Green Fluorescent Protein-Expressing *Escherichia coli* as a Selective Probe for HOCl Generation within Neutrophils†

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ABSTRACT: *Escherichia coli* were transformed by electroporation to introduce a plasmid harboring a GFP gene-containing vector. The fluorescence of the purified GFP isolated from the transformant was quenched by myeloperoxidase (MPO)-generated HOCl, by peroxynitrous acid (ONOOH) and by enzymatically or radiolytically generated $NO_2^{\cdot}$ but not by other putative neutrophil-generated oxidants. Fluorescence from the bacterium was effectively quenched by HOCl but not peroxynitrite, oxidizing radicals derived from its O−O bond homolysis, or the other oxidants under study. Exposure of serum-opsonized bacteria to human neutrophils resulted in extensive loss of GFP fluorescence; fluorescence microscopy revealed that phagocytosed bacteria were completely quenched but that bacteria remaining in the external media were unquenched. Addition of sodium azide to the medium to inhibit MPO prevented neutrophil-mediated fluorescence quenching. Because the amount of HOCl required to inhibit bacterial fluorescence was an order of magnitude greater than required to inhibit colonial growth, these results imply that sufficient HOCl was formed within the neutrophil phagosome to kill the microbe.

Neutrophils undergo dramatic physiological changes in response to agonist binding at specific external receptor sites, prominent among which are activation of a dormant respiratory chain (the NADPH oxidase), phagocytosis of adherent particles, and degranulation, i.e., migration and assimilation of cytosolic lysosomal particles within the developing phagosomal vacuole (*1*). Among the granule components is the enzyme myeloperoxidase (MPO),[1] which is estimated to be as much as 5% of the total cellular mass (*2*). In the conventional view of leukocyte function, MPO is assigned the role of enzymatic generation of the potent microbicide (*3*) hypochlorous acid (HOCl) from the reaction between Cl^- and NADPH oxidase-generated O_2^- and/or H_2O_2 (*4*, *5*). Indirect evidence supporting this viewpoint is the general observations that cell-free MPO−H_2O_2−Cl^- enzymatic systems are universally microbicidal (*1*, *3*) and that many microorganisms are killed less efficiently by MPO-deficient than by normal neutrophils (*3*). In one quantitative study, selective inhibitors of MPO and the NADPH oxidase were used in conjunction with superoxide dismutase-conjugated *Staphylococcus aureus* to show that the major pathway for leukocytic killing was both respiration- and MPO-dependent (*6*). Furthermore, studies in our laboratory utilizing fluorescein-conjugated microspheres to trap HOCl as chlorofluorescein products have indicated that HOCl is indeed generated at microbicidal levels within the neutrophil phagosome following stimulation of the NADPH oxidase (*7*). Collectively, these data support the assigned biological function of MPO as a catalyst for HOCl generation from respiration-derived reactive oxygen species.

This model of microbicidal action in neutrophils has been challenged by Segal and co-workers who, in recent provocative papers (*8*, *9*), have suggested that the true function of the NADPH oxidase is to polarize the phagosomal membrane by electrogenic transmembrane electron transport, thereby driving an influx of K^+ ion. Elevation of the internal K^+ concentration levels, in turn, activates granule-derived proteases that are the causative microbicidal agents. In this model, the primary assigned role of MPO within the phagosome is to catalyze H_2O_2 disproportionation, i.e., to function as a catalase, thereby minimizing H_2O_2-mediated oxidative inactivation of the proteases. Thus, despite the need for respiratory activation, the actual microbicidal mechanisms are viewed as nonoxidative in character, and the *in vitro* oxidative microbicidal reactions that have been described for HOCl and cell-free MPO−H_2O_2−Cl^- enzymatic systems (*3*) are unimportant within the phagosomal microenvironment. However, this model as described (*8*) appears inconsistent with our studies utilizing artificial particulate fluorescein probes in which substantial HOCl formation within the phagosome was demonstrated (*7*). To further probe HOCl involvement in physiologically relevant systems, two groups have recently examined the extent of formation and distribution of chlorinated tyrosines in bacterial and neutrophil-derived proteins following phagocytic killing.[2] From their studies (*13*), Winterbourn and associates concluded that the bulk of intraphagosomally generated HOCl reacts with neutrophil proteins, rather than entrapped bacteria, implying that HOCl-mediated killing might be relatively unimportant. The opposite conclusion was drawn by Rosen, Heinecke,

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[1] Abbreviations: MPO, myeloperoxidase; GFP, green fluorescent protein; HBSS, Hank's Balanced Salt Solution; LB, Luria broth; NOS, nitric oxide synthase; PBS, phosphate-buffered saline.

[2] Although tyrosine is relatively unreactive toward HOCl and chloramines, 2-chlorotyrosine and 2,5-dichlorotyrosine are thought to be selective markers for these chlorinating agents in biological systems (*10*−*13*).

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and co-workers from similar studies that utilized a different microbe *(Escherichia coli* versus *S. aureus*), who reasoned that the extent of chlorination of bacterial protein tyrosyl groups attending phagocytic killing was consistent with the accepted oxidative model (*14*).

To aid in resolving these issues, we have constructed an intraphagosomal bacterial probe by expressing green fluorescent protein (GFP) within the cytosol of a plasmid-bearing *E. coli*; this study was prompted by results from preliminary screening with various putative phagocyte-generated oxidants that had established that isolated GFP was highly sensitive to HOCl-induced loss of fluorescence. The capacity for fluorescence detection provided by GFP allows sensitive monitoring of both the temporal course of any damage occurring to the protein and the topographic location of bacteria relative to the neutrophil in mixed-cell populations. As reported herein, GFP fluorescence losses following exposure of this microbe to respiring human neutrophils occurred only within the phagosome and were MPO-dependent. Because *in vitro* experiments established that considerably greater amounts of HOCl were required to quench fluorescence than were needed to kill the bacterium, one infers that microbicidal amounts of HOCl are generated within the phagosome.

EXPERIMENTAL PROCEDURES

Materials. A plasmid harboring the pMEK91 GFP gene-containing vector (*15*) was transformed into a capsular serum-resistant strain of *E. coli* (ATCC 11775) by electroporation at 2.5 kV using a Biorad MicroPulser apparatus; explicit procedures are described in the Biorad manual (catalog number 165-2100). Transformed cells were selected by growth on kanamycin-containing nutrient agar plates; the construct readily expressed GFP without induction. The GFP plasmid-containing *E. coli* were subsequently grown on Luria broth (LB) at 37 °C and were replated every 2 weeks to maintain healthy colonies. For daily experiments, cells were grown from isolated colonies on the nutrient plate in two steps. An overnight culture (∼10 h) in 25 mL of LB was first grown at 37 °C, followed by a 100-fold dilution in the same medium; subsequent growth was monitored turbidimetrically at 540 nm using a HP 1452A diode array spectrophotometer. For most experiments, growth was stopped in mid-log phase (corresponding to an OD_{540} = 0.65−0.85), which was achieved in ∼3 h. Cells were harvested by centrifugation, washed once in 50 mM phosphate at pH 7.4, and resuspended in the same buffer. The final suspension was kept on ice and used immediately following harvesting. To isolate recombinant GFP, cells in suspension were ruptured by freeze−thaw cycling and the protein was isolated by ammonium sulfate precipitation followed by a series of chromatographic separations. The specific procedures followed closely a previously published method (*16*) with the following exceptions: DNase was included in the original cell suspension buffer, which was 20 mM Tris; an octyl Sepharose 4 B column was used for the first chromatographic step; the GFP-containing fractions were dialyzed against 5 mM Tris at pH 8.0 prior to the second chromatographic step, which, in this case, was gel filtration on Sephacryl S-100. Purity was confirmed by PAGE and MALDI−TOF mass spectrometry, which gave a single peak at 26 660 Da, corresponding to an estimated mass for monomeric GFP of ∼27 kDa (*16*). The A_{488}/A_{280} absorbance ratio of 1.3 was also consistent with ratios measured for GFP purified from other sources (*16*). The amount of GFP in the final reagent solution was 1.4 mg/mL, as determined by the Biorad protein assay, corresponding to [GFP] 50 μM. Neutrophils were isolated from EDTA-anticoagulated blood from individual donors by dextran sedimentation and centrifugation through Histopaque 1077 using specific protocols developed by Dr. Henry Rosen (University of Washington Medical School, Seattle, WA) (*17*). The purified neutrophils were suspended in Hank's Balanced Salt Soution (HBSS) buffered with 20 mM HEPES at pH 7.4 and kept on ice until use, which was within 1 h following isolation; cell densities were determined by counting on a hemocytometer slide using 0.1 mg/mL methylene blue in 3% acetic acid as a visual aid. Myeloperoxidase was isolated from bovine spleens by column chromatography (*18*); the purified enzyme had an RZ value of 0.70 and specific activity of 340 units/mg of protein as measured by the guaiacol assay (*19*). HOCl was purified by vacuum distillation from commercial bleach solutions after adjusting the pH to 7−8 with phosphoric acid; reagent concentrations were determined by spectrophotometric analysis of appropriately diluted pH ∼12 solutions using $\epsilon_{292}(OCl^-) = 350\ M^{-1}\ cm^{-1}$ (*20*). Human leukocyte elastase and cathepsin G were purchased from Sigma and were assayed for activity prior to their use by determining rates of hydrolysis of the nitroanilides, Suc-Ala-Ala-Ala-$NPhNO_2$ and Suc-Ala-Ala-Pro-Phe-$NPhNO_2$, respectively (*21*, *22*). Substrates were dissolved in dimethyl sulfoxide at 20 mM and then diluted 10-fold in 100 mM HEPES at pH 7.5. After addition of the enzyme, the reaction was monitored spectrophotometrically at 410 nm using $\epsilon_{410} = 8.8 \times 10^3\ M^{-1}\ cm^{-1}$; one unit of activity is defined as the hydrolysis of 1 μmol of substrate/min. Chloramine (NH_2Cl) was prepared by flow-mixing HOCl in 50 mM phosphate at pH 7.4 with an ∼10-fold excess of NH_3 in H_2O through a Gibson-type 12-jet tangential mixer; the reagent concentration was determined spectrophotometrically using $\epsilon_{242}(NH_2Cl) = 429\ M^{-1}\ cm^{-1}$ (*23*). Peroxynitrous acid (ONOOH) was prepared using tandem 4-jet mixers by flow-mixing $NaNO_2$ with acidic solutions of H_2O_2 followed by an alkaline quench (*24*); spectrophotometric determination of reagent concentrations was made using $\epsilon_{302}(ONOO^-) = 1670\ M^{-1}\ cm^{-1}$. These solutions also contained ∼20% NO_2^- as an unavoidable contaminant, as determined by the Griess colorimetric assay (*25*). Other chemicals were best-available-grade from commercial suppliers and used as received; in-house deionized water was further purified by reverse osmosis/deionization using a Barnstead E-pure system.

Methods. Bacteria were opsonized by incubation in a polypropylene tube for 30 min at 37 °C in 2 mL of HBSS buffer supplemented with 10% of the donor's serum, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$; when inhibition of MPO was desired, 1 mM NaN_3 was also included. Phagocytosis was initiated by mixing suspensions of neutrophils and bacteria that had been prewarmed to 37 °C in amounts sufficient to give *E. coli*/neutrophil ratios ranging from 5:1 to 20:1.[3] Phagocytosis and fluorescence changes in the bacterial GFP were monitored visually using a Nikon Microphot FX fluorescence microscope equipped with a Nikon Microphot Epi-fl attachment and a Chroma 31001 filter cube set, which

gave excitation and emission band-passes of 465−497 and 516−558 nm, respectively. Two types of experiments were undertaken. In one, conducted at room temperature, 10 μL of the mixed cell suspension was transferred to a polylysine-coated microscope slide and photographs of the slide were taken at 15 min intervals; in the other, 10 μL aliquots were transferred to the slide at 15 min intervals from a rotating suspension maintained at 37 °C and both light and fluorescence photographs were immediately taken. Overlayment of these photomicrographs allowed one to correlate positions of the GFP bacteria and neutrophils. The time course of fluorescence intensity changes upon exposure to various oxidants at 37 °C was determined using a SPEX Fluorolog-3 instrument whose cell-holder temperature was regulated with a Peltier control unit; excitation was at 395 nm, with emission scans being recorded from 450 to 550 nm (maximal λ_{em} = 509 nm). The reactions of purified GFP and GFP-*E. coli* suspensions with chemical and enzymatically generated oxidants were also monitored spectrofluorimetrically, generally at [GFP] =17−50 nM, which was sufficient to give $\sim 10^6$ cps or at $\sim 7 \times 10^7$ cells/mL. Reactions with HOCl, NH_2Cl, and ONOOH were investigated by serial addition of increasing amounts of the oxidants into vortexing solutions of GFP or GFP *E. coli* contained in 3 mL fluorescence cuvettes, followed immediately by measurement of the fluorescence intensity. Some experiments with ONOOH were made in argon-purged solutions to minimize potential side reactions arising from formation of nitrosoperoxycarbonate (i.e., the $ONOO^-$-CO_2 adduct) (*26*); in other experiments, 25−100 mM $NaHCO_3$ was deliberately added to the buffer to ensure complete intermediary formation of the adduct. For H_2O_2, whose reaction rates with protein are expected to be slower, fluorescence intensities were monitored for 30 min between additions of the oxidant; similarly, for reactions in which the oxidant was slowly generated, fluorescence intensities were monitored for periods of up to several hours after initiating the reactions. Reactions by the Fenton system, ascorbate−Cu−H_2O_2 (*24*), were initiated by adding 80 μM H_2O_2 to argon-purged 50 mM sodium phosphate at pH 7.4 containing 100 mM ascorbate, 10 μM $CuSO_4$, and GFP or bacteria. Enzymatic generation of HOCl by the cell-free MPO−H_2O_2−Cl^- system was initiated by adding 80 μM H_2O_2 to 75 nM MPO and the GFP target in phosphate-buffered saline (PBS; 50 mM phosphate at pH 7.4 plus 100 mM NaCl; for studies with the proteases, elastase, and cathepsin G, KCl was substituted for NaCl). MPO-catalyzed generation of $NO_2^{\cdot}$ was initiated by adding 20−400 μM H_2O_2 to 5−10 nM MPO in 0.1 M sodium phosphate at pH 7.4 containing 10 mM $NaNO_2$ and either pure GFP or the bacteria. All reactions were run at 25 °C; the enzymatic reactions were also run at 37 °C, as indicated. Radiolysis studies were conducted using the ^{60}Co source available at the Washington State University nuclear reactor facility. Solutions of GFP or suspensions of the GFP *E. coli* were placed in nitric-acid-washed test tubes, then sealed with rubber septa, and bubbled with N_2O for 10 min. The samples were placed in the reactor for timed periods, and fluorescence intensities and cell viabilities were compared to reference standards that had been treated identically but not exposed to γ irradiation. The radiation dose was 1.1−1.5 krad/min, as determined by Fricke dosimetry (*27*). In N_2O-saturated phosphate buffer, the oxidant generated is almost exclusively $OH^{\cdot}$; the $H^{\cdot}$ atom is also produced but at ∼5% of the concentration of the hydroxyl radical (*27*) Addition of 1 mM KNO_2 or 100 mM $NaHCO_3$ to the medium causes near-quantitative conversion of $OH^{\cdot}$ to $NO_2^{\cdot}$ and $CO_3^{\cdot -}$, respectively (*28*). Pour-plate analysis on nutrient agar (Difco) was routinely used to determine cell survival in oxidant-exposed suspensions of GFP *E. coli*.

RESULTS

Oxidant-Induced Loss of Fluorescence from Purified GFP. To ascertain the susceptibility of GFP to various potential neutrophil-generated oxidants, fluorescence losses were measured following exposure of 17.5 nM of the purified protein to biologically relevant concentrations of the oxidants. GFP was insensitive to hydrogen peroxide, chloramine, and the Cu-catalyzed Fenton system described in the Experimental Procedures, exhibiting only ∼10% overall loss in fluorescence intensity following serial additions of 200 μM H_2O_2 and 1.5 μM NH_2Cl to concentrations totaling 2 mM and 200 μM, respectively, and exposure to the Fenton system for up to 4 h. Similarly, there was no loss in fluorescence intensity when 25 nM GFP was exposed to radiolytically generated $OH^{\cdot}$ radical or $CO_3^{\cdot -}$ at cumulative dose levels as high as 400 μM radicals.

In marked contrast to these results, exposure to ∼17 μM HOCl caused immediate and complete loss of fluorescence from the protein. Titrimetric studies using ∼3 μM GFP indicated that the chromophore absorption band at 488 nm was lost in parallel with fluorescence, indicating that quenching was caused by direct oxidative bleaching of the chromophore by HOCl; the reaction stoichiometry determined from the titration curves was ∼200 HOCl molecules/chromophore. Exposure to the cell-free MPO−H_2O_2−Cl^- enzymatic system using the same amount of H_2O_2 as limiting reagent that was used in the Fenton system (80 μM) caused complete quenching of fluorescence within 10 min; however, fluorescence intensity was not lost if any of the enzyme assay components were deleted or if 1 mM sodium azide (NaN_3) was added to inhibit the enzyme. ONOOH, which is a potential physiological source of the oxidizing radicals, $NO_2^{\cdot}$ and $OH^{\cdot}$ (*29*), also quenched fluorescence at comparable oxidant levels. However, the titrimetric profiles of the two oxidants were quantitatively different. Specifically, the response to HOCl followed a sigmoidal curve, indicating that multiple reactions with the protein were required before fluorescence was lost. In contrast, fluorescence losses in titrations involving peroxynitrite appeared hyperbolic (Figure 1) and displayed a persistent residual fluorescence, even upon addition of a total of 2 mM NaOONO, the highest concentration investigated (data not shown). When NaOONO was added to bicarbonate-containing buffers to generate $NO_2^{\cdot}$ and

[3] Research from several laboratories has shown that phagocytosis of bacteria and other particles by neutrophils in buffer suspensions occurs within a short period after mixing (*7, 75*). The uptake can be described as a first-order process, with $t_{1/2} \sim 5$ min (*75*); after ∼3 half-lives, phagocytosis essentially ceases. The GFP *E. coli* used in the present study exhibited the same behavior, which was confirmed by an additional experiment. Neutrophils were challenged with bacteria at a 5:1 bacterium/neutrophil ratio to initiate phagocytosis; subsequent challenges made 20−30 min later with the same cell densities of bacteria led to only minor increases in the number of ingested cells, indicating that the newly added cells were not appreciably taken up. Thus, phagocytosis is *de facto* nearly synchronous in these experiments.

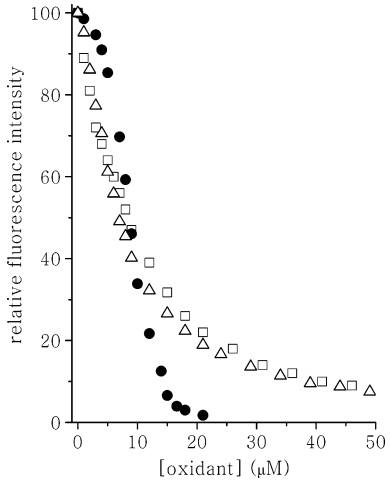


FIGURE 1: Effect of oxidants on purified recombinant GFP. GFP (17.5 nM) in 100 mM phosphate at pH 7.4; serial additions of HOCl (●), NaOONO (□), or NaOONO in Ar-purged solutions (△). λ_{ex} = 395 nm, and λ_{em} = 509 nm. The data displayed are for single experiments, which were repeated 3−7 times with very similar results.

$CO_3^{\cdot-}$ via nitrosoperoxycarbonate ($ONOOCO_2^-$) (*26*, *30*), fluorescence quenching was no longer observed. However, unlike $OH^{\cdot}$ and $CO_3^{\cdot-}$, radiolytically generated $NO_2^{\cdot}$ effectively quenched GFP fluorescence. In these reactions, the fluorescence intensity decreased exponentially with increasing time of exposure to γ irradiation; 50% inhibition was observed upon cumulative exposure to ∼60 μM $NO_2^{\cdot}$, and a maximal inhibition of 92% was achieved after cumulative exposure to ∼400 μM radicals. Generation of $NO_2^{\cdot}$ by the MPO−H_2O_2−NO_2^- enzymatic system (*31*) led to the dose-dependent loss of GFP fluorescence that was remarkably similar to the radiolysis results. In these studies, ∼50 μM H_2O_2 (corresponding to formation of ∼100 μM $NO_2^{\cdot}$) was required to inhibit fluorescence by 50% and a maximal inhibition of 80% was achieved at ∼100 μM H_2O_2, beyond which there were no further changes in fluorescence intensity. The inability of $ONOOCO_2^-$ to quench fluorescence noted above can be attributed to rapid recombination of the simultaneously generated $NO_2^{\cdot}$ and $CO_3^{\cdot-}$ radicals to form NO_3^- and CO_2.

Oxidant-Induced Loss of GFP Fluorescence in Bacteria. A qualitatively similar response was seen when GFP-expressing *E. coli* 11775 were exposed to these oxidants; i.e., of the set, the only oxidants found to be capable of significantly reducing the fluorescence from the cells were HOCl, peroxynitrite (in CO_2-free buffers), and MPO-generated $NO_2^{\cdot}$. Specifically, no change was observed upon serial additions of 200 μM H_2O_2 to suspensions of 6.6 × 10^7 cells/mL over a 3 h period to a total concentration of 2 mM; a 10% decrease in fluorescence was initially observed when (1−7) × 10^7 cells were exposed to the ascorbate−Cu−H_2O_2 system, but the fluorescence intensity subsequently returned to its initial value over the course of a 4 h incubation. No change in intensity was observed upon γ irradiation of 2.7 × 10^8 cells/mL in media where the primary oxidizing radical was $OH^{\cdot}$, $CO_3^{\cdot-}$, or $NO_2^{\cdot}$ at dose levels of these radicals as high as 400 μM. These doses were severalfold higher than that required to kill the cells. In contrast, sequential additions of 20 μM HOCl to 6.6 × 10^7 cells/mL caused a sigmoidal-shaped loss in intensity that

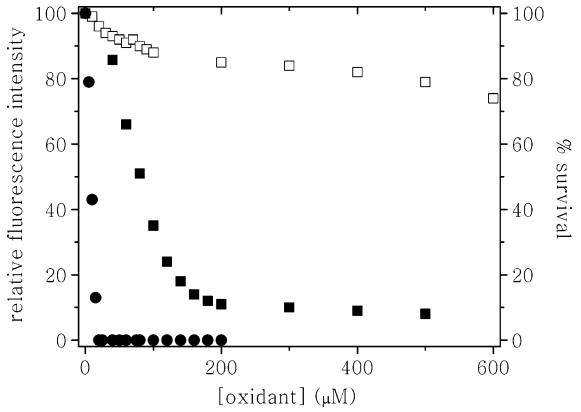


FIGURE 2: Oxidation of GFP-expressing *E. coli* by HOCl and NaOONO. Cell survival following serial additions of HOCl to 6.6 × 10^7 cfu/mL in 100 mM phosphate at pH 7.4 (●), corresponding fluorescence changes (■), and fluorescence changes after serial additions of NaOONO to Ar-purged suspensions (□). Data are for individual cultures; experiments were repeated 5−10 times with very similar results.

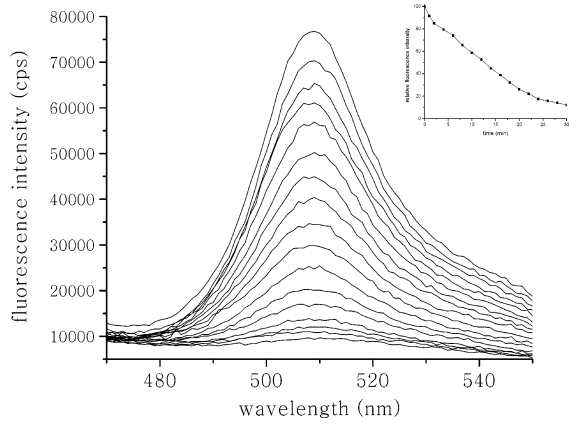


FIGURE 3: Effect of cell-free MPO-H_2O_2-Cl^- enzymatic system on the fluorescence from GFP-expressing *E. coli*. Conditions: 1 ×10^7 cfu/mL, 130 μM H_2O_2, 100 mM NaCl, ∼50 nM MPO, reaction at 37 °C. Spectral scans were made every 2 min following initiation of the reaction (λ_{ex} = 395 nm); the inset shows the temporal loss of the fluorescence intensity at 509 nm. This experiment was repeated 3 times with very similar results.

reached 90% when the total added amount exceeded ∼140 μM (Figure 2); this corresponds to exposure of each cell to ∼1 × 10^9 HOCl molecules. For comparison, the LD_{90} of these cells was determined to be ∼1 × 10^8 HOCl molecules/cell (Figure 2), which is similar to that previously reported for other bacteria under comparable conditions (*13*, *32*−*36*). GFP fluorescence within the bacteria was also completely quenched by the cell-free MPO−H_2O_2−Cl^- system; specifically, nearly all fluorescence from ∼10^7 cells/mL was lost after 30 min in a reaction medium containing 130 μM H_2O_2 and ∼50 nM (4.3 μg/mL) MPO (Figure 3). No fluorescence changes were observed when any of the reaction components (MPO, H_2O_2, or Cl^-) were deleted or when sufficient NaN_3 was added to inhibit the enzyme.

Peroxynitrite was much less effective at reducing fluorescence from the cells than from the purified protein (Figures 1 and 2). Incremental 100 μM additions to ∼10^7 cells/mL caused only a slow progressive loss in intensity, with >20% residual remaining after a total addition of 2 mM peroxynitrite. This amount of peroxynitrite is far greater than could reasonably be achieved in physiological environ-

Table 1: Oxidant Toxicities and GFP Fluorescence Quenching Capabilities in *E. coli* 11775 and Purified Protein

oxidant	LD_{90}[a] ($\times 10^9$)	I_{90}/LD_{90}[b] (cytosolic GFP)	I_{90}[c] ($\times 10^3$) (purified GFP)
HOCl	0.1	11	1.1
NH_2Cl	0.08	$\gg 3000$[d]	$\gg 300$[e]
NaOONO (aerobic)	1.4	16	6.0
NaOONO (Ar purged)	1.4	13	5.9
NaOONO + 25 mM HCO_3^-	14	$\gg 10$[f]	$\gg 6000$[g]
NaOONO + 100 mM HCO_3^-	13	$\gg 4$[h]	$\gg 5000$[g]
H_2O_2	20	$\gg 45$[i]	> 80[j]
$OH^{\cdot}$	0.1	$\gg 6$[k]	$\gg 20$[k]
$NO_2^{\cdot}$	0.2	$\gg 4$[k]	10
$CO_3^{\cdot -}$	0.2	$\gg 4$[k]	$\gg 20$[k]

[a] Number of oxidant molecules required to kill 90% of the bacteria. [b] Ratio of oxidant molecules required to decrease the fluorescence intensity at 509 nm by 90% to LD_{90}. [c] Number of oxidant molecules per protein molecule required to reduce the fluorescence intensity by 90%. [d] A total of 2.3×10^{11} molecules of NH_2Cl/cfu *E. coli* decreased the intensity by ~1%. [e] This amount of NH_2Cl decreased the intensity by ~5%. [f] A total of 1.4×10^{11} molecules of NaOONO/cfu *E. coli* decreased the intensity by ~5%. [g] This amount of NaOONO decreased the intensity by 2−5%. [h] A total of 6.0×10^{10} molecules of NaOONO/cfu *E. coli* decreased the intensity by ~10%. [i] A total of 1.4×10^{11} molecules of H_2O_2/cfu *E. coli* decreased the intensity by ~1%. [j] This amount of H_2O_2 decreased the intensity by ~20%. [k] No loss in fluorescence intensity at the highest applied doses (400 μM).

ments and corresponds to $\sim 6 \times 10^{10}$ ONOOH/cell. The apparent LD_{99} determined for these cells under these conditions was $\sim 5 \times 10^9$ ONOOH/cell.[4] When the medium contained 25−100 mM bicarbonate ion, no loss of fluorescence intensity was observed from cells exposed to 100 μM incremental additions of NaOONO totaling 2 mM. In the presence of 25 mM total carbonate (~1 mM CO_2 plus ~24 mM HCO_3^-), the LD_{99} was $\sim 6 \times 10^{10}$ peroxynitrite/cell, approximately 10-fold greater than the value in the absence of bicarbonate ion. These results are consistent with earlier demonstrations that CO_2 catalyzes isomerization of $ONOO^-$ to the innocuous NO_3^- ion, thereby limiting intracellular oxidations by both ONOOH and the membrane-impermeable $CO_3^{\cdot -}$ anion (*37*, *38*).

The microbicidal MPO−H_2O_2−NO_2^- enzymatic system was also markedly less effective at quenching fluorescence in the intact cells than the pure protein. No loss in fluorescence was observed at 100 μM added H_2O_2, although at higher oxidant levels, a slow linear dose-dependent loss was observed, reaching ~40% inhibition at 400 μM H_2O_2, the highest concentration examined. All of the cells were killed at these high oxidant doses. Toxicities of the various oxidants examined and their effects upon GFP fluorescence from both purified protein and cells are summarized in Table 1.

Effect of Neutrophil Proteases and Medium Acidity upon GFP Fluorescence. Cathepsin G and elastase, microbicidal proteases found in leukocyte granules, were investigated as potential sources of GFP degradation. Incubation of either 17 nM purified GFP in PBS with either 180 μM cathepsin G or 670 μM elastase for 1 h at 37 °C gave no loss in fluorescence intensity. No changes in fluorescence intensities or cell viabilities were noted when GFP-expressing *E. coli* at 1×10^7 cells/mL were incubated with the same concentrations of these enzymes for 2 h. The specific activities of the enzymes used in these studies were ~50 units/mg of elastase and ~60 units/mg of cathepsin G (*21*, *22*). Activities measured in the bacterial suspension buffer (50 mM PBS at pH 7.4) under standard reaction conditions were 80 and 97%, respectively, of the activities measured in standard assay buffers. When 1×10^7 cells/mL *E. coli* were added, the residual activity of elastase toward its nitroanilide substrate was 72% of the activity measured in the absence of cells; the activity of cathepsin G declined to 22% of the value measured in the absence of cells. These assays confirm that the enzymes were active in the GFP-*E. coli* suspensions.

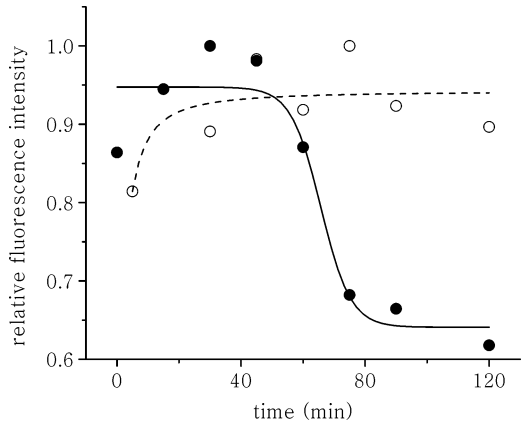
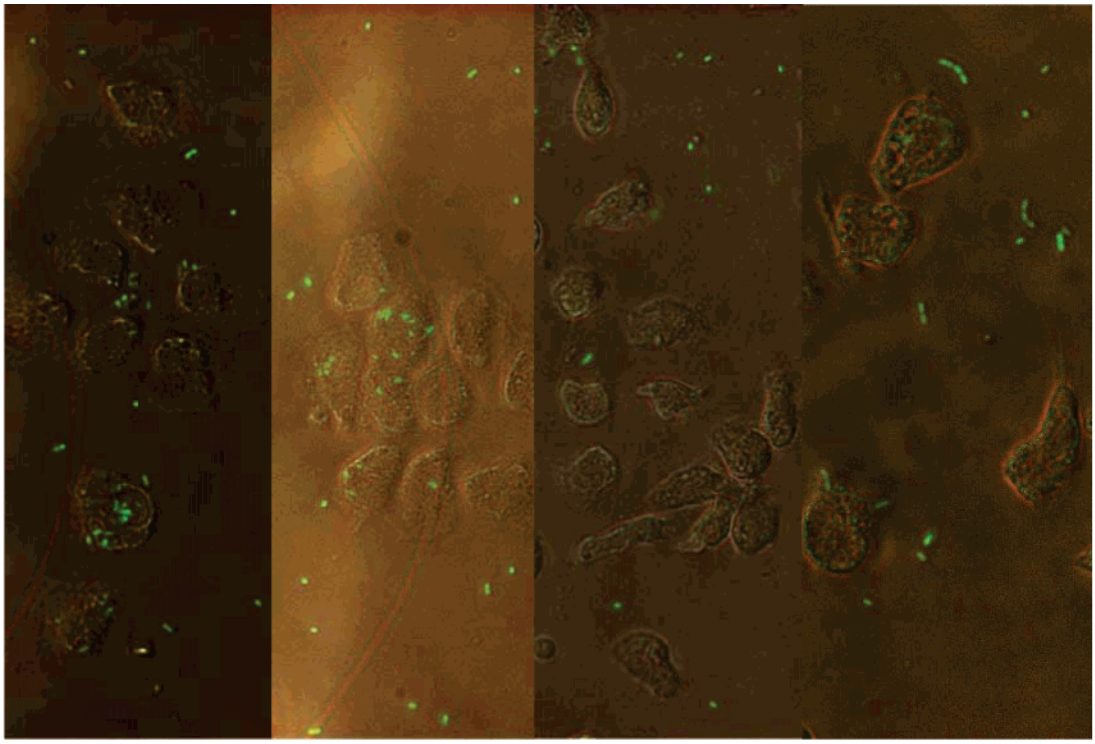


FIGURE 4: Fluorescence intensity changes following phagocytosis of opsonized stationary-phase GFP-expressing *E. coli* by human neutrophils. A 5:1 *E. coli*/neutrophil ratio was used, leading to ~50% phagocytosis of the bacteria. Data are for media containing no added NaN_3 (●) or 1 mM NaN_3 (○); each data point is the fluorescence emission average at 509 nm from nine 500−520 nm scans ($\lambda_{ex} = 395$ nm). The solid line is a sigmoidal fit to the solid circles; the dashed line is a logistic fit to the open circles. Data are the results for a single experiment, which was repeated 4 times with very similar results.

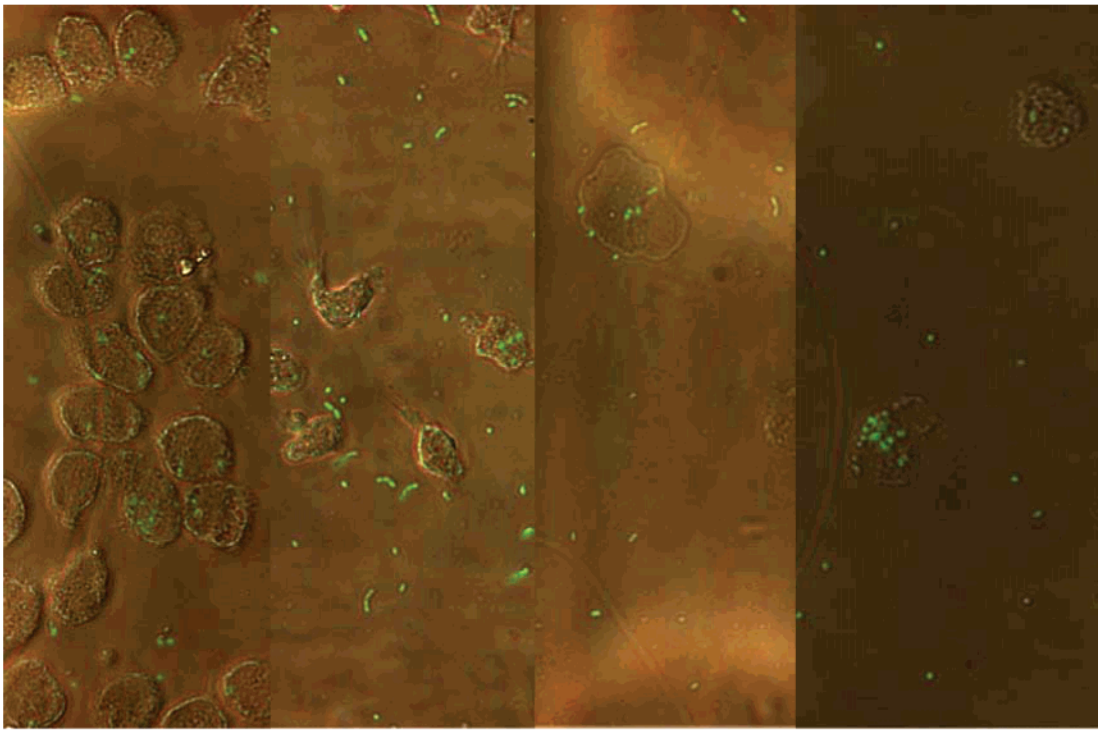
The fluorescence intensity of the GFP protein was pH-independent over the range pH 7−8, but decreased progressively with acidification below pH 7.0 in phosphate-buffered solutions, declining to ~60% of the pH 8.0 value at pH 6.0. These changes occurred immediately upon addition of the acid (buffer at pH 5) and were nearly completely reversed upon restoring the initial pH; following the initial drop, the fluorescence intensity underwent no further changes over a period of 2−3 h.

Fluorescence Changes in Neutrophil-Phagocytosed E. coli. A measurable decrease in GFP fluorescence intensity was observed at 45 min following incubation of serum-opsonized bacteria with neutrophils in a 5:1 ratio. The fluorescence continuously declined with time for an additional 30 min to ~50% of the initial fluorescence, following which no further changes occurred. These fluorescence losses could be completely inhibited by adding 1 mM NaN_3 to the medium prior to phagocytosis (Figure 4). At this concentration level, N_3^- has no detectable effect upon bacterial phagocytosis, NADPH oxidase-dependent respiration, or degranulation by the neutrophils (*6*, *7*, *39*). Visual inspection using a fluorescence microscope indicated that ~$^1/_2$ of the bacteria were phagocytosed under the experimental conditions. Similar experiments, utilizing a higher (20:1) bacteria/neutrophil ratio

[4] Unlike most oxidants, the toxicity of peroxynitrous acid does not scale linearly with *E. coli* cell densities; this unusual feature has been modeled by assuming that most of the ONOOH decays to nitrate ion. The LD_{50} estimated for *E. coli* ATCC 25922 from the model is 10^6−10^7 ONOOH/cell (*76*).



(a) 15 min 45 min 70 min 90 min



(b) 15 min 45 min 70 min 90 min

FIGURE 5: Fluorescence microscope images of 20:1 suspensions of opsonized stationary-phase GFP-expressing *E. coli* and human neutrophils taken at various times following initiation of phagocytosis. (a) NaN_3 absent. (b) NaN_3 (1 mM) added to the buffer. This experiment was repeated 4 times with equivalent results.

in which $\sim^1/_3$ of the bacteria were phagocytosed, indicated that nearly all fluorescence losses occurred within the subpopulation of cells that had been phagocytosed (Figure 5a). Specifically, a comparison of standard and fluorescence photomicrographs revealed that <5% of the external bacteria had lost fluorescence over the entire course of the experi-

ments, whereas nearly all of the internalized bacteria were nonfluorescent. As in the other experiments, addition of NaN_3 during the incubation period prevented fluorescence quenching in the phagocytosed cells (Figure 5b). These experiments were conducted by periodically sampling the cells from a rotating suspension maintained at 37 °C, as described in the Experimental Procedures. Similar results were obtained when mixtures of phagocytosing cells were adhered to polylysine-coated microscope slides and subsequent changes were monitored visually, in this case at ~23 °C. After ~2 h, decreases in the fluorescence intensity of the phagocytosed *E. coli* were noticeable; after ~3 h, the fluorescence was completely quenched. In contrast, fluorescence intensities from bacteria that had not been phagocytosed appeared unchanged. The slower rate of decline in fluorescence intensity observed with the immobilized cells may have been due to the lower temperature at which these experiments were conducted.

DISCUSSION

Neutrophil-Generated Oxidants. The neutrophil possesses numerous microbicidal macromolecules among its granule components (*1*, *3*, *40*−*43*); these have been categorized as "oxidative" or "nonoxidative" based primarily upon their presumed mechanisms of action. Central among the oxidative set is the peroxidase, MPO, which is capable of using the respiratory end products of the NADPH oxidase (*44*) to generate, among other oxidants, HOCl from Cl^- and $NO_2^{\cdot}$ from NO_2^- (*1*, *4*, *5*, *45*, *46*). Intraphagosomal levels of Cl^- are expected to be comparable to those found in serum (~0.15 M), and NO_2^- may be elevated at sites of infection through catabolism of nitric oxide synthase (NOS)-generated NO to concentrations where it could be a competitive MPO substrate (*5*, *46*); both MPO-generated HOCl (3) and $NO_2^{\cdot}$ (*47*) are potent microbicides. In the biological milieu, HOCl can react directly with bacteria or generate as secondary oxidants chloramines, chloramides, nitryl chloride (NO_2Cl), and hydroxyl radical via reaction with endogenous amines and amides (*23*, *48*), NO_2^- (*49*, *50*), and $O_2^{\cdot -}$ (*51*), respectively. Although OH could further generate $CO_3^{\cdot -}$ and $NO_2^{\cdot}$ by reaction with HCO_3^- and NO_2^- (*28*), respectively, $NO_2^{\cdot}$ is a relatively stable radical that can oxidize and nitrate biological compounds but does not generate additional inorganic radicals from medium components. Hydrogen peroxide, formed during the respiratory burst, is not particularly toxic in the absence of redox metal ions (*52*), but *E. coli* in suspension are highly susceptible to oxidative inactivation by an ascorbate−Cu^{II}−H_2O_2 microbicidal system (*52*) and considerable indirect evidence has been advanced in support of similar lethal reactions involving $O_2^{\cdot -}$-initiated H_2O_2 oxidations catalyzed by bacterially derived iron (*53*). Peroxynitrous acid, which might be formed by the reaction between NOS-generated $NO^{\cdot}$ and NADPH oxidase-generated $O_2^{\cdot -}$ (*54*), is a strong oxidant (*55*) that is capable of directly oxidizing numerous biomolecules (*56*, *57*), although its primary reactions in physiological environments appear to be peroxo O−O bond homolysis to generate the same set of oxidizing radicals, i.e., $OH^{\cdot}$, $CO_3^{\cdot -}$, and $NO_2^{\cdot}$ (*26*, *29*, *30*).

Bactericidal Mechanisms. Bacteria vary widely in their susceptibility to individual microbicidal components of neutrophil granules, as has long been known from comparisons of neutrophil function under aerobic versus anoxic conditions or by using functionally impaired neutrophils (*1*, *3*, *58*), as well as from examination of the microbicidal capabilities of isolated components in *ex vivo* biological assays (*1*, *3*). Moreover, studies in which the microbicidal effectiveness of normal, MPO-deficient, and NADPH oxidase-deficient cells are compared have fostered the notion that redundant or parallel microbicidal mechanisms exist. A pertinent example is the recent work of Rosen and Michel (*59*), who showed that killing of *E. coli* ATCC 11775 by human neutrophils did not require respiratory activation or an active peroxidase; nonetheless, inhibition of bacterial DNA synthesis, certainly a lethal event (*60*), was by far more efficient in unimpaired, i.e., respiring MPO positive, neutrophils. Thus, it appears that nonoxidative mechanisms were sufficient to kill this organism and thereby masked the MPO-dependent oxidative microbicidal mechanism occurring in parallel in the normal neutrophil. In contrast, indirect evidence for synergism between oxidative and nonoxidative mechanisms has recently been obtained by using genetically altered mice. Animals deficient in either MPO or neutrophil elastase were considerably more susceptible to virulent *Klebsiella pneumoniae* than wild-type strains (*61*), implying that both types of mechanisms are required for protection. Similarly, results from two separate studies suggest that both MPO (*62*) and elastase (*8*) are necessary to protect mice from fungal infection by *Candida albicans*. Comparisons of toxicities of neutrophil proteinases toward *E. coli* suggest that bactericidal mechanisms may even be strain-specific. Thus, although elastase has been reported to kill virulent strains of *E. coli* (*63*) and *K. pneumoniae* (*61*), neither this enzyme nor cathepsin G appeared to be capable of killing the capsular strain of *E. coli* (ATCC 11775) used in our studies.

In developing their model of neutrophil function based upon respiration-dependent activation of granule proteases (*8*, *9*), Segal and co-workers have argued that the role of MPO in generating oxidative toxins has been grossly overestimated. In their view, MPO functions in the phagosome primarily as a catalase, thereby protecting neutrophil-derived proteases from inactivation by respiration-generated H_2O_2 (and, *de facto*, from HOCl (*61*, *64*−*67*) or other MPO-generated oxidants). This notion can be tested by determining the extent of chlorination or MPO-dependent oxidation occurring within the phagosome. Specifically, the amount of HOCl required to kill a bacterium, as measured by inhibition of colonial growth, appears to be fairly constant. Dose-dependence survival curves for bacteria with very differing structural composition and metabolic capabilities (*S. aureus* ATCC 27217, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus lactis* 7962, and the *E. coli* strains ATCC 25922, ATCC 11775, AN718, AN1460, and ML-35) are all sigmoidal with LD_{90} values that fall within the range of $0.2-1.5 \times 10^8$ HOCl molecules/bacterium (*13*, *32*−*36*). A demonstration that bacteria were chlorinated or oxidized in MPO-dependent reactions within the phagosome to levels in excess of this amount would therefore provide strong evidence that MPO does indeed function to generate lethal levels of bactericidal oxidants.

In their studies using tyrosine chlorination as a marker for reactions between HOCl and *S. aureus* 27217 within the human neutrophil, Winterbourn and colleagues concluded that 5 nmol of HOCl/10^8 cells or $\sim 3 \times 10^7$ HOCl molecules/

bacterium had reacted with the entrapped bacteria by the point at which they were killed (*13*). In contrast, the measured LD_{90} for this organism was $\sim 1.5 \times 10^8$ HOCl molecules/bacterium. In similar studies using *E. coli* 11775 as a bacterial probe, Rosen and colleagues isolated (3−4)-fold higher levels of bacterial chlorotyrosine under optimal conditions (*14*). Using fluorescein-conjugated 1-μm-sized polyacrylamide spheres as bacterial mimics, we had previously demonstrated trapping of $\sim 6 \times 10^7$ HOCl molecules/particle within the phagosome (*7*). This amount, which was ~10% of the O_2 consumed by the NADPH oxidase in the respiratory burst, was considered to be a lower limit to the actual MPO-generated HOCl formed in the phagosome because the rate constant for fluorescein chlorination is low relative to those for reactions between HOCl and the susceptible biological targets associated with killing (*7*, *32*).

Collectively, these studies clearly establish that MPO-catalyzed chlorination of phagocytosed particles is extensive in human neutrophils, in contradistinction to the suppositions of Segal and co-workers. However, because the amount of isolated chlorinated reaction products requires an amount of HOCl that is very similar to that needed to inhibit colonial growth, it is not clear from these data whether HOCl formation alone can account for MPO-dependent killing within the phagosome.

GFP E. coli as an HOCl-Selective Probe. Among the various potential phagocyte-generated oxidants investigated, only HOCl, ONOOH, and $NO_2^{\cdot}$ were capable of quenching fluorescence in the isolated GFP at physiologically reasonable levels of the oxidants (Table 1). Furthermore, GFP localized within the bacterial cytosol was quenched far more efficiently by HOCl than either ONOOH (Figure 3) or $NO_2^{\cdot}$. Because these oxidants can permeate bilayer membranes by passive diffusion (*37*, *68*, *69*), it is likely that this discrimination arises primarily from differing target specificities within the bacterial cytosol. Our results concerning the reactivity of peroxynitrite are very similar to those of a previous study in which analogous reactions involving GFP-expressing MCF-7 (human breast carcinoma) cells (*70*) were examined. These researchers found that fluorescence quenching of purified recombinant GFP required bolus addition of relatively large amounts of ONOOH, that this reaction was prevented by addition of HCO_3^-, and that slow infusion of peroxynitrite or controlled synthesis in ONOOH-generating systems had no effect whatsoever on GFP fluorescence. Consistent with these results, the loss of fluorescence from the GFP-expressing cells was negligible to concentration levels of ONOOH that caused membrane lysis. Addition of MPO but not horseradish peroxidase caused extensive fluorescence quenching; although this reaction was not identified, the authors ascribed it to oxidation by MPO-generated HOCl. They also noted that heme peroxidase-catalyzed oxidation of NO_2^- by H_2O_2 caused the loss of fluorescence in solutions of purified GFP, a reaction that they ascribed to $NO_2^{\cdot}$ formation. In any event, the quenching of GFP fluorescence within the bacterial cytosol observed in our studies appears to be a reaction uniquely associated with HOCl; in *in vitro* assays, ~10 times as much HOCl was required to inhibit fluorescence as was required to inhibit colonial growth (Figure 2).

Role of HOCl in Intraphagosomal Killing. Use of GFP-expressing bacteria as an alternative probe offers the advantages that real-time analysis is possible and that isolation and quantification of product species from the cellular milieu is not required to assess the extent of MPO involvement in intraphagosomal bactericidal reactions. The results displayed in Figures 4 and 5 establish unequivocally that GFP fluorescence in phagocytosed bacteria is quenched by neutrophils in MPO-dependent reactions. These reactions are almost certainly oxidative in character; although fluorescence intensity in the purified protein was partially lost upon acidification of the medium, the magnitude of this effect cannot account for the complete loss of fluorescence from entrapped bacteria. Furthermore, several studies have established that the newly formed phagosome first undergoes slight alkanization prior to slowly acidifying and falls below pH 7 only 1 h post-phagocytosis (*7*, *39*, *71*, *72*). These temporal changes in intraphagosomal acidities were not perceptibly altered by addition of NaN_3 (*7*).

The most plausible neutrophil-generated oxidant is HOCl; although the extent to which nonoxidative components might participate in a symbiotic manner was not determined, the reaction characteristics are consistent with HOCl being a major, if not the sole, contributor to fluorescence inhibition. Specifically, the sigmoidal nature of the titrimetric fluorescence decay curve (Figure 1), the relatively large I_{90}/LD_{90} value (Table 1), and the abrupt loss of fluorescence intensity within the phagosome that occurred relatively late during the respiratory burst (Figure 4) are all features that are expected from earlier work on reactions of HOCl and MPO−H_2O_2−Cl^- with bacteria. The fluorescence inhibition seen in titrimetric curves in the present study parallels earlier results in which the loss of activity in vulnerable cytosolic molecules upon exposure to HOCl was compared to the loss of cfu (*40*). This behavior is a consequence of the very high selectivity exhibited by HOCl for nucleophilic centers within biopolymers (*40*); i.e., the externally generated oxidant reacts first with a limited number of susceptible target sites on the bacterial envelope, and only after they have been oxidized does significant inactivation of vulnerable biomolecules occur within the cytosol. Earlier work had also established that the loss of cfu correlates with irreversible inactivation of proteins on the plasma membrane involved with metabolite transport and energy transduction (*40*). Because considerably more HOCl is required to quench GFP fluorescence than inhibit cell division (Figure 2), one infers from the data that the bacteria entrapped within the neutrophil phagosome have been exposed to sufficient HOCl to cause cellular death by this mechanism.

One remarkable feature of the selectivity of purified GFP toward various chemical oxidants is the complete absence of fluorescence quenching by NH_2Cl. A similar selectivity for HOCl over chloramines was previously found for fluorescence quenching of fluorescein conjugated to zymosan (*39*), which presumably reflects the greater oxidizing potential of HOCl. Nonetheless, chloramine and HOCl exhibit comparable toxicities toward bacteria (e.g., Table 1), and inhibition of specific bacterial proteins require very similar dose levels of these oxidants (*40*). Thomas and co-workers have suggested that chloramines, formed as secondary oxidants by reaction of endogenous amines with MPO-generated HOCl, are the actual neutrophil-generated toxins (*48*, *73*, *74*). These reactions are very rapid under physiological conditions (*40*), so that intraphagosomal accumula-

tion of chloramines to toxic levels might precede significant HOCl-mediated loss of GFP fluorescence. The loss of GFP fluorescence therefore does not allow one to distinguish between HOCl and RNHCl species as the actual toxins. In either case, however, it does reinforce our conclusion that MPO-generated chlorine oxidants constitute a primary vector of bactericidal action in the phagosome.

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