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REVIEW

Mechanisms of Hemolysis During Sepsis

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Abstract— Cell-free hemoglobin is increasingly playing a more central role in the pathogenesis of sepsis being proved to be a potent predictor of patient's outcome. It is crucial, hence, to further investigate the mechanisms of sepsis-induced hemolysis with the aim of deriving possible therapeutic principles. Herein, we collected the most important previously known triggers of hemolysis during sepsis, which are (1) transfusion reactions and complement activation, (2) disseminated intravascular coagulation, (3) capillary stopped-flow, (4) restriction of glucose to red blood cells, (5) changes in red blood cell membrane properties, (6) hemolytic pathogens, and (7) red blood cell apoptosis.

KEY WORDS: sepsis; systemic inflammation; hemolysis; disseminated intravascular coagulation; erythrocytes; lipopolysaccharide; pore formation; transfusion reactions.

INTRODUCTION

Both clinical [1–4] and experimental [5–8] studies have shown that sepsis and systemic inflammation lead to a massive release of hemoglobin from red blood cells (hemolysis) being accompanied with an increased risk of death [1–4, 8, 9]. In critically ill patients with sepsis, thus, elevated concentrations of circulating cell-free hemoglobin are measurable [1–4]. In animal experiments, the administration of lipopolysaccharide (LPS) to induce systemic inflammation leads to a significant increase in plasma concentration of cell-free hemoglobin as well [5–8].

Cell-free hemoglobin and its prosthetic group heme can contribute to organ dysfunction and death [1–4, 9–12]; the pathological mechanisms include nitric oxide consumption, vasoconstriction, oxidative injury to lipid membranes, activation of the transcription factor NF-κB, endothelial injury as well as iron-driven oxidative inhibition of glucose metabolism [10–14]. Thus, hemolysis can act as a kind of amplifier of the complex response to an infection or injury [8, 15] and worsen the outcome from animals and patients with systemic inflammation, sepsis, or trauma [1–4, 10].

Pathogenesis of Sepsis

Sepsis is the leading cause of death in industrialized countries after cardiovascular and oncological diseases [16]. Despite all advances in intensive care, the mortality for patients with sepsis and septic shock is still high with reported probabilities of 30 to 50% [16–20].

Sepsis recently was defined as "life-threatening organ dysfunction caused by a dysregulated host response to an infection" (Sepsis-3 definition), whereas a septic shock was described as "subset of sepsis with circulatory and cellular/metabolic dysfunction" [21, 22]. Sepsis definitions (*post hoc* referred to as "Sepsis-1 definition" [23]) stated in 1992 by a "Consensus Conference" [24] still differentiated between *sepsis*, *severe sepsis*, and *septic shock*. Crucial for a *sepsis*, thus, was the presence of at least two of four criteria of a systemic inflammatory response syndrome (SIRS), which includes (1) fever (≥ 38.0 °C) or hypothermia (≤ 36.0 °C), (2) tachycardia (heart rate ≥ 90/min), (3) tachypnea (frequency ≥ 20/min) or hyperventilation, and

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(4) leukocytosis (white blood cells $\geq 12{,}000/mm^3$) or leukopenia (white blood cells $\leq 4000/mm^3$). However, the proof of infection was not mandatory; a suspicion of infection was sufficient [23, 24]. A *severe sepsis* further was defined as sepsis with organ dysfunction and a *septic shock* as severe sepsis with cardiovascular collapse that does not respond to fluid intake [23]. In 2001, sepsis definition (*post hoc* referred to as "Sepsis-2 definition" [23]) has been modified by the "International Sepsis Definitions Conference" [25]. The term *sepsis*, thus, described a clinical picture defined by a range of general parameters (*e.g.*, fever, tachypnea, and hyperglycemia), inflammatory parameters (including white blood cell count, plasma C-reactive protein, and plasma procalcitonine), hemodynamic parameters (*e.g.*, cardiac index, organ function parameters, platelet count, and coagulation parameters) as well as tissue perfusion parameters (such as plasma lactate level) [25]. With the new Sepsis-3 definitions, the term *severe sepsis* should not be used anymore since—according to the new definitions—every form of sepsis is associated with organ dysfunction [22, 23]. The diagnosis of *septic shock*, moreover, requires a lactate serum concentration of > 2 mmol/L following volume replacement in addition to circulatory changes. So, changes in cellular metabolism are crucial, too [22].

Pathogenesis of sepsis is complex and only partly explained, being a multi-factorial interplay between protective and repair mechanisms (generalized immune response) and the intravascular coagulation system [26, 27]: The cause of sepsis is primarily an exaggerated, generalized inflammatory response to an extrinsic stimulus. Mechanistically, so-called PAMPs (pathogen-associated molecular patterns) lead to the activation of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and C-type lectin receptors (CLRs) [28, 29]. Thus, the organism reacts to extrinsic stimuli including bacterial toxins (*e.g.*, lipopolysaccharide, LPS—the cell membrane constituents of Gram-negative bacteria released among others as a result of the use of antibiotics) [27] with the activation of immune cells (granulocytes and macrophages) and the release of inflammatory mediators (cytokines) [28, 29]. The cytokines released can further lead to pronounced peripheral vasodilation with arterial hypotension by activating the inducible nitric oxide (NO) synthase (iNOS) and the subsequent formation of NO [30]. Hemodynamics will be impaired, which on the one hand can lead to the production of oxygen radicals and thus directly to tissue damage. On the other hand, impaired microcirculation in the tissue usually causes local ischemia (circulatory disorders and lack of oxygen in the tissue) and often results in multiple organ failure [31].

Furthermore, a massive release of cytokines will shift the balance between pro- and anti-coagulatory factors in the blood, which will lead to increased coagulation of the blood (coagulopathy). This is called a disseminated intravascular coagulation (DIC), which also causes microvascular perfusion disorders [26, 32]. An excessive coagulation activation may, in turn, lead to a so-called consumption coagulopathy in the later course of sepsis, which complicates the rehabilitation of the infection source due to the risk of internal and external bleeding complications [19, 26].

In recent years, it has also been shown that toll-like receptors and other pattern recognition receptors are activated not only by extrinsic factors but also by intrinsic stimuli (so-called damage-associated molecular patterns, DAMPs) that are released when the host cell is damaged [27, 29]. These endogenous receptor agonists are indicators of tissue injury and referred to as "danger molecules." Well-known danger molecules include HMGB1 (high-mobility group box 1), ATP, hyaluronic acid, mitochondrial DNA, heat shock proteins, uric acid, and S100 proteins [29]. Various experimental studies showed that cell-free hemoglobin also has the characteristics of a danger molecule [33, 34].

A sole consideration of the immunological processes for the understanding of the pathogenesis of sepsis means the excessive inflammatory response including the so-called cytokine storm—as it was made during the "Sepsis-1" and "Sepsis-2" definitions—is no longer sufficient to capture the complexity of the disease. Rather, with the new "Sepsis-3" definition, the host response and resulting inflammation-driven organ dysfunction has become key in the understanding of sepsis. Thus, the collapse of the otherwise tightly regulated metabolic and energetic homeostasis is a significant pathophysiological event during sepsis. Due to a higher need of fatty acids, amino acids, and glucose for providing acute phase proteins and mediators during sepsis, for example, metabolism is switched to catabolism [35–37]. However, the metabolic response to an infection also includes alterations in heme metabolism; a strategy to restrain pathogens to access iron would be the induction of hypoferremia [14, 38].

Pathogenesis of Hemolysis

In the course of intravascular hemolysis (destruction of red blood cells within the vascular compartment), hemoglobin will be released into the plasma. Normally, cell-free hemoglobin will dimerize and rapidly be bound by its hemoglobin scavengers haptoglobin and hemopexin [12]. Massive hemolysis, however, may result in saturation and depletion of these hemoglobin removal systems and

consequently in an accumulation of hemoglobin and heme in plasma [12].

Cell-free hemoglobin has characteristics of a "danger molecule." Adverse effects associated with massive intravascular hemolysis can be attributed to several biochemical properties of the molecule hemoglobin including the following mechanisms: (1) After dimerization of the tetrameric hemoglobin, dimers are able to translocate to the extravascular space and access extravascular tissues. Thus, adverse reactivity of hemoglobin and heme is not limited to the intravascular space [39]. (2) One of the mechanisms by which cell-free hemoglobin exerts its detrimental effects is its ability to effectively scavenge nitric oxide (NO), which in turn leads to perfusion disorders and an increased arterial and pulmonary arterial pressure [39, 40]. (3) Another pathogenic mechanism involves the release of iron from cell-free hemoglobin with consecutive radical formation, which in turn can modify lipids, proteins, and DNA, leading to inflammation [39]. (4) Heme released from cell-free hemoglobin has been described to be an activator of TLR-4 [39, 41, 42]. Heme/TLR-4 signaling, moreover, was found to activate NF-κB and trigger vaso-occlusion [42]. (5) Furthermore, the released iron may favor the growth of bacteria [43, 44].

The pathophysiological significance of cell-free hemoglobin is also illustrated by the fact that the organism has developed complex mechanisms to remove cell-free hemoglobin, heme, and iron from its circulation. Once cell-free hemoglobin was bound by its scavenger haptoglobin, the resulting haptoglobin–hemoglobin complex will bind to CD163 on the surface of macrophages/monocytes to initiate endocytosis and degradation of the complex [12, 39]. Heme released from cell-free hemoglobin on oxidation is bound by hemopexin and degraded by hepatocytes in the liver [12].

Central Role of Cell-Free Hemoglobin During Sepsis

In infectious diseases, such as malaria and sepsis, high amounts of cell-free hemoglobin and heme were found [8], suggesting that hemolysis during sepsis and systemic inflammation is of pathophysiological relevance. Hemolysis, on the on hand, would mean a higher iron access for the invading pathogens (counteracting the host iron metabolic response [38]). On the other hand, however, cell-free hemoglobin is the "frontline defense molecule during infections by hemolytic microbes" [45]. By interacting with/binding to lipopolysaccharide (LPS), cell-free hemoglobin was described to structurally change and, thus, activate its redox activity to generate "microbicidal free radicals" [45].

Moreover, animal studies have shown a synergistic effect of hemoglobin on LPS-induced inflammation (*e.g.*, TNF-α) [33, 34]. Thus, the intravenous administration of hemoglobin in LPS-pretreated mice leads to a higher TNF-α concentration and an increased mortality; in turn, these effects could be inhibited by hemoglobin antibodies [33, 34]. Further studies showed that hemoglobin just like LPS binds to toll-like receptors and, thus, causes synergism [11, 41, 46]. It is also known that a reduced hemoglobin degradation in heme oxygenase-deficient mice during experimental sepsis leads to increased mortality compared to the wild type [8].

Cell-free hemoglobin is clinically important since its concentration is positively correlated with increased mortality in patients with sepsis. This was proven by two independent clinical studies [1, 2]. Thus, cell-free hemoglobin is an important predictor of survival in sepsis. Already in 1979, intravascular hemolysis was suggested as prognostic sign in septicemia [47]. Twenty years later, in 1990, Cooper *et al.* found mortality in patients with polymicrobial bacteremia to be predicted by hemolysis [48]. However, not only plasma levels of cell-free hemoglobin were associated with mortality of sepsis patients [1, 2] but also plasma levels of haptoglobin [49] and hemopexin [8]. Interestingly, plasma levels of cell-free hemoglobin measured in septic patients (median concentration 150 μg/mL) were significantly higher than the concentrations that showed synergistic effects with LPS in *in vitro* experiments (30 μg/mL) [50].

POSSIBLE MECHANISMS OF HEMOLYSIS DURING SEPSIS

Given the increasing evidence for the pathophysiological importance of cell-free hemoglobin in sepsis/systemic inflammation [3, 8], it is important to study the mechanisms of inflammation-induced hemolysis. Possible mechanisms of red blood cell death and hemolysis during sepsis and systemic inflammation include transfusion reactions and complement activation, disseminated intravascular coagulation, capillary stopped-flow, invasion of the amphiphilic LPS into the red blood cell membrane with corresponding changes in membrane properties, hemolytic pathogens and pore-forming toxins, red blood cell starvation (restriction of glucose), and eryptosis (red blood cells apoptosis). However, the latter rather seems to be a mechanism to counteract hemolysis.

Transfusion Incidents

"*Storage Lesion*" *Hemolysis*

Transfusion of packed red blood cells has always been a debatable issue, not just in sepsis. For instance,

various studies depict unfavorable effects of the transfusion of stored blood. Thus, red blood cells might be damaged by metabolic, enzymatic, and oxidative products after prolonged storage [51]. The so-called storage lesion was described to be associated with immune-modulation [52], an increased risk of infection [53], and hemolysis [54–56]. Transfusion of elderly blood (stored more than 14 days) in dogs and mice were reported to be associated with a negative outcome, a massive hemolysis, and an increased mortality [56–58]. In critically ill patients, likewise, it has been shown retrospectively that transfusion of red blood cells is related to increased morbidity and mortality, which may increase with prolonged storage before transfusion [54, 59]. In patients undergoing cardiac surgery (*e.g.*, coronary artery bypass graft surgery, pediatric cardiac surgical repair/palliation with cardiopulmonary bypass, aortic cross-clamp), duration of storage of red blood cells was associated with an increased risk of postoperative complications and a reduced survival [53, 60, 61]. Purdy and co-workers reported a correlation of mortality in septic patients with the age of packed red blood cells transfused [62]. Moreover, longer storage duration of red blood cells is associated with an increased risk of acute lung injury in patients with sepsis [63]. A nice overview reviewing the association of red blood cell storage to clinical outcomes was given by Tinmouth *et al*. in 2006 [64].

On the contrary to the studies mentioned above, other studies do not confirm the influence of red blood cell storage on the outcome [64]. The study of Vamvakas and Carven, for instance, did not corroborate the reported link between transfusion of elderly blood and increased morbidity in patients undergoing cardiac surgery [65]. Another group concluded that, based on their study, no justification could be found for the use of a maximum storage time for red blood cell transfusions in patients undergoing coronary artery bypass graft surgery [66]. Heddle and co-workers compared the effect of short-term *versus* long-term blood storage on mortality after transfusion using a general hospital patient population and found no difference [67]. However, this may not be true for critically ill patients [68].

Taken together, transfusions of red blood cells in the ill and especially patients with sepsis remain controversial [68]. Thus, it is not surprising that red blood cell transfusion has only made it into the latest Surviving Sepsis Guidelines as a restricted recommendation [21]: "We recommend that RBC transfusion occur only when hemoglobin concentration decreases to < 7.0 g/dL in adults in the absence of extenuating circumstances, such as myocardial ischemia, severe hypoxemia, or acute hemorrhage (strong recommendation, high quality of evidence)." However, although the clinical consequences of blood storage in the critically ill are unclear [64], the association between blood storage and hemolysis as a consequence of red blood cell damage remains undisputed [51].

Complement-Dependent Immune-Hemolysis

During blood transfusion, there may always be incidents due to "incompatible red blood cell transfusion" [69]. Like every cells, red blood cells carry a wide variety of surface molecules on the membrane. These antigens are also called agglutinogens because they cause the red blood cells to agglutinate with the corresponding antibodies, which are also called agglutinins. Most "hemolytic transfusion reactions" can be attributed to ABO antibodies (ABO incompatibility of red blood cells) leading to intravascular hemolysis [69, 70] as a consequence of robust complement activation [71]. Extravascular hemolysis, however, results from Rh incompatibility of red blood cells [72] and is complement independent [71]. During extravascular hemolysis, the IgG-coated red blood cells are degraded in the so-called reticuloendothelial systems such as liver, spleen, and lymph nodes. Since no hemoglobin is released into the circulation, no haptoglobin is consumed to scavenge the cell-free hemoglobin [70].

Complement activation can occur in three primary cascades: the classical pathway, the lectin pathway, and the alternative pathway. The lectin and alternative pathways of complement activation play a role in immunity. However, during hemolytic transfusion, the classical antibody-mediated pathway is crucial. However, regardless of the initial stimulus, each pathway ends in formation of an enzyme complex that converts the complement component C3 into the active products C3a and C3b. Ultimately, activation of the complement cascade results in formation of the terminal complement complex C5b-9, the so-called membrane attack complex, and consequently a pore formation resulting in osmotic lysis of the target [71]. In the case of red blood cells, hemolysis will result.

Apart from antibodies, the remaining triggers of "immune hemolysis" are the complement system right away as well as the immune cells [70]. The complement system has been shown to play a significant role in the pathogenesis of various inflammatory processes. During sepsis/systemic inflammation, the complement system is activated excessively, with the primary goal to recognize pathogen-associated molecular patterns on invading microorganisms or danger-associated molecular patterns of damaged tissue [73]. However, excessive complement activation as a response to excessive inflammation can quickly

turn from a local to a systemic event. And uncontrolled complement activation, again, can also result in the destruction of healthy host tissue [73]. Thus, the complement system may be causally involved in the onset of hemolysis during sepsis [74] by directly damaging the red blood cells upon activation as a result of detecting pathogen structures [73]. Thus, as in the case of the antibody-mediated pathway, the membrane attack complex might be directed against the red blood cells and result in hemolysis [75]. However, also the anaphylatoxins C3a and C5a may lead to cellular and organ disturbances [75].

Inhibition of the terminal complement cascade by eculizumab (inhibits the cleavage of C5 into C5a und C5b and thus the formation of the membrane attack complex 8, MAC C5b-C9) for the treatment of hemolytic paroxymal nocturnal hemoglobinuria (PNH) significantly prevented PNH-related symptoms in patients including abnormal thrombophilia, red blood cell destruction, and the extent of hemolysis [76]. Studies of Huber-Lang *et al.* showed that treatment with an anti-C5a antibody [77] or a specific C5a receptor antagonist [78] improves survival in septic mice using the cecal ligation and puncture (CLP) model. However, they found no significant differences in hemolysis, neither *in vivo* (in septic mice) nor *in vitro* (using sensitized sheep red blood cells), in presence or absence of the anti-C5a antibody [77]. In 1982, Hitomi and Fujii described that inhibition of the complement-dependent hemolysis could be prevented by a synthetic inhibitor of the complement system *in vitro* (using sensitized sheep red blood cells), but they gave no indication of an hemolysis-reducing effect *in vivo* (in mice treated with endotoxin), although the inhibitor strongly protected endotoxemic mice from death [79]. However, the substrate specificity of this protease inhibitor (FUT-175) is very broad since FUT-175 inhibits various proteases both of the complement and the coagulation system.

Recently, our own collaboration could exclude the complement system as well as pro-inflammatory cytokines as hemolysis triggers in *in vitro* experiments on human red blood cells [80, 81]. From hemolytic uremic syndrome (HUS), we know that damage to the endothelium (endothelial lesions) might be the primary cause of hemolysis. During HUS, endothelial lesions cause a complement-dependent activation of immune response and local thrombus formation—attachment of fibrin and platelets to the endothelial lesions and consequently disseminated intravascular coagulation (DIC)—and further mechanical destruction of the red blood cells in the fibrin mesh resulting in hemolysis [82]. Similar to HUS, during sepsis an activation not just of the complement system but also of the coagulation system has been described (essentially in consequence of the so-called pro-coagulant shift of the endothelial cells), which offers us the next possible cause of hemolysis during sepsis: destruction of the red blood cells in the fibrin mesh. Even though Heidemann *et al.* showed in 1979 that complement activation following infusion of endotoxin in dogs occurs early and before activation of coagulation and hemolysis could be measured [83]. However, the model used merely reflects an anaphylactic than an endotoxin shock [83].

Disseminated Intravascular Coagulation and Microangiopathy

Sepsis/systemic inflammation is frequently associated with disseminated intravascular coagulation (DIC) being a predictor of mortality in septic patients [84, 85]. DIC is characterized by a systemic intravascular coagulation, formation of microvascular thrombi, insufficiently compensated consumption of platelets and coagulation factors, and eventually bleeding tendency [84]. Furthermore, fibrinolysis (dissolution of a blood clot) is also regularly inhibited in the early stages of sepsis [86]. Essential for the development of DIC during sepsis is the so-called pro-coagulatory shift of the endothelial cells, caused among others by an increased expression of tissue factor and adhesion molecules especially by damaged endothelial cells [87]. During DIC, fibrin strands within the fibrin mesh formed could cut red blood cells, resulting in the formation of schistocytes (strongly deformed red blood cells or fragments of red blood cells) and the release of hemoglobin. That mechanism of hemolysis was described the first time in the early 1970s as "microangiopathic hemolysis" [88, 89]. In a case report, Bull and Kuhn presented the pathogenesis of microangiopathic hemolytic anemia in a patient with an infiltrating adenocarcinoma [88]. In that patient, they found large numbers of micro-clots composed of fine fibrin strands in his vasculature. Moreover, peripheral blood was characterized by the absence of platelets and the presence of schistocytes [88]. They concluded that intravascular coagulation must be the most likely cause of this microangiopathic hemolytic anemia. But tragically, the patient received a total of 23 U of blood [88]. However, 6 years later, Heyes and co-workers demonstrated in an experimental study in rats that infusions of thrombin induce DIC accompanied with hemolysis and schistocytosis [89]. This study further supports the concept that fibrin deposition in the blood vessels as a result of DIC might contribute to red blood cell fragmentation and, in turn, hemolysis [89].

There are various other studies demonstrating a relationship between DIC and intravascular hemolysis. In a prospective study on septic children with DIC (without any transfusion), fragmented red blood cells and microangiopathic hemolysis could be observed [90], likewise in adult sepsis patients with DIC [91–94]. Recently, using a rat model of lipopolysaccharide (LPS)-induced systemic inflammation, our own collaboration could show that argatroban (a specific direct thrombin inhibitor and consequently an inhibitor of coagulation) abolishes DIC, schistocyte formation, and hemolysis. Interestingly, inhibition of coagulation is capable of diminishing DIC and hemolysis but not antiplatelet therapy—treatment with eptifibatide (an antiplatelet drug of the glycoprotein IIb/IIIa inhibitor class) failed to reduce LPS-induced DIC, schistocyte formation, and hemolysis.

However, the association between hemolysis and activation of coagulation is also discussed in reverse. In 1971, Jacobi *et al*. posed the opposite hypothesis that intravascular hemolysis must be the trigger of consumption coagulopathy in the ill [95]. Using rabbits, they found that a slow injection of a red blood cell hemolysate (obtained from freezing of rabbit red blood cells) results in consumption coagulopathy in some rabbits, but not a rapid injection [95]. Helms and co-workers also reported an effect of hemolysis on hemostasis (*in vitro* platelet activation) after injection of a red blood cell hemolysate (obtained from mechanical shear of human red blood cells) [96]. However, hemolysate injection hardly can reflect the pathophysiological conditions known from intravascular hemolysis—a limitation Jacobi *et al*. set up themselves [95]. On the contrary, Dale and Aasen showed that intravascular hemolysis occurred after the initiation and progress of DIC using a model of lethal administration of endotoxin in dogs [97, 98]. They purposed that intravascular hemolysis could not have influenced the development of DIC [97, 98].

Finally, a bi-directional crosstalk between hemolysis and coagulation was postulated with induction of tissue factor by cell-free hemoglobin as potentially central mechanism for hemolysis to trigger coagulation [87]. The link between hemolysis, coagulation, and innate immunity, moreover, seems to be evolutionarily conserved from the invertebrate hemocyanin to the vertebrate hemoglobin [87].

Capillary Stopped-Flow and Microvascular Stasis

One crucial factor in pathogenesis of systemic inflammation/sepsis is an impaired microcirculation [99]. Alterations in microvascular perfusion are accompanied by reduced perfused capillary density and red blood cell velocity, even under normotensive conditions [100–102]. An increased amount of capillaries with low/no flow is also called as “capillary stopped-flow” [103] or microvascular stasis [104, 105].

In his overview of the participation of microcirculation during sepsis/septic shock, Hinshaw formulated that changes in vessel diameter and concomitant rheological changes to blood cells result in a release of cell components from blood cells (*e.g.*, hemoglobin) [106]. That implies first a mechanical damage of blood cells (not just red blood cells but also white blood cells) by altered flow properties during sepsis. Since capillary stopped-flow or microvascular stasis is characterized by vessels filled with “tightly packed” blood cells [104, 107], resting time and close contact of red blood cells to white blood cells are increased. Besides a mechanical damage, an enzymatic damage of red blood cells by white blood cells and their components would also be possible [104]. Fundamentally, that would be nothing less than a different kind of “storage lesion” hemolysis [51].

There are various studies that show a relationship between microvascular stasis and intravascular hemolysis. Already in 1940, Mumme described that renal stasis causes hemolysis [108]. Almost 30 years later, McKay and Whitaker found hemolysis during epinephrine infusion in rabbits, monkeys, and dogs to be ultimately due to fragmentation of red blood cells in consequence of stasis with local agglutinate formation in various capillary areas [109, 110]. A similar relationship—hemolysis as a consequence of red blood cell accumulation in liver sinusoids and their subsequent fragmentation—has been described by Dale and co-workers during lethal canine endotoxin shock [97]. Recently, our own collaboration could demonstrate that hemolysis in course of LPS-induced systemic inflammation should be initiated by an early small intestinal microvascular stasis [111, 112].

There are various studies, however, that demonstrate that the presence of cell-free hemoglobin causes microvascular stasis—*vice versa*. A group of researchers around Wim Buurman found acute hemolysis (induced by infusion of water or pre-lysed red blood cells) as well as intraoperative hemolysis (during major aortic surgery) to be associated with an impaired renal, hepatic, and intestinal microvasculature [113–115]. Vinchi and co-workers proved that the hemoglobin scavenger hemopexin prevents from hepatic microvascular stasis induced by intravascular hemolysis (using a mouse model of heme overload in wild-type mice compared to hemopexin-null mice) [116]. Similar to that, Belcher *et al*. found a relationship between microvascular stasis and total plasma heme concentrations (comparison of different treatments to induce hemolysis and heme overload in transgenic sickle mice) [42]. They further demonstrated that intravascular hemolysis during sickle cell disease triggers microvascular stasis *via* TLR-4 signaling [42].

In any case, there is a relationship between the release of hemoglobin from red blood cells and the microvascular stasis in abdominal organs. Causality seems to apply in both directions. So, hemolysis—above a certain threshold of approximately 10–20 μmol/L—appears to be a kind of amplifier of microvascular disorders [111, 115].

Metabolic Disorders and Glucose Restriction

Energy metabolism and glucose hemostasis are tightly regulated in healthy individuals [117]. During sepsis, however, for providing acute phase proteins and mediators, a higher need of metabolites forces a switch to catabolism (also called septic auto-cannibalism) including a rapid mobilization of glucose, fatty acids, and amino acids [35–37]. A higher glucose requirement is covered, then, by increased glycogenolysis and gluconeogenesis [37, 118]. However, glycogen depots are quickly exhausted and glucose supply can only be achieved by an increased gluconeogenesis in the liver. If glucose consumption further exceeds glucose production or uptake, finally hypoglycemia occurs [118]. Red blood cells are the one who suffer the most since red blood cells are dependent on glucose as sole source of energy (anaerobic glycolysis) [119, 120]. Different to the brain, red blood cells cannot replace its energy source glucose by ketone bodies [37]. Without sufficient glucose supply, red blood cells will starve and perish and cytoplasmic components will release. Hemolysis will be the consequence [120]. Recently, our own collaboration could show that moderate glucose supply reduces hemolysis in rats treated with LPS to induce systemic inflammation [121]. This is in line with the *in vitro* study of Hendry in 1951, who described a delayed hemolysis of human red blood cells in solutions of glucose [122].

Nevertheless, the crosstalk between glucose and heme metabolism in sepsis is bidirectional since an excessive accumulation of cell-free heme following hemolysis influences the glucose metabolism by iron-driven oxidative inhibition of the glucose-6-phosphatase (a liver enzymes being important for endogenous glucose production *via* gluconeogenesis and glycogenolysis) [14].

Membrane Interaction and Deformability

Lipopolysaccharide (LPS)—the main constituent of the outer cell wall of Gram-negative bacteria—is known to bind to the pathogen recognition receptor TLR-4 and to activate the innate immune and hemostatic systems. Similar to the amphiphilic membrane phospholipids, LPS has both hydrophilic and lipophilic properties. Due to these properties, LPS can easily be incorporated into the membrane of (red blood) cells, alter their membrane properties, and thus promote cell death [123, 124].

It has long been described that deformability of red blood cells is reduced in both clinical sepsis and experimental systemic inflammation [124–126]. Our own collaboration recently could show that LPS molecules quickly insert into model membranes from 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), increase membrane fluctuation amplitudes and significantly weaken mechanical stiffness of DMPC membranes by decreasing molecular order [123]. We found, moreover, a decreased osmotic resistance and membrane stiffness of washed red blood cells treated with LPS [80, 81]. We further proved that LPS evokes hemolysis independent of the TLR-4 pathway and the complement system but *via* direct membrane effects [80, 81].

Lipopolysaccharides consist of three sub-regions: the lipid A (forming the inner lipophilic area of LPS and anchoring into the outer bacterial membrane), the core region (containing carbohydrate and non-carbohydrate components), and the polysaccharide or *O*-antigen (forming the outer hydrophilic region of LPS and determining the surface properties of the bacterium). Up to now, it is unclear which sub-regions are necessary to alter membrane properties in red blood cells. Lipid A alone, however, does not appear to affect the osmotic resistance of red blood cells [127].

Besides the insertion of LPS into phospholipid bilayers, a loss of phospholipids from the red blood cell membranes was discussed to result in osmotic instability of the red blood cells as well (formation of so-called spherocytes) [128, 129]. Already in 1941, Macfarlane and collaborators described hemolysis due to loss of lecithin from the red blood cell membrane in consequence of an infection with *Clostridium perfingens* [128, 130]. Responsible for the hydrolysis of lecithin from the membrane is lecithinase, the alpha-toxin of *Clostridium perfingens* [130]. More recent cases also describe sepsis patients with *C. perfingens* infections that develop hemolysis [131].

Pore-Forming Toxins and Hemolysins

Some pathogens are capable of causing hemolysis by cytolytic toxins. Several bacterial pathogens, both Gram-positive and Gram-negative, have been described to produce so-called pore-forming toxins (PFTs) [132–135], which are "one of Nature's most potent biological weapons" [132]. The toxic effect is mediated by the perforation of mammalian, insect, or even bacterial cell membranes by pores, whereupon the homeostasis collapses and the cell will perish [134]. Thus, pore-forming toxins are important pathogenicity factors [134, 136]. About 25% of all known bacterial protein toxins are

PFTs [134]. However, pore-forming toxins are also produced by cnidarians (*e.g.*, sea anemones, and hydra), mushrooms, plants, or worms [134, 135].

Bacterial pore-forming toxins are divided into several groups (*e.g.*, alpha-PFTs such as diphtheria toxin and beta-PFTs such as aerolysin), which differ in their structure and mode of action [132–135]. "The earliest documented effect of PFTs is their ability to rapidly kill red blood cells through osmotic lysis" [134]. Therefore, some pore-forming toxins are also named hemolysins (*e.g. Staphylococcus aureus* alpha-hemolysin or *Escherichia coli* hemolysin E) [132–135]. Isolated hemolysins are capable of causing *in vitro* hemolysis of red blood cells [136, 137]. This allows among others the microbiological classification of pathogenic bacteria such as *Staphylococcus aureus* [138]. This *in vitro* activity, however, does not necessarily mean that hemolysin-producing pathogens cause significant hemolysis during infection *in vivo* [138]. In 1986, Hacker and co-workers systematically analyzed the influence of cloned *E. coli* hemolysin genes on the pathogenicity in different animal models (chicken embryo assay, *E. coli* intranasal, subcutaneous, intravenous, or intraperitoneal injection in mice [139]. They proved that all the hemolysin-positive *E. coli* strains cause symptoms of hemoglobinuria in several *in vivo* models [139].

Suicidal Death of Red Blood Cells (Eryptosis)

As a consequence of aging and upon injury, red blood cells can undergo an apoptosis-like cell death, which is referred to as eryptosis [140, 141]. Eryptosis is tightly regulated and triggered by a wide range of (endogenous) mediators and stimuli such as calcium signaling, ceramide formation, complement activation, energy depletion, eicosanoid release, hemolysin, and heme [140–142]. Eryptotic red blood cells undergo degradation in the reticuloendothelial system. Thus, physiologically, eryptosis is considered as a "preemptive measure to curtail premature hemolysis" of injured red blood cells [141]. During sepsis as well as malaria, HUS, and osmotic shock, eryptosis is increased [140, 141, 143] suggesting that eryptosis may protect against hemolysis upon injury. On the other hand, eryptosis is associated with anemia, microcirculatory derangement, and thrombosis [142, 144].

CONSEQUENCES AND POSSIBLE THERAPEUTICS

These mechanisms all seem to be mutually dependent, to reinforce and influence each other. Energy depletion to the red blood cells, for example, on the one hand, triggers hemolysis [121]. On the other hand, however, it is one of many stimuli to induce eryptosis and to avoid hemolysis [142, 144]. The same applies to hemolysin. For one thing, the pore-forming toxin hemolysin is one the pathogens' tools of causing hemolysis or releasing hemoglobin and poorly available iron [139]; then again it trigger eryptosis, one mechanism of protecting against hemolysis [142].

By reducing metabolic disorders or disseminated intravascular coagulation, hemolysis will not be totally removed during sepsis. A proportion of the direct membrane effect of lipopolysaccharides still exists, as is the proportion of the microvascular stasis during septic shock. Nevertheless, considering that hemolysis worsens the patients' outcome, a reduction of hemolysis at a certain percentage is another step to minimize mortality during sepsis and systemic inflammation.

Inhibition of the terminal complement cascade by eculizumab was the breakthrough for the treatment of hemolysis during PNH [76]. However, the mechanisms of sepsis-induced hemolysis (unlike hemolysis during PNH) are more complex. The magic bullet for the treatment of hemolysis during sepsis has yet to be found. The most promising therapeutic approach so far would be to minimize the toxicity induced by cell-free hemoglobin and heme, *e.g.*, by using (natural) scavengers such as haptoglobin and hemopexin [39].

CONCLUSION

The pathological mechanism of intravascular hemolysis in classical hemolytic diseases has been well studied. There are initial studies showing that hemolysis during sepsis is associated with the patient's outcome. Therefore, it seems plausible that hemolysis is also a causal factor in sepsis, contributing to the poor outcome of septic patients. Future studies will need to show if a reduction of hemolysis can improve the prognosis of sepsis.

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