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Red Blood Cell Clearance in Inflammation

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Summary
Anemia is a frequently encountered problem in the critically ill patient. The inability to compensate for anemia includes several mechanisms, collectively referred to as anemia of inflammation: reduced production of erythropoietin, impaired bone marrow response to erythropoietin, reduced iron availability, and increased red blood cell (RBC) clearance. This review focuses on mechanisms of RBC clearance during inflammation. We state that phosphatidylserine (PS) expression in inflammation is mainly enhanced due to an increase in ceramide, caused by an increase in sphingomyelinase activity due to either platelet activating factor, tumor necrosis factor-α, or direct production by bacteria. Phagocytosis of RBCs during inflammation is mediated via RBC membrane protein band 3. Reduced deformability of RBCs seems an important feature in inflammation, also mediated by band 3 as well as by nitric oxide, reactive oxygen species, and sialic acid residues. Also, adherence of RBCs to the endothelium is increased during inflammation, most likely due to increased expression of endothelial adhesion molecules as well as PS on the RBC membrane, in combination with decreased capillary blood flow. Thereby, clearance of RBCs during inflammation shows similarities to clearance of senescent RBCs, but also has distinct entities, including increased adhesion to the endothelium.

Schlüsselwörter
Erythrozyten-Clearance · Seneszenz · Entzündung

Zusammenfassung
Anämie ist ein häufig auftretendes Problem bei schwerkranken Patienten. Die Unfähigkeit, die Anämie zu kompensieren, umfasst mehrere Mechanismen, die unter dem Begriff Entzündungsanämie zusammengefasst werden: reduzierte Erythropoetin-Produktion, vermindertes Ansprechen des Knochenmarks auf Erythropoetin, reduzierte Eisenverfügbarkeit sowie erhöhte Erythrozyten-Clearance. Diese Übersichtsarbeit beschäftigt sich mit den Mechanismen der Erythrozyten-Clearance im Zustand der Entzündung. Wir behaupten, dass die gesteigerte Phosphatidylserin(PS)-Expression im Zuge von Entzündungsprozessen hauptsächlich durch einen Anstieg des Ceramid-Spiegels bedingt wird, der wiederum die Folge einer erhöhten Sphingomyelinase-Aktivität ist, die durch den plättchenaktivierenden Faktor, den Tumornekrosefaktor-α oder die direkte Produktion durch Bakterien generiert wird. Phagozytose von Erythrozyten bei Entzündungsvorgängen wird durch das Erythrozytenmembranprotein, Bande 3 vermittelt. Verminderte Deformierbarkeit der Erythrozyten scheint ein wichtiges Entzündungsmerkmal zu sein, das ebenfalls von Bande 3 sowie Stickoxid, reaktiven Sauerstoffradikalen und Sialinsäure-Resten vermittelt wird. Des Weiteren ist die Haftung der Erythrozyten am Endothel bei Entzündungsvorgängen gesteigert, was höchstwahrscheinlich die Folge einer erhöhten Expression von endothelialen Adhesionsmolekülen sowie PS an der Erythrozytenmembran in Kombination mit verminderter kapillärer Durchblutung ist. Damit zeigt die Erythrozyten-Clearance im Zustand der Entündung Ähnlichkeiten mit der Clearance alternder Erythrozyten, aber auch individuelle Eigenschaften wie die gesteigerte Endotheladhäsion.

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Introduction

It is commonly known that anemia is a frequently encountered problem in the critically ill patient. Almost 95% of patients admitted to the intensive care unit (ICU) have a hemoglobin level below normal after 3 days of ICU admission [1]. As a consequence, critically ill patients are frequently transfused. The CRIT study showed that 44% of the patients received at least 1 or more units of red blood cells (RBCs). In sepsis patients, transfusion rates even reach 73% [2]. The association between transfusion and adverse outcome found in a number of observational studies in several critically ill patient populations [3–6] calls for a thorough understanding of the causes of anemia. In this review, we discuss the causes of anemia in sepsis patients, focusing on anemia of inflammation. We discuss mechanisms of anemia of inflammation, with a special emphasis on increased RBC clearance.

Causes of Anemia in Sepsis

In sepsis, pre-existent factors which contribute to chronic anemia before admission to the ICU are often present, including poor nutritional status, co-morbidities such as renal failure, or intensive treatment for malignancies. These factors not only contribute to anemia, but at the same time put these patients at increased risk of sepsis. Causes of anemia in sepsis are multifactorial and are summarized in table 1. One cause of anemia which stands somewhat separate is blood loss. Increased blood loss can occur via the gastrointestinal tract, from surgical procedures, or through repeated phlebotomy [7]. In total, a median blood loss of 128 ml/day has been calculated in critically ill patients [8]. Healthy individuals donating blood can compensate a loss of about 10 ml of RBCs per day [9]. Critically ill patients, however, are impaired in their ability to regenerate these losses. Causes of this inability to correct for deficiencies are the consequence of inflammatory processes, collectively referred to as anemia of inflammation. This type of anemia was once known as anemia of chronic disease. However, it turned out not to be restricted to chronic disease but also occur in patients with acute inflammation [7]. Moreover, some of the chronic disorders that were covered by the term led to anemia through a different mechanism, for example chronic kidney failure. Therefore, by 1983 it was advocated to change the term to anemia of inflammation [10].

Pathogenesis of Anemia of Inflammation

The pathogenesis of anemia of inflammation comprises 4 different mechanisms: reduced production of erythropoietin (EPO), impaired bone marrow response to EPO, reduced iron availability, and increased RBC clearance.

Table 1. Causes of anemia in sepsis

Pre-existing conditions
Renal failure
Poor nutritional status
Chemotherapy
Nutritional deficiency
Iron deficiency
Folate deficiency
Blood loss
Gastrointestinal tract
Surgical procedures
Repeated phlebotomy
Anemia of inflammation
Reduced production of erythropoietin
Impaired bone marrow response to erytropoietin
Reduced iron availability
Increased RBC clearance
Phosphatidylserine exposure
Erythrocyte phagocytosis
Reduced deformability
Adherence to endothelium
Hemolysis
Diffuse intravascular coagulation

Decreased Production of Erythropoietin

Reduced erythropoiesis is a result of reduced maturation of erythroid precursors. When hemoglobin levels drop, the normal response is an increase in EPO production. In sepsis, however, the rise in EPO levels in response to anemia is blunted, corresponding with low hematocrit [11]. The blunted endogenous EPO response to anemia in critically ill patients is irrespective of the presence of renal failure [12]. As low levels of EPO are found in several subsets of patients, including trauma and sepsis [13, 14], the common denominator most likely is the presence of an inflammatory condition.

Impaired Bone Marrow Response to Erythropoietin

Critically ill patients are able to respond to EPO administered exogenously in terms of an increase in reticulocyte count [12] and decreased need for blood transfusion [15]. However, the erythropoid response to exogenously administered EPO is also blunted, as doses of EPO given to generate a response are higher when compared to doses administered to patients with renal failure. At present, a recent meta-analysis of the efficacy of EPO has not led to the recommendation to supply EPO to the critically ill [16]. The cause of bone marrow hyporesponsiveness to EPO is not known but is likely to include inflammatory pathways. EPO binds to a specific receptor, resulting in proliferation and differentiation of erythroid progenitors. This process may be counterbalanced by death signals, resulting in impaired RBC production. In line with this, increased apoptosis of bone marrow erythroid precursors was demonstrated in sepsis patients. Also, erythroid progenitors can be inhibited by incubation with serum of septic patients

[17]. This suggests that inflammation directly suppresses erythroid precursors. Alternatively, as the amplification of erythropoiesis that results from the administration of EPO increases the need for iron, a lack of iron may underlie the blunted response to EPO. In anemia of chronic kidney disease, it is well recognized that response to EPO is greatly enhanced by giving intravenous iron. It should be noted that only 1 study in the meta-analysis administered intravenous iron in association with EPO. In that study, the effect of EPO treatment was greater in terms of a reduced need for blood transfusion and increase in hemoglobin concentrations [18].

Reduced Iron Availability

Iron for erythropoiesis comes from dietary sources through absorption in the gut. However, this accounts for only a small part of our daily iron need. The majority of iron used for erythropoiesis comes from lysis of aged RBCs, which generates free heme which is then degraded to iron and recycled towards the circulation or stored in ferritin molecules. Hepcidin is a major regulator of the iron metabolism, which acts by binding to the iron exporter ferroportin, causing internalization of iron and inhibiting the release of iron from tissue macrophages. Thus, hepcidin reduces the concentration of iron in the blood. The production of hepcidin is upregulated in response to elevated serum iron levels. Inflammation also induces hepcidin, which is a fast response resulting in a drop in iron levels within hours [19]. The induction of hepcidin synthesis by inflammation is not fully understood but depends on IL-6, as infusion of IL-6 into human volunteers induces increased hepcidin synthesis with a decrease in plasma iron levels [20]. Indeed, hepcidin levels have been found to be elevated in critically ill trauma patients, correlating with the duration of the anemia [21], as well as in critically ill patients not suspected for iron deficiency [22]. Thereby, it is reasonable to assume that hepcidin is elevated in inflammatory conditions, contributing to anemia. However, given the complexity of separating iron deficiency from anemia of inflammation, more research is needed in this area.

Increased Red Blood Cell Clearance

Although some microbial agents can elicit severe hemolytic reactions in the course of sepsis, increased hemolysis in general is thought to play a minor role in anemia of inflammation, as parameters of hemolysis are usually not disturbed. The decreased RBC lifespan in inflammation is rather thought to be due to an altered morphology of the RBCs, resulting in increased adherence to the endothelium and clearance from the circulation. The following chapters describe these changes in morphology in detail. As changes occurring during inflammation to some extend are similar to those observed during RBC ageing, a short description of changes during ageing is given first.

Senescence of Red Blood Cells

Phosphatidylserine Exposure

Apoptosis is the term used for the suicidal cell death of nucleated cells and is characterized by loss of cellular K^+ with subsequent cell shrinkage, nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell membrane blebbing, and phosphatidylserine (PS) exposure at the cell surface [23]. However, since RBCs are devoid of nuclei and mitochondria, the term eryptosis was introduced to describe the process of apoptosis in these cells [24]. Eryptosis is triggered by an increased cytosolic Ca^{2+} concentration due to activation of cation channels by a number of different causes [23, 25]. In an in vivo experiment, an increase in PS expression with increasing age of the RBCs has been found, which correlated with the rate of RBC removal from the circulation [26]. It was long believed that after increased PS expression, macrophages eliminate these apoptotic RBCs by recognizing them through their specific PS receptor [27, 28]. However, various receptors have been identified recently, such as Tim1, Tim4 and Stabilin-2, that can mediate binding and phagocytosis of apoptotic cells by the recognition of PS on these cells [29, 30]. In addition, several plasma proteins, such as lactadherin, GAS6 and protein S, have been described to bind to PS and act as bridging molecules to direct PS to receptors on phagocytes, $\alpha_v\beta_{3/5}$ integrins, and receptors of the Axl family and mediate clearance of PS-positive cells [31].

Erythrocyte Phagocytosis – Band 3 and CD47

In contrast to eryptosis, RBCs can also be phagocytosed directly without being apoptotic first. There are 2 distinct mechanisms of erythrophagocytosis due to ageing. Naturally occurring antibodies (NAbs) that bind to band 3 are implicated in the clearance of senescent RBCs [32]. Band 3 is an abundant RBC integral membrane protein which has 2 different domains: the membrane-spanning domain catalyzes anion exchange and is recognized by NAbs, whereas the cytoplasmic domain binds different proteins and thereby regulates the structure and function of the RBCs [33, 34]. Band 3 undergoes a conformational change in senescent RBCs, although no consensus exists over the exact mechanism leading to this change. It is thought that oxidative damage to hemoglobin, which occurs during senescence, and the subsequent formation of hemichromes which bind to band 3, can eventually lead to clustering of band 3 into large aggregates. These clusters show enhanced affinity for NAbs [35–39]. Indeed, a mutual correlation has been shown to exist between the amount of membrane-bound hemichromes, percentage of aggregated band 3, and phagocytosis intensity [40, 41]. Another hypothesis is that proteolytic degradation of band 3 is required to form the band 3 epitope(s) recognized by NAbs [42, 43]. NAbs are not efficient opsonins, due to their low affinity and low circulating numbers. Their efficiency is increased by the activation of the classical complement pathway, which signifi-

Table 2. Identified mechanisms of erythrocyte clearance in physiologic senescence and inflammation

	Physiologic senescence	Inflammation
Phosphatidylserine exposure	+/–	+
Erythrocyte phagocytosis	+	+/–
Reduced deformability	+	+
Adherence to endothelium	–	+

cantly lowers the amount of NAbs needed for induction of phagocytosis [44–46]. For example, an in vitro experiment showed that phagocytosis of sheep RBCs was at least 10-fold more effective when opsonized with C3b immunoglobulin G (C3b-IgG) compared to opsonization with IgG alone [47]. NAbs form complexes with C3b, which are more resistant to inactivation by factors H and I than free C3b. Furthermore, the activation of C3 convertase by these complexes is more potent than by immobilized C3b [45]. However, the exact mechanism how opsonization with C3B-IgG increases phagocytosis compared to opsonization with IgG or C3B alone remains unclear. An alternative mechanism of erytrophagocytosis involves the regulation of expression of 'eat me' and 'don't eat me' signals. CD47, one of the membrane proteins expressed by RBCs, has an inhibitory effect on erythrocyte phagocytosis by macrophages [48–52]. CD47 binds to SIRPα on the macrophage, eventually leading to inhibition of phagocytosis [53–56]. However, CD47-SIRPα can, through an unknown mechanism, also promote phagocytosis of apoptotic cells [57, 58]. Recently, it was shown that SIRPα plays a role in the removal of aged RBCs through CD47 binding [53]. CD47 undergoes a conformational change in response to oxidative damage due to ageing, after which CD47 binds thrombospondin-1 [59, 60] and is subsequently recognized as an 'eat me' signal by SIRPα [118].

Reduced Deformability

To be able to pass through capillaries with a diameter 2–3 μm while their own diameter is 8 μm, RBCs are highly deformable. The ability to deform relies on different characteristics of the RBC, including membrane composition, cellular geometry, and cytoplasmic viscosity [61, 62]. The filtering of senescent RBCs from the circulation is performed by the spleen as a consequence of its unique structure. Arterial blood passes the red pulp that contains many macrophages, and then on to the venous sinuses which are eventually drained into the vena lienalis. To reach these sinuses, the blood from the cords is forced through very small slits that are formed by stress fibers running parallel to the endothelial cells. This passage is more difficult for senescent RBCs which have stiffening membranes such that they stick in the cords and are phagocytosed by the earlier mentioned red pulp macrophages [63, 64]. Decreased RBC deformability is not only observed in senescence, but also occurs in a wide variety of pathological conditions, such as malaria [65], hereditary spherocytosis [66], sickle cell disease [67], and sepsis [68–73]. In the following chapter, the effects of inflammation on RBC clearance are discussed by comparing mechanisms occurring in ageing with inflammatory pathways (table 2).

Effects of Inflammation on Clearance of Red Blood Cells

Phosphatidylserine Exposure in Inflammation

PS exposure has been claimed to be involved in accelerated RBC clearance during inflammation. This is thought to be due to an increase in the plasma concentrations of sphingomyelinase, an enzyme that converts sphingomyelin into ceramide [74]. Ceramide enhances the sensitivity of RBCs to an already increased intracellular Ca^{2+} concentration, and thereby enhances PS exposure [75]. Sphingomyelinase normally resides in the lysosomes of macrophages, but can be secreted into plasma [76]. Many factors can lead to an increase in plasma levels of sphingomyelinase, and some of these are also implicated in sepsis, such as tumor necrosis factor-alpha (TNF-α) [77] and platelet activating factor (PAF) [78]. Furthermore, bacteria such as *Staphylococcus aureus* can also produce sphingomyelinase [79]. In vitro experiments show that PS exposure is induced by incubation of RBCs with plasma of sepsis patients but not by incubation with plasma of healthy volunteers [79]. Furthermore, PS exposure is induced after treatment with supernatant from cultured *S. aureus* with sphingomyelinase activity, but not after exposure to supernatant from mutated *S. aureus* lacking sphingomyelinase activity [79].

Erythrocyte Phagocytosis in Inflammation – Band 3 and CD47

As in ageing, band 3 may be involved in mediating erythrocyte phagocytosis in inflammation. It is shown that the RBC band 3/α-spectrin ratio increased in septic mice compared to non-septic mice in a cecal ligation and puncture (CLP) model [80]. In a different experiment, but using the same in vivo model, the same group found an increase in band 3 phosphorylation in septic mice compared to non-septic mice [81]. However, no evidence exists linking these changes in band 3 directly to an increased RBC clearance in a sepsis model. No evidence has been published concerning the CD47-thrombospondin-1 combination that is recognized as an 'eat me' signal by SIRPα. However, since this is a recently elucidated mechanism, additional research is needed.

Reduced Deformability in Inflammation

In vitro, lipopolysaccharide (LPS) induces a change in the deformability in RBCs after whole blood stimulation, but not in isolated RBCs [68]. Also, in patients with sepsis due to both Gram-negative and Gram-positive bacteria, reduced RBC de-

formability has been shown when compared to healthy controls [69–73]. This may implicate that other factors besides the erythrocyte itself may be needed to induce reduced deformability. Several factors are involved in the decrease of RBC deformability during sepsis, which we will discuss here. As in aging, band 3 may be involved in reduced deformability in inflammation. It was found in an in vivo CLP mouse model that a higher RBC band 3/α-spectrin ratio was associated with decreased RBC deformability [80]. Also, in vitro, RBC deformability was found to depend on the band 3 phosphorylation state [82, 83]. Reactive oxygen species (ROS) influence RBC deformability in inflammation. ROS can lead to protein degradation in RBCs in vitro [84], in particular membrane proteins such as band 3 and spectrin [85]. A clear link between ROS and loss of deformability was established in an in vitro experiment showing that human RBCs undergo loss of deformability after exposure to H_2O_2 [86]. Also, in an in vivo CLP rat model, decreased RBC deformability was found in septic rats when compared to rats that underwent sham surgery. Decreased RBC deformability was prevented by pretreating the rats with the ROS scavenger α-tocopherol [73]. Another mechanism that is implicated in the reduced deformability encountered in inflammation is nitric oxide (NO). NO is a mediator that is released by vascular endothelial cells and acts mainly as a vasodilator [87–89]. Small amounts of NO are present in the blood under physiological conditions, but during inflammation and infection its concentration may increase by 10-fold [90]. Several in vitro experiments showed that NO causes a decrease in RBC deformability [91, 92], and this was also shown in an in vitro sepsis model in which this loss of RBC deformability was attenuated by the NO inhibitor N-monomethyl arginine [93]. Furthermore, a selective inhibitor of NO synthase prevented overproduction of NOS, accumulation of NO within the RBC, as well as a decrease in RBC deformability in murine sepsis [94]. Another factor of importance in clearance of RBCs during inflammation are sialic acid residues (SA) which are bound to glycophorin and account for the negative force of the RBC membrane [95]. Due to this negative force, RBCs have repellent properties. When the SA content is cleaved from glycophorin after treatment with neuraminidase, RBCs have a reduced mean curvature [96]. A reduction in SA content was found in RBCs from critically ill patients when compared to RBCs from healthy volunteers, associated with a decrease in RBC deformability [97].

Increased Adherence of Red Blood Cells to the Endothelium in Inflammation

In sepsis, there are profound disturbances in microcirculation, already occurring in the early phase [98–100] and clearly contributing to adverse outcome [99–101]. The resistance of microcirculatory disorders to vasodilators [102] and the apparent independence of the mean arterial blood pressure and cardiac output [98, 99, 103] raises the hypothesis that these disorders may be caused by an obstruction due to adherence of cells to the endothelium and may not be solely due to a low flow state. Indeed, in diffuse intravascular coagulation which occurs in 25% of patients with sepsis [104], increased aggregation of cells with formation of microthrombi is apparent. However, RBC adherence to the endothelium may also play a role. Although not extensively investigated, there is some evidence for this phenomenon. Incubation of both endothelial cells and RBCs with endotoxin increased adherence of RBCs to endothelial monolayers [105]. This effect was also observed after stimulation with TNF-α [106]. The mechanisms mediating RBC adhesiveness to the endothelium during inflammation are not well characterized. Most knowledge comes from specific diseases characterized by the presence of vascular pathology, including sickle cell disease, diabetes mellitus, and malaria. Specific ligand-receptor interactions have been identified in enhanced RBC adherence to the endothelium in sickle cell disease [107]. In diabetes, advanced glycation end products (AGE) expressed on diabetic RBCs ligate with the receptor for AGE (RAGE) expressed on endothelial cells. Also, AGE-RAGE interactions have been found in other inflammatory states, including trauma [108]. Of interest, AGE-RAGE interactions may play a role in adverse effects of blood transfusion, as AGE formed in stored RBCs was found to ligate to endothelial bound RAGE, resulting in endothelial damage [109]. Whether specific ligand-receptor interactions play a role in other inflammatory states is not known. However, there is evidence that non-receptor cytoadhesion is mediated by exposure of PS on the RBC membrane. RBCs expressing PS on their outer membrane are more prone to adhere to endothelial cells, irrespective of the cause of the band 3/α-spectrin ratio PS exposure [110–116]. A definite role for PS in adherence of RBCs to endothelium has been shown by a reversal of adhesion following blocking of PS by PS liposomes [110]. As discussed before, inflammatory conditions are able to induce PS exposure on RBCs [79]. Besides specific RBC-endothelial interactions, low flow may contribute to increased RBC adhesiveness. Flow in the microcirculation in sepsis is diminished, with a decrease in the number of perfused capillaries which display intermittent flow and differences in RBC velocities. This led to the hypothesis that flow may have an effect on adhesion of RBCs to vascular endothelium. Indeed, it was found that higher flow rates reduced and lower flow rates increased RBC adherence to the endothelium [117]. Taken together, similar to the Virchow's triad in thrombosis, both alterations in the microcirculatory flow as well as activation of endothelial adhesion markers may contribute to increased RBC adherence to the endothelium in sepsis.

Conclusion

Anemia is a common feature in sepsis due to several inflammatory pathways collectively referred to as anemia of inflam-

mation. RBC clearance is likely to contribute significantly to anemia of inflammation. We postulate that besides mechanisms that also play a role in RBC clearance during senescence, RBC-endothelial interactions are important features underlying the clearance of RBCs from the circulation during inflammation.

Disclosure Statement

The authors declare that they have no conflict of interest relating to the material covered in this manuscript.

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Erratum

Transfus Med Hemother 2012;39:Č×1

In the article

Straat M, van Bruggen R, de Korte D, Juffermans NP: Red blood cell clearance in inflammation. Transfus Med Hemother 2012;39:353–360

there was a mistake.

Correction Statement
We are very sorry to note an error that has slipped into our above mentioned review article, which was pointed out to us by Dr. H.U. Lutz.
In the last sentence on page 355, the ‘classical complement pathway’ should be replaced by the ‘alternative complement pathway’.

Marleen Straat, Nicole Juffermans