractions from the sucrose gradients (fractions 5 and 6). Band 3 was also dramatically decreased in these fractions, while, surprisingly, GPC was practically unaffected. It is shown here for the first time that p55, a protein involved in the linkage between GPC and the membrane skeleton, was present both in the DRMs and in the soluble membrane fraction, and was heavily proteolyzed in the lower fractions of the "washed" samples (Fig. 2C, compare fractions 5 and 6 of "washed" *vs* "filtered"). Protein 4.1 and ankyrin were also completely degraded after 16 h centrifugation at 4 °C.

The Coomassie-stained gels (Fig. 2D) revealed that the low-density fraction from washed erythrocytes contained membrane-skeletal proteins, mostly spectrin and actin, which were absent in the DRMs from filtered erythrocytes, but also contained a series of bands not immediately recognizable as intact erythrocyte membrane proteins (for instance bands "a" and "b" in fraction 2 of "washed" samples). Spectrin appeared distributed in various fractions of the washed erythrocytes, while it was confined mainly in fraction 6 of filtered erythrocytes. Densitometric analysis of the total spectrin bands in fractions 2–6 of washed erythrocytes and fraction 5 and 6 of filtered erythrocytes (Fig. 2D) revealed an overall decrease of approximately 40% of β-spectrin in the samples of washed erythrocytes. This was due to proteolytic conversion of β-spectrin to a band of lower molecular mass (Fig. 2D, asterisk).

Interestingly, with this type of staining, two bands, corresponding to glycophorin A (GPA) dimer (approximately 95 kDa, PAS1) and monomer (approximately 36 kDa, PAS2), were negatively stained. In sharp contrast with the high stability of GPC (Fig. 2C), GPA (both dimer and monomer) appeared to be completely digested in washed erythrocytes (Fig. 2D: compare the negatively stained bands in fractions 5 and 6 in "washed" and "filtered" samples).

Contrary to the situation for washed erythrocytes, in filtered and DFP-pre-treated erythrocytes no sign of proteolysis, even of the most sensitive proteins, was evident.

3.4. Leukodepletion of blood and its impact on the integrity of erythrocyte membrane proteins during DRM preparation

Since filtration does not completely remove granulocytes, we next examined the effects of low levels of contaminating PMNs in erythrocyte suspensions subjected to detergent treatment, as described in Fig. 3 legend. The image of the gradients after ultracentrifugation is shown in Fig. 3A. The translucent band above the 40% sucrose solution (asterisk) corresponds to the membrane skeletons with DRMs associated to them. DRMs were not released, due to the low levels of contaminating PMNs.

The integrity of protein 4.1 and ankyrin was examined in fractions 5 and 6 from the gradients. At 1 PMN/10^6 erythrocytes, both proteins appeared significantly proteolyzed and reached almost complete degradation at 50 PMNs/10^6 erythrocytes (Fig. 3B). Breakdown products of variable size were evident in the Western blots of ankyrin and protein 4.1, representing different levels of degradation of the two proteins, depending on the degree of contamination by PMNs. Band 3 was also significantly proteolyzed. GPC, which we initially described to undergo partial fragmentation (Fig. 1B), showed remarkable stability in the subsequent experiments (Figs. 2C and 3B). p55 appeared to be equally distributed between fractions 5 and 6 (Fig. 3B). Fraction 5 presented a lower p55 signal because this fraction was collected as half volume of the sample-containing 40% sucrose step and half volume of the 30% sucrose solution step.

The spectrin skeletons retained their integrity, as indicated by both the presence of the translucent band in the sucrose gradients (Fig. 3A, asterisk), and by the absence of spectrin degradation, as shown in Fig. 3C.

4. Discussion

Despite the fact that erythrocytes have been the model of choice for the study of cell membranes, experiments studying their lipid rafts are based on procedures designed for studies on other cell types [1,29]. As early as 1973, Yu *et al.* [12] reported the existence of a sphingolipid-enriched moiety that remained associated with the membrane skeleton in Triton-extracted erythrocyte ghosts. Similar results were later reported by Sheetz [14], who used DFP-pre-treated erythrocytes. Although the importance of the original observations of Yu *et al.* is acknowledged [30], their implication for the study of lipid rafts in erythrocytes have not been fully appreciated. In fact, the "sphingolipid segments" of Yu *et al.* [12], which may now be identified as the lipid raft fraction, remained tightly associated with the membrane skeleton, and would have been impossible to isolate as a fraction of low buoyant density. Nevertheless, the original definition of lipid rafts (as membrane domains resistant to extraction in cold 1% Triton X-100 and floating in the upper half of a 5–30% sucrose density gradient) is widely accepted as the method of choice for isolation from cells (including erythrocytes) and can be used to characterise the composition of these structures. This procedure, however, did not work in our hands [10,15,16], due to the relatively low level of

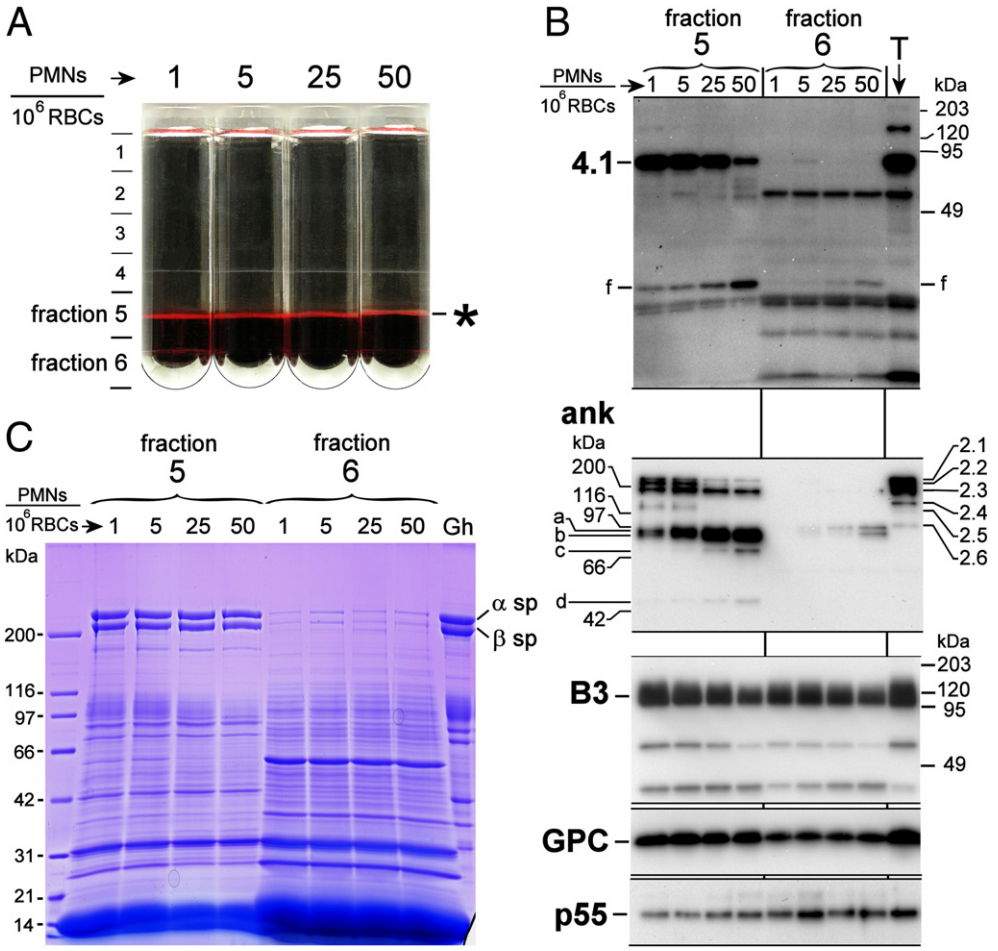

Fig. 3. Effect of low-level contamination by PMNs on erythrocyte membrane proteins. (A) Filtered erythrocytes were pre-treated with 1 mM DFP, washed free of the excess DFP with HNM, washed one last time with HKM and divided in four aliquots. To these, known amounts of autologous purified granulocytes were added, at levels of 1, 5, 25 and 50 PMNs per 10^6 erythrocytes. The four suspensions were treated with Triton X-100 in HKM and subjected to ultracentrifugation for 16 h in sucrose gradients. Carbonate was not added to the sample, and a low-density, DRM-like fraction was not obtained, as expected for a PMN contamination below 100 PMNs/10^6 erythrocytes. The DRMs remained associated with the membrane skeleton and migrated at the interface between the 40% and 30% sucrose solutions (asterisk). Fractions 5 and 6 from each tube were collected, treated with DFP before addition of SDS–PAGE sample buffer and subjected to SDS–PAGE and Western blotting. (B) The Western blots of protein 4.1, ankyrin, Band 3, GPC and p55 are shown, respectively, for fractions 5 and 6 of the four gradients. Loading of samples "T" is explained in the legend of Fig. 2. Band "f" in the protein 4.1 blot and bands "a", "b", "c" and "d" in the ankyrin blot are degradation products of the respective protein. (C) Coomassie blue-stained gel evidencing the absence of spectrin breakdown. Gh = sample of purified ghost membranes.

residual contaminating leukocytes in samples of filtered erythrocytes, as we have documented here.

While several studies have taken into account the problem of erythrocyte purity and inhibition of proteolysis [14,17,18,20,31–35], most of the published work on erythrocyte lipid rafts has been performed on erythrocytes obtained by simply washing the blood and eliminating the "buffy coat", which results in substantial, but incomplete removal of leukocytes. However, the "buffy coat" is comprised mainly of mononuclear cells (lymphocytes and monocytes) and residual white cells (mainly granulocytes) are difficult to separate from erythrocytes by simple centrifugation in physiologic solutions because of the similar buoyant density of the two cell types. If 4.5×10^9/l and 4.5×10^{12}/l are taken as average counts for PMNs and erythrocytes, respectively, in normal blood [36], then the number of PMNs/10^6 erythrocytes is approximately 1000. The levels of PMNs that we measured in washed blood was always above 100 PMNs/10^6 erythrocytes. It is evident that washing affords only, at best, a ten-fold purification of erythrocytes from PMNs. The residual, relatively high levels of PMNs may escape detection by conventional methods [35,37,38] and induce extensive proteolysis of erythrocyte proteins, via the liberation of proteases from the secretory granules of PMNs following the action of detergents.

Even as few as 1 PMN per million erythrocytes (corresponding to <5 PMNs/μl of blood), which is the target level adopted in transfusion medicine for efficient filtration systems [37], can induce the damage of several protease-sensitive membrane proteins. Because filtration through cellulose does not guarantee absolute leukodepletion, inhibition of the proteolytic activities from residual contaminating granulocytes can only be obtained by pre-treating the erythrocyte suspensions with DFP, a membrane-permeable serine esterase inhibitor, before subjecting the samples to lysis with the detergent.

Although DFP is a rather non-specific inhibitor of a wide range of hydrolases, and PMN granules contain multiple hydrolase, lipase and esterase activities [39], our results point to a major contribution of proteolysis in the observed events. In fact, DRMs are released only when contaminating PMNs are present above a certain threshold, and proteolysis begins to affect β-spectrin. Support for this conclusion comes from two lines of evidence. First, proteolysis of ankyrin and protein 4.1 was already significant at low levels of PMN contamination (1–50 PMNs/10^6 erythrocytes), when no release of DRMs was observed (Fig. 3). Second, when eglin-C was used in samples of washed erythrocytes instead of DFP, as a selective inhibitor of neutrophil elastase and cathepsin G (the most highly expressed serine proteases in neutrophils) [40], DRM release was inhibited at

0.1 μM eglin-C, and protein 4.1 and ankyrin were heavily proteolyzed, but spectrin was completely unaffected (not shown). It appears that protein 4.1 and ankyrin are not involved, directly or indirectly, in the linkage of lipid rafts to the membrane. In fact, ankyrin links spectrin to band 3, and protein 4.1 binds to GPC, but neither band 3 nor GPC could be detected in the DRM fractions, as we have confirmed in this study.

The results presented here suggest a scenario whereby the supramolecular assembly of the membrane skeleton, with its associated lipid rafts, is preserved even when ankyrin and protein 4.1 are proteolyzed. Protein 4.1 increases the affinity of spectrin for actin, but the spectrin-actin complex is relatively stable even when protein 4.1 is proteolyzed. When proteolysis begins to affect β-spectrin, membrane-skeletal fragments are produced. Those fragments that are associated with lipid rafts can be isolated as low-density DRMs, contaminated by membrane proteins. A centrifugation time of at least 4 h is necessary to isolate this material, although considerable variability was observed. Evidently, the length of time necessary to release the DRMs is inversely related to the degree of contamination of the original sample by PMNs, which, in turn, depends on the variable outcome of the washing procedure and "buffy coat" removal. Many of the published articles on lipid raft isolation, including the original article itself [1], contemplate long centrifugation times (15–22 h). It would be interesting to verify whether, and to what extent, proteolysis could contribute to the isolation of lipid rafts also in other cell models.

Only serine proteases, but not metalloproteases, are likely responsible for the observed effects, probably because the latter require pre-activation and different conditions for expressing their catalytic activity. The inhibitory effect of eglin-C on DRM isolation supports this view.

A summary of the various conditions that do or do not result in the isolation of DRMs is shown in Fig. 4. When neutrophil proteases are inactive, because filtered erythrocytes are used, or washed cells are pre-treated with DFP, DRMs can be obtained only after addition of carbonate. Concerning the mechanism of action of carbonate, it is conceivable that by increasing the pH and ionic strength of the medium, electrostatic interactions between the DRMs and the membrane skeleton are weakened. Although the fast kinetics of carbonate action (30 min at 4 °C, Fig. 1C) would argue in favour of this view, other mechanisms cannot be ruled out. A study is under way to evaluate the possible contribution of S-acylated (palmitoylated) proteins in the membrane skeleton [41,42], such as p55, spectrin and ankyrin, for the anchoring of DRMs, through the insertion of their saturated palmitoyl groups into the liquid-ordered phase of these microdomains: the alkalinization brought about by carbonate might be sufficient for hydrolysing these relatively weak thioester bonds.

We previously reported that the integral proteins band 3 and GPC were present at low, but significant levels in DRMs, and speculated that they could be responsible for the anchoring of DRMs to the membrane skeleton [15]. However, results presented here contradict this notion, as the new protocol of DRM isolation with high Mg^{2+} and high K^{+}, HEPES buffers and potassium carbonate, and pre-treatment with DFP, allowed the isolation of DRMs completely devoid of not only membrane-skeletal proteins, but also of band 3 and GPC.

In summary, the present results confirm that the sphingolipid-rich, detergent-resistant lipid moiety of the erythrocyte membrane is bound to the membrane skeleton, from which it can be dissociated only by increasing the pH and the ionic strength of the medium. Electrostatic interactions between the DRMs and the membrane skeleton are likely responsible for this association. Ankyrin- and protein 4.1-mediated vertical linkages, anchoring the spectrin skeleton to the integral membrane proteins band 3 and GPC, appear not to be involved in these interactions. On the other hand, as DRMs have been shown to contain aminoglycerophospholipids to a significant extent [43], and phosphatidylserine and phosphatidylethanolamine liposomes bind to spectrin at regions that have been mapped in the sequence of both α- and β-spectrin subunits [44–46], it could be hypothesized that such association involves a direct interaction between the spectrin skeleton and phospholipid head groups.

The artefactual condition whereby proteases from contaminating neutrophils are responsible for the release of DRMs without the need for carbonate, proved useful in suggesting a possible role for spectrin in the DRM/membrane skeleton association. It revealed that a proteolytic cleavage of β-spectrin, limited to less that 50% of total β-spectrin monomers, strongly correlated with the liberation

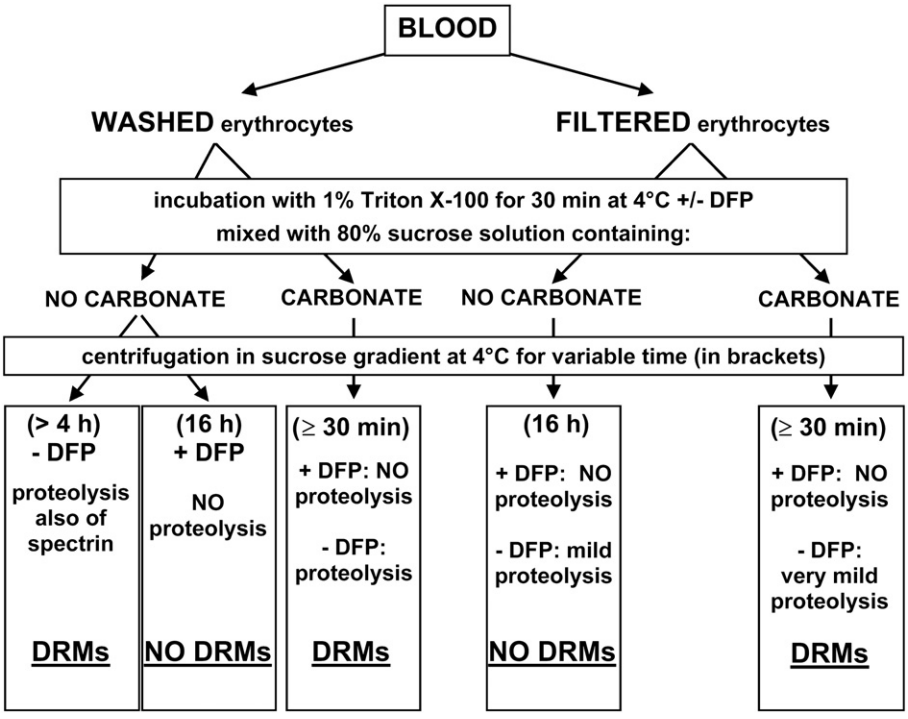

Fig. 4. Schematic representation of the various conditions that do or do not result in the isolation of low-density DRMs from washed or filtered erythrocytes.

of the lipid raft fraction. Our tentative interpretation of this result is that the proteolytic cleavage is in the tetramerization domain of β-spectrin, resulting in the release of spectrin dimers that bear lipid rafts associated with them and are free to float in the low-density region of sucrose gradients.

The identification of DRMs with lipid rafts has been called into question because there is evidence that the detergent itself might be responsible for DRM formation. This was suggested forty years ago with the suspicion of "demixing following detergent action" of a sphingolipid-rich fraction of the erythrocyte membrane [12]. Such action of Triton X-100 in the formation of DRMs cannot be ruled out. However, it is still compatible with the existence of liquid-ordered micro/nano domains in the plane of the membrane, on which certain detergents may have a demixing effect [3].

The association of a significant portion of the lipid bilayer, in the form of lipid rafts, to the underlying membrane skeleton may represent an additional vertical linkage that confers robustness to the cell membrane. It would be appropriate to evaluate the relevance of this association on the stability and other properties of the erythrocyte membrane, whether erythrocytes from other species also exhibit this feature, and if naturally occurring defects of this linkage exist, that may be related to abnormalities in the architecture of the erythrocyte membrane.

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References

[1] D.A. Brown, J.K. Rose, Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface, Cell 68 (1992) 533–544.
[2] S. Munro, Lipid rafts: elusive or illusive? Cell 115 (2003) 377–388.
[3] D. Lichtenberg, F.M. Goñi, H. Heerklotz, Detergent-resistant membranes should not be identified with membrane rafts, Trends Biochem. Sci. 30 (2005) 430–436.
[4] L.J. Pike, The challenge of lipid rafts, J. Lipid Res. 50 (2009) S323–S328.
[5] T. Nebl, K.N. Pestonjamasp, J.D. Leszyk, J.L. Crowley, S.W. Oh, E.J. Luna, Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes, J. Biol. Chem. 277 (2002) 43399–43409.
[6] M. Grzybek, A. Kozubek, P. Dubielecka, A.F. Sikorski, Rafts—the current picture, Folia Histochem. Cytobiol. 43 (2005) 3–10.
[7] B.F. Lillemeier, J.R. Pfeiffer, Z. Surviladze, B.S. Wilson, M.M. Davis, Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton, Proc. Natl. Acad. Sci. USA 103 (2006) 18992–18997.
[8] L.D. Walensky, M. Narla, S.E. Lux, Disorders of the red blood cell membrane, in: R.I. Handin, S.E. Lux, T.P. Stossel (Eds.), Blood: principles and pratice of hematology, second edition, Lippincott Williams and Wilkins, Philadelphia, 2003, pp. 1709–1858.
[9] V. Bennett, A.J. Baines, Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues, Physiol. Rev. 81 (2001) 1353–1392.
[10] A. Ciana, C. Balduini, G. Minetti, Detergent-resistant membranes in human erythrocytes and their connection to the membrane-skeleton, J. Biosci. 30 (2005) 317–328.
[11] M. Grzybek, A. Chorzalska, E. Bok, A. Hryniewicz-Jankowska, A. Czogalla, W. Diakowski, A.F. Sikorski, Spectrin-phospholipid interactions. Existence of multiple kinds of binding sites? Chem. Phys. Lipids 141 (2006) 133–141.
[12] J. Yu, D.A. Fischman, T.L. Steck, Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents, J. Supramol. Struct. 1 (1973) 233–248.
[13] T.L. Steck, The organization of proteins in the human red blood cell membrane. A review, J. Cell Biol. 62 (1974) 1–19.
[14] M.P. Sheetz, Integral membrane protein interaction with Triton cytoskeletons of erythrocytes, Biochim. Biophys. Acta 557 (1979) 122–134.
[15] C. Crepaldi Domingues, A. Ciana, A. Buttafava, C. Balduini, E. de Paula, G. Minetti, Resistance of human erythrocyte membranes to Triton X-100 and C12E8, J. Membr. Biol. 227 (2009) 39–48.
[16] C.C. Domingues, A. Ciana, A. Buttafava, B.R. Casadei, C. Balduini, E. de Paula, G. Minetti, Effect of cholesterol depletion and temperature on the isolation of detergent-resistant membranes from human erythrocytes, J. Membr. Biol. 234 (2010) 195–205.
[17] E. Beutler, C. West, K.G. Blume, The removal of leukocytes and platelets from whole blood, J. Lab. Clin. Med. 88 (1976) 328–333.
[18] E. Beutler, T. Gelbart, The mechanism of removal of leukocytes by cellulose columns, Blood Cells 12 (1986) 57–64.
[19] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature (London) 227 (1970) 680–685.
[20] G. Fairbanks, T.L. Steck, D.F.H. Wallach, Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane, Biochemistry 10 (1971) 2606–2617.
[21] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[22] A. Böyum, Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g, Scand. J. Clin. Lab. Invest. Suppl. 97 (1968) 77–89.
[23] E. Melloni, S. Pontremoli, M. Michetti, O. Sacco, B. Sparatore, F. Salamino, B.L. Horecker, Binding of protein kinase C to neutrophil membranes in the presence of Ca2+ and its activation by a Ca2+-requiring proteinase, Proc. Natl. Acad. Sci. USA 82 (1985) 6435–6439.
[24] S.K. Chintala, R. Sawaya, B.B. Aggarwal, S. Majumder, D.K. Giri, A.P. Kyritsis, Z.L. Gokaslan, J.S. Rao, Induction of matrix metalloproteinase-9 requires a polymerized actin cytoskeleton in human malignant glioma cells, J. Biol. Chem. 273 (1988) 13545–13551.
[25] G.S. Herron, M.J. Banda, E.J. Clark, J. Gavrilovic, Z. Werb, Secretion of metalloproteinases by stimulated capillary endothelial cells. II. Expression of collagenase and stromelysin activities is regulated by endogenous inhibitors, J. Biol. Chem. 261 (1986) 2814–2818.
[26] C. Heussen, E.B. Dowdle, Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates, Anal. Biochem. 102 (1980) 196–202.
[27] P.A. Snoek-van Beurden, J.W. Von den Hoff, Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors, Biotechniques 38 (2005) 73–83.
[28] V.M. Fowler, V. Bennett, Erythrocyte membrane tropomyosin: purification and properties, J. Biol. Chem. 259 (1984) 5978–5989.
[29] L.J. Pike, Lipid rafts: heterogeneity on the high seas, Biochem. J. 378 (2004) 281–292.
[30] D.A. Brown, E. London, Structure of Detergent-Resistant Membrane Domains: Does Phase Separation Occur in Biological Membranes? Biochem. Biophys. Res. Commun. 240 (1997) 1–7.
[31] S.K. Srivastava, Y.C. Awasthi, S.P. Miller, A. Yoshida, E. Beutler, Studies on gamma-glutamyl transpeptidase in human and rabbit erythrocytes, Blood 47 (1976) 645–650.
[32] H.U. Lutz, Vesicles isolated from ATP-depleted erythrocytes and out of thrombocyte-rich plasma, J. Supramol. Struct. 8 (1978) 375–389.
[33] V. Bennett, P.J. Stenbuck, Human erythrocyte ankyrin. Purification and properties, J. Biol. Chem. 255 (1980) 2540–2548.
[34] A. Brovelli, C. Seppi, A. Bardoni, C. Balduini, H.U. Lutz, Re-evaluation of the structural integrity of red-cell glycoproteins during aging in vivo and nutrient deprivation, Biochem. J. 242 (1987) 115–121.
[35] J.F. Hoffman, A. Wickrema, O. Potapova, M. Milanick, D.R. Yingst, Na pump isoforms in human erythroid progenitor cells and mature erythrocytes, Proc. Natl. Acad. Sci. USA 99 (2002) 14572–14577.
[36] P.W. Marks, Data of importance in hematology, in: R.I. Handin, S.E. Lux, T.P. Stossel (Eds.), Blood: principles and pratice of hematology, second edition. Lippincott Williams and Wilkins, Philadelphia, 2003, pp. 2193 and 2216.
[37] P. Rebulla, L. Porretti, F. Bertolini, F. Marangoni, D. Prati, C. Smacchia, M. Pappalettera, A. Parravicini, G. Sirchia, White cell-reduced red cells prepared by filtration: a critical evaluation of current filters and methods for counting residual white cells, Transfusion 33 (1993) 128–133.
[38] S. Dzik, G. Moroff, L. Dumont, A multicenter study evaluating three methods for counting residual WBCs in WBC-reduced blood components: Nageotte hemocytometry, flow cytometry, and microfluorometry, Transfusion 40 (2000) 513–520.
[39] S.W. Edwards, Biochemistry and physiology of the neutrophil, Cambridge University Press, Cambridge, UK, 1994.
[40] U. Seemüller, H. Fritz, M. Eulitz, Eglin: elastase-cathepsin G inhibitor from leeches, Meth. Enzymol. 80 (1981) 804–816.
[41] M. Mariani, D. Maretzki, H.U. Lutz, A tightly membrane-associated subpopulation of spectrin is 3H-palmitoylated, J. Biol. Chem. 268 (1993) 12996–13001.
[42] S. Arni, S.A. Keilbaugh, A.G. Ostermeyer, D.A. Brown, Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues, J. Biol. Chem. 273 (1998) 28478–28485.
[43] K.S. Koumanov, C. Tessier, A.B. Momchilova, D. Rainteau, C. Wolf, P.J. Quinn, Comparative lipid analysis and structure of detergent-resistant membrane raft fractions isolated from human and ruminant erythrocytes, Arch. Biochem. Biophys. 434 (2005) 150–158.
[44] X. An, X. Guo, H. Sum, J. Morrow, W. Gratzer, N. Mohandas, Phosphatidylserine binding sites in erythroid spectrin: location and implications for membrane stability, Biochemistry 43 (2004) 310–315.
[45] A.F. Sikorski, K. Białkowska, Interaction of spectrins with membrane intrinsic domain, Cell. Mol. Biol. Lett. 1 (1996) 97–104.
[46] A. Hryniewicz-Jankowska, E. Bok, P. Dubielecka, A. Chorzalska, W. Diakowski, A. Jezierski, M. Lisowski, A.F. Sikorski, Mapping of an ankyrin-sensitive, phosphatidylethanolamine/phosphatidylcholine mono- and bi-layer binding site in erythroid beta-spectrin, Biochem. J. 382 (2004) 677–685.