Anemia in sepsis: the importance of red blood cell membrane changes

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SUMMARY

Anemia is a common problem in acutely ill patients, especially in those who develop sepsis. There are many factors contributing to the development of anemia in these patients, including blood sampling and other losses, decreased red blood cell (RBC) synthesis, and possibly increased destruction. Increased RBC uptake may be due to changes in RBC morphology and the RBC membrane during inflammatory processes. In particular, a rapid increase in RBC sphericity correlated with a decreased surface carbohydrate membrane content and alterations in the lipid bilayer with increased peroxidation and phosphatidylserine exposure in the outer leaflet of the membrane. Better understanding of these alterations could facilitate new strategies to prevent the development of anemia in sepsis.

ANEMIA IN THE CRITICALLY ILL

Anemia is very common in acutely ill patients, to the extent that about one-third of intensive care unit (ICU) patients receive a red blood cell (RBC) transfusion at some point during their ICU stay.[1] A large observational study including 3534 patients in Western European ICUs[2] indicated that the mean admission hemoglobin concentration was 11.3 g/dL, with 63% of patients having an admission hemoglobin concentration less than 12 g/dL and 29% less than 10 g/dL. In a prospective monocenter observational cohort study, Chohan *et al.*[3] observed that 55% of all patients had a hemoglobin concentration less than 9 g/dL.

The etiology of anemia in critically ill patients is multifactorial and includes blood losses (trauma, blood sampling, surgical procedures and occult gastrointestinal bleeding), decreased RBC production by functional

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iron deficiency,[4] and altered erythropoiesis (apoptosis of erythroid precursors and lower erythropoietin concentrations for a given hematocrit).[5,6] Blood sampling is probably the main factor in the development of anemia in the critically ill, especially in patients with sepsis. Indeed, 37 to 65 mL of blood is drawn daily;[2,7,8] better blood sugar control may have contributed to increased blood sampling in many ICUs.

Hemodilution, by abundant intravenous infusions, can also reveal anemia in the critically ill.[7] Perhaps a poorly understood cause of anemia in ICU patients, especially in septic patients, is the increased uptake of altered RBCs by the reticuloendothelial system. Indeed, alterations in RBC membrane composition and morphology, such as seen during the senescence process, can trigger RBC uptake by macrophages of the spleen and/or the liver.[9–13] This factor is, however, likely to be a relatively minor cause of anemia.

In this review, we will focus on the effects of sepsis on alterations of RBC morphology and membrane composition and their possible contribution to the development of anemia in septic patients. We will first describe RBC shape and membrane composition in healthy individuals and then review the alterations that occur in sepsis.

RBC PHYSIOLOGY

RBC shape

The human RBC adopts a biconcave-discoid shape *in vivo* (Figure 1). This shape represents an equilibrium between two opposite extremes of shape: the stomatocyte (membrane internalization) and the echinocyte (membrane externalization with formation of multiple spikes on the RBC surface).[14] Between these two reversible morphological variations, a multitude of shapes were already described by Bessis 35 years ago;[15] some of them are characteristic of various hematologic diseases.[15]

This biconcave disk shape is based on static and dynamic properties of the membrane, arising from its chemical composition, molecular organization and physical properties of its major components: lipids, proteins and carbohydrates. Why the RBC should have this particular profile remains unresolved. It has been suggested that this shape maximizes the surface area to volume ratio and thus expedites diffusion. In the blood,

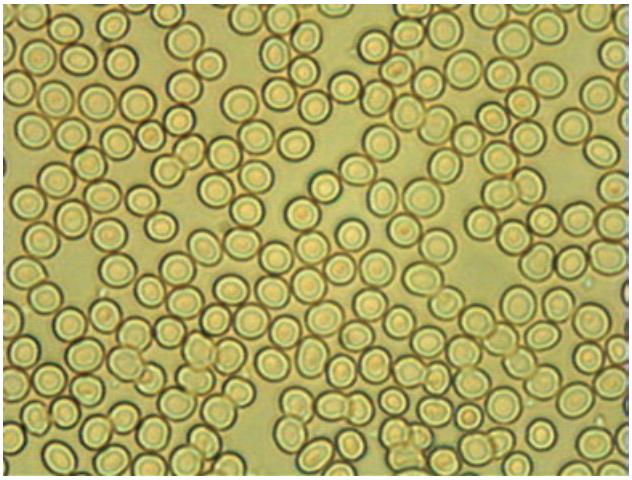

Figure 1. Optical microscopy of a blood smear from a healthy volunteer. Magnification ×50. RBCs show a typical discoid shape. RBC, red blood cell.

the RBC can deform with the action of large external forces, but returns to its resting shape when these forces are removed (concept of 'RBC shape memory').[16] The resting shape is not influenced by the liquid cytoplasm but only by the predominant role of its membrane. For these reasons, the study of RBC membrane abnormalities is essential to understand the changes in RBC shape.

RBC membrane

The RBC membrane is composed of proteins (52% in weight), lipids (40%) and carbohydrates (8%). Membrane proteins are divided into two classes, depending on their relation to the lipid bilayer. The first family consists of 'integral', membrane spanning proteins, especially band 3 protein, membrane channels or transporters, glycophorins (in particular A and C) and glycoproteins; carbohydrate moieties coupled to 'integral' proteins form an additional leaflet, also called 'glycocalyx'. Sialic acids, in particular N-acetylneuraminic acid (SA), an acidic carbohydrate, are bound to glycophorin A and are responsible for 60% to 90% of the surface negative charge of the RBC membrane. A second family is composed of 'peripheral' proteins constituting the inner membrane skeleton. These include spectrin (α and β subunits), actin, protein 4.1, protein 4.2, tropomyosin, adducin, myosin and ankyrin.[14,16,17] Membrane elasticity – and thereby deformability – depends on the

structural interactions between the outer plasma membrane and the underlying protein cytoskeleton.

Lipids, including phospholipids, glycolipids and cholesterol, are arranged as a bilayer and distributed unevenly between the two leaflets of this bilayer, giving a transverse membrane asymmetry. Indeed, the glycolipids and the choline phospholipids (phosphatidylcholine, sphingomyelin) are oriented toward the outer surface of the membrane, while the aminophospholipids [phosphatidylserine (PS), phosphatidylethanolamine and phosphoinositolphospholipids] are oriented toward the cytoplasmic surface.[14] This repartition of PS into the lipid bilayer is important and modifications by exposure of these PS in the outer leaflet serve as a trigger for erythrophagocytosis.[12]

RBCs IN SEPSIS

Sepsis is a complex pathophysiological process that involves alterations in the microcirculation (vessels with a diameter <100 μm) and changes in the biochemical and physiological characteristics of the blood constituents.[17,18] Sepsis can alter the microcirculation by several mechanisms including the increased release of vasoactive substances, altered control of vasomotricity, formation of microthrombi and interstitial edema. These mechanisms can alter arteriolar diameter, increase the number of stopped-flow and high-velocity capillaries, and enhance the heterogeneity of capillary transit time and spatial distribution of perfused capillaries.[19,20] Sepsis can alter RBC morphology and rheology (viscosity, aggregation and deformability),[17,21,22] and these alterations are likely to contribute to the microvascular changes in these patients.[18,19,23] While the role of white blood cells (WBCs) has been the focus of many studies in sepsis, the role of altered RBC shape, biochemistry and rheology has only recently been investigated.

Alterations of RBC deformability

Several studies have demonstrated alterations in RBC deformability in animal models of septic shock and in septic humans.[21,22,24–26] Several factors could be implicated in this altered deformability, including increased intracellular calcium and decreased adenosine triphosphate concentrations, the effects of nitric oxide on RBC membrane fluidity, and a decrease in some RBC membrane components like SA.[17] Interactions with other cellular blood components could also decrease RBC deformability and alter blood flow. Baskurt and Meiselman[27] observed decreased RBC deformability only if WBCs were present, and this alteration was more pronounced when WBCs were activated by two different stimuli (tumor necrosis factor and N-formyl-methionyl-leucyl-phenylalanine). These alterations in RBC deformability were prevented by incubation with superoxide dismutase (SOD) or catalase, suggesting a role of reactive oxygen species (ROS) produced by the activated WBCs.

Mechanisms of alterations in RBC deformability

Alterations of RBC morphology in sepsis

Relatively few studies have been devoted to the changes in RBC morphology in sepsis.[17,28–30] In an optical microscopic examination of blood smears from septic patients, we observed, using the Bessis classification,[15] an increased number of abnormal RBCs, including echinocytic and spheroechynocytic transformations, associated with an increased aggregation process (Figure 2).[28] These alterations are potentially important because they could be implicated in the microvascular alterations observed in septic patients.[19–23] Reinhart and

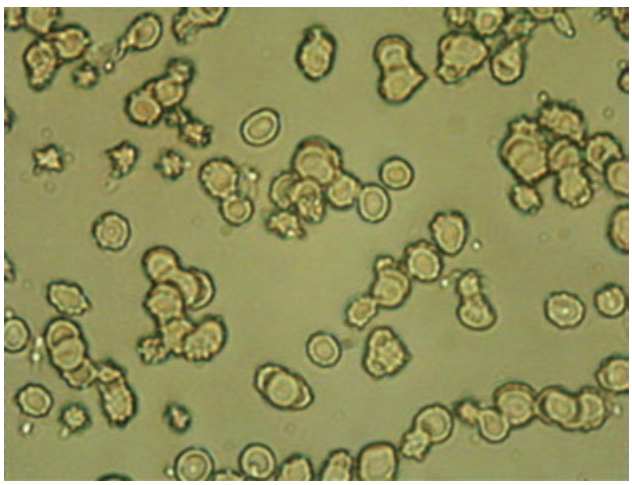

Figure 2. Optical microscopy of a blood smear from a patient with septic shock due to bacteremia and pneumonia. Magnification ×50. Different RBC morphologies were observed with resting typical discoid shape, echinocytes (spikes on the RBC membrane) and other abnormal RBC shapes. RBC, red blood cell.

Chien[31] reproduced stomatocytic and echinocytic transformations *ex vivo* by the addition of either chlorpromazine or salicylates to RBCs from healthy volunteers; there was decreased deformability capacity in the stomatocytic RBCs and an increased viscosity in the blood containing a majority of echinocytes.[31]

Alterations of the RBC membrane in sepsis

Inflammatory states are characterized by an increased production of ROS as well as by a decrease in antioxidant defenses. Damage occurs when the production of ROS exceeds the antioxidant defenses of the tissues.[32] ROS, which include superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), produced by the WBCs can also damage hemoglobin and induce hemolysis.[32,33] Uyesaka *et al.*[33] demonstrated that RBCs exposed to O_2^- displayed pronounced degradation of membrane proteins (band 3 and spectrin) with formation of new protein bands. This reorganization of membrane proteins can decrease RBC deformability. ROS can also affect the lipid part of the RBC membrane by induction of lipid peroxidation. Huet *et al.*[34] recently showed that RBC membrane lipid peroxidation was increased in patients on the first day of septic shock, as reflected by significantly increased levels of thiobarbituric acid-malondialdehyde concentrations. Moreover, the antioxidant defenses of these RBCs was also reduced, as reflected by a decreased glutathione content and reduced activities of SOD and catalase.[34]

Binding of endotoxin (lipopolysaccharide, LPS) to the RBC membrane could play a role in the alterations in RBC rheology, although data on this effect are conflicting.[25,35] Washed RBCs suspended in phosphate buffered saline showed no change in deformability after incubation with LPS,[24,25] whereas RBCs incubated with LPS in whole blood exhibited markedly altered deformability.[35] These results underline the important role of the activation of leukocytes with generation of cytokines and ROS, which can alter the RBC membrane. Interestingly, Pöschl *et al.*[25] observed an inverse relationship between the amount of hydroxymyristic acid (a component of the lipid A) measured in the RBC membrane and the membrane deformability assessed by a laser-diffraction shearing device at high shear stress (60 Pa).[25]

We investigated the relationship between the SA content of the RBC membrane and RBC shape estimated by a flow cytometry technique in critically ill patients.[29] We compared blood samples from ICU patients on the first day of severe sepsis or septic shock with RBCs from patients without sepsis and from healthy volunteers. We excluded patients with hematologic disease, with recent RBC transfusion, or with diseases known to induce alterations in RBC rheology (e.g. diabetes mellitus, cirrhosis, terminal renal failure). To determine RBC shape by flow cytometry, we adapted the technique from Rolfes-Curl *et al.*[36] Briefly, in iso-osmolality, biconcave RBCs from volunteers appear essentially as two populations of cells, and the forward light scatter channel histograms show a typically bimodal distribution of RBCs. On this histogram, it is possible to calculate the second moment of Dissymmetry of Pearson (PCD; $3 \times (\text{mean-median})/\sigma$), which expresses the sphericity of the RBC (Figure 3).

The PCD value obtained in healthy volunteers is around –0.8 and a PCD value of zero represents a perfect spherical RBC shape. In this study, the PCD values were significantly reduced in all ICU patients suggesting a right shift in the histogram (septic -0.48 ± 0.2, non-septic -0.52 ± 0.23 and volunteers -0.70 ± 0.15) (Figure 4). Moreover, RBCs from septic patients failed to modify their shape in a hypo-osmolar solution. Interestingly, we also observed a significant correlation between RBC shape and the SA content of the RBC membrane in the ICU patients ($r^2 = 0.15$; $P = 0.015$).[29] We could not exclude an artefact for RBC shape estimation with the flow cytometry technique because we decreased the osmolality of the RBC samples from the septic patients, and this could induce swelling of the RBC and modify its spherical shape. However, when we compared the data with a matched non-septic population with similar calculated osmolality, we observed the same differences in the moment values.[29] From this study, we concluded that already on the first day of sepsis, RBCs are characterized by a more spherical shape, a decreased capacity to assume the spherical shape in hypo-osmolar solution, and a reduction in the SA content of the RBC membrane.[29]

We recently compared the RBC shape in blood from septic patients assessed by the same flow cytometry technique to RBCs from patients with diseases characterized by abnormalities in RBC rheology, including diabetes mellitus and terminal renal failure requiring hemodialysis.[30] We confirmed that the RBCs became rapidly more spherical in septic patients than in healthy volunteers (PCD: -0.58 ± 0.22 *vs.* -0.89 ± 0.12; $P < 0.05$) but similar alterations were observed in

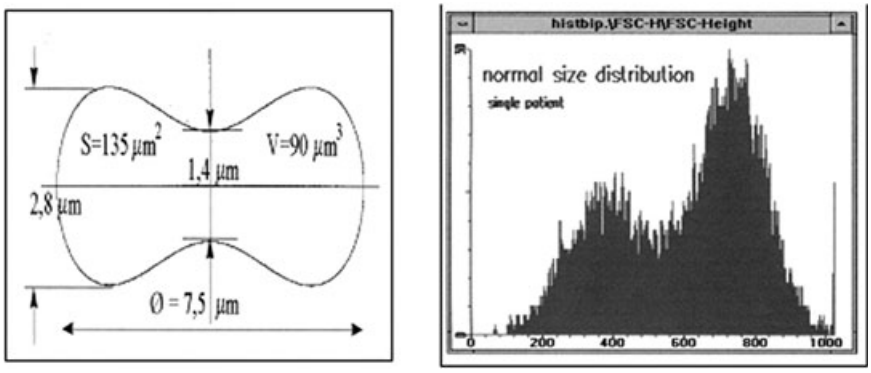

Figure 3. Schematic representation of a human RBC behind the flow cytometer (left side). RBC forward light scatter distribution in isotonicity. Example of an RBC analysis in a healthy volunteer. *X* axis represents the RBC size (in arbitrary units) and the *Y* axis, the number of events limited to 15,000 cells. The histogram showed a double peak with PCD values of around –0.8 (right side). PCD, Dissymmetry of Pearson; RBC, red blood cell.

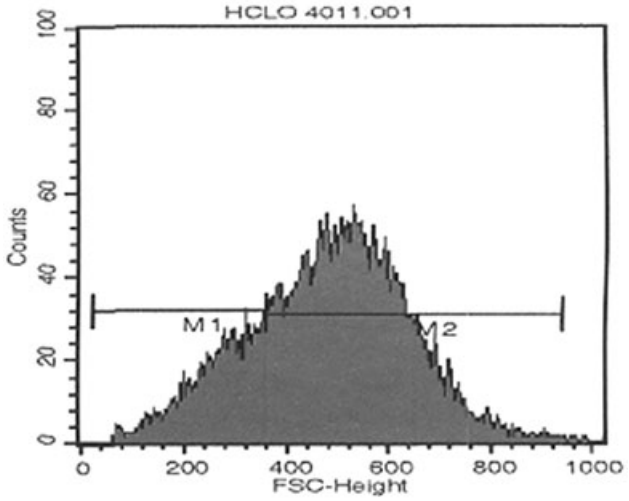

Figure 4. RBC forward light scatter distribution from RBCs from a septic patient. RBC, red blood cell.

patients with terminal renal failure (PCD: –0.56 ± 0.14; $P < 0.05$) and diabetes mellitus (PCD: –0.59 ± 0.23, $P < 0.05$). Moreover, by multivariate analysis, including underlying disease, age, RBC, WBC and platelet counts, blood sugar, C-reactive protein, sodium and urea concentrations, and calculated osmolality, only the underlying disease was the principal cause of the alterations in RBC shape.[30]

In association with sphericity, we also observed a significant decrease in the SA content of the RBC membrane in septic patients.[29] On the other hand, alterations in the membrane surface were not associated with a decrease in the integral RBC membrane protein (glycophorin A) which binds the SA.[29] These results were in agreement with a study by Nieuwland *et al.*[37] on the microparticles derived from WBCs, endothelial cells and platelets in patients with meningococcal sepsis. Blood glycophorin A content was unaltered in these patients during the first 36 hours of sepsis.[37] Thus, it seems that RBCs from septic patients present membrane alterations with decreased SA content but no alteration of the other membrane proteins. Nevertheless, in a mouse model of septic shock induced by cecal ligature and puncture, Spolarics *et al.*[26] observed an increased B3/α spectrin ratio, suggesting a possible alteration of the membrane integral/peripheral ratio. These observations need confirmation in humans, as there are interspecies differences in RBC shape, membrane composition and rheology.[38] Further studies with electrophoresis of human RBC membrane proteins are needed to evaluate the possible leakage of these proteins during sepsis.

To confirm the early development of the desialylation process, we studied the time course of free SA concentrations in a sheep model of septic shock induced by peritonitis.[39] We observed a significant increase in serum free SA concentrations already 15 hours after the induction of peritonitis.[39] As SA decreased rapidly on the RBC membrane surface,[29] and also in circulating proteins[39] and serum free SA concentrations increased in septic patients, we hypothesized that neuraminidase activity

was rapidly increased in sepsis. We recently tested this hypothesis in patients with sepsis.[40] Modifications of the lipid bilayer, especially the exposure of PS, have been reported in RBCs from septic patients. Kempe *et al.*[41] recently observed that incubation of RBCs from healthy volunteers in plasma from septic patients triggers PS exposure highlighted by increased fixation of annexin V on the surface of these RBCs.[41] The same effect was also observed when RBCs from healthy volunteers were incubated with the supernatant of pathogens.[41] The effect of patient plasma on RBC annexin V was paralleled by the formation of ceramide and a significant increase in intraerythrocytic calcium. This, in turn, could activate the calcium-sensitive K+ channel, which, together with chloride channels, can allow KCl to leave the cell, thus resulting in cell shrinkage. Increased intraerythrocytic calcium concentrations also activate the protease calpain leading to the degradation of the RBC membrane and cell membrane blebbing.[41–43]

LINKS BETWEEN ALTERATIONS IN RBCs AND THE DEVELOPMENT OF ANEMIA IN SEPSIS

There is some suggestion – but no proof in sepsis – that altered RBCs are more rapidly cleared from the circulation. First, the SA content of the RBC membrane plays an essential role in RBC survival. Indeed, several studies have indicated that treatment of RBCs with neuraminidase facilitates uptake by the reticuloendothelial system.[10,44] More than 25 years ago, Durocher *et al.*[44] showed in rats and rabbits a rapid clearance of ^{51}Cr desialylated erythrocytes, with sequestration by the liver. Simchon *et al.*[10] observed in rats that more than 70% of neuraminidase-treated injected RBCs disappeared from the circulating blood in 30 minutes compared with less than 2% of normal RBCs; the relative distributions of neuraminidase-treated RBCs to normal RBCs, as determined by radioactivity counting, were significantly greater than 1 in the spleen (5.65 ± 0.97, mean ± SD), the liver (2.84 ± 0.21), the lung (1.48 ± 0.31) and the kidney (1.49 ± 0.27), indicating a preferential trapping of neuraminidase-treated RBCs in these regions. In the same model, measurement of regional blood flow by the microsphere technique indicated that blood flow was reduced in the organs where the neuraminidase-treated RBCs were trapped.[10] Recently, Ensinck *et al.*[45] observed increased erythrophagocytosis by monocytes of human RBCs treated with high concentrations of neuraminidase (10 mIU/mL neuraminidase from *Clostridium perfringens*). These results may be explained by a lower negative surface charge potential of the neuraminidase-treated RBCs, thus facilitating their contact with phagocytic cells.[45]

Second, exposure of PS at the cell surface facilitates binding by PS receptors expressed by macrophages.[46] Modifications of the phospholipid membrane asymmetry, as also observed in sickle cell RBCs,[13] also trigger increased clearance by the reticuloendothelial system.[11–13]

Regrettably, there are no data concerning a possible increased erythrophagocytosis of RBCs from septic patients. We observed that these RBCs from septic patients were rapidly altered with decreased SA RBC membrane content and increased PS membrane exposure in sepsis. Nevertheless, further studies are necessary to make the link between these alterations in RBCs and the development of anemia in the critically ill. A better understanding of the development of anemia may lead to new therapeutic strategies to prevent it.

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