Human Neutrophils Use the Myeloperoxidase-Hydrogen Peroxide-Chloride System to Chlorinate but Not Nitrate Bacterial Proteins during Phagocytosis*

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The generation of extracellular oxidants by neutrophils has been widely investigated, but knowledge about the chemical reactions that occur in the phagolysosome, the cellular compartment that kills pathogens, is more limited. One important pathway may involve the production of potent halogenating agents such as hypochlorous acid (HOCI) by the myeloperoxidase-hydrogen peroxide-halide system. However, explorations of the oxidation chemistry of phagolysosomes have been hampered by the organelle’s inaccessibility. To overcome this limitation, we recovered Escherichia coli that had been internalized by human neutrophils. We then analyzed the bacterial proteins for 3-chlorotyrosine, a stable marker of damage by HOCI. Mass spectrometric analysis revealed that levels of 3-chlorotyrosine in E. coli proteins increased markedly after the bacteria were internalized by human neutrophils. This increase failed to occur in E. coli exposed to neutrophils deficient in NADPH oxidase or myeloperoxidase, implicating H2O2 and myeloperoxidase in the halogenation reaction. The extent of protein chlorination by normal neutrophils paralleled bacterial killing. Our observations support the view that the phagolysosome of human neutrophils uses the myeloperoxidase-hydrogen peroxide-chloride system to chlorinate bacterial proteins. In striking contrast, human neutrophils failed to nitrate bacterial proteins unless the medium was supplemented with 1 mM nitrite, and the level of nitration was low. Protein chlorination associated with bacterial killing was unaffected by the presence of nitrite in the medium. Nitration required NADPH oxidase but appeared to be independent of myeloperoxidase, suggesting that neutrophils can nitrate proteins through a pathway that requires nitrite but is independent of myeloperoxidase.

The generation of oxidants by neutrophils is critical to host defenses against microbial pathogens (1, 2). Oxidant production begins with a cytoplasmic membrane-associated NADPH oxidase, which reduces molecular oxygen to superoxide. Dismutation of superoxide then yields hydrogen peroxide (H2O2), but both superoxide and H2O2 are relatively nontoxic to bacteria. However, activated neutrophils also secrete the heme protein myeloperoxidase, which constitutes ∼5% of cellular protein (3). Klebanoff (4) proposed more than 30 years ago that halogenating intermediates generated by this enzyme are of major importance in killing bacteria.

At plasma concentrations of halide, the major action of myeloperoxidase is to convert chloride (Cl−) to hypochlorous acid (HOCI), the most potent bacterial cytotoxin known to be generated by phagocytes (5–7).

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\text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{HOCI} + \text{H}_2\text{O} \quad (\text{Eq. 1})
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In vitro studies indicate that HOCI reacts with the tyrosine residues of proteins to form 3-chlorotyrosine (8–10). Because 3-chlorotyrosine is stable, it can serve as a marker for oxidative damage by ephemeral HOCI (11).

Recent studies have revealed that nitrite also is a substrate for myeloperoxidase (12). A system containing millimolar concentrations of nitrite, myeloperoxidase and H2O2 exhibits potent microbial activity against Escherichia coli (13). Activated neutrophils nitrate phenolic compounds by a pathway that requires nitrite, H2O2, and myeloperoxidase (12, 14–16). Both nitryl chloride and nitrogen dioxide radical have been proposed as the nitrating intermediates in this reaction. However, the physiological relevance of these reactions is not yet known.

Most of the work on the myeloperoxidase system of neutrophils has examined the generation of oxidants by cell-free or externally secreted enzyme. However, bacterial killing takes place in the phagolysosome, a specialized intracellular compartment of unknown composition (1, 2). Both myeloperoxidase and H2O2 are secreted into this compartment. A study using phagocytosable probes suggests that chlorination is important in this location, although nitration was undetectable in medium supplemented with very high concentrations of nitrite (15). These observations suggest that myeloperoxidase may promote different reactions in the phagolysosome than in the extracellular milieu.

Despite the key role of the phagolysosome in microbicidal activity, little is known about its chemical and enzymatic activities (1, 2). To further investigate the oxidative chemistry of the organelle, we devised a way to recover and chemically analyze bacteria that had been internalized. First, we incubated human neutrophils with E. coli in medium supplemented with human serum. Serum promotes opsonization and phagocytosis of the bacterium and inhibits a number of extracellular oxidative reactions (17–19). We then reisolated the bacteria that had been phagocytosed by the neutrophils. By analyzing the bacteria by isotope dilution gas chromatography/mass spec-
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**EXPERIMENTAL PROCEDURES**

**General Methods—**E. coli (ATCC 11775) were maintained as frozen (−80 °C) stock in trypticase soy broth (Difco) supplemented with 20% fetal calf serum. Periodically, aliquots were streaked on nutrient agar plates, grown overnight at 37 °C, and stored for up to 1 month at 4 °C. Single colonies were inoculated into tubes containing 10 ml of trypticase soy broth and incubated overnight with end-over-end tumbling at 37 °C. Bacteria were pelleted for 15 min at 2,500 × g and washed once or twice in 0.1 M sodium sulfate. Washed bacteria were suspended to the required turbidity at 540 nm in HHBSS (modified Hanks balanced salts without calcium, magnesium, bicarbonate, or phenol red, supplemented with 20 mM HEPES, pH 7.45), on ice, and used within 30 min of preparation. Human neutrophils were obtained from venous blood as described previously (20) and were suspended in ice-cold HHBSS at 5 × 10⁷ cells/ml. Donors with chronic granulomatous disease (CGD) (21–23) and hereditary complete myeloperoxidase deficiency (24) have been characterized previously. Glucose oxidase (Type VS from Aspergillus niger, Sigma) was used as received, and the supplier's specification of activity was assumed to be correct. Monochlorodimedon (Sigma) was prepared as a 10 mM stock solution in ethanol and stored at 4 °C. Phorbol myristate acetate (Sigma) was prepared as a 100 μg/ml stock solution in dimethyl sulfoxide and stored at −20 °C in aliquots that were used individually for experiments.

**Bacterial Killing—**Phagocytosis and killing assays were performed in 3 ml of HHBSS supplemented with 1 mM CaCl₂, 0.5 mM MgCl₂ and 10% (v/v) fresh autologous serum (25). Neutrophils (final concentration 5 × 10⁶ cells/ml) and bacteria (final concentration 2.5 or 5 × 10⁷ cells/ml) were added. Incubations were carried out in 4 ml of sterile polypropylene test tubes tumbled end-over-end at 37 °C. In some instances, bacteria were supplemented with additional 10 mM glucose without discernible effects on neutrophil microbicidal activity, and they were terminated by transferring the mixtures to an ice-water bath. The bacterial viability was determined by the pour-plate method (25). Per cent killing of bacteria by neutrophils was calculated as 100 × (C₁ − C₂)/C₁, where C₁ and C₂ represent the concentrations of viable E. coli at the beginning of the incubation and at sample time t/τ (C).

**Recovery of Phagocytosed Bacteria from Neutrophils—**Phagocytosed bacteria were released from neutrophils by lysing the neutrophils, but not the bacteria, with a hypertonic detergent solution. The lysate was enriched for bacterial proteins by differential and density gradient centrifugation. Incubation mixtures (3 ml) were transferred to a 50-ml conical bottom polystyrene tube containing 27 ml of 0.1% Triton X-100 and vortexed vigorously for 1 min at 37 °C to lyse neutrophils. Samples of the detergent lysate (20 μl) were removed for determination of neutrophil-mediated bacterial killing (26). The remaining lysate was centrifuged for 5 min at 400 × g at room temperature to remove intact neutrophils, nuclei, and cellular debris. The supernatant was centrifuged for 30 min at 27,000 × g at 4 °C to recover bacteria. The bacterial pellet was resuspended in 200 μl of distilled water, layered onto a 1-ml cushion of 20% sucrose in a microcentrifuge tube, and centrifuged for 5 min at 16,000 × g. The supernatant layers were aspirated, and the bacterial pellets were resuspended in 200 μl of distilled water and stored at −20 °C for subsequent analysis.

**Purity of Recovered Bacterial Proteins—**Bacterial proteins were isolated from neutrophils as described above, using bacteria that were cultured in medium supplemented with 2.5 μM/ml of ubiquitously labeled l-[14C]amino acids (Amersham Biosciences). The specific activity of protein recovered when bacteria were incubated with neutrophils plus serum was compared with that from bacteria incubated under neutrophil-free conditions. The abundance of reisolated bacterial proteins was estimated to be 42% of total protein (35 and 49%) in two independent analyses.

**Amino Acid Isolation and Derivatization—**Neutrophils, E. coli, or reisolated E. coli were thawed and delipidated three times with methanol-water-washed ether (7:3, v/v). Following the addition of internal standards (50 nmol of L-[1-14C]tyrosine, 30 pmol of 3-nitro[13C6]tyrosine, and 30 pmol of 3-chloro[13C6]tyrosine), the protein pellet was resus- pended in 0.5 ml of HBr (48%) containing 1% phenol and heated at 110 °C for 20 h (27). The acid hydrolysate was dried under N₂ and brought up in 50 mM ammonium acetate (pH 7–8). The solution was added to anion exchange resin (AG1-X8; BioRad)/10 mM ammonium acetate (1:1, w/w) and heating for 1 h at 70 °C. Samples were stored at room temperature for up to 48 h. They were then evaporated to dryness under N₂, and the heptafluorobutyryl derivatives were prepared with reaction of heptafluorobutyric acid with L-amino acids (9, 30, 70 °C). Gas Chromatography/Mass Spectrometry (GC/MS)—The gas chromatograph was a Varian 3400 equipped with a DB-17 column (30 m, 0.2 μm i.d., 0.25 μm methyl silicone film coating, from P. J. Cobert, St. Louis, MO). Mass spectra were acquired on a Finnigan SSQ 7000 Series mass spectrometer (San Jose, CA) operated in the negative ion electron capture mode (27). The ions used for detecting analyte and internal standard were: tyrosine, m/z 417 and 423 ions; 3-chloro- tyrosine, m/z 451 and 457 ions; 3-nitrotyrosine, m/z 762 and 768 ions. Samples were injected into the GC/MS system with a 20:1 split. The injector temperature was 250 °C, and the transfer lines were maintained at 250 °C. The GC oven was maintained at 150 °C for 5 min after the injection and was then increased at a rate of 20 °C/min to a final temperature of 270 °C. Emission current was set at 300 μA, the electron energy was 100 eV, and the source temperature was 200 °C. The reagent gas was methane.

**Effect of Serum on Neutrophil-mediated Chlorination—**Chlorination was measured as loss of absorbance at 290 nm when monochlorodimedon was chlorinated to the dichloro compound (ε = 19,000 M⁻¹ cm⁻¹) (29, 30). Neutrophils (5 × 10⁶ cells) were incubated in 1 ml of HHBSS with 0.5 mM CaCl₂, 0.5 mM MgCl₂, and 35 μM monochlorodimedon. The cells were activated by adding a particulate stimulus (zymosan 4 mg/ml, 15 min incubation at 37 °C (30, n = 3) or a soluble stimulus (phorbol myristate acetate, 100 mg/ml from a 100 μg/ml stock in dimethyl sulfoxide, 10 min incubation at 37 °C, n = 2). Zymosan (40 mg/ml HHBSS) was suspended by vigorous homogenization, opsonized for 30 min at 37 °C with an equal volume of fresh human serum, washed twice by centrifugation in HHBSS, and resuspended to the original volume in HHBSS. Where indicated, 100 μl of complement-inactivated serum (56 °C, 30 min) replaced an equal volume of HHBSS. Reactions were terminated by transferring the mixtures with an ice-water bath. Samples (4 °C) were supplemented with an increment of 100 μl HHBSS or complement-inactivated serum, as appropriate, to ensure that all samples contained equal amounts of serum during subsequent analysis. Particulate material (zymosan and zymosan) was removed by centrifugation (30 s at 11,000 × g, room temperature). Supernatants were dialyzed 2-fold with HHBSS, and absorbance at 290 nm was determined. Absorbance of samples was corrected using blanks containing no monochlorodimedon.

**Quantifying Phagocytosis—**Opsonized bacteria were incubated with neutrophils for 30 min or 2 h at 37 °C as described above. Samples were diluted 10-fold with HHBSS or 0.1% Triton X-100 and vortexed vigorously. Then 0.1 ml of each vortexed sample was diluted 5-fold in HHBSS and deposited by centrifugation onto a charged glass microscope slide (ProbeOn Plus, Fisher Biotech). Air-dried slides were heat-fixed and stained briefly (30 s) with 0.5% safranin. The number of internalized bacteria (200 neutrophils) was determined by light microscopy. After removing the labeled (as described above) and unlabeled bacteria were incubated with neutrophils for 120 min. The fraction of radiolabel that sedimented during low-speed centrifugation (400 × g × 5 min) was used as an index of phagocytosis by neutrophils. Under these conditions, 75 ± 4% of the radiolabel was recovered in the cellular pellet. Only 21 ± 19% of the label sedimented when bacteria were incubated with serum alone (n = 4).

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1 The abbreviations used are: HHBSS, Hanks’ balanced salt solution without calcium, magnesium or phenol red, supplemented with 20 mM HEPES pH 7.45; CGD, chronic granulomatous disease; GC, gas chromatography; MS, mass spectrometry.
RESULTS

We incubated human neutrophils with E. coli for 2 h at 37 °C in HBBSS at a bacteria:neutrophil ratio of 5–10 to 1. The medium was supplemented with 10% autologous serum to promote opsonization and phagocytosis of the bacteria (Fig. 1a) (20, 25) and to inhibit HOCl-mediated chlorination of extracellular bacteria (31). Under these conditions, 10% serum strongly inhibited zymosan-stimulated chlorination of monochlorodimedon (30). Thus, in the absence of serum, 5 × 10^6 zymosan-stimulated neutrophils generated 19 ± 3 nmol dichlorodimedon (mean ± S.E. for three independent experiments)/15 min. This value fell to 3 ± 0.3 nmol/15 min when 10% serum was present. When the neutrophils were exposed to phorbol myristate acetate, a soluble agent that stimulates monochlorodimedon chlorination by neutrophils, serum was completely inhibitory (33 nmol dichlorodimedon/10 min in the absence of serum versus 0 nmol/10 min in the presence of serum, n = 2).

Neutrophils rapidly internalized E. coli in the presence of serum. Quantification by light microscopy demonstrated that >95% of the bacteria were internalized by the neutrophils within 30 min. Analysis of phagocytosed radiolabel by differential centrifugation indicated that, after a 2-h incubation, 80% of the bacteria were associated with neutrophils. Light microscopy of the preparations suggested that this decrease probably resulted from lysis of phagocytes engorged with bacteria (not shown). Serum-mediated phagocytosis of E. coli was associated with the expected reduction in microbial viability (5.1 ± 2.5% of the initial inoculum; n = 18).

To isolate phagocytosed bacteria, we lysed neutrophils with hypotonic 0.1% Triton X-100 detergent. Importantly, this procedure did not affect the viability of E. coli, indicating that the bacteria remained intact. Unlysed neutrophils and cellular debris were removed by low-speed centrifugation, and bacteria were recovered from the supernatant by high-speed centrifugation. Light micrographs of E. coli isolated by this procedure revealed that ~80% of the bacteria were associated with neutrophils (i.e. they had been phagocytosed) after a 2-h incubation in the presence of serum (Fig. 1a). Lysing the cells with detergent resulted in bacteria that were essentially free of intact neutrophils (Fig. 1b). When these bacteria were isolated by sequential low- and high-speed centrifugation, there was little morphologic evidence of neutrophil proteins (Fig. 1c). Evaluating the purity of the preparation by using radiolabeled bacteria indicated that 42% (n = 2) of the recovered protein was bacterial in origin.

To determine whether 3-chlorotyrosine was present in phagocytosed bacteria, we assayed E. coli that had been prepared from neutrophils by detergent lysis and differential centrifugation as described above. When amino acids from acid hydrolysates of bacteria were isolated and derivatized with n-propanol and heptafluorobutyric anhydride and then analyzed by GC/MS in the negative ion electron capture mode, we detected a compound that exhibited major ions and retention time identical to authentic 3-chlorotyrosine. Selected ion monitoring demonstrated that the ions derived from the amino acid co-eluted with ions derived from authen-

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**Fig. 1.** Light microscopy of E. coli incubated with neutrophils. E. coli cells were incubated for 2 h at 37 °C with human neutrophils in the presence of 10% autologous fresh serum. a, micrograph of bacteria at the end of the incubation. b, micrograph of bacteria lysed with 0.1% Triton X-100. Most bacteria are free of neutrophils, but neutrophil debris can be seen. c, micrograph of bacteria isolated by sequential low- and high-speed centrifugation. Bacteria are highly concentrated, and only occasional neutrophil debris is visible. Incubation and staining conditions were as described under “Experimental Procedures.”

**Fig. 2.** Detection of n-propyl heptafluorobutyryl derivative of 3-chlorotyrosine in proteins of bacteria by negative ion electron capture GC/MS with selected ion monitoring. Neutrophils isolated from a control subject (top), a CGD subject (middle), or a myeloperoxidase-deficient subject (bottom) were incubated with E. coli as described in Fig. 1. The bacteria were then recovered from detergent-treated neutrophils, delipidated, and subjected to acid hydrolysis. Amino acids in the hydrolysate were isolated, reduced, derivatized, and subjected to isotope dilution GC/MS analysis. Note that the ion current is approximately the same (~10 × 10^6) for the internal standard (3-chloro[13C4]tyrosine) in all of the analyses. In contrast, the ion current of 3-chlorotyrosine varies markedly in bacteria isolated from control (14 × 10^6), CGD (0.7 × 10^6), and myeloperoxidase-deficient (1.3 × 10^6) neutrophils.

**Statistical Analysis**—Simultaneous comparisons among several groups were performed by one-way analysis of variance using Dunnet’s method to compare groups (SigmaStat, Jandel Scientific, San Rafael, CA). Comparison between two groups was performed using Student’s t test. A p value of <0.05 was considered significant.
neutrophils alone were incubated with serum for 2 h and myeloperoxidase-deficient neutrophils, suggesting that H₂O₂
teins of bacteria isolated from control, CGD, and
30466 experiments.

3-chlorotyrosine (3CT) and 3-nitrotyrosine (3NT) were
determined by negative ion electron capture GC/MS with selected ion monitoring for bacterial proteins by human neutrophils.

Reisolated bacterial proteins were analyzed by isotope dilution GC/MS. After a 2-h incubation, bacteria that had been fed to neutrophils and immediately reisolated exhibited 1170 ± 180 μmol of 3-chlorotyrosine/mol of tyrosine (n = 6) (Fig. 3). This was 8 times the level (150 ± 150 μmol of 3-chlorotyrosine/mol of tyrosine; n = 4) seen when neutrophils alone were incubated with serum for 2 h and neutrophils from patients with CGD (CGD PMN + EC), or hereditary complete myeloperoxidase deficiency (~MPO PMN + EC). Results are the mean ± S.E. of n determinations.

Almost 6 times the level (200 ± 150 μmol of 3-chlorotyrosine/mol of tyrosine; n = 6) seen when E. coli alone were incubated under the same conditions (Fig. 3). Moreover, the time course and extent of 3-chlorotyrosine formation correlated strongly with the time course and extent of bacterial killing (r = 0.83; two independent experiments) (Fig. 4). However, when neutrophils from patients with CGD (n = 3) or hereditary complete myeloperoxidase deficiency (n = 3) were substituted for normal neutrophils, the level of 3-chlorotyrosine in proteins isolated from phagocytosed E. coli were similar to those observed in E. coli incubated without neutrophils or normal neutrophils incubated without bacteria (Fig. 3).

We used isotope dilution GC/MS to estimate the relative susceptibility of bacterial and neutrophil proteins to chlorination. Bacterial proteins were labeled with L-[13C₆]tyrosine by growing bacteria in medium containing the amino acid. The bacteria were then incubated with serum alone or with neutrophils under conditions described in Fig. 1. Using isotope dilution GC/MS, we then quantified the levels of 3-chlorotyrosine/tyrosine and 3-chloro[13C₆]tyrosine/[13C₆]tyrosine in total protein from pellets obtained after centrifugation for 5 min at 2500 × g. When bacteria were incubated in serum alone, there was no detectable chlorination of bacterial tyrosines. When bacteria were phagocytosed by neutrophils, bacterial tyrosines were chlorinated to 2530 μmol/mol of tyrosine. Chlorination of the general tyrosine pool, predominantly neutrophil, was 430 μmol/mol of tyrosine. Assuming that bacterial proteins were 42% pure after detergent lysis and differential centrifugation (“Experimental Procedures”), we estimate that at least 85% of the chlorotyrosine measured in experiments described in the tables and figures was of bacterial origin. When bacteria were incubated under nonphagocytic conditions (complement-inacti-
vated serum) with neutrophils activated for oxidative metabolism with phorbol myristate acetate, the extent of chlorination of bacterial tyrosines was just one-sixth (500 μmol/mol) of that for phagocytosed bacteria.

Neutrophils isolated from patients with CGD fail to generate substantial amounts of H2O2 in response to a phagocytic stimulus (1, 32, 33). We therefore determined whether H2O2 generated continuously by glucose and glucose oxidase would allow CGD neutrophils to chlorinate bacterial proteins (25, 34). When we provided this system to normal neutrophils that were ingesting E. coli, the level of 3-chlorotyrosine in the bacterial proteins increased 3-fold (Table I). Under the same conditions, CGD neutrophils increased 3-chlorotyrosine production from a negligible level to one that matched the elevated level of normal neutrophils.

In contrast to 3-chlorotyrosine, 3-nitrotyrosine did not become significantly enriched in the proteins of bacteria exposed to human neutrophils (Fig. 4). Levels of 3-nitrotyrosine were similar in E. coli alone, neutrophils alone, or neutrophils mixed with E. coli (Table II). Similarly, replacing normal neutrophils with those from patients with CGD or hereditary complete myeloperoxidase deficiency failed to decrease tyrosine nitration (Table II). When opsonized bacteria were exposed to normal neutrophils in medium supplemented with 1 mM sodium nitrite, however, a modest increase in tyrosine nitration was observed (p < 0.05, analysis of variance). CGD neutrophils failed to nitrate bacterial proteins when the medium was supplemented with 1 mM nitrite. In contrast, under these conditions myeloperoxidase-deficient and control neutrophils nitrated bacterial proteins to a similar degree.

**DISCUSSION**

The generation of extracellular oxidants by neutrophils has been widely investigated, but the chemical and enzymatic reactions of the phagolysosome, the principal site of microbial killing, are less well understood because of the relative inaccessibility this compartment. To address this limitation, we recovered bacteria that had been internalized by human neutrophils and subjected them to viability tests and chemical analyses.

In the expected fashion, serum-opsonized E. coli that were ingested by neutrophils progressively lost viability (25). Their demise correlated with a marked increase in the formation in their proteins of 3-chlorotyrosine, a marker for oxidation by HOCl. There was a strong correlation between protein chlorination and bacterial death, although the issue of whether chlorination contributes to microbial activity is not resolved. The increase in 3-chlorotyrosine required both bacteria and neutrophils. It also was dependent upon myeloperoxidase and H2O2 because 3-chlorotyrosine levels failed to rise in bacteria exposed to neutrophils isolated from patients with hereditary myeloperoxidase deficiency or CGD. Two observations suggest that H2O2 was the limiting factor in halogenation. First, the level of protein-bound 3-chlorotyrosine tripled in E. coli exposed to normal neutrophils and glucose plus glucose oxidase, a peroxide-generating system. Second, CGD neutrophils chlorinated E. coli proteins as well as normal human neutrophils did when they were supplemented with the same system. Collectively, these observations indicate that human neutrophils use myeloperoxidase and H2O2 to chlorinate bacterial proteins in the phagolysosome. There was a strong association between protein chlorination and bacterial killing, consistent with in vitro studies showing that HOCl is a potent microbicidal agent.

Neutrophils failed to nitrate bacterial tyrosine when the medium was not supplemented with extra nitrite. With 1 mM nitrite supplementation, however, the level of 3-nitrotyrosine in bacterial proteins increased 3-fold. Protein tyrosine nitration appeared to require H2O2 because the level of 3-nitrotyrosine failed to rise in bacteria exposed to CGD neutrophils. In contrast, myeloperoxidase deficiency had little effect on protein nitration. It is important to note that neutrophils from our patient with hereditary complete myeloperoxidase deficiency are able to mount a prolonged respiratory burst in response to a phagocytic challenge but are completely devoid of myeloperoxidase (24). These observations suggest, for the first time, that human neutrophils nitrate bacterial proteins in the phagolysosome by a pathway that requires H2O2 and nitrite but not myeloperoxidase.

Our results indicate that halogenation reactions catalyzed by myeloperoxidase are more important than nitration reactions for modifying bacterial proteins within the neutrophil phagolysosome. Although neutrophils appear to support modest amounts of protein tyrosine nitration, this reaction requires relatively large amounts of nitrite in the extracellular medium. The amount that would actually enter the phagosome is unknown, but the volume of extracellular fluid incorporated into this compartment is likely to be small (35, 36). The low levels of nitration that we observed with E. coli are consistent with theoretical calculations (37-39) and with the observations of Jiang et al. (15, 40), who used fluoresceinated polystyrene beads to trap chlorinating and nitrating agents formed within phagocytic vacuoles of neutrophils. It is noteworthy that nitration requires H2O2 and nitrite but not myeloperoxidase, raising the possibility that pathways not involving myeloperoxidase enable phagocytes to generate low levels of reactive nitrogen species in vivo.

Chapman et al. (41) used *Staphylococcus aureus* labeled with [13C6]tyrosine and GC/MS detection to demonstrate that neutrophils produce 3-chloro[13C6]tyrosine, providing the first compelling evidence that HOCl generated by myeloperoxidase reacts with bacterial proteins in the phagolysosome. Interestingly, and in contrast to our findings, chlorinated neutrophil proteins accounted for more than 90% of total chlorinated tyrosine residues formed during phagocytosis (41). Presumably, much of this chlorinated neutrophil protein was removed in our

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**Table II**

**Influence of nitrite on nitration of bacterial tyrosine by neutrophils**

| Condition | 3-Nitrotyrosine<sup>a</sup> | Fold change |
|-----------|----------------|-------------|
| Normal neutrophils plus E. coli | 148 ± 25 (15) | 2.9<sup>b</sup> |
| Chronic granulomatous disease neutrophils plus E. coli | 172 ± 78 (4) | 1.2 |
| Myeloperoxidase-deficient neutrophils plus E. coli | 88 ± 50 (3) | 3.4<sup>b</sup> |
| E. coli only | 122 ± 91 (6) | 0.8 |
| Neutrophils only (all types) | 77 ± 35 (4) | 1.9 |

<sup>a</sup> Results are the mean ± S.E. of n (in parentheses) determinations.

<sup>b</sup> p < 0.05 comparing “No supplement” with “1 mM NaNO2.”

<sup>c</sup> Results from normal, chronic granulomatous disease, and myeloperoxidase-deficient neutrophils were pooled.
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A protocol for bacterial recovery as described in Fig. 1. Further, we have recently identified elevated concentrations of 3-chloro-tyrosine by mass spectrometric analysis of peritoneal inflammatory fluid from septic wild-type mice. Levels of 3-chloro-tyrosine did not rise in septic myeloperoxidase-deficient mice, mice that are more susceptible than wild-type to mortality from polymicrobial infection (42). The correlation between chlorination and microbicidal activity strongly supports the hypothesis, first proposed by Klebanoff (4) more than 30 years ago, that halogenation reactions executed by myeloperoxidase play a key role when neutrophils kill bacterial pathogens.

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REFERENCES

1. Klebanoff, S. J., and Clark, R. A. (1978) The Neutrophil, North-Holland Publishing Co., Amsterdam
2. Hurst, J. K., and Barrette, W. C., Jr. (1989) CRC Crit. Rev. Biochem. Mol. Biol. 24, 271–328
3. Bainton, D. F., Ullrey, J. L., and Farquhar, M. G. (1971) J. Exp. Med. 134, 907–934
4. Klebanoff, S. J. (1987) J. Exp. Med. 166, 1063–1078
5. Harrison, J. C., and Schultz, J. J. (1976) J. Biol. Chem. 251, 1371–1374
6. Foote, C. S., Goyne, T. E., and Lehrer, R. I. (1983) Nature 304, 715–716
7. Albrich, J. M., McCarthy, C. A., and Hurst, J. K. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 210–214
8. Domigan, N. M., Charlton, T. S., Duncan, M. W., Winterbourn, C. C., and Kettle, A. J. (1995) J. Biol. Chem. 270, 16542–16548
9. Hazen, S. L., Hsu, F. F., Mueller, D. M., Crowley, J. R., and Heinecke, J. W. (1990) J. Clin. Invest. 86, 1283–1289
10. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2075–2081
11. Heinecke, J. W. (1999) FASEB J. 13, 1113–1120
12. van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1999) J. Biol. Chem. 272, 7617–7620
13. Klebanoff, S. J. (1993) Free Radic. Biol. Med. 14, 351–360
14. Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B., and van der Vliet, A. (1996) J. Biol. Chem. 271, 19199–19208
15. Jiang, Q., and Hurst, J. K. (1997) J. Biol. Chem. 272, 32767–32772
16. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1998) Nature 391, 393–397
17. Koush, P., Gressier, B., Luyckx, M., Brune, C., Dine, T., Cazin, M., and Cazin, J. C. (1999) Fimraco (Rome) 54, 695–699
18. Dularay, B., Yao, C. M., and Elson, C. J. (1991) Am. Rheum. Dis. 50, 383–388
19. Wasil, A., Halliwell, B., Hutchison, D. C., and Baum, H. (1987) Biochem. J. 243, 219–223
20. Rosen, H., Michel, B. R., and Chait, A. (1991) J. Immunol. Methods 144, 117–125
21. Dinauer, M. C., Curnutte, J. T., Rosen, H., and Orkin, S. H. (1989) J. Clin. Invest. 84, 2012–2016
22. Hurt, J. K., Loehr, T. M., Curnutte, J. T., and Rosen, H. (1991) J. Biol. Chem. 266, 1627–1634
23. Heyworth, P. G., Curnutte, J. T., Nauseef, W. M., Volpp, B. D., Pearson, D. W., Rosen, H., and Clark, B. A. (1991) J. Clin. Invest. 87, 352–356
24. Rosen, H., and Klebanoff, S. J. (1976) J. Clin. Invest. 58, 60–69
25. Rosen, H., and Michel, B. R. (1997) Infect. Immun. 65, 4173–4178
26. Klebanoff, S. J., Waltersdorph, A. M., and Rosen, H. (1984) Methods Enzymol. 105, 399–403
27. Heinecke, J. W., Hsu, F. F., Crowley, J. R., Hazen, S. L., Leeuwenburgh, C., Mueller, D. M., Rasmussen, J. E., and Turk, J. (1999) Methods Enzymol. 309, 124–144
28. Crowley, J. R., Yarasheski, K., Leeuwenburgh, C., Turk, J., and Heinecke, J. W. (1998) Anal. Biochem. 259, 127–135
29. Hager, L. P., Morris, D. R., Brown, F. S., and Eberwein, H. (1966) J. Biol. Chem. 241, 1709–1777
30. Kettle, A. J., and Winterbourn, C. C. (1991) Free Radiac. Res. Commun. 12–13 Pt 1, 47–52
31. Hazen, S. L., Gauth, J. P., Hsu, F. F., Crowley, J. R., d’Avincenzo, A., and Heinecke, J. W. (1997) J. Biol. Chem. 272, 16990–16998
32. Babior, B. M., Kipnes, R. S., and Curnutte, J. T. (1973) J. Clin. Invest. 52, 741–744
33. Babior, B. M. (1999) Blood 93, 1464–1476
34. Baehner, R. L., Nathan, D. G., and Karnovsky, M. L. (1970) J. Clin. Invest. 45, 865–870
35. Stossel, T. P., Pollard, T. P., Mason, R. J., and Vaughan, M. (1971) J. Clin. Invest. 50, 1745–1757
36. Rozenberg-Arsko, M., Salters, M. E., van Strijp, J. A., Guze, J. E., and Verhoeff, J. (1983) Infect. Immun. 65, 852–859
37. Lymar, S. V., and Hurst, J. K. (1998) Chem. Res. Toxicol. 11, 714–717
38. Lymar, S. V., and Hurst, J. K. (1995) J. Am. Chem. Soc. 117, 8867–8888
39. Lymar, S. V., and Hurst, J. K. (1995) Chem. Res. Toxicol. 8, 833–840
40. Jiang, Q., Griffin, D. A., Barosky, D. F., and Hurst, J. K. (1997) Chem. Res. Toxicol. 10, 1080–1089
41. Chapman, A. L., Hampton, M. B., Senthilmohan, R., Winterbourn, C. C., and Kettle, A. J. (2002) J. Biol. Chem. 277, 9757–9762
42. Gauth, J. P., Yeh, G. C., Tran, H. D., Byun, J., Henderson, J. P., Richter, G. M., Belaaoua, A., Hotchkiss, R. S., and Heinecke, J. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11961–11966
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