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Effect of Iron on Leukocyte Function: Inactivation of H_2O_2 by Iron

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We investigated the effect of $FeSO_4$ on phagocytosis-associated, increased oxidative metabolism via the hexose monophosphate shunt, with special attention to its effect on H_2O_2 levels. The availability of glutathione peroxidase and glutathione reductase for H_2O_2 disposal and hexose monophosphate shunt stimulation also are evaluated. The results show an impairment of phagocytosis-associated hexose monophosphate shunt activity together with an increase both of resting and phagocytosing formate oxidation. These apparently paradoxical findings are resolved by demonstrating a direct enhancement of formate oxidation by $FeSO_4$ in a cell-free system. In addition, measurement of H_2O_2 concentrations via scopoletin fluorescence shows reduction of H_2O_2 by $FeSO_4$. There is no effect on either glutathione peroxidase or glutathione reductase activities. These data suggest that one mechanism of $FeSO_4$ impairment of microbicidal activity is by its removal of H_2O_2.

The discovery that phagocytosing leukocytes undergo the phenomenon of degranulation (11), where lysosomes fuse with and discharge their contents into phagosomes, has focused attention on their role in host defense against infection (41, 42). The impaired microbicidal and fungicidal activity of leukocytes with abnormal lysosomes lends support to this hypothesis (16, 19, 34-36). Gladstone and Walton (8, 9) further confirmed that purified lysosomal contents possess antibacterial activity. However, their studies demonstrated a marked inhibition of this microbicidal activity in the presence of $FeSO_4$ and hematin due to Fe^{2+} binding of cationic proteins (8). Continued investigation showed that whole leukocytes, in the presence of Fe^{2+}, either in rotating suspensions or in clot culture, had markedly decreased microbicidal activity against staphylococci and other organisms (9). Other investigations into the role of iron in host defense recently reviewed by Weinberg (38) suggest that iron stimulates bacterial growth, impairs leukocyte antibacterial activity, enhances or suppresses bacterial secondary metabolism, and detoxifies bacterial toxins. Other work has suggested that during infection there is a fall in the serum iron, decreasing the percent saturation of transferrin and increasing the storage of iron (4, 39).

Although the antibacterial role of the lysosomal contents seems clear, a more serious defect in host defense against bacterial invaders occurs when the phagocytosis-associated burst in oxidative metabolism is impaired (21, 33). The classic example of this phenomenon is seen in chronic granulomatous disease of children (12), where leukocytes ingest bacteria normally, appear to degranulate adequately, and have normal lysosomal contents, but fail to kill catalase-positive bacteria. Significantly, these leukocytes also fail to show a phagocytosis-associated enhancement of hexose monophosphate shunt (HMPS) activity, and their production of H_2O_2 during phagocytosis is impaired markedly. Recent work has demonstrated that the antibacterial activity of polymorphonuclear leukocytes correlates with the production of H_2O_2 and its interaction with myeloperoxidase and halide ions (15, 16, 21, 26, 27, 32).

Because the phagocytosis-associated burst in oxidative metabolism plays such an important role in the leukocytes' antibacterial activity, it was of interest to investigate the effect of $FeSO_4$ in this system. To do this, we studied HMPS activity and also the effect of $FeSO_4$ on H_2O_2 production both in the leukocyte and cell-free preparations. Our studies suggest an Fe^{2+}-associated removal of H_2O_2 with reduced HMPS activity.

MATERIALS AND METHODS

Leukocytes. Rabbit polymorphonuclear leukocytes were obtained, as described previously, through the intraperitoneal infusion of physiological saline (500 to

700 ml), with paracentesis of the exudate 12 to 18 h later (9).

The cells were suspended in modified Krebs-Ringer phosphate buffer (14) containing 4.6 mM glucose, 1.5 mM sodium formate, and 20% normal rabbit plasma (previously collected and stored at −60 C). Human polymorphonuclear leukocytes were obtained from the heparinized venous blood of normal, healthy donors and prepared as described previously (14). The cells were suspended as above, with fresh autologous plasma substituted for the normal rabbit plasma.

Particles. *Staphylococcus aureus* P66 was prepared as described previously, heat killed at 60 C for 20 min, and suspended at a concentration of 3×10^9 to 6×10^9 bacteria per ml (9). The killed organisms were divided into 1- to 2-ml volumes and stored frozen at −20 C.

Latex particles, 1.03 μm in diameter (Dow Chemical Co.), were used without processing.

Fe^{2+}. A neutralized solution of 23 mM $FeSO_4$ in 30 mM sodium citrate was freshly prepared every 2nd day and added to the incubating cell suspension to give final concentrations of 1.84, 0.92, and 0.09 mM Fe^{2+}.

Scintillation cocktail. Toluene containing 5 g of 2,5-diphenyloxazole and 0.245 g of 1,4-bis-(5-phenyloxazolyl)-benzene per liter, obtained from Nuclear Enterprises Ltd., Sighthill, Edinburgh 11, Scotland, was used as the scintillation cocktail.

Radiochemicals. Radiochemicals were obtained from Amersham/Searle Corp. [1-^{14}C]glucose for the HMPS studies and [^{14}C]sodium formate for the H_2O_2 experiments were dissolved in normal saline at concentrations of 2 and 5 μCi/ml, respectively.

Metabolic studies. All studies were conducted with 25-ml Erlenmeyer flasks in a shaking water bath (Gallenkamp) at 37 C. The flasks contained 3 ml of the leukocyte suspension containing 1×10^7 to 6×10^7 leukocytes and were incubated in quadruplicate with and without Fe^{2+} at each concentration used. Duplicate blank flasks without cells also were set up each time. After 15 min of incubation, 0.2 μCi of [^{14}C]glucose for evaluation of HMPS activity or 0.5 μCi of [^{14}C]formate for evaluation of H_2O_2 formation was added to each flask, and 0.1 ml of latex was added to half of the control flasks and half of the flasks at each concentration of Fe^{2+}.

The effect of the presence or absence of 1.84 mM $FeSO_4$ on the H_2O_2-associated oxidation of formate to CO_2 was evaluated in two similar cell-free systems. The first had: sodium formate, 8.8 nmol; glucose, 16.6 nmol; glucose oxidase, 17.6 U; catalase, 70 U; and [^{14}C]formate, 0.2 μCi. The second system omitted catalase and, rather than generating H_2O_2 by means of glucose and glucose oxidase, H_2O_2 was added directly at a concentration of 0.5 μmol/ml. The total volume in each system was 1 ml.

The flasks then were closed with Suba Seal rubber stoppers (Gallenkamp) from which a plastic center well was suspended (Kontes Glass Co., Vineland, N.J.). In some instances, a Durham fermentation tube (8 by 35 mm) was placed within the center well. Hyamine hydroxide (0.3 ml) was placed in this container. At the conclusion of the incubation period (30 to 60 min), 0.3 ml of 6 N H_2SO_4 was added to terminate the experiment and facilitate the release of all $^{14}CO_2$. After an additional 30 min of incubation, the hyamine-containing center wells or Durham tubes were transferred to glass counting vials to which 10 ml of scintillation cocktail was added. These vials were closed and shaken, and their radioactivity was determined in a Nuclear-Chicago (730 series) liquid scintillation system or a Packard Tri-Carb liquid scintillation spectrometer, model 574. Results are expressed in nanomoles of glucose or formate converted to $^{14}CO_2$ per 0.5 h, with the significance of differences between means determined by Student's t test. The percent change of HMPS activity or of formate oxidation was calculated as follows:

$$100 - \frac{\Delta \text{ activity with iron}}{\Delta \text{ activity without iron}} \times 100$$

Fluorometric determination of H_2O_2. The effect of $FeSO_4$ (1.84 mM) on H_2O_2 levels was determined by the disappearance, due to H_2O_2 oxidation, of the fluorescent peroxidase substrate scopoletin (6-methyl-7-hydrolyl-1,2-benzopyrone) according to the method of Andreae (1). The reaction mixture in a 1-ml volume consisted of 10^{-2} M citrate buffer (pH 4.5), 2.5×10^{-8} mol of scopoletin, and 2.5×10^{-8} mol of H_2O_2. The reaction was started with the addition of 6.25×10^{-9} mol of horseradish peroxidase at pH 4.5 and allowed to proceed for 5 min. Borate buffer (pH 10, 9 ml) then was added, and the fluorescence of a small volume was determined with a Turner fluorometer at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. Results are expressed as the concentration of residual fluorescent scopoletin remaining after incubation with H_2O_2 in the presence and absence of $FeSO_4$.

Enzyme studies. Glutathione reductase activity was determined in the 10,000 × g supernatant from whole-cell lysates prepared by the freezing (dry ice-acetone) and thawing (37 C) three times of a leukocyte suspension containing 2×10^3 to 4×10^3 leukocytes per ml. The enzyme activity of the lysate was assayed by following the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm in a Cary model 14 spectrophotometer for 5 min (28). Glutathione peroxidase was determined from lysates prepared as above and assayed by the method of Paglia and Valentine (24). These results are expressed as nanomoles of NADPH oxidized per minute per milligram of protein. The protein concentrations of the cell lysate supernatants were determined by the method of Lowry et al. (20).

RESULTS

There was no significant effect on resting HMPS activity by ferrous ions (Fe^{2+}) at any of the concentrations used either in rabbit or in human leukocytes. Resting rabbit cells were slightly more active than resting human cells. With phagocytosis, however, there was a significant reduction of the HMPS at Fe^{2+} concentrations of 1.84 and 0.92 mM in rabbit cells of 43 and 32%. In human cells the reduction at 1.84 mM Fe^{2+} was 52% (Table 1). There was no

reduction of formate oxidation either at rest or with phagocytosis at any of the concentrations of iron used. Rather, there was an increase in the oxidation of formate to CO_2 at rest, which was further stimulated with phagocytosis both in rabbit and human cells (Table 2). The effects of $FeSO_4$ on glutathione reductase and glutathione peroxidase are shown in Table 3. These results fail to show a significant alteration from normal values in the presence of 1.84 mM $FeSO_4$. Since neither glutathione reductase nor glutathione peroxidase activity was significantly reduced by $FeSO_4$, the effect of $FeSO_4$ on the H_2O_2 oxidation of formate in the presence of catalase was investigated. These results, where glucose and glucose oxidase generated H_2O_2, are indicated in Table 4 and show a marked enhancement of formate oxidation in the presence of $FeSO_4$.

In the absence of catalase and with the direct addition of H_2O_2 rather than the use of H_2O_2 generation, there was little H_2O_2 oxidation of formate, but H_2O_2 together with $FeSO_4$ caused a large increase in formate oxidation ($P < 0.001$). The presence of $FeSO_4$ alone also increased formate oxidation ($P < 0.05$) but to a somewhat lesser degree than $FeSO_4$ and H_2O_2 together (Table 4). The effect of $FeSO_4$ on H_2O_2 concentrations as a function of scopoletin fluorescence is shown in Table 5. Equimolar concentrations of H_2O_2 and scopoletin result in almost complete disappearance of scopoletin fluorescence in the presence of horseradish peroxidase. When $FeSO_4$ was added, however, H_2O_2-associated scopoletin oxidation occurred to a much lesser degree. Since $FeSO_4$ had no effect on scopoletin fluorescence by itself, it may be concluded that its effect in the presence of H_2O_2 was due to $FeSO_4$ associated decomposition of H_2O_2.

DISCUSSION

Activation of the HMPS with phagocytosis is known to be necessary for the microbicidal activity of leukocytes (21, 33). Its impairment in the presence of $FeSO_4$ could, at first glance, be interpreted as contributing to the leukocytes' reduced microbicidal capacity; however, the mechanism by which HMPS activation potentiates bacterial killing is not direct and involves a number of related reactions. The HMPS involves the conversion of glucose-6-phosphate to

TABLE 1. *Effect of $FeSO_4$ on [1-^{14}C]glucose oxidation*[a]

Species	Particle	Control	1.84 mM $FeSO_4$	0.92 mM $FeSO_4$	0.09 mM $FeSO_4$
Rabbit	−	13.4 ± 1.7 (5)	11.4 ± 2.1, $P < 0.5$ (5)	11.3 ± 3.4, $P = 0.5$ (4)	15.8 (1)
Rabbit	+	126.7 ± 12.5 (5)	75.5 ± 3.7, $P < 0.01$ (5)	87.3 ± 9.1, $P = 0.05$ (4)	125.2 (1)
Human	−	8.3 ± 3.1 (7)	7.8 ± 1.3, $P > 0.5$ (7)		
Human	+	195.0 ± 16.6 (7)	98.8 ± 22.8, $P < 0.01$ (7)		

[a] Nanomoles of $^{14}CO_2$ produced per 10^7 leukocytes per 30 min ± standard error of the mean. P values refer to significance of difference between $FeSO_4$ treated cells and controls. Numbers in parentheses indicate number of experiments.

TABLE 2. *Effect of $FeSO_4$ on [^{14}C]sodium formate oxidation*[a]

Species	Particles	Control	1.84 mM $FeSO_4$	0.92 mM $FeSO_4$	0.09 mM $FeSO_4$
Rabbit	−	0.36 ± 0.09 (5)	1.81 ± 0.39 (5), $P < 0.01$	1.88 ± 0.45 (4), $P < 0.01$	1.16 ± 0.44 (4), $P = 0.05$
Rabbit	+	1.31 ± 0.13 (5)	4.12 ± 0.94 (5), $P < 0.02$	5.34 ± 1.40 (4), $P < 0.01$	2.46 ± 0.44 (3), $P = 0.01$
Human	−	1.7 ± 0.5 (10)	4.8 ± 1.1 (20), $P < 0.02$		
Human	+	4.0 ± 0.6 (10)	7.2 ± 0.09 (10), $P < 0.01$		

[a] Nanomoles of $^{14}CO_2$ produced per 10^7 leukocytes per 30 min ± standard error of the mean. P values refer to significance of difference between $FeSO_4$-treated cells and controls. Numbers in parentheses indicate number of experiments.

ribose-5-phosphate and requires glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase for this conversion. These enzymes, in turn, require NADP, which along with glucose-6-phosphate is rate limiting for the reaction (3). During HMPS activity, NADP is converted to NADPH, which then must be reconverted to NADP for continued activity of the HMPS. Investigation into the mechanisms of NADP formation has provided a spirited divergence of opinions (5, 7, 25, 29, 30, 40). Briefly, these involve the relative importance of NADH oxidase, indirectly leading to NADP (5, 7, 28), or of NADPH oxidase, directly resulting in NADP (25, 30, 40). The reaction of both NADPH and NADH oxidases results in the formation of H_2O_2, which has a central role in the leukocytes' antibacterial function. The H_2O_2 formed also may play a role in the link between NADH oxidase and NADP formation through reactions involving glutathione where:

$$H_2O_2 + 2\,GSH \xrightarrow{\text{glutathione peroxidase}} GSSG + 2\,H_2O \quad (1)$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{glutathione reductase}} 2\,GSH + NADP \quad (2)$$

(See references 21, 23, and 37.)

In addition, superoxide (O_2^-), a highly reactive compound produced in leukocytes and stimulated during phagocytosis, may be involved in bacterial killing by leukocytes (2). Superoxide is converted to H_2O_2 and oxygen by the enzyme superoxide dismutase (36).

The central importance of H_2O_2 to the microbicidal activity of leukocytes was established by Klebanoff (15–18) and explored by Sbarra et al. (21, 22, 26, 27, 32, 33). Their studies elucidated the interaction of H_2O_2, myeloperoxidase, and halide ion for effective microbicidal activity by leukocytes. When iodide is the cofactor, killing is associated with iodination of bacteria (15). When chloride is the cofactor, killing appears to be related to the oxidative decarboxylation of amino acids to aldehydes, which possess antibacterial properties (26, 27). More recently, Selvaraj et al. have shown that oxidative cleavage of peptide bonds also occurs (32).

In view of the importance of H_2O_2 to the cells' microbicidal activity, the apparent increased H_2O_2, as measured by formate oxidation, together with lower HMPS activity in the presence of $FeSO_4$ was difficult to explain or correlate with the impaired microbicidal activity shown by Gladstone and Walton. If glutathione peroxidase or reductase were inhibited by $FeSO_4$, the coexistence of reduced HMPS activity and enhanced H_2O_2 quantities could perhaps be reconciled since one of the mechanisms of H_2O_2 removal would be inoperative. This, however, would not explain the decreased bacterial killing, and evaluation of glutathione reductase failed to show any impairment of activity in the presence of $FeSO_4$.

The apparently paradoxical inhibition of the HMPS associated with increased formate oxidation appears to be due to the fact that formate oxidation, although commonly used to measure H_2O_2 formation (13), is not specific for H_2O_2, and in this case the increased formate oxidation was due to its oxidation to CO_2 by the iron in the medium. Ferrous iron, even complexed to citrate, may be readily oxidized by H_2O_2 to ferric iron and water (10) and, in the presence of formate, CO_2 is formed. Therefore, it is not H_2O_2 itself that is responsible for the increased formate oxidation; rather, H_2O_2 is itself reduced by Fe^{2+}. Since H_2O_2 is reduced, there is less

TABLE 3. *Effect of $FeSO_4$ (1.84 mM) on glutathione reductase and peroxidase activities*[a]

$FeSO_4$	Glutathione reductase	Glutathione peroxidase
−	13.6 ± 2.2 (7)	9.3 ± .9 (7)
+	13.7 ± 3.2 (6)	8.5 ± 1.3 (7)

[a] Nanomoles of NADPH oxidized per minute per milligram of protein ± standard error of the mean. Numbers in parentheses indicate number of experiments.

TABLE 4. *Effect of $FeSO_4$ on H_2O_2-related oxidation of sodium formate to CO_2*[a]

Additions to sodium formate (8.8 nmol)	n[b]	Without $FeSO_4$	With $FeSO_4$
None	12	Blank	151 ± 50 ($P < 0.05$)
Glucose (16.6 nmol), glucose oxidase (17.6 U), catalase (70 U)	2	25	110
H_2O_2 (0.5 μmol/ml)	12	34.5 ± 23	265 ± 42 ($P < 0.001$)

[a] Nanomoles of $^{14}CO_2$ produced ± standard error of the mean.
[b] n, Number of experiments.

TABLE 5. *Effect of $FeSO_4$ on H_2O_2-associated scopoletin oxidation*

Additions to scopoletin (2.5×10^{-8} mol)	n^a	Residual scopoletin concn (mol)
HPO[b], 6.5×10^{-9}	6	2.5×10^{-8}
$FeSO_4$, 1.84×10^{-6} HPO, 6.5×10^{-9}	6	$2.3 \times 10^{-8} \pm 0.06$[c]
H_2O_2, 2.5×10^{-8} HPO, 6.5×10^{-9}	5	$0.14 \times 10^{-8} \pm 0.095$
H_2O_2, 2.5×10^{-8} $FeSO_4$, 1.84×10^{-6} HPO, 6.5×10^{-9}	5	$1.35 \times 10^{-8} \pm 0.1$, $P < 0.005$

[a] *n*, Number of experiments.
[b] HPO, Horseradish peroxidase.
[c] Standard error of the mean.

of it available for its microbicidal interaction with myeloperoxidase. Probably then it is this factor, along with the inactivation of cationic proteins by $FeSO_4$, that reduces the cells' ability to kill bacteria. Also, with decreased H_2O_2, less GSH will be oxidized to GSSG through the glutathione peroxidase reaction and, therefore, less NADP will be formed through the GSSG-NADPH-linked glutathione reductase reaction, with consequent reduction of HMPS activity. Since NADP also is formed through the reaction of NADPH oxidase and probably through the NADPH-NAD transhydrogenase, the partial HMPS activation observed in the presence of iron can be accounted for. In the presence of $FeSO_4$, at least two important microbicidal pathways are impaired. H_2O_2 is reduced to harmless water, and cationic proteins, perhaps including myeloperoxidase (31), are bound and rendered inactive, resulting in a critical reduction of microbicidal capacity. That the cell's entire bactericidal capacity is not eliminated may be due to the quantity of iron involved versus the amount of material with which it interacts and to the presence of alternative microbicidal pathways.

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