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Inhibition of Cell-Free Oxidative Bactericidal Activity by Erythrocytes and Hemoglobin

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Sickle cell anemia and other chronic hemolytic anemias are associated with an increased frequency of bacterial infections. There is evidence to suggest that in hemolytic states massive erythrocyte (RBC) ingestion by macrophages interferes with their antibacterial function, thereby predisposing infection. Stimulated by this possibility, we recently demonstrated that erythrophagocytosis by macrophages markedly inhibited intracellular killing of bacteria, and that zymosan-stimulated superoxide generation and chemiluminescence were also suppressed by RBC ingestion. We examined the effects of RBC components on generation of chemiluminescence, superoxide, and bactericidal activity by cell-free oxidative systems. Generation of chemiluminescence by hypoxanthine-xanthine oxidase was depressed in the presence of human RBC lysate or column-fractionated hemoglobin but not crystallized human hemoglobin (methemoglobin) (peak cpms of 15,522 [P = 0.00024], 28,360 [P = 0.0088], and 50,041 [P = 0.37], respectively, compared with 59,898 for positive controls). Similarly, hypoxanthine-xanthine oxidase production of superoxide was inhibited in the presence of column-fractionated human hemoglobin (43.8 verus 17.4 nmol per tube, P = 0.000001). A cell-free bactericidal system, acetaldehyde and xanthine oxidase with or without myeloperoxidase and Cl^-, was markedly inhibited by column-purified hemoglobin. For example, after 2 h of incubation, surviving numbers of *Staphylococcus aureus* were: control (buffer only), 2.5×10^6/ml; bactericidal system, none; bactericidal system plus hemoglobin, 2.2×10^6/ml ($P \leqq 0.03$, bactericidal system versus other systems). Our studies have documented that interactions between RBC (hemoglobin) and reactive products of oxygen metabolism inhibit oxidative bactericidal mechanisms in cell-free systems as well as in macrophages. Erythrophagocytosis-induced macrophage dysfunction may predispose to bacterial infections in chronic hemolytic states such as sickle cell anemia.

Serious bacterial infections are frequent complications of sickle cell anemia (SCA) (1). There is evidence that impaired macrophage function might account for certain host defense abnormalities and predispose to infection in SCA (11, 14). Defects of splenic macrophage function in SCA (14) and of reticuloendothelial system function in experimental hemolytic anemia (11) have been reported. It has been proposed that extensive erythrophagocytosis by macrophages may compromise antibacterial activity and thus predispose to infection in these hemolytic states (7, 11). Stimulated by these observations, we recently examined the effects of erythrocyte (RBC) ingestion on antibacterial function and oxidative bactericidal mechanisms in macrophages (8). These studies revealed that erythrophagocytosis by macrophages inhibits intracellular bactericidal activity and that zymosan-induced superoxide generation and chemiluminescence were also suppressed by RBC ingestion. To determine the mechanism of this phenomenon, we examined the effects of RBC components on cell-free oxidative systems. These investigations demonstrated that RBCs and hemoglobin inhibit generation of chemiluminescence, superoxide, and bactericidal activity by oxidative systems employing xanthine oxidase (XO) and appropriate substrates.

MATERIALS AND METHODS

Bacteria. An isolate of *Staphylococcus aureus* 502A was stored at −70°C. For the bactericidal studies ca. 10^3 bacteria were inoculated into Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and cultured overnight at 37°C. Organisms were then washed, diluted, and used in the assays described below.

RBC and hemoglobin preparations. RBCs used in the studies were fresh human RBCs from peripheral venous blood and sheep RBCs coated with immunoglobin G antibody to these cells (sheep EA) (Cordis Laboratories, Miami, Fla.). For some experiments human RBC lysate was prepared by hypotonic lysis in deionized water. Purified human hemoglobin was obtained by fractionation of human RBC lysate on a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (18). Crystallized (2×) human hemoglobin (Miles Laboratories, Inc., Kankakee, Ill.) was used in assays of chemiluminescence generation. Determinations of total hemoglobin and methemoglobin concentrations in fresh human RBC lysate, Sephadex G-200 column-fractionated hemoglobin, and commercial crystallized human hemoglobin were performed by standard methods (6, 9, 12).

Chemicals and enzymes. Acetaldehyde was obtained from Fisher Scientific Co., Fair Lawn, N.J., and EDTA was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Other reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

Cell-free (hypoxanthine-XO) chemiluminescence. The hypoxanthine-XO reaction generates superoxide, which can interact with chemical substances (such as luminol) to produce chemiluminescence (8, 10). To examine the effects of RBCs, RBC lysate, or hemoglobin on light emission during this reaction, we performed the following studies. All procedures were carried out in the dark. Samples (2 ml) of a solution containing 1.5×10^{-4} M hypoxanthine and 1×10^{-4} M EDTA in 0.05 M potassium phosphate buffer, pH 7.8, were placed in dark-adapted scintillation vials. Luminol (2.5×10^{-9} M) was then added to the vials. Sheep EA, human

TABLE 1. The influence of RBC components on chemiluminescence generated by the hypoxanthine-XO reaction

RBC component added	Peak chemiluminescence (cpm)[a]
None (control)	59,898 ± 7,593 (7)
Human RBC lysate	15,522 ± 2,478 (7)[b]
Column-fractionated human hemoglobin	28,630 ± 4,487 (6)[c]
Crystallized human hemoglobin (methemoglobin)	50,401 ± 6,218 (7)[d]

[a] Values are the mean ± the standard error of the mean; the number of experiments is given in parentheses.
[b] Significantly less than controls (hypoxanthine-XO only), P = 0.00024.
[c] Significantly less than controls (hypoxanthine-XO only), P = 0.0088.
[d] Not different from controls (hypoxanthine-XO only), P = 0.37.

RBCs, human RBC lysate (all at 10^6 cells per ml), crystallized human hemoglobin, or column-purified human hemoglobin (both at 50 μg/ml) were added to some samples. The vials were placed in a scintillation counter, operated in the off-coincidence mode, and baseline counts were obtained. XO (1.25 mU/ml) was added to the vials, which were counted in continuous 12-s cycles until chemiluminescence peaked and returned toward baseline. Results were expressed as peak counts per minute minus baseline counts. Controls included vials without hypoxanthine, vials without XO, and vials to which superoxide dismutase (SOD) had been added.

Cell-free (hypoxanthine-XO) superoxide anion generation. Production of superoxide by the hypoxanthine-XO reaction was measured directly by reduction of ferricytochrome *c* (4), and the effect of purified human hemoglobin on this reaction was determined. A 3-ml amount of a solution containing 1.5 $\times 10^{-4}$ M hypoxanthine, 10 μM ferricytochrome *c*, and 1 $\times 10^{-4}$ M EDTA in 0.05 M potassium phosphate buffer (pH 7.8) was placed in 6-ml plastic tubes. Purified human hemoglobin (500 μg/ml, equivalent to 10^7 RBCs per ml) was added to some tubes. XO (2.25 mU/ml) was then added to the tubes, which were incubated in a 37°C water bath for 2 h. After centrifugation at 500 × *g* for 12 min at 4°C, the optical density of the supernatants was measured at 550 nm in a Carey 219 spectrophotometer (Varian, Houston, Tex.). Controls included tubes without XO and tubes to which SOD was added.

Cell-free (acetaldehyde-XO) bactericidal activity. We examined the ability of a cell-free bactericidal system, acetaldehyde and XO with or without myeloperoxidase and chloride, to inactivate *S. aureus* (15, 16). The reaction between acetaldehyde and XO produces both hydrogen peroxide and superoxide and is especially effective in killing *S. aureus* when myeloperoxidase is present. To evaluate the effects of RBCs or hemoglobin on this system, the following studies were performed. *S. aureus* cells (1×10^6 to 5×10^6) were suspended in tubes containing 0.1 M phosphate buffer, pH 7.0, with 2×10^{-5} M EDTA–1×10^{-5} M ferrous sulfate–4×10^{-3} M ammonium sulfate–4.3×10^{-3} M sodium sulfate–1×10^{-2} M acetaldehyde in a final volume of 0.5 ml. When myeloperoxidase was added to the system, acetaldehyde was reduced to 10^{-4} M, and sodium chloride was substituted for an isotonically equivalent amount of sodium sulfate (3.3×10^{-2} M). A total of 5×10^6 sheep EA (10^7 RBCs per ml) or 250 μg of purified human hemoglobin (500 μg per ml) were added to some tubes. XO (10 mU) was added before incubation of the tubes in a 37°C water bath for 2 h. At that time samples from each tube were appropriately diluted and cultured on Trypticase soy agar for quantitation of viable organisms. Controls included tubes without acetaldehyde, tubes without XO, and tubes to which catalase, SOD, or both were added.

Statistical analysis of data. Differences between experimental groups were determined by means of a two-sample *t* test, using a Tektronix 4051 computer.

RESULTS

Cell-free (hypoxanthine-XO) chemiluminescence. Generation of superoxide by the hypoxanthine-XO reaction is associated with chemiluminescence, which is inhibited by SOD. The effects of sheep EA, human RBCs, human RBC lysate (97% hemoglobin [oxyhemoglobin] and 3% methemoglobin), Sephadex G-200-fractionated human hemoglobin (100% hemoglobin [oxyhemoglobin]) and crystallized human hemoglobin (100% methemoglobin) on this reaction are shown in Table 1. RBC or hemoglobin preparations (lysate or column-fractionated) had a marked inhibitory effect on the generation of chemiluminescence. In contrast, crystallized hemoglobin (methemoglobin) produced no significant reduction in light emission.

Cell-free (hypoxanthine-XO) superoxide anion generation. Direct quantitation of superoxide anion generation during the hypoxanthine-XO reaction was accomplished by assay of cytochrome *c* reduction. SOD-inhibitable cytochrome *c* reduction was markedly reduced in the presence of human column-fractionated hemoglobin. Thus, the amount of cytochrome *c* reduced by superoxide dropped from 43.8 to 17.4 nmol per tube when hemoglobin was added to the incubation mixture (Fig. 1).

Cell-free (acetaldehyde-XO) bactericidal activity. The reaction between acetaldehyde and XO, which produces both superoxide and hydrogen peroxide, reduced the number of viable *S. aureus* cells by at least three logs (99.9%) during a 1- or 2-h incubation period. The addition of either SOD or catalase abolished the bactericidal activity, indicating that both superoxide and hydrogen peroxide were necessary for this system to function. Similarly, the addition of either RBCs (sheep EA) or column-fractionated hemoglobin very effectively protected staphylococci from the antibacterial oxida-

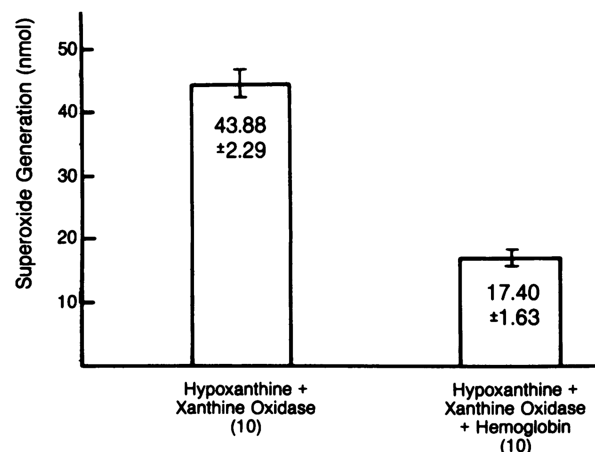

FIG. 1. Effect of exposure to Sephadex G-200-fractionated human hemoglobin on superoxide generation by the hypoxanthine-XO reaction during a 2-h incubation period. Values are expressed as the mean ± the standard error of the mean for each group. Numbers of experiments are in parentheses. The difference in superoxide production between the two groups is highly significant (P = 0.000001).

TABLE 2. Bactericidal activity of a cell-free system containing acetaldehyde + XO, with or without myeloperoxidase

Incubation conditions[a]	Viable *S. aureus* ($\times 10^6$) at:[b]	
	1 h	2 h
Starting inoculum	2.04 ± 0.49 (7)	3.37 ± 0.66 (9)
After incubation		
Buffer only	2.08 ± 0.48 (7)	2.51 ± 0.48 (9)
Acet + XO	0.0003 ± 0.0002 (7)[c]	0.0016 ± 0.0007 (9)[d]
Acet + XO + SOD	1.94 ± 0.52 (7)	2.06 ± 0.94 (9)
Acet + XO + RBCs	—[e]	2.58 ± 0.59 (9)
Acet + XO + RBC	2.80 ± 0.67 (6)	1.80 ± 0.16 (8)
Acet + XO + hemoglobin	2.44 ± 0.58 (7)	1.47 ± 0.52 (9)
Acet + XO + MPO + Cl^-	—	0 (9)[f]
Acet + XO + MPO + Cl^- + SOD	—	0 (9)[f]
Acet + XO + MPO + Cl^- + catalase	—	1.60 ± 0.47 (7)
Acet + XO + MPO + Cl^- + hemoglobin	—	2.17 ± 0.93 (8)

[a] Acet, Acetaldehyde; MPO, myeloperoxidase.
[b] Values are the mean ± the standard error of the mean $\times 10^6$; the number of experiments is given in parentheses.
[c] Significantly less than control (buffer only), $P = 0.0015$.
[d] Significantly less than control (buffer only), $P = 0.00084$.
[e] —, Not done.
[f] Significantly less than control (buffer only); $P = 0.00016$.

tive products generated during the enzymatic reaction (Table 2).

The acetaldehyde-XO bactericidal system was much more efficient in the presence of myeloperoxidase. The myeloperoxidase-containing preparation inactivated all exposed organisms within a 2-h incubation period. SOD had no inhibitory effect on this bactericidal capacity, whereas catalase (or catalase and SOD) virtually eliminated the antistaphylococcal activity. As in the experiments without myeloperoxidase, the enzyme-mediated oxidative antibacterial system was completely inhibited in the presence of purified human hemoglobin, obtained by Sephadex G-200 fractionation (Table 2).

DISCUSSION

Bacterial infections are frequent, serious and often fatal, complications of SCA. Substantial evidence suggests that impaired reticuloendothelial macrophage function may be at least in part responsible for major host defense abnormalities in SCA (7, 11, 14). The concept that marked erythrocyte ingestion by these phagocytes might compromise cellular antimicrobial function and subsequently predispose to infection in sickle cell disease has been proposed (7, 11). Certainly, extensive erythrophagocytosis by macrophages of the liver, and especially the spleen, is well documented in SCA (1). Thus, we recently evaluated the effects of erythrocyte ingestion on the capacity of macrophages to kill bacteria (8). Those studies showed that erythrophagocytosis by peritoneal and alveolar macrophages inhibits the intracellular killing of *Salmonella typhimurium* and *S. aureus*. Furthermore, exposure of alveolar macrophages to RBCs depressed the production of superoxide and the generation of chemiluminescence during zymosan ingestion. Finally, we demonstrated that intact RBCs and RBC lysate reduced the generation of superoxide by a cell-free oxidative system which employed XO and hypoxanthine.

Curtailment of these oxidative phenomena in macrophages and in cell-free oxidative reactions by intact RBCs and RBC lysate could potentially be due to the SOD present in erythrocytes (13) or to interactions between other RBC components and oxygen reduction products. In the present study, we examined the effects of RBC components on cell-free oxidative reactions in assays with XO and appropriate substrates. These studies revealed that intact and lysed RBCs (sheep and human) as well as Sephadex G-200-fractionated human hemoglobin inhibited the generation of chemiluminescence, superoxide, and bactericidal activity against *S. aureus*, an organism reported to cause serious infection in SCA (2, 5). In contrast with the inhibition by hemoglobin (Fe^{2+}), methehemoglobin (Fe^{3+}) had no effect on the generation of chemiluminescence in the cell-free system. Since the column-purified hemoglobin preparations do not contain SOD, it is probable that hemoglobin interacts in a direct fashion with reactive oxidative products. In both macrophages and the cell-free system, this interaction compromises bactericidal activity. In other words, the "scavenging" of oxygen reduction products by hemoglobin protects bacteria from the harmful effects of these products.

Precise definition of the interactions between hemoglobin and reactive oxidative products is lacking. However, our data from cell-free oxidative systems suggests that hemoglobin is able to interact with both superoxide anion and hydrogen peroxide. For example, hemoglobin directly inhibited the generation of superoxide by the hypoxanthine-XO reaction. Similarly, cell-free chemiluminescence and acetaldehyde-XO-mediated bactericidal activity, both of which require production of superoxide (activity abolished by SOD), were essentially negated by the addition of hemoglobin. Furthermore, the myeloperoxidase-mediated bactericidal system, which requires the participation of hydrogen peroxide but not superoxide, was also completely inhibited by hemoglobin. Whether hemoglobin also interacts directly with hydroxyl radical, in addition to limiting the reactants (superoxide and hydrogen peroxide) required for its production, is unknown.

In any event it is apparent that RBC components inhibit oxidative bactericidal mechanisms, both in macrophages and in cell-free systems. This effect is due to interactions between hemoglobin and reactive products of oxygen metabolism. Macrophage dysfunction induced by hemoglobin of ingested erythrocytes may predispose to bacterial infections in SCA and in certain other hemolytic states, such as hemoglobin SC disease (17) and β thalassemia major (3).

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LITERATURE CITED

1. **Barrett-Conner, E.** 1971. Bacterial infection and sickle cell anemia. Medicine **50:**97–112.
2. **Charache, S., J. C. Scott, and P. Charache.** 1979. 'Acute chest syndrome' in adults with sickle cell anemia. Arch. Intern. Med. **139:**67–69.
3. **Corry, J. M., W. C. Marshall, L. A. Guthrie, A. G. Peerless, and R. B. Johnston, Jr.** 1981. Deficient activity of the alternative pathway of complement in β thalassemia major. Am. J. Dis. Child. **135:**529–531.
4. **Crapo, J. D., J. E. McCord, and I. Fridovich.** 1978. Preparation and assay of superoxide dismutase. Methods Enzymol. **53:**382–393.
5. **Eeckels, R., F. Gatti, and A. M. Renoirte.** 1967. Abnormal distribution of haemoglobin genotypes in Negro children with severe bacterial infections. Nature (London) **216:**382.
6. **Evelyn, K. A., and H. T. Malloy.** 1938. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. Biol. Chem. **126:**655–662.
7. **Gill, F. A., D. Kaye, and E. A. Hook.** 1966. The influence of erythrophagocytosis on the interaction of macrophages and Salmonella in vitro. J. Exp. Med. **124:**173–183.
8. **Hand, W. L., and N. L. King-Thompson.** 1983. Effect of erythrocyte ingestion on macrophage antibacterial function. Infect. Immun. **40:**917–923.
9. **Hanks, G. E., M. Cassell, R. N. Ray, and H. Chaplin, Jr.** 1960. Further modification of the benzidine method for measurement of hemoglobin in plasma. J. Lab. Clin. Med. **56:**486–498.
10. **Hodgson, E. K., and I. Fridovich.** 1976. The mechanism of the activity-dependent luminescence of xanthine oxidase. Arch. Biochem. Biophys. **172:**202–205.
11. **Kaye, D., F. A. Gill, and E. W. Hook.** 1966. Factors influencing host resistance to Salmonella infections: the effects of hemolysis and erythrophagocytosis. Am. J. Med. Sci. **254:**205–215.
12. **Makarem, A.** 1974. Hemoglobins, myoglobins, and haptoglobins, p. 1111–1214. *In* R. J. Henry, D. C. Cannon, and J. W. Winkelman (ed.), Clinical chemistry: principles and technics, 2nd ed. Harper & Row, Publishers, Inc., Hagerstown, Md.
13. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. **244:**6049–6055.
14. **Pearson, H. A., R. P. Spencer, and E. A Cornelius.** 1969. Functional asplenia in sickle-cell anemia. N. Engl. J. Med. **281:**923–926.
15. **Rosen, H., and S. J. Klebanoff.** 1979. Bactericidal activity of a superoxide anion-generating system. A model for the polymorphonuclear leukocyte. J. Exp. Med. **149:**27–39.
16. **Rosen, H., and S. J. Klebanoff.** 1981. Role of iron and ethylenediaminetetraacetic acid in the bactericidal activity of a superoxide anion-generating system. Arch. Biochem. Biophys. **208:**512–519.
17. **Topley, J. M., L. Cupidore, S. Vaidya, R. J. Hayes, and G. R. Serjeant.** 1982. Pneumococcal and other infections in children with sickle cell-hemoglobin C (SC) disease. J. Pediatr. **101:**176–179.
18. **Wilson, W. A., and E. J. Thomas.** 1979. Activation of the alternative pathway of human complement by haemoglobin. Clin. Exp. Immunol. **36:**140–144.