Red blood cell dysfunction in septic glucose-6-phosphate dehydrogenase-deficient mice

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Submitted 28 November 2003; accepted in final form 27 January 2004

Spolarics, Zoltán, Michael R. Condon, Muhammad Siddiqi, George W. Machiedo, and Edwin A. Deitch. Red blood cell dysfunction in septic glucose-6-phosphate dehydrogenase-deficient mice. Am J Physiol Heart Circ Physiol 286: H2118–H2126, 2004. First published January 29, 2004; 10.1152/ajpheart.01085.2003.—Glucose-6-phosphate dehydrogenase (G-6-PDH) deficiency is the most common known human genetic polymorphism. This study tested the hypothesis that G-6-PDH deficiency worsens sepsis-induced erythrocyte dysfunction. Sepsis (24 h) was induced by cecal ligation and puncture in wild-type (WT) and G-6-PDH-deficient (G-6-PDH activity 15% of WT) mice. Erythrocyte responses were tested in whole blood as well as in subpopulations of circulating erythrocytes. Whereas erythrocyte deformability was similar in unchallenged deficient and WT animals, sepsis decreased erythrocyte deformability that was more pronounced in deficient than WT animals. Sepsis also resulted in anemia and hemolysis in deficient compared with WT animals. Mean corpuscular hemoglobin content and erythrocyte deformability decreased in younger erythrocyte subpopulations from septic deficient compared with WT animals. Sepsis decreased the reduced-to-oxidized glutathione ratio in erythrocytes from both deficient and WT animals; however, plasma glutathione increased more in deficient than in WT animals. Erythrocyte content of band 3 associated with the cytoskeleton was elevated in deficient compared with WT erythrocytes. The antioxidant N-acetyl-l-cysteine in vivo alleviated the sepsis-induced decrease in erythrocyte deformability in deficient animals compared with sham-operated control animals. This study demonstrates that a mild degree of G-6-PDH deficiency (comparable to the human class III G-6-PDH deficiencies) worsens erythrocyte dysfunction during sepsis. Increased erythrocyte rigidity and tendency for hemolysis together with alterations in band 3-spectrin interactions may contribute to the immunomodulatory effects of G-6-PDH deficiency observed after major trauma and infections in humans.

INFECTION-INDUCED DECREASE in red blood cell (RBC) deformability has been demonstrated in several independent studies using animal models as well as in human investigations (4, 5, 16, 26, 52). Decrease in RBC deformability may contribute to the multiple organ dysfunction syndrome in septic patients (35, 40, 46). Decreased RBC deformability may cause microcirculatory dysfunction by worsening blood congestion in capillaries and compromising oxygen exchange (28). RBC deformability is also decreased during the normal aging process of RBC, resulting in increased interactions between aged RBC and the mononuclear phagocyte system predominantly residing in spleen and liver (8). Oxidative stress caused by superoxide anion generation within the RBC or reactive oxygen and nitrogen species derived from phagocytes has been shown to play a role in the development of RBC deformability decrease during infections (3, 5, 13, 52). This study tested the effects of glucose-6-phosphate dehydrogenase (G-6-PDH) deficiency on sepsis-induced changes in RBC dysfunction.

G-6-PDH is a ubiquitous enzyme that plays a central role in the support of cellular redox balances. G-6-PDH is the first and rate-limiting step of the hexose monophosphate shunt, a primary source of cellular NADPH that is consumed by reactive oxygen detoxifying pathways (through glutathione). G-6-PDH is under tissue-specific regulation, and its cellular activity can be induced by a variety of physiological and pathological challenges in nucleated cells (6, 27). In contrast, circulating RBCs lack the capacity of de novo protein synthesis, and thus erythrocytes rely on the level of G-6-PDH activity that is present at the end of their maturation in the bone marrow. Thus G-6-PDH deficiency primarily affects RBC functions (7, 34).

G-6-PDH deficiency is one of the common human genetic polymorphisms, affecting over 400,000 people worldwide (7, 34). G-6-PDH deficiency protects against malaria infection, and the prevalence of the defects may reach 10–25% of the population in malaria-endemic regions. The commonest forms (class III) of the defects, including the Mediterranean (3% residual G-6-PDH activity) and African (10–15% residual activity) forms, do not cause symptoms in otherwise healthy individuals. However, the deficient phenotype may manifest on intoxications or pathological challenges. The clinical manifestations of the class III defects are neonatal jaundice and fava bean- or drug-induced acute hemolysis (7, 34). Additionally, our clinical investigations on trauma patients with multiple organ injuries (48) demonstrated that G-6-PDH deficiency predisposes to anemia, increased incidence of sepsis, and augmented monocyte activation compared with nondeficient patients with similar injuries. An increased degree of anemia and blunted anti-inflammatory cytokine responses were also demonstrated in G-6-PDH-deficient compared with nondeficient trauma patients after moderate injuries (29).

The effects of G-6-PDH deficiency on RBC functions during polymicrobial sepsis are not known. Therefore, in the current study, we tested the hypothesis that a moderate degree of G-6-PDH deficiency would increase the magnitude of sepsis-induced RBC deformability decrease and dysfunction. We also tested whether the sepsis-induced decrease in RBC deformability is associated with changes in glutathione redox status or band 3-cytoskeleton interactions and whether subpopulations...
of circulating RBCs are affected at different degrees in G-6-PDH deficiency. To elucidate these questions, we used a G-6-PDH-deficient mouse model that displays a degree of G-6-PDH deficiency (10–15% of normal) similar to that presented by the most common class III human G-6-PDH deficiencies (36, 43, 44).

MATERIALS AND METHODS

Animals. Breeding pairs of G-6-PDH-mutant mice (24, 36, 43) were purchased from the Medical Research Council (MRC) of the United Kingdom (Frozen Embryo and Sperm Archive Mammalian Genetics Unit, MRC, Chilton, UK). Initial breeding pairs were established in quarantine at Taconic Farm (Germantown, NY). Offspring were genotyped and breeding colonies were established at Taconic Farms. Animals were shipped to our institute and housed in our animal facility under a 12:12-h light-dark cycle. Animals were fed with standard rodent chow.

Male G-6-PDH-deficient (y/−) and normal (WT, y+/+) 10- to 12-wk-old animals were used in the experiments to allow full maturation of all RBCs in these animals (RBC life span is ~40 days in mice). Animals used in the experiments were phenotyped by G-6-PDH activity in whole blood with a kit (48) as well as being genotyped by DNA analysis. The studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205] and were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School.

Genotyping. The mutant G-6-PDH variant was identified as described previously (36, 43). The particular mutation in the G-6-PDH gene results in the disappearance of a DdeI restriction site in the mutant gene. DdeI results in full digestion of the amplified PCR product in normal males or females, whereas it does not cut the PCR product from deficient (hemizygous) male or homozygous female animals; partial digestion occurs in heterozygous females. Briefly, 100 ng of total genomic DNA was isolated from tail clippings and the target gene was amplified with sense (GGAAACTGGCTGTGCGC) and antisense (TCAGCTCCGGCTCTCTTCTG) primers. PCR products were visualized with ethidium bromide staining and polyacrylamide gel electrophoresis.

Cecal ligation and puncture and in vivo treatments. Polymicrobial sepsis was induced with the cecal ligation and puncture (CLP) model as described previously (1, 19). Briefly, animals were anesthetized by a subcutaneous injection of Nembutal (5 mg/100 g body wt). Animals were anesthetized and blood was collected by 10.220.32.246 on October 23, 2017 http://ajpheart.physiology.org/ Downloaded from
Glutathione determinations. GSH and GSSG in RBCs and plasma were determined using the protocol of Tietze (49). In brief, after the in vivo treatments, blood was drawn into heparinized tubes. From the freshly obtained blood, RBCs were sedimented and aliquots of the RBC pellet or plasma (5 μl of cell suspension, 0.1 ml of plasma) were precipitated in 3% sulfosalicylic acid at 4°C. To trap GSH, parallel samples were diluted in potassium phosphate buffer (100 mM, pH 6.5) in the absence or presence of 10 mM N-ethylmaleimide (NEM) and incubated for 15 min at room temperature. Precipitates were sedimented by centrifugation. Unreacted NEM was removed from the samples by chromatography to prevent interference with the subsequent recycling assay for glutathione measurements. Aliquots (100 μl) of the supernatants from samples treated or not treated with NEM were loaded onto disposable C18 chromatography columns and run in parallel (Waters, Milford, MA). Samples were eluted by the addition of 1.2 ml of phosphate buffer. C18 columns were washed with methanol and water repeatedly and then equilibrated with phosphate buffer (100 mM, pH 6.5) before chromatography. The recycling assay for the determination of glutathione content was performed under the same conditions and with buffers and reagents as described in detail previously (49). Cellular and plasma GSH and GSSG contents were calculated from dilution factors and from a glutathione standard assay run in parallel with the specimen assays.

Plasma hemoglobin was determined by the measurement of absorbance at 405-, 410-, 415-, and 430-nm wavelengths. Plasma hemoglobin concentrations were calculated with the molar extinction coefficient of oxyhemoglobin E 415 (1.25 × 10⁵). Calculation of plasma hemoglobin concentrations from the absorbance at 405, 410, and 430 nm with the corresponding molar extinction coefficients showed similar results.

RBC protein distribution. The distribution of major protein constituents of circulating RBCs was determined from water- and Triton-soluble sample extracts prepared from RBC ghosts as follows. RBC...
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RESULTS

Characterization of mouse model. Figure 1A shows G-6-PDH activity in whole blood in animals derived from the breeding colony and in two commonly used mouse strains. G-6-PDH activity in deficient hemizygous (−/y) males or homozygous (−/−) females was 15–20% of that in WT (+/+) males or (+/+). Heterozygous females (−/+)) showed an intermediate value. G-6-PDH activity of C3H and C57BL/6 mice was similar to that of WT animals of the breeding colony. The same relative difference was found in isolated peritoneal

was sedimented from heparinized blood samples (0.5 ml) by centrifugation at 760 g for 10 min at 4°C. After removal of plasma and buffy coat, RBCs were washed three times and then suspended in Dulbecco’s PBS. RBCs were lysed at 4°C by diluting 1:10 in (in mM) 5 sodium phosphate, 1 EGTA, 0.1 sodium orthovanadate, and 0.1 PMSF, pH 8.0. Ghosts were isolated from the hemolysate by centrifugation at 20,000 g for 30 min at 4°C. White RBC ghosts were obtained by three successive washes in same buffer (20,000 g for 10 min at 4°C). Membrane proteins were extracted from RBC ghosts with 0.3% Triton X-100 in (in mM) 25 HEPES, 1 EGTA, 0.1 PMSF, and 0.1 sodium orthovanadate with 0.3 M NaCl and 1 μg/ml aprotinin at pH 7.4 by gentle mixing at 4°C for 15 min. Extracts were centrifuged at 4°C for 30 min at 20,000 g. After phase separation, samples were dissolved in 2% SDS. Sample protein content was determined by the bicinchoninic acid method. Samples were mixed 1:1 with SDS sample buffer (Bio-Rad), heated for 5 min at 98°C, and subjected to SDS-PAGE (9.5% gel; 0.02 mg protein/lane) according to the method of Laemmli. Gels were stained with Bio-Safe Coomassie (Bio-Rad), photographed, and scanned with an IS-1000 digital imaging system (Alpha Innotech).

Reagents. When applicable, cell culture-grade buffers, media, and reagents were used. Hanks’ balanced salt solution, without phenol red, and Dulbecco’s PBS were purchased from Life Technologies (Grand Island, NY). Buffers were sterile filtered and degassed before use.

Statistical analysis. Statistical calculations were performed with JMP software (SAS Institute, Cary, NC). Results were analyzed by ANOVA followed by t-test for pairwise comparisons or Tukey-Kramer’s test for multiple comparisons. Statistically significant difference was concluded at $P < 0.05$.

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Fig. 3. Comparison of RBC deformability between G-6-PDH-deficient and WT animals after sepsis. Erythrocyte deformability in blood from septic or naive G-6-PDH-deficient and WT animals was determined and compared. A: shear stress in logarithmic or linear (inset) scale. After Lineweaver-Burk conversion, the values of shear stress causing half-maximal cell elongation ($K_{EI}$, B) and maximal cell elongation ($EI_{max}$, C) were calculated and compared among the experimental groups. Values are means ± SE; $n = 8$ in each group. Statistically significant differences *compared with WT septic and & compared with naive in corresponding genotypes are shown. Absence of error bars indicates that SE is within the size of the symbol.

Fig. 4. Comparison of erythrocyte subpopulations between G-6-PDH-deficient and WT animals after sepsis. Whole blood samples from naive or septic (24 h after CLP) animals were subjected to centrifugal elutriation, and aliquots of fractionated cells (elutriation fractions 1–5) were analyzed as described in MATERIALS AND METHODS. Samples from naive (left) and septic (right) animals are compared. A and B: mean corpuscular volume (MCV) of erythrocyte subpopulations. C and D: cell distribution among fractions. E and F: mean corpuscular hemoglobin content (MCH). G and H: $K_{EI}$ values determined from the shear response curves. I and J: G-6-PDH activity in RBC subpopulations (U/10¹² cells). Values are means ± SE; $n = 6$ in each group. Statistically significant differences *compared with WT septic and & compared with elutriation fraction 1 are shown. Absence of error bars indicates that SE is within the size of the symbol.
The macrophage G-6-PDH activity between deficient and WT animals (data not shown). Figure 1B depicts a typical finding of genotyping: DdeI digestion of the amplified DNA product generated by primers centered on the mutation site resulted in partial digestion in heterozygous (lane 3), lack of digestion in hemizygous (lane 5) and full digestion in WT (lane 7) animals. In the present investigations all animals were genotyped as well as tested for G-6-PDH activity. Either littermates or animals bred in parallel and matched by age were used in the experiments.

**Sepsis-induced changes in hematologic parameters and RBC deformability.** Baseline values of circulating RBC counts and blood hemoglobin content were similar in unchallenged deficient and WT animals. After sepsis (24 h), RBC counts and blood hemoglobin content were decreased in G-6-PDH-deficient compared with WT animals (Fig. 2, A and B). Septic G-6-PDH-deficient animals also displayed elevated plasma hemoglobin content compared with WT septic animals (Fig. 2C). Baseline values of circulating white blood cells and platelets were similar in deficient and WT animals, with the exception of lymphocyte numbers, which were slightly greater in deficient animals (Fig. 2, D–F). Sepsis resulted in marked decreases in the number of circulating neutrophils, lymphocytes, and platelets. In contrast to the observed anemia, the 24-h sepsis-induced changes in white blood cell and platelet counts were similar in G-6-PDH-deficient and WT animals (Fig. 2, D–F).

Figure 3 compares the sepsis-induced changes in RBC deformability. Whereas RBC deformability shear stress response curves overlapped in naive unchallenged deficient and WT animals, sepsis resulted in significant decrease in RBC deformability in both groups. However, the degree of RBC deformability decrease was more pronounced in septic G-6-PDH-deficient animals compared with septic WT animals (Fig. 3A). Using the Lineweaver-Burk conversion of the shear stress response curves, we also calculated the shear stress causing half-maximal RBC elongation ($K_{EI}$) and maximal cell elongation ($EI_{max}$) after these treatments. As shown, $K_{EI}$ was significantly elevated after sepsis in both groups compared with controls. The increase in $K_{EI}$ was more pronounced and significantly different in septic deficient vs. septic WT animals (Fig. 3B; note that the increase in $K_{EI}$ indicates a decrease in cell deformability). Additionally, $EI_{max}$ was decreased in septic deficient animals compared with septic WT animals (Fig. 3C).

**Alterations in RBC subpopulations and band 3 abundance.** Circulating erythrocytes can be separated into different subpopulations. With centrifugal elutriation, smaller-sized (older) cells elute at lower and larger-sized (younger) cells at higher flow rates. Figure 4 compares the characteristics of RBC subpopulations isolated by centrifugal elutriation in G-6-PDH-deficient and WT naive (Fig. 4, A, C, E, G, and J) and septic (Fig. 4, B, D, F, H, and J) animals. Mean corpuscular volume of erythrocytes (MCV) gradually increased in parallel with the increasing elutriation flow rates (elutriation fractions 1–5). As expected, MCV was similar in corresponding elutriation fractions in naive as well as septic deficient and WT animals (Fig. 4, A and B).

The pattern of cell yield distribution was slightly different between naive deficient and WT animals (Fig. 4C), and this pattern difference became more pronounced after sepsis, resulting in statistically significant increase in cell yield in fraction 2 and decrease in fraction 4 of deficient compared with WT animals (Fig. 4D). Mean corpuscular hemoglobin (MCH) was lower in cells from fraction 4 of septic G-6-PDH-deficient compared with septic WT animals (Fig. 4F). Determination of the $K_{EI}$ values of isolated RBC subpopulations from the shear stress response curves revealed decreased RBC deformability in all RBC cell populations after sepsis. In G-6-PDH-deficient animals, the most pronounced increase in $K_{EI}$ was observed in RBC fractions 4 and 5 and was significantly increased compared with corresponding fractions from WT animals (Fig. 4H). Finally, determination of G-6-PDH activity in isolated RBC subpopulations revealed a gradual increase in G-6-PDH activity in parallel with increasing cell size in WT as well as deficient animals. However, G-6-PDH activity was doubled in the largest- compared with the smallest-sized cells in deficient animals whereas this difference was ~30% in WT animals (Fig. 4I). After sepsis, the increase in G-6-PDH activity in RBC subpopulations of increasing size becomes blunted in G-6-PDH deficient animals, whereas the pattern of G-6-PDH activity increase in the eluted fractions was not altered in septic WT animals (Fig. 4J).

Because alterations in band 3 protein organization have been shown to be important in oxidative RBC damage, aging, and clearance, we tested the effects of G-6-PDH deficiency and sepsis on the association between band 3 and the cytoskeleton. Figure 5A shows band 3 content in RBC Triton extracts from deficient and WT animals after sepsis. Band 3 normalized to sample α-spectrin content was elevated in naive G-6-PDH-deficient and septic WT-deficient RBCs. Triton-soluble protein distribution was determined in RBC ghosts as described in MATERIALS AND METHODS. A: summary from the analysis of 8 animals in each group. Intensity of band 3 (B3) normalized to the intensity of α-spectrin recovered from the same sample is shown (means ± SE). B: a typical finding from 2 animals in each group. Arrows indicate proteins of the cytoskeletal network including actin 4.1, 4.1b, and 4.2 as identified by their electrophoretic migration.
deficient compared with WT animals. After sepsis, the band 3-to-α-spectrin ratio was significantly elevated in WT animals (Fig. 5A). In septic animals, this ratio remained significantly elevated in G-6-PDH deficient compared with WT animals (Fig. 5).

Role of oxidative stress. In a separate set of experiments, we compared sepsis-induced alterations in blood glutathione metabolism between deficient and WT animals (Fig. 6). Sepsis resulted in an increase in plasma total glutathione (predominantly GSSG) content. The increase in plasma glutathione was greater in septic G-6-PDH-deficient than WT animals (Fig. 6A). Sepsis also resulted in a decrease in the intracellular ratio of GSH to GSSG in erythrocytes (Fig. 6B). This decrease in the GSH-to-GSSG ratio was the result of an increase in RBC GSSG content without major changes in intracellular GSH (Fig. 6, C and D). The sepsis-induced decrease in the GSH-to-GSSG ratio and increase in GSSG were similar in G-6-PDH-deficient and WT animals (Fig. 6, B–D).

The elevated plasma glutathione levels indicated that sepsis-induced oxidative stress was greater in G-6-PDH-deficient than WT animals. To further elucidate the potential role of oxidative stress in the observed alterations in RBC deformability, we tested the effect of the antioxidant NAC in vivo. NAC was administered postoperatively, followed by a second injection at 22 h to CLP-treated as well as sham-operated animals. Figure 7 indicates that, in deficient animals, NAC treatment alleviated the sepsis-induced decrease in RBC deformability as reflected in the increase in EI determined at 1.69 and 3.0 Pa in NAC-treated septic vs. vehicle-treated septic animals (Fig. 7, A and B). Furthermore, calculating the $K_{EI}$ from the shear stress response curves also indicated a beneficial effect of NAC treatment in septic deficient animals (Fig. 7C). In septic WT animals, the effect of NAC treatment did not reach statistically significant levels (data not shown).

DISCUSSION

This study demonstrates for the first time that G-6-PDH deficiency exacerbates the magnitude of decreased RBC deformability after polymicrobial septic challenge. G-6-PDH deficiency also predisposes to the development of anemia, increases the tendency for hemolysis, and augments the release of cellular GSSG after sepsis. The alterations in the pattern of the distribution of RBC subpopulations in G-6-PDH-deficient animals suggest elevated erythrocyte removal and indicate a role of younger erythrocytes in the development of erythrocyte dysfunction during sepsis. The described observations on RBC dysfunction in G-6-PDH deficiency may explain some of the immunomodulatory effects observed in the human G-6-PDH deficiency after infections (29, 48).

It is well known that the life span of circulating erythrocytes is ~60 days (half of normal) in the class III human deficiencies, even in otherwise healthy individuals (34). In mice, the life span of circulating erythrocytes is ~40 days, and it is likely that the G-6-PDH-deficient mice display a shortening in erythrocyte life span similar to that observed in the human deficiencies. Decreases in cell deformability and hemoglobin content, increase in the membrane content of lipid peroxidation products, and alterations in band 3 membrane assembly represent phenotypic changes of erythrocyte aging (8, 9). In this context, the comparison of erythrocyte subpopulations between deficient and WT animals has resulted in important observations. It was evident that the cell distribution was slightly different between nonmanipulated naive deficient and WT control mice.

Fig. 6. Sepsis-induced alterations in RBC glutathione content in G-6-PDH-deficient and WT animals. GSH and GSSG were determined in RBC as described in MATERIALS AND METHODS. A: total plasma glutathione (predominantly GSSG). B: GSH-to-GSSG ratio in RBC. C and D: GSSG (C) and GSH (D) in RBC. Values are means ± SE; $n = 8–10$ in each treatment group. Statistically significant differences *compared with WT septic and #compared with naive in corresponding genotypes are shown.
although the differences did not reach statistically significant levels (Fig. 4C). However, after sepsis, the difference in the distribution pattern became evident between deficient and WT animals (Fig. 4D), indicating that smaller-sized erythrocyte subpopulations are more abundant in the circulation of G-6-PDH-deficient animals. This suggests an “accelerated aging” process of deficient erythrocytes during sepsis. Additionally, the fact that the difference in cellular G-6-PDH activity between the oldest and youngest cells was evident in naive deficient animals (Fig. 4I), whereas this difference disappeared in septic deficient animals (Fig. 4J), is also consistent with an accelerated life cycle of circulating erythrocytes in deficient compared with WT animals during sepsis.

The more pronounced decrease in erythrocyte deformability in septic deficient vs. WT animals was readily detectable in whole blood (Fig. 3) as well as after comparing erythrocyte subpopulations (Fig. 4H). However, the differences in decreased deformability between deficient and WT animals were more pronounced in the larger-sized (younger) subpopulations of erythrocytes. Furthermore, the decrease in hemoglobin content was also evident in these larger-sized cell populations from deficient animals (Fig. 4F). These observations indicate that the younger cell populations, presumably freshly released from the bone marrow, are also adversely affected by sepsis in G-6-PDH deficiency. The fact that the differences in deformability and hemoglobin content between septic deficient and WT animals were more pronounced in the larger-sized subpopulation of erythrocytes may also be the result of accelerated removal of smaller-sized (older) erythrocytes in septic G-6-PDH-deficient animals. This possibility is consistent with the mild degree of anemia and with the increased G-6-PDH activity in the smallest-sized erythrocytes at 24 h after CLP in deficient compared with WT animals.

Elevated membrane abundance of band 3 tetraters is accompanied by increased associations between band 3 and the cytoskeleton. Elevated band 3 association with the cytoskeleton has been shown to parallel the decrease in RBC deformability (21, 39) and to participate in triggering the removal of old circulating erythrocytes by the mononuclear phagocyte system (9). Thus the observation that band 3-spectrin associations were significantly elevated in naive as well as septic deficient compared with WT animals suggests that G-6-PDH deficiency potentiates the removal of erythrocytes from the circulation. Because band 3 also functions as an anion exchanger, it remains to be tested whether the observed alterations in band 3 are associated with changes in the ion milieu of RBC in deficient animals and after sepsis.

The role of oxidative stress in causing decreased RBC deformability during the physiological aging process of erythrocytes is well documented (5, 13). In fact, during the normal process of oxygen exchange, erythrocytes are exposed to oxidative stress because a small portion of oxygen escapes from the hemoglobin-oxygen complex, resulting in the release of superoxide anion and formation of methemoglobin. However, during the inflammatory response, erythrocytes are further exposed to reactive oxygen and nitrogen species released from activated macrophages. Macrophage- and neutrophil-derived oxidants and nitric oxide have been shown to cause decreased erythrocyte deformability (3, 5, 13). Our results showing a decrease in the ratio of cellular GSH to GSSG in both deficient and WT septic animals, compared with nonmanipulated controls, adds further support to the notion that erythrocytes are exposed to oxidative stress during the septic response. However, because the sepsis-induced decrease in the GSH-to-GSSG-ratio and increase in cellular GSSG content were similar in deficient and WT animals, it appears that the intracellular oxidative stress, primarily associated with oxygen exchange, was tolerated similarly in deficient and WT erythrocytes during sepsis. Therefore, the observed decrease in RBC deformability cannot be directly accounted for by the compromised intraerythrocyte glutathione status of the deficient cells during sepsis. However, septic animals also displayed elevated plasma content of GSSG that was more pronounced in the deficient animals. The release of GSSG from cells exposed to oxidative stress is a protective mechanism that supports the maintenance of the intracellular redox potential. Whereas GSSG in the plasma may be derived from a variety of cells during sepsis, the fact that plasma glutathione was elevated more in septic G-6-PDH-deficient than WT animals indicates an aggravated oxidative stress in deficient animals during sepsis. These observations support the potential role of exogenous oxidative stress in causing the observed aug-
mentation of decreased RBC deformability in septic G-6-PDH-deficient animals. These conclusions are also consistent with in vitro studies in G-6-PDH-deficient RBCs showing an increased sensitivity of deficient erythrocytes to neutrophil-induced cell damage (25).

The notion that oxidative stress is greater in G-6-PDH-deficient animals is further supported by our findings that NAC alleviated the decrease in erythrocyte deformability in septic deficient animals. However, this observation must be interpreted with caution because NAC has also been shown to serve not only as a reducing equivalent but also as a potent inhibitor of macrophage activation (22, 38). Furthermore, because G-6-PDH is a single-copy gene and it is expressed in all cells of the body, G-6-PDH deficiency may also alter the redox balance toward the prooxidant side in macrophages and neutrophils during sepsis (47). Thus additional studies using alternative antioxidants and testing phagocyte responses are needed to elucidate whether alterations in phagocyte activation and redox balance are part of the mechanism causing the pronounced decrease in RBC deformability after sepsis in G-6-PDH deficiency. Nevertheless, the elevated plasma levels of GSSG and the beneficial effects of NAC on RBC deformability clearly support the hypothesis that an increased oxidative stress in G-6-PDH deficiency contributes to the deformability decrease in septic G-6-PDH-deficient animals.

It is evident from our findings that polymicrobial sepsis resulted in a similar decrease in the number of circulating neutrophils, lymphocytes, and platelets in deficient and WT animals at 24 h after CLP, although the G-6-PDH-deficient animals developed a detectable anemia 24 h after the septic challenge. This suggests that hemolysis or elevated elimination of circulating RBCs occurred in G-6-PDH-deficient animals during the early phase of sepsis. A potential role of hemolysis is supported by the fact that free plasma hemoglobin levels were elevated in the septic G-6-PDH-deficient compared with WT animals.

Infection-induced anemia in critically ill patients is a common occurrence with important clinical implications (37, 45, 48, 50). In previous studies using the CLP septic model, anemia was observed in BALB/c mice (20) and young (4–5 wk old) C57BL/6 mice (16). In this current investigation, using mice on a 101/E1/CH3 background, anemia was observed only in septic (24 h) G-6-PDH-deficient animals. This discrepancy is most likely the result of the fact that the current study used 10- to 12-wk-old animals to allow erythrocytes of the entire age spectrum to appear in the circulation (the life span of erythrocytes is 40 days in mice; Ref. 23). Younger mice also have elevated erythrocyte counts compared with older animals. Thus age and strain differences and the severity of sepsis may influence erythrocyte removal or bone marrow activity accounting for different onset of anemia in septic rodent models.

Previous investigations on severely injured G-6-PDH-deficient trauma patients indicated that these patients had an increased incidence of sepsis, a longer duration of the systemic inflammatory response syndrome, augmented monocyte activation, blunted IL-10 production, and a more pronounced anemia compared with nondeficient patients with similar injuries (48). In this context, increased RBC damage may have contributed to these human observations because the immunomodulatory role of damaged erythrocytes has been documented in animal as well as human investigations (15, 30, 33, 41).

Furthermore, interaction of oxidatively damaged erythrocytes with macrophages has been shown to augment cytokine responses (30, 41) and to interfere with phagocytic functions (15, 33). Likewise, hemoglobin and its derivatives, including methemoglobin and heme, have also been shown to activate phagocytes as well as endothelial cells (2, 11, 32, 51). It remains to be elucidated whether the observed decrease in erythrocyte deformability alters macrophage responses to sepsis and whether this is an important component of the altered inflammatory response in G-6-PDH deficiency.

The role of G-6-PDH deficiency in the protection against malaria is well accepted; however, the mechanism of protection remains only partially elucidated (10, 12, 42). Our current observations may have potential implications in this context as well. Studies on malaria-infected patients indicated decreased erythrocyte deformability that correlated with the clinical severity of malaria infection (17, 18), suggesting that the decrease in erythrocyte deformability is part of the mechanism of infected erythrocyte removal from the circulation. We propose that the preconditioning effect of G-6-PDH deficiency to increase RBC rigidity during infections is part of the potential mechanism that contributes to the protection against malaria infection by elevating removal of erythrocytes and modulating macrophage activation.

Together, our observations indicating a pronounced decrease in RBC deformability in septic G-6-PDH-deficient animals suggest a role of RBC dysfunction in the immunomodulatory effects of G-6-PDH deficiency. The observed RBC dysfunction may aggravate microcirculatory disturbances and organ dysfunction and may also contribute to the modulation of macrophage responses during severe infections in G-6-PDH deficiency.

ACKNOWLEDGMENTS

We thank Pietro Antoneli for technical assistance in the studies.

GRANTS

This study was supported by National Institute of General Medical Sciences Grant GM-55005.

REFERENCES

Sepsis, G-6-PDH Deficiency, and Erythrocyte Dysfunction

Cavinato RA, Bastos KRB, Sardinha LR, Elias RM, Alvarez JM, and Chiu DTY and Liu TZ.

Giger U, Sticher B, Naef R, Burger R, and Lutz HU.


