Increased Respiratory Burst in Myeloperoxidase-Deficient Monocytes

By Richard M. Locksley, Christopher B. Wilson, and Seymour J. Klebanoff

Studies of the respiratory burst in myeloperoxidase (MPO) deficient monocytes were undertaken to assess the physiologic consequence of the absence of MPO in these cells. As previously demonstrated with neutrophils, MPO-deficient monocytes had a greater initial rate, duration, and total superoxide production in response to phagocytosis of zymosan than did normal monocytes. Introduction of purified eosinophil peroxidase (EPO) into the phagosome by binding the enzyme to the surface of the zymosan particles changed the hypermetabolic characteristics of superoxide production in MPO-deficient cells to more closely resemble normal cells, but had no effect on superoxide generation by the normal monocytes. Further, inactivation of the bound EPO before ingestion restored the supranormal respiratory burst by the MPO-deficient cells. Lodination by MPO-deficient monocytes was significantly depressed as compared to normal monocytes following the ingestion of zymosan (1.9 versus 10.1 n mole l^-1/10^7 monocytes/30 min; p < 0.01). In contrast, lodination was markedly augmented in MPO-deficient cells compared to normal cells after ingestion of zymosan coated with EPO (208 versus 70 n mole l^-1/10^7 monocytes/30 min; p < 0.005), presumably reflecting the greater amounts of hydrogen peroxide formed by MPO-deficient cells. There were no differences in the levels of endogenous scavengers of reactive oxygen products (catalase, superoxide dismutase, glutathione peroxidase and reductase, and total glutathione) in MPO-deficient and normal monocytes that would account for the enhanced respiratory burst of MPO-deficient cells. These findings support a role for peroxidase in the termination of the respiratory burst of monocytes.

Patients with hereditary myeloperoxidase deficiency lack peroxidase in neutrophils and monocytes. This enzyme, which is normally present in cytoplasmic granules, is released into the phagosome following microbial ingestion, where it participates in the destruction of the microorganism through reaction with H_2O_2 and a halide to form a potent antimicrobial system. Although some patients with MPO deficiency have been noted to have an increased number of infections, most remain well despite the presence of a microbicidal defect in their neutrophils and monocytes in vitro. This may be due to a number of factors. First, phagocytes contain a variety of antimicrobial systems, some requiring oxygen and others not, and their combined effects appear to be in excess of the needs of the cell under most circumstances. Further, when MPO is absent, there appears to be an increase in the respiratory burst of neutrophils, with the potential for the formation, in greater than normal amounts, of oxygen-derived products with MPO-independent antimicrobial activity. Past studies of the increased respiratory burst of MPO-deficient phagocytes have been limited to neutrophils. In this article we report on the increased respiratory burst of MPO-deficient monocytes as measured by superoxide anion (O_2^-) production and by lodination associated with the ingestion of peroxidase-coated particles. Measurement of scavengers of toxic oxygen metabolites indicated that the enhanced respiratory burst in MPO-deficient monocytes was not due to a deficiency of these compounds.

Materials and Methods

Special Reagents

Horse eosinophil peroxidase (EPO) was purified and stored as described. The final preparation was in 0.05 M sodium acetate buffer, pH 4.7, 1.0 M sodium chloride. Peroxidase activity was assayed by guaiacol oxidation. One unit is the amount of enzyme that oxidizes 1 μmole of guaiacol/min at 25°C, using a molar absorbancy for the product, tetraguaiacol, of 2.66 x 10^5 cm^-1 at 470 nm. Catalase (bovine liver, 75,000 U/mg; Worthington Biochemical Corp., Freehold, NJ) was dialyzed against water and stored at 4°C until use. Human serum albumin (essentially fatty-acid-free) that oxidizes 1 μmole of guaiacol/min at 25°C was obtained from Sigma Chemical Co., St. Louis, MO, and 125I-iodide and 131I-iodide (carrier-free in 0.1 M NaOH) from New England Nuclear Corp., Boston, MA.

Preparation of Monocytes

Venous blood collected in 0.2% K^-EDTA from normal volunteers and from two patients with hereditary complete MPO deficiency was diluted in normal saline and separated over Ficoll (Winthrop Laboratories, New York, NY)-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). The mononuclear cells at the interface were harvested, washed twice in Ca^++-Mg^++-free Hanks' balanced salt solution (HBSS; GIBCO Laboratories, Grant Island, NY), and resuspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 5.5 mM glucose (KRPG). Cell preparations contained 24% ± 6% monocytes as determined by phagocytosis of neutral red particles and by staining for nonspecific esterase, less than 1.5% polymorphonuclear leukocytes (range 0.6%–1.4%) as determined from microscopic examination of smears stained with Diff-Quik.
Cytometer and adjusted to 5 x 10⁶ monocytes/ml in KRPG. Cells were ≥98% viable as assessed by trypan blue exclusion.

In two experiments, washed mononuclear cells were resuspended at 10⁶ monocytes/ml in RPMI 1640 (GIBCO Laboratories) containing 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine (RPMI medium) with 10% autologous serum, and distributed to 35-mm diameter tissue culture dishes (Falcon Labware, Oxnard, CA). After incubation for 1 hr at 37°C in humidified 5% CO₂-air, the nonadherent cells were removed by washing with warm (37°C) HBSS, and the monolayers were assayed for O₂⁺ production (see below). The total protein content of the monolayers was determined as described.¹¹

Preparation of Zymosan

Zymosan (ICN Pharmaceuticals, Cleveland, OH), after suspension at 25 mg/ml in water at 4°C with a Potter-Elvehjem homogenizer, was boiled for 10 min, washed 3 times in 0.1 M phosphate-buffered saline, pH 7.2 (PBS), resuspended at 25 mg/ml in PBS, and stored at 4°C. On the day of the experiment, zymosan was opsonized by incubation at 37°C for 30 min in a shaking bath with human AB serum and washed 3 times in PBS at 4°C. Two aliquots were taken, centrifuged (1,000 g for 20 min), and each resuspended in a minimal volume (20–60 μl) of 0.1 M sodium phosphate buffer, pH 7.2 (PB), and sonicated in a bath sonicator (Branson Sonic Power Co., Danbury, CT) to facilitate dispersion of the particles. One aliquot was incubated in a shaking bath at 37°C with 4.5 U EPO/25 mg opsonized zymosan. The other aliquot was incubated with an equivalent volume of 0.05 M sodium acetate buffer, pH 4.7–1.0 M sodium chloride. After 15 min incubation, the zymosan preparations were washed 3 times in iced (4°C) PB and resuspended in KRPG.

In some experiments, EPO was iodinated by preincubation for 15 min at 37°C with 0.35 μCi carrier-free ¹²⁵I and 10⁻⁴ M hydrogen peroxide (H₂O₂, 30%; American Scientific and Chemical Co., Portland, OR) in a final volume of 250 μl of PB in a shaking bath. The reaction was stopped by the addition of 60 μg/ml catalase in iced PB. Zymosan was added to the reaction mixture and zymosan with ¹²⁵I-EPO bound to its surface was prepared as described above. After 4 washes in large volumes at PB at 4°C, there was no further ¹²⁵I detectable in the supernatant, and the radiolabeled zymosan was suspended in KRPG.

To inactivate EPO bound to zymosan, particles were incubated at 37°C for 15 min in a shaking bath with 10⁻¹ M sodium azide (Matheson, Coleman and Bell, Norwood, OH), 10⁻³ M glucose, and 2 U glucose oxidase (Type V, Sigma) in a final volume of 0.5 ml in PB. The zymosan was washed 3 times and resuspended in KRPG. Zymosan coated with ¹²⁵I-EPO was similarly treated with azide plus glucose plus glucose oxidase to determine whether loss of peroxidase activity was due to enzyme inactivation or the loss of EPO from the particle surface. An aliquot of the reaction mixture was layered over 0.5 ml of silicone oil (Versilube F50, General Electric Co., Waterford, NY) and centrifuged at 8,000 g for 2 min. The upper aqueous phase was aspirated, placed into a scintillation vial containing Aquasol-2 (New England Nuclear) and counted in a liquid scintillation counter to determine the loss of ¹²⁵I from the zymosan. The bottom of the tube containing the pellet was cut off and placed in a scintillation vial containing 10 ml of Aquasol-2. After sonication in a bath sonicator to facilitate dispersion of the pellet, vials were counted in a liquid scintillation counter.

Iodination by Zymosan-EPO

The components of the reaction mixture (see legend Table I) were added to 12 x 75 mm polystyrene test tubes (Falcon Labware) and rotated end-over-end 16 times/min (Roto-Rack; Fisher Scientific Co., Pittsburgh, PA) at 37°C for 20 min. One milliliter of cold (4°C) 10% trichloroacetic acid (TCA) was added to stop the reaction, and the precipitate was collected by centrifugation (1,900 g for 5 min at 4°C) and washed 4 times with cold 10% TCA. The tube containing the precipitate was placed in a counting tube and the radioactivity determined in a well scintillation counter (Auto-Gamma Scintillation Spectrometer, Packard Instrument Co., Downers Grove, IL). Less than 0.5% of the total added radioactivity was TCA-precipitable in controls performed without added zymosan. Results are expressed as nmol iodide per hour, calculated as described.¹¹

¹²⁵I-iodide was routinely employed for the measurement of iodination. However, ¹¹I-iodine was used when zymosan coated with ¹²⁵I-EPO was employed as the catalyst. In this instance, controls were run without added ¹¹I and the counts subtracted from the total. Under the conditions employed, the counts due to ¹²⁵I were 14.9%–16.4% of the total observed with ¹¹I.

Iodination by Monocytes

The reaction mixture contained 4 x 10⁻³ M sodium phosphate buffer, pH 7.4, 1.28 x 10⁻¹ M NaCl, 1.2 x 10⁻³ M KCl, 10⁻³ M CaCl₂, 2 x 10⁻³ M MgCl₂, 8 x 10⁻³ M NaI (0.05 μCi ¹²⁵I), 2 x 10⁻³ M glucose, 1.25 x 10⁶ monocytes, and 2.5 mg opsonized zymosan in a final volume of 0.5 ml in 12 x 75 mm polystyrene test tubes. After incubation at 37°C on a rotary rack for the periods indicated, the reaction was stopped and the TCA precipitate collected as described above. Resting iodination was determined in the absence of zymosan but with the addition of 3% human AB serum.

Assay for Superoxide Anion

Superoxide anion generation by monocytes was determined by superoxide dismutase-inhibitable ferricytochrome-C (Sigma Chemical Co.) reduction, as described.¹² The reaction was initiated by the addition of opsonized zymosan with or without bound EPO to duplicate tubes or monolayers and O₂⁻ production determined at the time points indicated. Each reaction tube was run in parallel with an identical tube, except for the addition of superoxide dismutase (SOD, 2,500 U/ml, Sigma Chemical Co.) at a final concentration of 25 μg/ml. Control tubes containing media alone, cells alone, or zymosan alone were assayed in parallel; O₂⁻ release in these tubes

Table 1. Iodination by EPO Bound to Zymosan*  

<table>
<thead>
<tr>
<th>Additions</th>
<th>Iodination (nmole/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete zymosan-EPO system</td>
<td>9.93 ± 0.52 †</td>
</tr>
<tr>
<td>H₂O₂ omitted</td>
<td>0.02 ± 0.01 †</td>
</tr>
<tr>
<td>H₂O₂ omitted, glucose plus glucose oxidase added</td>
<td>9.21 ± 0.62</td>
</tr>
<tr>
<td>EPO omitted</td>
<td>0.02 ± 0.01 †</td>
</tr>
<tr>
<td>Catalase added</td>
<td>0.11 ± 0.05 ‡</td>
</tr>
<tr>
<td>Heat-adapted catalase added</td>
<td>8.66 ± 0.82</td>
</tr>
<tr>
<td>Azide added</td>
<td>0.07 ± 0.04 †</td>
</tr>
<tr>
<td>Aminothiol oxidized</td>
<td>0.14 ± 0.04 †</td>
</tr>
</tbody>
</table>

*The complete zymosan-EPO system consisted of 0.1 M sodium phosphate buffer, pH 7.0, 10⁻⁴ M H₂O₂, 2.5 mg zymosan-EPO, 0.5 mg albumin, and 8 x 10⁻⁴ M sodium iodide (0.05 μCi ¹²⁵I) in a final volume of 0.5 ml. Catalase, 60 μg/ml azide, 10⁻² M, aminothiol, 5 x 10⁻³ M, glucose, 10⁻² M, and glucose oxidase, 2 U, were added where indicated. The reaction was started by the addition of zymosan-EPO (or zymosan in EPO omitted).

†Mean ± SE of 3–5 experiments.
‡Significantly different from iodination by complete zymosan-EPO system p < 0.01.
was <2 nmole over the duration of the assay in all experiments. The total protein content of the monolayers was assayed in duplicate from monolayers that were not exposed to zymosan.

Determination of numbers of cell-associated zymosan particles was assessed by microscopic examination of stained aliquots removed at the designated time points.

Monocyte Enzyme and Glutathione Assays

Mononuclear cells harvested and washed after Ficoll-Hypaque separation were suspended in HBSS at approximately 5 × 10^7 mononuclear cells/ml and underlayered with a 1:1 volume of 100% heat-inactivated calf serum (Grand Island Biologicals, Grand Island, NY). Cells were centrifuged at 200 g for 15 min, and centrifugation through calf serum was repeated. The subsequent pellet of cells, essentially platelet-free, was washed twice in HBSS and resuspended in RPMI medium with 10% heat-inactivated fetal bovine serum (FBS; Sterile Systems Inc., Logan, UT), at 5 × 10^6 mononuclear cells/ml. Cells were distributed to 100 x 15 mm Petri dishes (6 ml/dish) and allowed to adhere for 1 hr at 37°C in humidified 5% CO2-air. Monolayers were washed 8 times with warm (37°C) HBSS, containing Ca2+ and Mg2+ to remove nonadherent cells, and overlayed with 6 ml of RPMI 1640 medium containing 15% FBS and 20 mM lidocaine (preservative-free; Astra Pharmaceuticals, Worcester, MA). After incubation for 15 min at 25°C, the cells were collected by gentle pipetting and scraping with a rubber policeman and washed once in RPMI medium containing 15% FBS and once in PBS. Cells prepared in this fashion were 100% mononuclear cells, 89% ± 3% nonspecific esterase positive, 77% ± 3% neutral red positive, 79% ± 3% MPO positive, and were 92% ± 2% viable as assessed by trypan blue exclusion. There were no polymorphonuclear leukocytes or platelets evident on Diff-Quik-stained smears.

Cells were adjusted to 10^7/ml in PBS, supported in an ice bath, and sonicated 3 times using 20-sec bursts at a power setting of 1.5 (Sonicator Cell Disruptor; Heat Systems-Ultrasonics, Inc., Plainview, NY). Octyl phenoxy polyethoxyethanol (Triton X100; Sigma Chemical Co.) was added to the suspension to achieve a final concentration of 0.4%, and the mixture was incubated for 10 min at 4°C. After centrifugation (700 g for 10 min at 4°C), an aliquot of the supernatant was deproteinized with 2.5% sulfosalicylic acid for 10 min at 4°C, cleared by centrifugation (8,000 g for 2 min at 4°C), and stored at −70°C before analysis for total glutathione by the method of Tietze.23 The remainder of the supernatant was diluted 1:1 with glycerol and stored in aliquots at −70°C. Aliquots were analyzed within 1 wk for catalase,32 superoxide dismutase,37 glutathione peroxidase,36 glutathione reductase,36 and for protein by a modification of the Lowry technique using bovine serum albumin as a standard. In preliminary experiments, the activity or amount of these substances remained stable under our conditions for at least 1 wk.

Statistical Analysis

Results are expressed as the mean ± SE of the numbers of experiments designated in the figures and tables. Each experiment was performed using duplicate determinations, which were meant to form a single n. Probability differences were determined using the Student’s two-tailed t test for independent means.

RESULTS

Zymosan Preparations

In this study, either opsonized zymosan, opsonized zymosan with EPO on its surface, or opsonized zymosan with inactivated EPO on its surface was used as the stimulus of the respiratory burst of monocytes from normal individuals or patients with hereditary MPO deficiency. EPO is a strongly basic protein that binds firmly to negatively charged surfaces.17,31 When opsonized zymosan was incubated with EPO and multiply washed to remove unbound enzyme, a portion of the peroxidase was retained on the surface of the particle. This was demonstrated in two ways. First, zymosan preincubated with EPO, like free EPO, converted 125I-iodide to a TCA-precipitable form in the presence of H2O2 (or the H2O2-generating system, glucose + glucose oxidase) and albumin (Table 1). The addition of catalase or the hemeprotein inhibitors, azide or aminothiazole, abolished iodination, implicating the peroxidase system. Opsonized zymosan, which had not been preincubated with EPO (EPO omitted in Table 1), was ineffective as the catalyst. EPO bound to opsonized zymosan retained its iodinating activity for at least 4 hr when stored at 4°C.

The second method employed for the demonstration of the binding of EPO to zymosan utilized 125I-EPO. Zymosan incubated with 125I-EPO and washed extensively retained 15% ± 3% of the added 125I. 125I-EPO bound to zymosan was enzymatically active, as indicated by its catalysis of the iodination of albumin in the presence of added 115I-iodide and H2O2. Iodination was at a rate (9.17 ± 0.71 nmole/hr), comparable to that observed with unlabeled EPO bound to zymosan (9.93 ± 0.52 nmole/hr), suggesting that the peroxidatic activity of bound 125I-EPO was unaltered by the radiolabeling procedure.

When the zymosan–EPO complex was preincubated with azide, glucose, and glucose oxidase and then washed, the complex no longer catalyzed the iodination reaction (Table 2). Preincubation with azide alone or glucose plus glucose oxidase alone had no effect. The inhibition of iodination by preincubation with azide, glucose, and glucose oxidase was not due to the loss of EPO from the zymosan particles, since (1) no peroxidatic activity appeared in the supernatant of the Table 2. Inactivation of EPO Bound to Zymosan*

<table>
<thead>
<tr>
<th></th>
<th>Iodination (nmole/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymosan–EPO</td>
<td>9.93 ± 0.52†</td>
</tr>
<tr>
<td>Preincubation</td>
<td></td>
</tr>
<tr>
<td>Azide plus glucose/glucose oxidase</td>
<td>0.32 ± 0.30‡</td>
</tr>
<tr>
<td>Azide</td>
<td>9.14 ± 0.61</td>
</tr>
<tr>
<td>Glucose/glucose oxidase</td>
<td>9.49 ± 0.38</td>
</tr>
</tbody>
</table>

*The components of the reaction mixture were as described for the complete system in Table 1, except that the zymosan–EPO was preincubated with the substances indicated, as described in Materials and Methods.
†Mean ± SE of 3 experiments.
‡p < 0.001 when compared to zymosan–EPO; all others not significant.
reaction mixture as assessed by guaiacol oxidation; and
(2) when $^{125}$I-EPO bound to zymosan was used, the
radiolabel remained associated with the zymosan par-
ticles with less than 5% of the total radioactivity
appearing in the supernatant fluid.

Superoxide Production by Normal and
MPO-Deficient Monocytes

In preliminary studies, the monocyte concentration
was varied from 1 to $7.5 \times 10^5$/ml, and the zymosan
corcentration from 0.5 to 10 mg/ml; optimal results
were achieved with final concentrations of $2.5 \times 10^4$
monocytes/ml and 5 mg opsonized zymosan/ml (data
not shown). These concentrations were used in all
subsequent experiments unless otherwise noted. As
shown in Fig. 1A, $O_2^\cdot$ formation by MPO-deficient
monocytes incubated with opsonized zymosan was
significantly greater than that of normal monocytes at
all time points studied. The initial rate of $O_2^\cdot$
production, as measured during the first 15 min, was 1.7 and
2.8 n mole/10$^6$ cells/min ($n = 7; p < 0.05$) for normal
and MPO-deficient monocytes, respectively; $O_2^\cdot$
production by MPO-deficient monocytes increased to a
maximum of 108 n mole/10$^6$ cells at 75 min following
the addition of particles, as compared to a total value
of 58 n mole/10$^6$ cells for normal monocytes observed
at 45 min. With neither cell population was the cess-
ation of $O_2^\cdot$ production due to loss of monocyte viability,
which remained >94% at the end of the assay as
assessed by trypan blue exclusion. Nor was the cess-
ation of $O_2^\cdot$ production due to complete ferricyto-
chrome-C consumption, since the addition of fresh
monocytes resulted in further $O_2^\cdot$ production, but the
addition of ferricytochrome-C did not.

When the zymosan–EPO complex was used to trig-
ger the respiratory burst, $O_2^\cdot$ generation by MPO-
deficient monocytes more closely resembled that of
normal monocytes (Fig. 1B). Although the initial rate
(first 15 min) of $O_2^\cdot$ production by MPO-deficient
monocytes remained higher than that of normal mono-
cytes (2.8 versus 1.5 n mole/10$^6$ cells/min; $p < 0.05$),
both the peak $O_2^\cdot$ production and the duration of the
burst by MPO-deficient cells were not significantly
different than that of normal monocytes. Superoxide
production by MPO-deficient monocytes during the
initial 30 min was comparable with zymosan (Fig. 1A)
or zymosan coated with EPO (Fig. 1B), but at all
subsequent time points, $O_2^\cdot$ production was signifi-
cantly less when zymosan-EPO was used. Viability of
the monocytes was >93% in all experiments at the
conclusion of the assay.

The prolonged duration of the respiratory burst and
greater peak $O_2^\cdot$ production by MPO-deficient, as
compared to normal, monocytes was again observed
when the EPO bound to zymosan was inactivated by
preincubation with azide, glucose, and glucose oxidase
(Fig. 1C). In all experiments, the numbers of cell-
associated zymosan particles were comparable with
each of the 3 zymosan preparations and with both
monocyte populations (data not shown).

In the experiments described above, mononuclear
cells prepared by Ficoll-Hypaque separation (which
contained lymphocytes as well as monocytes) were
employed in suspension. The percentages of lympho-
cytes in the normal and MPO-deficient mononuclear
cell preparations were comparable, suggesting that
potential scavenging, or other, effects of lymphocytes
did not account for the observed differences in $O_2^\cdot$
production. This was further supported by two experi-
ments assessing $O_2^\cdot$ production by adherent mononu-
clear cells after the nonadherent lymphocytes had been
removed by vigorous washing (Table 3). As with
mononuclear cells in suspension, $O_2^\cdot$ production by
zymosan-treated MPO-deficient monocytes was
greater than that of similarly treated normal monocytes, and the presence of EPO on the zymosan surface decreased $O_2^-$ production by MPO-deficient cells following the initial 30 min of incubation.

**Iodination by Normal and MPO-Deficient Monocytes**

Optimum iodination by phagocytes requires both a product of the respiratory burst, i.e., $H_2O_2$ peroxidase, and as a result, might be expected to be depressed when MPO-deficient monocytes are employed. Such was found to be the case (Fig. 2A). Iodination by normal monocytes was evident 5 min following the addition of opsonized zymosan and increased to reach a level of 10 nmole/10⁷ cells in 30 min. In contrast, iodination by MPO-deficient monocytes was significantly depressed throughout the 30-min incubation period (1.9 nmole at 30 min; $p < 0.01$).

When opsonized zymosan coated with EPO was employed as the particle, iodination by normal monocytes was increased, reaching a level of 70 nmole/10⁷ cells at 30 min. The initial rate of iodination by MPO-deficient monocytes was comparable to that of normal monocytes with zymosan–EPO as the particle; iodination by both was evident at 2 min and reached the same level at 5 min. However, following this initial period, iodination by MPO-deficient cells continued at a considerably higher rate than that of normal cells, reaching a level at 30 min of 209 nmole/10⁷ cells. When zymosan coated with inactivated EPO was used as the stimulus, iodination by normal and MPO-deficient monocytes was comparable to that observed with zymosan (data not shown).

**Scavenging Systems in Monocytes**

Scavengers such as catalase and the glutathione cycle components may be important in the protection of phagocytes from damage by the oxygen products generated during the respiratory burst. As shown in Table 4, MPO-deficient and normal monocytes contained comparable levels of catalase, superoxide dismutase, and the glutathione cycle components ($p > 0.05$, MPO-deficient versus normal).

**DISCUSSION**

Previous studies had demonstrated that neutrophils from patients with complete hereditary MPO deficiency have an augmented respiratory burst as measured by oxygen consumption, $O_2^-$ production, $H_2O_2$ release, and hexose monophosphate shunt activity. The studies reported here demonstrate that these patients' blood monocytes, which also lack granule MPO, likewise have an increased respira-

---

**Table 3. Superoxide Production by Adherent Monocytes**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time (min)</th>
<th>Zymosan</th>
<th>Normal</th>
<th>MPO-def</th>
<th>Difference</th>
<th>Zymosan–EPO</th>
<th>Normal</th>
<th>MPO-def</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>268</td>
<td>287</td>
<td>19</td>
<td></td>
<td>613</td>
<td>690</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>594</td>
<td>613</td>
<td>19</td>
<td></td>
<td>715</td>
<td>690</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>642</td>
<td>633</td>
<td>9</td>
<td></td>
<td>993</td>
<td>705</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>314</td>
<td>299</td>
<td>15</td>
<td></td>
<td>403</td>
<td>377</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>620</td>
<td>570</td>
<td>50</td>
<td></td>
<td>882</td>
<td>615</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>675</td>
<td>622</td>
<td>53</td>
<td></td>
<td>1105</td>
<td>647</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

*Monolayers containing comparable numbers of normal or MPO-deficient monocytes were stimulated with zymosan or zymosan–EPO and the nmole O$_2$ produced/mg monolayer protein was determined as described in the text. Different subjects were used in each experiment.
Respiratory burst as measured by the rate, peak, and duration of $O_2^-$ production following the phagocytosis of opsonized zymosan.

Jandl et al., using 27,000-g neutrophil particles isolated at various periods after stimulation of the cells with zymosan, observed substantially increased $O_2^-$ forming capacity when particles were prepared in the presence of hemoprotein inhibitors or in the absence of oxygen. They concluded that the respiratory burst was terminated, in part, by the inactivation of the $O_2^-$ forming system by MPO. The modulation of the respiratory burst by MPO was further suggested by its ability to interact with stimulated normal neutrophils, although caution has been urged in the interpretation of such studies. We used EPO bound to opsonized zymosan to explore the relationship of peroxidase to the respiratory burst in monocytes.

We have previously shown that EPO, a strongly basic protein, adheres firmly to the surface of microorganisms with retention of peroxidatic activity. Further, mononuclear phagocytes can ingest peroxidase-coated organisms, and the exogenous peroxidase, which can be visualized histochemically in phagolysomes, can be utilized to enhance microbicidal activity. As shown here, EPO also binds to zymosan with retention of peroxidatic activity as indicated by its ability to catalyze the iodination reaction. When zymosan-coated with EPO was ingested by MPO-deficient monocytes, the respiratory burst more closely resembled that of normal cells; the initial rate of $O_2^-$ formation continued to be greater, but the peak level and the duration of the burst were not different from normal monocytes.

Azide forms a complex with the iron of hemoproteins, resulting in a loss of enzyme activity. In studies with catalase, it has been shown that the iron remains in the ferric form and that the complex is reversible. However, when $H_2O_2$, the heme-iron is reduced to the ferrous form, the azide is oxidized, and a product of that oxidation stabilizes the catalase in the ferrous form. If EPO reacts similarly, then irreversible inactivation on exposure to azide and $H_2O_2$ might be expected, since the enzymatic activity of peroxidases generally requires ferric iron. When EPO-coated zymosan particles were exposed to azide and a constant flux of $H_2O_2$ for 15 min, the EPO was inactivated, as measured by the absence of iodinating capacity. Inactivation was irreversible under our conditions. Using radiolabeled EPO, we were able to demonstrate that the inactivation of EPO was not an artifact due to detachment of the enzyme from the opsonized zymosan. When the particles with inactivated EPO on their surface were used to stimulate the respiratory burst of MPO-deficient monocytes, supranormal $O_2^-$ production was again observed. The burst of normal monocytes was comparable whether zymosan, zymosan coated with EPO, or zymosan coated with inactivated EPO was used, suggesting that the differences observed using MPO-deficient cells were not due to interference with the $O_2^-$ assay by EPO.

Iodination by normal phagocytes is largely dependent on the release of the granule MPO and the production of $H_2O_2$ by the respiratory burst; the two interact to oxidize iodide to a form that binds in covalent linkage to iodine acceptor groups of protein and possibly lipids. In addition, peroxidase-independent iodination mediated by hydroxyl radicals may occur. The neutrophils of patients with MPO deficiency have decreased iodination, as do MPO-deficient monocytes ingesting heat-killed Candida albicans. We confirm the decreased iodination by normal phagocytes is largely dependent on the release of the granule MPO and the production of $H_2O_2$ by the respiratory burst; the two interact to oxidize iodide to a form that binds in covalent linkage to iodine acceptor groups of protein and possibly lipids.

Further, mononuclear phagocytes can ingest peroxidase-coated organisms, and the exogenous peroxidase, which can be visualized histochemically in phagolysomes, can be utilized to enhance microbicidal activity. As shown here, EPO also binds to zymosan with retention of peroxidatic activity as indicated by its ability to catalyze the iodination reaction. When zymosan-coated with EPO was ingested by MPO-deficient monocytes, the respiratory burst more closely resembled that of normal cells; the initial rate of $O_2^-$ formation continued to be greater, but the peak level and the duration of the burst were not different from normal monocytes.

Azide forms a complex with the iron of hemoproteins, resulting in a loss of enzyme activity. In studies with catalase, it has been shown that the iron remains in the ferric form and that the complex is reversible. However, when $H_2O_2$, the heme-iron is reduced to the ferrous form, the azide is oxidized, and a product of that oxidation stabilizes the catalase in the ferrous form. If EPO reacts similarly, then irreversible inactivation on exposure to azide and $H_2O_2$ might be expected, since the enzymatic activity of peroxidases generally requires ferric iron. When EPO-coated zymosan particles were exposed to azide and a constant flux of $H_2O_2$ for 15 min, the EPO was inactivated, as measured by the absence of iodinating capacity. Inactivation was irreversible under our conditions. Using radiolabeled EPO, we were able to demonstrate that the inactivation of EPO was not an artifact due to detachment of the enzyme from the opsonized zymosan. When the particles with inactivated EPO on their surface were used to stimulate the respiratory burst of MPO-deficient monocytes, supranormal $O_2^-$ production was again observed. The burst of normal monocytes was comparable whether zymosan, zymosan coated with EPO, or zymosan coated with inactivated EPO was used, suggesting that the differences observed using MPO-deficient cells were not due to interference with the $O_2^-$ assay by EPO.

Iodination by normal phagocytes is largely dependent on the release of the granule MPO and the production of $H_2O_2$ by the respiratory burst; the two interact to oxidize iodide to a form that binds in covalent linkage to iodine acceptor groups of protein and possibly lipids. In addition, peroxidase-independent iodination mediated by hydroxyl radicals may occur. The neutrophils of patients with MPO deficiency have decreased iodination, as do MPO-deficient monocytes ingesting heat-killed Candida albicans. We confirm the decreased iodination by normal phagocytes is largely dependent on the release of the granule MPO and the production of $H_2O_2$ by the respiratory burst; the two interact to oxidize iodide to a form that binds in covalent linkage to iodine acceptor groups of protein and possibly lipids.
greater levels of intraphagosomal peroxidase. Rather, it seems probable that the supranormal iodination of MPO-deficient monocytes ingesting zymosan coated with EPO reflects greater \( \text{H}_2\text{O}_2 \) production. Studies with \( \text{O}_2^\cdot \) production indicated that when zymosan coated with EPO was employed as the stimulus, the initial rate of the respiratory burst of MPO-deficient monocytes remained high, although the peak and duration were depressed to levels comparable to those of normal monocytes. Iodination appeared to be a more sensitive measure of the difference in the respiratory burst between normal and MPO-deficient monocytes, since even with zymosan coated with peroxidase as the particle, a major difference was still observed.

The levels of endogenous scavengers of \( \text{O}_2^\cdot \) (superoxide dismutase) and \( \text{H}_2\text{O}_2 \) (catalase or the components of the glutathione cycle) were comparable in MPO-deficient and normal monocytes, suggesting that the increased recovery of these oxygen products from MPO-deficient cells was due to enhanced production rather than to depressed catabolism by these scavengers. A number of cell types adapt to increased exposure to oxygen or its products with an increase in the level of scavengers. However, adaptation by MPO-deficient monocytes to the increased respiratory burst by the development of increased levels of protective scavengers might not be expected, since the number of times that phagocytic cells undergo a respiratory burst during their lifetime is limited. Studies with MPO-deficient PMN have also indicated normal levels of endogenous scavenging systems.

These data support the concept that peroxidase plays a role in the termination of the respiratory burst of monocytes. Thus, (1) MPO-deficient monocytes have a greater peak and duration of the respiratory burst than do normal monocytes; (2) introduction of peroxidase into the phagosome at the time of stimulation changes the hypermetabolic characteristics of the respiratory burst of MPO-deficient cells to more closely resemble normal cells; and (3) inactivation of the bound peroxidase before ingestion restores the hypermetabolic characteristics of the MPO-deficient monocytes. Both MPO and EPO are capable of inactivating a wide variety of biologic mediators. The inactivation of granule enzymes by the MPO system has been proposed, based on their inactivation by normal, but not MPO-deficient, neutrophils during phagocytosis, but whether inactivation of the NADPH-oxidase by peroxidase occurs is unknown.

Toxic oxygen metabolites generated by phagocytic cells have been implicated in the microbicidal activity of these cells by both peroxidase-dependent and independent mechanisms. Patients with MPO deficiency, although perhaps being at greater risk for disseminated fungal disease, are generally free from the recurrent infections that plague patients with chronic granulomatous disease. The experiments described here and elsewhere, which demonstrate a supranormal respiratory burst by MPO-deficient phagocytes, suggest a mechanism by which the MPO-independent antimicrobial activity of these cells might be increased to partially compensate for the deficiency of MPO.

ACKNOWLEDGMENT

We wish to thank Ann Waltersdorph and W. Michael Weaver for expert technical assistance, and Caroline Wilson for excellent secretarial skills.

REFERENCES

Respiratory Burst in MPO-Deficient Monocytes


Increased respiratory burst in myeloperoxidase-deficient monocytes

RM Locksley, CB Wilson and SJ Klebanoff

Updated information and services can be found at:
http://www.bloodjournal.org/content/62/4/902.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml