

Iron Deficiency and Neutrophil Function: Different Rates of Correction of the Depressions in Oxidative Burst and Myeloperoxidase Activity After Iron Treatment

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The polymorphonuclear granulocyte (PMN) kills ingested bacteria by mechanisms that include myeloperoxidase (MPO) and a sudden increase in oxygen consumption (the oxidative burst), both of which are iron dependent. The magnitude of the oxidative burst and activity of MPO were determined in PMNs during the progression of iron deficiency (ID) and following its treatment in rats. As ID developed, the oxidative burst after zymosan activation was less depressed than the activity of MPO. There was no change in the oxidative burst after activation with phorbol myristate acetate (PMA) or in the generation of superoxide (O_2^-) by NADPH oxidase-containing particles from PMNs. Following iron treatment, impairment of the oxidative

burst after zymosan activation was corrected after 1 day. In contrast, the deficit in MPO activity was not corrected until 7 days after initiation of iron treatment. The pattern of recovery in MPO activity after iron treatment corresponded to the prolonged period of maturation of the PMN primary granule since the formation of primary granules, which contain MPO, takes place only in the early, mitotic stages of maturation. The tendency of the PMN to maintain the oxidative burst allows the cell to preserve its capacity for bacterial killing during the progression of iron deficiency.

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IRON DEFICIENCY (ID) results in impairments of immune function, particularly in respect to cell-mediated immunity and the ability of polymorphonuclear granulocytes (PMNs) to kill ingested bacteria.¹⁻⁵ The PMN is of particular interest with respect to ID, since the cell has many iron-containing constituents. At least two morphologically and chemically distinct granule populations have been characterized in the cytoplasm of the PMN.^{6,7} The primary granule is the only granule that is formed early in maturation; it is produced mainly during the promyelocyte stage and contains myeloperoxidase (MPO).⁷ This iron-containing enzyme contributes to the antimicrobial function of the PMN,⁸ but the importance of this role is uncertain, since patients with a congenital deficiency of MPO are virtually asymptomatic.^{9,10} Other granules, including the specific granule, are formed during and subsequent to the myelocyte stage.⁷ The specific granule or a third type of granule is believed to contain cytochrome b, an iron-containing constituent that is believed to be required for the oxidative burst, the marked increase in oxygen consumption that occurs following phagocytosis.¹¹⁻¹⁴ The oxidative burst plays an important role in bacterial killing, as judged from the marked susceptibility to infection in chronic granulomatous disease, a congenital disorder in which the oxidative burst is absent or decreased.¹¹⁻¹⁴

In studies of PMN function in ID children reported by Chandra and Saraya,^{15,16} the nitroblue tetrazolium (NBT) test, which reflects the oxidative burst, was decreased. The

abnormality was reversed when patients were reinvestigated 4 to 7 days after treatment with iron.¹⁵ Most studies of bactericidal capacity in ID show it to be impaired.¹⁵⁻²⁰ Reversal of the abnormality was reported within 4 to 7 days by Chandra.¹⁵ Walter and colleagues observed partial recovery after 3 to 5 days and complete recovery by 15 days.²⁰ Correction of the abnormality was much slower in the study of Yetgin and co-workers,¹⁷ in which bacterial killing was partially improved after 1.5 months of iron treatment and completely reversed after 3 months of therapy. Experimental evidence in rats also showed an abnormal NBT test and decreased MPO activity in ID that had reversed when PMNs were reevaluated 2 months after initiation of iron treatment.²¹

Because the contrasting maturation patterns of the different types of PMN granules might result in correspondingly distinct rates of reversal of abnormalities in PMN enzymes and related functions during recovery from ID, we investigated MPO activity and the oxidative burst, abnormalities associated with the primary granules and the specific granules, respectively, before and after iron administration to rats with ID.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Bantin-Kingman, Fremont, CA) were obtained at weaning (21 days of age) and were randomly assigned to one of two dietary groups, the ID and the control group. ID rats received an ID diet (iron 3 to 9 mg/kg diet); control rats were given the same diet but with added ferric citrate (iron 60 to 63 mg/kg diet). Both diets were otherwise identical in composition to the American Institute of Nutrition diet proposed for growing rats with the exception that cellulose (which has a variable iron content) was omitted.²² Diets (Teklad, Madison, WI) and distilled water were provided ad libitum.

Protocol. Groups of 5 to 8 rats in each of the diet categories were studied after 1, 2 and 3 weeks of diet; 1, 2, 3, 5, and 7 days after iron administration for the MPO assay; and 1 and 3 days after iron administration for measurement of the oxidative burst. After 3 weeks of the diets (at 6 weeks of age), rats of the ID group received 10 mg of elemental iron as iron dextran (Imferon, Merrell Dow Pharmaceutical, Cincinnati) divided into two intramuscular injections of 0.1 mL each. Control rats received an injection of the same volume of 0.9% saline. Hemoglobin and body weight were measured

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on the day of each experiment. Hemoglobin was determined by the cyanmethemoglobin method (Data Medical, Arlington, TX).

Isolation of rat PMNs. Functionally active PMNs were obtained from the peritoneal cavity 4 hours after intraperitoneal (IP) injection of a casein suspension as described by Lemanske and colleagues.²³ Rats were killed by decapitation, and the peritoneal cavities were lavaged with a total of 40 mL of chilled Dulbecco's phosphate-buffered saline (PBS), pH 7.4, without calcium and magnesium (CMF).²⁴ The peritoneal exudates were collected separately from each rat and filtered through gauze into a 50-mL sterile centrifuge tube. The cells were kept at 4°C or on ice throughout the isolation procedure.

The PMN concentrates were centrifuged at 400 g for 15 minutes at 4°C. The resulting pellets were treated with 5 mL of ammonium chloride solution for 2 minutes with moderate agitation to lyse RBCs.²⁵ After 2 minutes, 40 mL of CMF was added to each tube and mixed thoroughly. The cells were then centrifuged and washed three times with CMF.

In a second experiment, PMNs were isolated from the peripheral blood as described by Babior and Cohen²⁴ with minor modifications. Peripheral blood from male Sprague-Dawley rats was collected in a syringe containing 1 to 1.5 mL of acid-citrate-dextrose solution (ACD). The anticoagulated blood was diluted with the same amount of CMF and mixed with half the amount of 4.5% dextran (mol wt 488,000) in 0.9% saline. The upper layer was removed after the mixture was placed upright for 1 hour at room temperature. The leukocyte-rich plasma was centrifuged, and resulting pellets were treated as described above. The cells were washed twice with CMF, pooled, and suspended in 20 mL of CMF. Then 10 mL of Histopaque-1077 was carefully injected under a 20-mL layer of leukocyte suspension in a 50-mL sterile centrifuge tube using a spinal needle. The tube was centrifuged at 400 g for 20 minutes, and the overlying fluid was aspirated.

The washed neutrophils obtained from either the peritoneal cavity or the peripheral blood were then resuspended in CMF and counted using a Coulter counter model ZBI (Coulter Electronics, Hialeah, FL). Differential counts were performed on Giemsa-stained blood smears to verify that >90% of the cell from the peritoneal cavity and 70% from the peripheral blood in each preparation were PMNs. Cell viability was >95% as determined with trypan blue in each preparation.

MPO was assayed to the method of Migler and DeChatelet with *p*-phenylenediamine as the hydrogen donor.²⁶ Isolated PMNs in a final concentration of 10⁷ cells/mL were subjected to sonication (model W-220, Heat Systemics-Ultrasonic, Plainview, NY) for two 8-second bursts in 0.25% Triton X-100. Disposable polystyrene cuvettes (Fisher Scientific, Pittsburgh) containing 2.75 to 2.92 mL of 10 mmol/L of *N*-[2-acetaamido]-iminodiacetic acid (ADA) pH 6.0, 30 μL of 0.075% H₂O₂, and 25 μL of 1% *p*-phenylenediamine in ethanol (wt/vol) were mixed thoroughly. The reaction was then started by addition of 25 to 200 μL of the PMN suspension (to a total 3.0-mL vol), with a final mixing by rapid inversions of the cuvette. The change in the absorbance at 485 nm was measured using a Hitachi double-beam spectrophotometer (Hitachi, Tokyo) with a chart recorder (Cole-Palmer Instrument, Chicago). The results were expressed as change in optical density per minute per 10⁶ cells.

Measurement of oxygen consumption by intact PMNs activated by zymosan or phorbol myristate acetate (PMA) was performed as described by Mackler and colleagues²⁷ in a polarographic chamber (Rank Bros, Cambridge, England) thermostated to 37°C. Preparation of opsonized zymosan was by the method of Markert and colleagues²⁸ using serum obtained from male rats of the same strain and from the same supplier as the experimental rats. The opsonized zymosan was washed twice with CMF and then suspended at a concentration of 22.5 mg/mL in CMF. The zymosan was prepared

fresh for each day's experiment. The assay mixture contained 0.80 mL of a solution of 0.25 mol/L of sucrose and 0.02 mol/L of Tris (pH 7.4), 0.20 mL freshly thawed rat serum (prepared as described above), 0.60 mL of Hank's solution and 0.20 mL of the intact PMNs suspended in CMF to yield a final concentration of 1.5 × 10⁷ cells/mL in a 2.0-mL total vol. Opsonized zymosan, 0.20 mL (22.5 mg/mL in CMF) or 4 mg of PMA in 0.20 mL of the sucrose was then added to the chamber to activate the cells. The maximal rate of O₂ consumption was determined after the addition. The measurement of O₂ by NADPH oxidase-containing particles from PMNs was measured by the method of Markert and colleagues.²⁸

Materials. Histopaque-1077, casein, zymosan A, *p*-phenylenediamine, Triton X-100, and 30% H₂O₂ were obtained from Sigma, St Louis.

Statistical analysis. Data were analyzed using the unpaired Student's *t* test.

RESULTS

Body weight and hemoglobin concentration. The body weight of the ID rats showed no significant difference after rats were on the diet for 1 week (Table 1). At 2 and 3 weeks, the mean weight of the iron-deficient group was below that of the control group by ~8% and ~20%, respectively. Recent studies under similar conditions have shown that the food intake of iron-deficient rats is essentially equivalent to that of control animals^{29,30} and hypothesize that the slower weight gain is due to metabolic alterations in the iron-deficient rat.³⁰ After iron treatment, the weight of the ID group increased rapidly for 1 to 2 days, but a significant difference of 12% between two groups remained after 7 days.

The ID rats had significantly lower hemoglobin levels as compared with the control rats even after 1 week on the diet. The differences became progressively greater with time, to a mean value that was 38% of the control after 3 weeks on the diet. After iron treatment, the hemoglobin levels of the ID group rose rapidly 1 to 5 days after treatment and were within 15% of control values 7 days after initiation of iron treatment (Table 1).

MPO activity. MPO activity did not differ in the ID group during the first 2 weeks of the regimen as compared with control animals (Table 2). During the progression of ID, the mean MPO activity of the ID group was not significantly reduced until after 3 weeks of diet, when it was significantly depressed, to 53% of the control activity. After iron treatment, the MPO activity of the deficient group did not change

Table 1. Body Weight and Hemoglobin Concentration

Week of Diet or Days After Treatment	Body Weight (g)		Hemoglobin (g/dL)	
	Iron-Deficient	Control	Iron-Deficient	Control
Week 1	90 ± 2	98 ± 2	8.1 ± 0.2*	12.4 ± 0.2
Week 2	132 ± 4*	149 ± 2	6.1 ± 0.3*	13.8 ± 0.1
Week 3 (day 0)	160 ± 3*	201 ± 3	5.3 ± 0.2*	13.8 ± 0.3
Day 1	171 ± 4*	196 ± 3	6.2 ± 0.2*	13.8 ± 0.3
Day 2	185 ± 5	208 ± 7	9.1 ± 0.3*	15.0 ± 0.4
Day 3	186 ± 5*	226 ± 6	9.8 ± 0.2*	13.8 ± 0.3
Day 5	193 ± 5*	237 ± 5	11.6 ± 0.2*	14.6 ± 0.2
Day 7	200 ± 5*	228 ± 8	12.2 ± 0.3*	14.3 ± 0.2

Means ± SEM are shown.

*Significant differences between two groups (*P* < .001).

Table 2. Myeloperoxidase Activity in PMNs During Progression of Iron Deficiency and Following Iron Treatment

Weeks of Diet or Days of Iron Treatment	Myeloperoxidase, Δ OD/min/ 10^6 PMNs				Percent of Control
	Rats per Group	Iron-Deficient Rats	Control Rats		
1 Week	8, 7	0.172 \pm 0.014	0.165 \pm 0.015		104
	8, 8	0.160 \pm 0.020	0.180 \pm 0.016		89
2 Weeks	8, 7	0.147 \pm 0.011	0.177 \pm 0.024		83
3 Weeks	8, 7	0.209 \pm 0.023*	0.396 \pm 0.057		53
1 Day	8, 7	0.172 \pm 0.013†	0.298 \pm 0.021		58
2 Days	8, 5	0.259 \pm 0.018*	0.435 \pm 0.032		60
3 Days	8, 8	0.141 \pm 0.009†	0.265 \pm 0.022		53
5 Days	7, 7	0.216 \pm 0.013*	0.270 \pm 0.021		80
	8, 8	0.232 \pm 0.013†	0.350 \pm 0.031		66
7 Days	8, 8	0.275 \pm 0.018	0.242 \pm 0.016		114

PMNs, polymorphonuclear granulocytes.

Additional groups of deficient and control rats were studied at two of the time points. Iron was administered to iron-deficient rats after 3 weeks of iron-deficient diet.

* $P < .01$.

† $P < .001$.

for 3 days and did not attain control levels until 7 days after iron treatment.

The oxidative burst. The changes in the rates of oxygen consumption of PMNs after stimulation with zymosan and PMA are shown in Table 3. No significant differences were found after 1 and 2 weeks of diet. After 3 weeks, however, there was a small but significant depression in oxygen consumption after zymosan activation in the ID group that was confirmed in an additional experiment. One and 3 days after iron treatment, the rate of oxygen consumption of the deficient PMNs was equal to that of the control PMNs. There was no difference in oxygen consumption after activation with PMA between iron-deficient and control rats studied after 3 weeks of the dietary regimens. O_2^- production by NADPH oxidase-containing particles from PMNs was not depressed after 3 weeks of the iron-deficient regimen (Table 4).

Peripheral blood PMNs. PMNs obtained from the peripheral blood were used to validate the results obtained on peritoneal PMNs in a second and smaller experiment on two

randomly assigned groups of rats that had been given the ID diet for 3 weeks. One group had been given intramuscular (IM) Imferon injection on the previous day, and the other group was untreated. The activity of MPO was not changed 1 day after treatment, whereas the rate of oxygen consumption after zymosan activation was significantly increased (Fig 1). Absolute values for oxygen consumption were considerably lower than in PMNs from peritoneal exudate, possibly due to the different method of isolation.

DISCUSSION

Clinical studies suggest that bacterial killing by PMNs depends primarily on the oxidative burst rather than on MPO. This is evident from a comparison between chronic granulomatous disease, an inherited abnormality in the O_2^- -forming oxidase or its activating system, and hereditary MPO deficiency. Chronic granulomatous disease, usually fatal, is characterized by recurrent infections caused by an inability to generate an oxidative burst to kill invading microorganisms.³¹ In contrast, the antibacterial defense in patients with MPO deficiency is relatively well preserved, with little evidence of morbidity other than a susceptibility to infection with *Candida*.^{9,10}

Our results in the ID rat indicate that the oxidative burst is less impaired than MPO activity after 3 weeks of an iron-deficient regimen. This presumably preserves the most vital function of the PMN. Mackler and co-workers²⁷ also reported a more profound depression of MPO than of the

Table 3. Oxygen Consumption After Activation of PMNs With Zymosan or PMA

Weeks of Diet or Days of Iron Treatment	Rats per Group	Oxygen Consumption, nmol O_2^- /min/ 10^6 PMNs		
		Iron-Deficient Rats	Control Rats	Percent of Control
Zymosan				
1 Week	8, 8	1.75 \pm 0.15	1.73 \pm 0.16	101
2 Weeks	8, 7	4.61 \pm 0.22	4.43 \pm 0.34	104
3 Weeks	9, 9	3.80 \pm 0.28*	4.73 \pm 0.32	80
	6, 7	4.32 \pm 0.34*	5.29 \pm 0.18	82
1 Day	8, 7	5.21 \pm 0.35	4.71 \pm 0.40	110
3 Days	8, 8	5.41 \pm 0.39	5.16 \pm 0.32	105
PMA				
3 Weeks	8, 7	4.51 \pm 0.21	4.40 \pm 0.23	103
1 Day	7	4.41 \pm 0.15	—	100

PMNs, polymorphonuclear granulocytes; PMA, phorbol myristate acetate.

* $P < .05$.

Table 4. Superoxide (O_2^-) Production by NADPH Oxidase-Containing Particles from PMNs

Group	Rats per Group	nmol O_2^- Produced/min/ 10^6 PMNs	Percent of Control
Control, 3 weeks	7	3.9 \pm 0.3	100
Iron-deficient, 3 weeks	8	4.4 \pm 0.4	113
Iron-deficient, 1 day treated	8	4.4 \pm 0.3	113

PMNs, polymorphonuclear granulocytes.

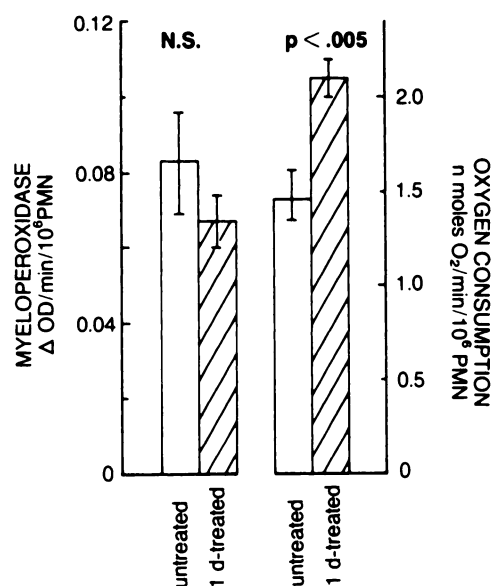


Fig 1. Myeloperoxidase activity and oxygen consumption after zymosan stimulation in 6 to 7 polymorphonuclear granulocyte (PMN) concentrates per group obtained from peripheral blood of 3 rats each in rats given an iron-deficient diet for 3 weeks, either saline treated (open bars) or iron treated (hatched bar). SEM is shown.

oxidative burst of PMNs in iron-deficient rats. They did, however, find a significant depression with PMA activation after 6 weeks of the dietary regimens. Surprisingly, PMN cytochrome b, which is thought to be essential for the oxidative burst,³⁰⁻³² was normal in concentration.²⁷

The generation of an oxidative burst is normally initiated by phagocytosis, which is followed by activation of the pentose shunt with a concomitant production of NADPH. The NADPH is then oxidized to generate O_2^- , which initiates a sequence of steps that result in bacterial killing. Zymosan is a particulate activator that mimics the effect of bacteria and other invasive organisms. It consists of yeast cell walls and must be ingested by the PMN to generate an oxidative burst. In contrast, PMA is a soluble stimulus that does not require phagocytosis. Our finding of a decreased oxidative burst in response to zymosan, but not following

PMA, suggests that phagocytosis may have been a limiting factor. Also in accord with this hypothesis is the maintenance of normal O_2^- production by PMN NADPH oxidase (Table 4). Additional evidence that impaired phagocytosis might explain a decrease in the zymosan-activated oxidative burst is the observation by Moore and Humbert²¹ that PMNs from iron-deficient rats show a slightly diminished phagocytosis of yeast cells (*C albicans*). The return to normal of the zymosan-activated oxidative burst within 1 day of iron administration might then indicate that such a mild defect in phagocytosis is rapidly reversed.

Our finding that the mild impairment of the oxidative burst after zymosan activation was corrected 1 day after iron treatment is compatible with Chandra's finding that NBT test in patients was normal 4 to 7 days after iron was given IM.¹⁵ In the study of Walter and colleagues, the abnormality in bacterial killing was partially improved after 3 to 5 days and was completely corrected 15 days after oral iron treatment.¹⁸ These results suggest somewhat slower recovery of the oxidative burst that might be due to the species difference, duration of prior iron deficiency, methodology, or the route of iron administration.

The pattern of PMN recovery in respect to MPO activity after iron treatment corresponds to the maturation of the primary granule. Reversal of deficient MPO production apparently takes place only in the early stages of PMN maturation, since recovery after iron treatment is not apparent until 5 days and not complete for 7 days. Kinetic studies of PMN maturation in the rat after in vivo labeling of the proliferating cells in the tibial bone marrow with (³H)thymidine show that 3 days elapse before peak labeling occurs in released PMNs.³⁵ MPO is synthesized during the early, mitotic stages of PMN maturation. The delay in reversal of MPO deficiency after iron treatment indicates that adequate iron must be present when the primary MPO-containing granules are produced and that correction of the deficit in MPO cannot take place at a later stage of cell maturation.

Our results indicate that the PMN in the ID rat tends to preserve its most vital function for bacterial killing and to correct this abnormality rapidly with iron treatment. Whether the sequence of recovery is the same in ID patients remains to be determined by more detailed investigations during the first 2 weeks of therapy.

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