Production of Brominating Intermediates by Myeloperoxidase

A TRANSHALOGENATION PATHWAY FOR GENERATING MUTAGENIC NUCLEOBASES DURING INFLAMMATION*

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The existence of interhalogen compounds was proposed more than a century ago, but no biological roles have been attributed to these highly oxidizing intermediates. In this study, we determined whether the peroxidases of white blood cells can generate the interhalogen gas bromine chloride (BrCl). Myeloperoxidase, the heme enzyme secreted by activated neutrophils and monocytes, uses H2O2 and Cl− to produce HOCl, a chlorinating intermediate. In contrast, eosinophil peroxidase preferentially converts Br− to HOBr. Remarkably, both myeloperoxidase and eosinophil peroxidase were able to brominate deoxycytidine, a nucleoside, and uracil, a nucleobase, at plasma concentrations of Br− (100 μM) and Cl− (100 mM). The two enzymes used different reaction pathways, however. When HOCl brominated deoxycytidine, the reaction required Br− and was inhibited by taurine. In contrast, bromination by HOBr was independent of Br− and unaffected by taurine. Moreover, taurine inhibited 5-bromodeoxycytidine production by the myeloperoxidase-H2O2-Cl2−-Br− system but not by the eosinophil peroxidase-H2O2-Cl2−-Br− system, indicating that bromination by myeloperoxidase involves the initial production of HOCl. Both HOCl-Br− and the myeloperoxidase-H2O2-Cl2−-Br− system generated a gas that converted cyclohexene into 1-bromo-2-chlorocyclohexane, implicating BrCl in the reaction. Moreover, human neutrophils used myeloperoxidase, H2O2, and Br− to brominate deoxycytidine by a taurine-sensitive pathway, suggesting that transelephantation reactions may be physiologically relevant. 5-Bromouracil incorporated into nuclear DNA is a well known mutagen. Our observations therefore raise the possibility that transelephantation reactions initiated by phagocytes provide one pathway for mutagenesis and cytotoxicity at sites of inflammation.

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Reactive oxidants generated by phagocytic white blood cells are critical to host defense because they kill invading patho-

gens (1–5). However, they are also potentially dangerous because they may damage tissues at sites of inflammation. The heme enzyme myeloperoxidase, synthesized and secreted by neutrophils and monocyte cells, is an important source of oxidants. It uses H2O2 generated by the phagocyte NADPH oxidase to produce potent cytotoxins. At plasma halide concentrations, its major initial product is HOCl (6, 7).

\[
\text{Cl}^- + \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \quad \text{(Eq. 1)}
\]

HOCl can oxidize sulfhydryl groups (8), halogenate and oxygenate unsaturated lipids (9, 10), and halogenate aromatic compounds (11–13). Myeloperoxidase-derived chlorinating agents also generate secondary oxidants such as monochloramines, dichloramines (14–16), and amino acid-derived aldehydes (17–19).

We previously demonstrated that HOCl generated by myeloperoxidase is in equilibrium with molecular chlorine (Cl2) through a reaction that requires chloride (Cl−) and H+ (12, 20).

\[
\text{HOCl} + \text{H}^+ + \text{Cl}^- \rightleftharpoons \text{Cl}_2 + \text{H}_2\text{O} \quad \text{(Eq. 2)}
\]

Cl2 generated by this pathway has been implicated in the production of 3-chlorotyrosine and 5-chlorodeoxycytidine by activated neutrophils (12, 20). Elevated levels of protein-bound 3-chlorotyrosine and myeloperoxidase are found in human atherosclerotic tissue, strongly suggesting that oxidative reactions involving HOCl damage proteins in this chronic inflammatory disorder (13, 21).

Chronic inflammation also increases the risk of cancer, raising the possibility that reactive intermediates generated by neutrophils, monocytes, and macrophages might damage nucleic acids and compromise the integrity of the genome (22–24). Genetic epidemiological studies have revealed that a polymorphism in the myeloperoxidase promoter region alters the risk for various cancers (25–30). These results suggest that myeloperoxidase may play an important role in carcinogenesis, perhaps by generating mutagenic oxidants during the inflammatory response.

A structurally related heme protein, eosinophil peroxidase, is released by activated eosinophils, which help kill invading parasites. This peroxidase contributes to the characteristic staining of eosinophils. At plasma concentrations of halide (100 mM Cl−, 20–100 μM bromide, <1 μM iodide; Refs. 31 and 32), eosinophil peroxidase preferentially oxidizes bromide (Br−) to produce the potent brominating agent HOBr (33–35).

\[
\text{Br}^- + \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{HOBr} + \text{H}_2\text{O} \quad \text{(Eq. 3)}
\]

Like HOCl, HOBr oxidizes biomolecules at sites of eosinophilic inflammation (36). DNA may be one important target because
eosinophil peroxidase brominates deoxycytidine in vitro. It is noteworthy that schistosomiasis, a chronic inflammatory disease characterized by an intense eosinophil granulomatous reaction to the eggs of the blood fluke *Schistosoma*, greatly increases the risk for cancer (reviewed in Refs. 37–39).

More than 100 years ago, inorganic chemists proposed the existence of interhalogens, which are combinations of different halogens (XeX e). Both binary (BrCl, IBr, and ICl) and ternary (ICl3) interhalogens have since been characterized. One pathway for their formation requires hypohalous acid (HOX) and halide ion (X; Refs. 40 and 41).

HOCI reacts with Br– by this mechanism to yield molecular BrCl. Anions of interhalogens and polyhalides are also known; they include ClBr2, BrCl2, Br2I–, Br2Cl–, and BrCl3. Chemically, interhalogens are extremely corrosive species that attack a wide range of other compounds (42).

In the current studies, we show that myeloperoxidase generates reactive brominating species that oxidize nucleobases by a reaction involving HOCI, Br–, and formation of BrCl, an interhalogen gas. We also found that human neutrophils used myeloperoxidase, Cl–, and Br– to brominate deoxycytidine, suggesting that transhalogenation reactions may be physiologically relevant. Our observations suggest that transhalogenation reactions executed by phagocytes may represent one pathway for mutagenesis and cytotoxicity at sites of inflammation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Organic solvents, H2O2, sodium hypochlorite, and sodium phosphate were obtained from Fisher. Bis(trimethylsilyl)trifluoroacetamide and silylation grade acetonitrile were from Regis Technologies, Inc. (Morton Grove, IL). All other materials were purchased from Sigma, except where indicated.

**Methods**

Myeloperoxidase and Eosinophil Peroxidase—Myeloperoxidase (A 405/348 > 0.8) was isolated from HL-60 cells by sequential lectin affinity, ion exchange, and size exclusion chromatographies (43, 44). Enzyme concentration was determined spectrophotometrically (ε405 = 178 mmоль cm–1; Ref. 45). Porcine eosinophil peroxidase (A405/A230 > 0.9) was provided by ExOxEnsis (San Antonio, TX).

Peroxidase Activity Assay—The purity of myeloperoxidase and eosinophil peroxidase were assessed by peroxidase activity using nonnaturating polyacrylamide slab gel electrophoresis and gel system 8 (46, 47). Glycerol (25 w/v) and cetyltrimethylammonium bromide (0.05% w/v) were included in all buffers. Riboflavin (0.024 mg/ml) was used as the polymerization catalyst, and the stacking gel was omitted. Peroxidase activity was visualized by incubating the gel in 400 mM tetramethylbenzidine, 10 mM sodium citrate, pH 5, 10 mM EDTA, 5 mM NaBr, and 200 μM H2O2.

Preparation of Hypohalous Acid and Taurine Bromamine—Chloride-free NaOCl was prepared by a modification of previously described methods. Sodium NaOCl (100 mM) mixed with ethyl acetate (1 ml per 100 ml of reaction mixture) was protonated by dropwise addition of concentrated phosphoric acid (final pH ≤ 6) with intermittent shaking. The organic phase containing HOCl was washed twice with water, and HOCl was extracted into the aqueous phase by dropwise addition of NaOH (final pH = 9). Residual ethyl acetate in the aqueous solution of chloride-free NaOCl was removed by bubbling with nitrogen gas. The concentration of NaOCl was determined spectrophotometrically (ε292 = 350 μM–1 cm–1; Ref. 48). Taurine monochloramine was prepared by addition of HOCl to taurine (1:100; mol/mol). Taurine monochloramine concentration was determined spectrophotometrically (ε292 = 429 μM–1 cm–1; Ref. 16).

Preparation of Hypobromous Acid and Taurine Bromamine—Bromide-free HOBBr was prepared as described (49). Briefly, silver nitrate solution was added to ~80 mM bromine water in a molar ratio of 1:5.1. The precipitate was removed by centrifugation, and 30 ml of the supernatant was distilled under vacuum using a foli-covered microscale distillation apparatus. The distillate was collected in a foil covered vial at 4 °C. Reagent taurine monobromamine was prepared by addition of reagent HOBBr to a 100-fold excess of taurine. HOBBr concentration was determined spectrophotometrically following formation of taurine monochloramine (1–2; Ref. 35).

Oxidation of Uracil and Deoxycytidine—All reactions were performed in gas-tight vials and initiated by addition with a gas-tight syringe of oxidents (H2O2 or HOCl/OCl–) through a septum while vortexing the sample. Reactions were terminated by addition of l-methionine to a final concentration of 6 mM. The concentration of H2O2 was determined spectrophotometrically (ε430 = 43.6 μM–1 cm–1; Ref. 50). The pH dependence of 5-bromouracil and 5-bromodeoxycytidine formation was performed using reaction mixtures containing phosphoric acid, monobasic sodium phosphate, and dibasic sodium phosphate (final concentration, 50 mM). The pH of the reaction mixture (which did not contain l-methionine) was determined at the end of the incubation.

Human Neutrophils—Neutrophils were prepared by density gradient centrifugation (51) and suspended in Dulbecco's phosphate-buffered saline supplemented with 1 mg/ml dextrose, 1 mM deoxycytidine, 100 μM NaBr, and 100 μM DTPA. pH 5.9. Differential cell counts revealed that neutrophil preparations contained 96–100% neutrophils and 0–4% eosinophils. Cells (3 ml) were activated with 200 nM phorbol myristate acetate, incubated at 37 °C for 60 min, and maintained in suspension with intermittent inversion. The reaction was terminated by addition of 6 M methionine and centrifugation at 400 × g for 10 min. The supernatant was concentrated to dryness under vacuum, dissolved in 0.4 ml of HPLC solvent A, centrifuged at 14,000 × g for 10 min, and the supernatant was subjected to HPLC analysis.

Reverse-phase HPLC of Reaction Products—Uracil and deoxycytidine reaction products were analyzed by reverse-phase HPLC with a C18 column (Beckman Porasil, 5 μm resin, 4.6 × 250 mm) at a flow of 1 ml/min and UV detection at 274 and 295 nm, respectively. Uracil reactions were analyzed by injection of 100 μl of reaction mixture onto the column followed by isocratic elution with 20% ammonium formate. For analysis of deoxycytidine reactions, 100 μl of the reaction mixture was injected on the column and eluted with a gradient of: 95% solvent A (0.1% trifluoroacetic acid, pH 2.5) and 5% solvent B (0.1% trifluoroacetic acid in methanol, pH 2.5) for 4 min, 5–100% solvent B over 20 min, and then 100% solvent B for 10 min. 5-Bromouracil and 5-bromodeoxycytidine yields were quantified by comparison of integrated peak areas to standard curves generated using commercially available compounds. For mass spectrometric analysis, HPLC fractions were collected and concentrated under vacuum. For NMR analysis, 10-fold concentrated reaction mixtures were fractionated on a semi- preparative C18 column (μPorasil, 5 μm resin, 10 × 250 mm; Beckman) at a flow rate of 2.5 ml/min with an isocratic gradient consisting of 90% 20 mM ammonium formate, pH 6.3, and 10% methanol. N-Chlorodeoxycytidine (retention time, 10 min) was prepared as described (20) and isolated with an analytical C18 column (μPorasil; 5 μm resin, 4.6 × 250 mm; Beckman) column using 5% methanol at a flow rate of 1 ml/min.

NMR Studies—Reaction products were isolated by HPLC, solubilized in D2O, and analyzed at 25 °C with a Varian Unity-Plus 500 spectrometer (499.843 MHz for H1) equipped with a Naloric indirect detection probe. 1H Chemical shifts were referenced to external sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 in D2O. Spectra were recorded from 8 transients with a 12-s pulse delay across a spectral width of 8000 Hz. Pyrimidine resonances of the brominated deoxycytidine (8.23 ppm; singlet, H6) and uracil (7.79 ppm; singlet, H6) reaction products were essentially identical to those of commercially available 5-bromodeoxycytidine and 5-bromouracil. When compared with substrate, the aromatic region of each product spectrum was notable for the lack of proton resonances at C-8, a downfield shift in the proton resonance at C-6, and conversion of the C-6 proton resonance from a doublet to a singlet, both of which are consistent with substitution of a bromine atom at the C-5 position.

Gas Chromatography-Mass Spectrometry (GC/MS)—After nucleobases were dried under vacuum, residual water was removed by forming an azo trope with 50 μl of pyridine and again drying the suspension under vacuum. DNA bases were converted to trimethylsilyl derivatives with excess bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane at 25 °C for 60 min. Aliquots (1 μl) were analyzed in the positive electron ionization mode using full mass scanning on either a Hewlett Packard 5890 Series II gas chromatograph.
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5-Bromodeoxycytidine Is the Major Product When the Myeloperoxidase-\(\text{H}_2\text{O}_2-\text{Br}^-\) System Oxidizes Deoxycytidine at Plasma Concentrations of Halide—To determine whether the enzyme can halogenate other pyrimidines, we incubated uracil with the myeloperoxidase-\(\text{H}_2\text{O}_2-\text{Cl}^-\) system. In the absence of added \(\text{Br}^-\), a new peak of material (retention time, 13.7 min) was detectable in the reaction mixture by HPLC analysis (Fig. 1B). In the presence of 100 \(\mu\text{M} \text{Br}^-\), we observed a second peak (retention time, 18 min). We isolated both peaks of material by HPLC and determined their structures by ultraviolet absorption spectroscopy, GC, and positive ion mass spectrometry, electrospray ionization tandem mass spectrometry, and \(^1\text{H}\) NMR spectroscopy (see “Methods”). We identified the early and late eluting materials as 5-chlorouracil and 5-bromouracil, respectively.

Production of 5-bromouracil by myeloperoxidase was dependent on the concentration of \(\text{Br}^-\) in the reaction mixture (Fig. 2). Increasing \([\text{Br}^-]\) from 0 to 100 \(\mu\text{M}\) in the presence of 100 \(\mu\text{M} \text{Cl}^-\) reduced uracil chlorination but increased uracil bromination. The apparent \(K_m\) values for \(\text{Cl}^-\) and \(\text{Br}^-\) binding by myeloperoxidase are 175 and 2 \(\mu\text{M}\), respectively, suggesting that the enzyme did not directly oxidize \(\text{Br}^-\) to generate material that migrated with a retention time distinct from that of 5-chlorodeoxycytidine (Fig. 1A). The new oxidation product was isolated by HPLC and identified as 5-bromodeoxycytidine on the basis of its HPLC retention time, ultraviolet absorption spectrum, GC retention time and positive ion mass spectrum, electrospray ionization tandem mass spectrum, and \(^1\text{H}\) NMR spectrum (see “Methods”).

Under these reaction conditions, 5-bromodeoxycytidine was also the major product when eosinophil peroxidase or lactoperoxidase oxidized deoxycytidine. It was therefore important to determine whether our myeloperoxidase preparation was contaminated by other peroxidases, even though we isolated the enzyme from HL-60 cells, a promyelocytic cell line that is not known to express other peroxidases. Myeloperoxidase was apparently pure as assessed by its heme spectrum and by denaturing polyacrylamide gel electrophoresis. Moreover, it yielded a single band of peroxidase activity that migrated with a retention time distinct from that of eosinophil peroxidase on nondenaturing gel electrophoresis. These observations indicate that the reactive intermediates that brominated deoxycytidine at plasma concentrations of \(\text{Cl}^-\) and \(\text{Br}^-\) resulted from the action of myeloperoxidase.

RESULTS

5-Bromouracil is the Major Product When the Myeloperoxidase-\(\text{H}_2\text{O}_2-\text{Br}^-\) System Oxidizes Deoxycytidine at Plasma Concentrations of Halide—We previously demonstrated that the myeloperoxidase-\(\text{H}_2\text{O}_2-\text{Cl}^-\) system (containing 100 \(\mu\text{M} \text{Cl}^-\)) oxidizes deoxycytidine to 5-chlorodeoxycytidine (20). When we supplemented this system with a plasma concentration of \(\text{Br}^-\) (100 \(\mu\text{M}\)), HPLC analysis detected a peak of...
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5-bromouracil. The relative yields of the halogenated pyrimidines also differed; under optimal conditions, the yield of 5-bromouracil was 5-fold greater than that of 5-chlorouracil.

We used HPLC to investigate the reaction requirements for generation of 5-bromodeoxycytidine by the myeloperoxidase-eosinophil peroxidase system (Table I). Both systems required enzyme and H_2O_2 and were blocked by catalase, a scavenger of H_2O_2. The product yield of the reaction was nearly quantitative relative to H_2O_2. Omitting Br^- did not completely prevent bromination, perhaps because the NaCl used as the salt contained up to 0.01% NaBr (up to a final [Br^-] of 10 μM). Two heme-enzyme inhibitors, cyanide and aminotriazole, also inhibited product formation. Both myeloperoxidase and eosinophil peroxidase brominated deoxycytidine in the absence of added Cl^-, which is consistent with the ability of myeloperoxidase to convert Br^- into HOBr (46). These results demonstrate that bromination of deoxycytidine by myeloperoxidase or eosinophil peroxidase requires active enzyme, Br^-, and H_2O_2.

The two enzymes differed in one important respect, however. Bromination by myeloperoxidase but not by eosinophil peroxidase was inhibited by taurine (Table I), which can react with HOCl or HOBr to form chloramines or bromamines (14, 35). This observation suggests that the enzymes brominate pyrimidines by different reaction pathways.

We used HPLC to establish the optimal reaction conditions for bromination of uracil (Fig. 3) and deoxycytidine (Fig. 4) by the myeloperoxidase-H_2O_2-Br^- system. Bromination of both substrates was proportional to [H_2O_2] from 0 to 100 μM. The yields of both products increased with rising [Br^-] over a physiologically relevant range. The pH dependence demonstrated a distinct optimum of 4.5–5.0 for 5-bromodeoxycytidine generation (Fig. 4C) and a broader range of 4.0–6.0 for 5-bromouracil generation (Fig. 3B). Both reactions were largely complete after 60 min.

Reagent HOCl Generates 5-Bromouracil and 5-Bromodeoxycytidine by a Reaction That Requires Br^-—Previous studies have indicated that myeloperoxidase preferentially oxidizes Cl^- to HOCl at plasma concentrations of Br^- and Cl^- . In contrast, eosinophil peroxidase selectively oxidizes Br^- to an HOBr-like species. To determine whether HOCl could be an intermediate when myeloperoxidase generates 5-bromouracil (Fig. 5) or 5-bromodeoxycytidine (Fig. 6), we compared the Br^-, oxidant, and pH dependences of product formation by reaction HOBr and HOCl. When we used HOBr, product yield was independent of [Br^-] (Figs. 5A and 6A). In striking contrast, HOCl required Br^- to brominate deoxycytidine.

Production of 5-bromouracil and 5-bromodeoxycytidine increased linearly with [HOBr] (Figs. 5B and 6B). In contrast, the yield of brominated products reached a plateau at around 75 μM of HOCl, a concentration similar to that of Br^- (100 μM) in the reaction mixture. The pH dependences of product generation by HOCl and HOBr were similar under acidic and neutral conditions. However, the yields of 5-bromouracil differed under alkaline conditions: at pH 8, HOBr but not HOCl generated large amounts of product (Fig. 5C). The decline in uracil bromination by HOCl at pH 8 was accompanied by an increase in uracil chlorination (data not shown). These results indicate that HOCl readily oxidizes Br^- to generate a brominating agent at physiologically plausible concentrations of halide.

Hypochlorous Acid and Myeloperoxidase Generate the Interhalogen Gas BrCl at Physiologic Concentrations of Bromide and Chloride—Previous studies indicate that reagent HOCl and HOBr have indicated that myeloperoxidase preferentially oxidizes brominated uracil by the myeloperoxidase-H_2O_2-Cl^- system. After a 60-min incubation at 37 °C, the reaction was terminated by adding 6 μmol of L-methionine, and products were quantified by reverse-phase HPLC.
To determine whether HOCl generates BrCl under physiologically plausible conditions, we added 50 \( \mu \text{M} \) of HOCl to a reaction mixture containing 100 \( \times \text{mM} \) Cl\(^-\) and 100 \( \times \text{mM} \) Br\(^-\), sparging the mixture continuously with nitrogen gas. The gas then was bubbled through cyclohexene, which contains a cis-double bond analogous to those found in pyrimidines and biological lipids. Because cyclohexene is an aprotic, nonpolar solvent that would prevent the proton and halide-dependent formation of BrCl shown in Equation 5, direct reaction of cyclohexene with BrCl from the reaction mixture would be the most likely source of any 1-bromo-2-chlorocyclohexane that appeared in this experiment (Scheme 1 and Ref. 52).

Mass spectrometric analysis of cyclohexene exposed to the gas sparged from the HOCl reaction mixture revealed a major peak of material with the mass-to-charge-ratio (Fig. 7A) and isotopic pattern expected for the molecular ion of 1-bromo-2-chlorocyclohexane. This material exhibited a retention time midway between that of authentic 1,2-dichlorocyclohexane and that of 1,2-dibromocyclohexane. It was not detectable when we omitted HOCl from the reaction mixture (Fig. 7A). When we replaced HOCl-Cl\(^-\) with the myeloperoxidase-H\(_2\)O\(_2\)-Cl\(^-\) system, we also observed a peak of material with the retention time and characteristic isotopic pattern of 1-bromo-2-chlorocyclohexane (Fig. 7B). Production of 1-bromo-2-chlorocyclohexane by myeloperoxidase required enzyme, H\(_2\)O\(_2\), Cl\(^-\), and Br\(^-\). These observations indicate that the myeloperoxidase pathway can produce BrCl by generating HOCl, which reacts with Br\(^-\) to form the interhalogen gas.

Physiologic Concentrations of Chloride Stimulate the Bromination of Deoxycytidine by Reagent HOCl—BrCl is hydrolyzed to HOBr and Cl\(^-\) in reaction mixtures containing H\(_2\)O.

\[
\text{BrCl} + \text{H}_2\text{O} \rightarrow \text{HOBr} + \text{Cl}^- + \text{H}^+ \quad \text{(Eq. 6)}
\]

Addition of Cl\(^-\) should drive this equilibrium toward the formation of BrCl. We therefore examined the effect of [Cl\(^-\)] on the formation of 5-bromodeoxycytidine by Br\(^-\) and chloride-free HOCl (Fig. 8). The yield was doubled by plasma concentrations of Cl\(^-\) (100 \( \times \text{mM} \)) and tripled by the highest concentration we examined (1,000 \( \times \text{mM} \) Cl\(^-\)). This increase in yield was not observed when chloride was replaced with perchlorate (ClO\(_4\)^\(^-\)), ruling out ionic strength as a cause of enhanced yield. Control experiments demonstrated that contamination of NaCl with NaBr also was not responsible for the increased yield of 5-bromodeoxycytidine. These observations support the hypothesis that BrCl is an important intermediate when myeloperoxidase brominates deoxycytidine.

Bromamines and Chloramines Produce 5-Bromodeoxycytidine—HOCl can react with amines to form BrCl, which then could react with Br\(^-\) to generate brominating species (52). Alternatively, chloramine formation might compete with Br\(^-\), inhibiting the formation of brominating species by HOCl. Our observation that taurine inhibits bromination by HOCl and Br\(^-\) (Table I) is consistent with the latter possibility. To distinguish between these two possibilities, we determined whether different haloamines can produce 5-bromodeoxycytidine (Fig. 9). Both N-chlorotaurine and N-bromotaurine generated high concentrations of 5-bromodeoxycytidine below pH 5 in the presence of 100 \( \times \text{mM} \) Br\(^-\) and 100 \( \times \text{mM} \) Cl\(^-\). Over the pH range 5.5–7, however, only N-bromotaurine generated significant levels of 5-bromodeoxycytidine.

Unlike N-chlorotaurine, N-chlorodeoxycytidine failed to convert cytosine to 5-bromocytosine except under strongly acidic conditions (<pH 3), and the yield was low even at pH 2 (Fig. 9). The chloramine of deoxycytidine therefore is unlikely to be an intermediate when myeloperoxidase brominates deoxycytidine. These observations indicate that primary haloamines can brominate pyrimidines in the presence of Br\(^-\). They also suggest that Br\(^-\) oxidation by HOCl is more rapid than nucleoside chloramine formation.

Activated Human Neutrophils Generate 5-Bromodeoxycytidine and 5-Bromouracil at Plasma Halide Concentrations—To determine whether oxidants generated by human neutrophils...
can brominate nucleobases, we stimulated the cells with phorbol myristate acetate in physiological salt solution supplemented with 100 μM NaBr and 1 mM deoxycytidine (Table II) or uracil (data not shown). HPLC and mass spectrometric analysis detected substantial quantities of 5-bromodeoxycytidine and 5-bromoouracil in the medium of the activated cells. Bromination required stimulation of the cells with phorbol ester and was inhibited by catalase and heme poisons, implicating H$_2$O$_2$ and a heme protein in the reaction (Table II). Omitting supplemental Br$^-$ significantly reduced but did not eliminate 5-bromodeoxycytidine production, most likely because the medium was contaminated with Br$^-$. Superoxide dismutase failed to affect the reaction.

Generation of 5-bromodeoxycytidine (Fig. 10, upper panel) by activated neutrophils was strongly affected by the pH of the culture medium. Acidification to the range observed in inflamed tissue and the phagolysosome significantly enhanced the cellular production of 5-bromodeoxycytidine. The progress curve of the reaction was essentially complete 30 min after the cells were activated with phorbol ester (Fig. 10, lower panel).

**Human Neutrophils Generate Brominating Intermediates by a Pathway Involving HOCl—In vitro studies demonstrated that N-bromotaurine, but not N-chlorotaurine, brominates deoxycytidine at pH 5.9 in the presence of 100 μM Br$^-$ and 100 mM Cl$^-$.** (Fig. 9). This observation suggests that reagent HOBr, but not HOCl, should brominate deoxycytidine in the presence of taurine under these conditions. Indeed, this reaction occurred when mM concentrations of taurine were included in the reaction mixture (Fig. 11A). In contrast, taurine inhibited bromination by HOCl under the same conditions, probably because it consumed the oxidant to form N-chlorotaurine, which is unreactive at pH 5.9. Taurine was also inhibitory (Fig. 11A) when the myeloperoxidase-H$_2$O$_2$ system replaced HOCl. In contrast, it had little affect on bromination by the eosinophil peroxidase-H$_2$O$_2$ system. It is noteworthy that, in the absence of taurine, product yields (relative to oxidant) of 5-bromodeoxycytidine were similar with reagent HOCl, reagent HOBr, myeloperoxidase, and eosinophil peroxidase. These observations indicate that reactive species generated by HOCl or HOBr brominate deoxycytidine with similar efficiency under these conditions. They also demonstrate that use of taurine, a potent scavenger of hypohalous acids, can distinguish between bromination reactions mediated by HOBr and HOCl.

We used taurine to determine whether human neutrophils use HOCl or HOBr to brominate deoxycytidine (Fig. 11B). We activated the cells with phorbol ester, incubated them for 60
min at pH 5.9 in buffer containing 100 μM NaBr, 100 mM NaCl, and 1 mM deoxycytidine, and used HPLC to determine whether 5-bromodeoxycytidine was produced. Taurine markedly inhibited the brominating ability of neutrophils (Fig. 11B), with an IC₅₀ similar to that of the myeloperoxidase-H₂O₂-Cl⁻-Br⁻ system (Fig. 11A). These observations strongly support the hypothesis that the major pathway by which human neutrophils generate reactive brominating species involves HOCl production followed by oxidation of Br⁻.

**DISCUSSION**

Our observations demonstrate that the myeloperoxidase system of human neutrophils generates brominating oxidants that convert uracil into 5-bromouracil and transform deoxycytidine into 5-bromodeoxycytidine (Scheme 2). Importantly, these pyrimidines become halogenated at plasma concentrations of Br⁻ (100 μM) and Cl⁻ (100 mM), suggesting that this pathway may be physiologically relevant.

Hypochlorous acid is thought to be the major product when myeloperoxidase is exposed to plasma halide concentrations. In contrast, eosinophil peroxidase preferentially converts Br⁻ to HOBr. It was therefore important to determine whether myeloperoxidase was oxidizing Cl⁻ or Br⁻ in our experiments. We obtained several lines of evidence to support the proposal that myeloperoxidase initially generates HOCl, which then is converted into reactive brominating species. First, reagent HOCl brominated uracil and deoxycytidine as effectively as HOBr. The HOCl-mediated reaction required Br⁻ and was optimal under acidic conditions. Second, taurine, which rapidly reacts with HOCl to form N-chlorotaurine, inhibited bromination of deoxycytidine by HOCl-Br⁻ or the myeloperoxidase-H₂O₂-Cl⁻-Br⁻ system. Importantly, kinetic studies indicate that chlorination of taurine by myeloperoxidase may involve an enzymatic intermediate, suggesting that taurine scavenges chlorinating species before they can diffuse out of the active site of the enzyme (14). In contrast, taurine had little effect on bromination by HOBr or the eosinophil peroxidase-H₂O₂-Cl⁻-Br⁻ system. Taurine thus selectively inhibits bromination mediated by HOCl under these experimental conditions. Finally, human neutrophils converted deoxycytidine to 5-bromodeoxycytidine by a reaction requiring H₂O₂ and Br⁻. Bromination was optimal under acidic conditions and was inhibited by taurine and heme poisons, implicating HOCl and myeloperoxidase in the reaction pathway. Collectively, these observations indicate that activated human phagocytes use myeloperoxidase to brominate pyrimidines at plasma concentrations of halide by reactions that initially require HOCl.

A key question is the mechanism by which HOCl then generates reactive brominating species. Based on the chemistry of the interhalogen compounds (40, 41), we propose that BrCl is...
Transhalogenation by Myeloperoxidase

![Diagram](attachment:image.png)

one potential intermediate in the pathway. BrCl is a stronger brominating oxidant than Br₂ or HOBr (42), suggesting that it halogenates pyrimidines. Alternatively, bromination may be mediated by HOBr or Br₂, which are in equilibrium with BrCl.

To directly detect BrCl, we sparged a reaction mixture containing HOCl-Br⁻ or the myeloperoxidase-H₂O₂-Cl⁻-Br⁻ system with nitrogen gas that we subsequently passed through cyclohexene. Mass spectrometric analysis of the resulting cyclohexene solution detected an ion with the expected mass-to-charge ratio, GC retention time, and isotopic abundance of 1-bromo-2-cyclohexane. Cyclohexene is an aprotic, nonpolar solvent that should not contain halide ions under these conditions. Thus, bromination of cyclohexene cannot involve a back-side nucleophilic attack of a bromonium ion intermediate by free Cl⁻ in solvent. Instead, it is likely to involve the concerted attack on the double bond by [Br⁻-Cl⁻] derived from molecular BrCl (Scheme 1). Detecting 1-bromo-2-cyclohexane therefore provides strong evidence that HOCl and myeloperoxidase generate the interhalogen gas BrCl.

Our demonstration that [Cl⁻] increased the yield of 5-bromo-2-deoxytocycinidine generation by reagent HOCl and Br⁻ is consistent with this proposal. Chloride ion is likely to stimulate bromination by reacting with HOBr to form BrCl (Equation 6). These observations further support the hypothesis that HOBr and HOCl are in equilibrium with BrCl under acidic conditions in the transhalogenation pathway (Scheme 3).

Another important question is whether the acidic conditions that are optimal for myeloperoxidase to generate brominating intermediates are likely to exist in vivo. We suggest that the acidic conditions that result from infection or tissue hypoxia at sites of inflammation may represent such an environment (54, 55). Also, cultured activated macrophages form phagocytic compartments in which the pH falls below 5 (46). Moreover, strongly acidic environments exist in the phagolysosome and stomach, where myeloperoxidase-derived HOCl (or chloramines) might generate brominated pyrimidines. Human neutrophils use a Cl₂-like species to generate 3-chlorotyrosine from tyrosine in vitro, and we have shown that 3-chlorotyrosine levels are markedly elevated in human atherosclerosis, a chronic inflammatory condition (13, 21). Therefore, inflammation may generate acidic environments in which myeloperoxidase can produce brominating oxidants.

Chronic inflammation is an important risk factor for cancer, and oxidants generated by phagocytic cells mutate bacteria and transform cultured mammalian cells (57, 58). Moreover, recent genetic epidemiological studies have found a relationship between cancer risk and a polymorphism in the promoter region of the myeloperoxidase gene. People with a polymorphism that increases myeloperoxidase expression were at increased risk for promyelocytic leukemia (26, 27). In contrast, those with polymorphisms that lower myeloperoxidase expression were at decreased risk for lung and laryngeal cancers (25, 28–30). These observations suggest that nucleobase halogenation might provide one mechanism for mutagenesis and cytotoxicity in vivo. Indeed, 5-bromouracil, one product of the myeloperoxidase-H₂O₂-Cl⁻-Br⁻ system, is incorporated into nuclear DNA as 5-bromodeoxyuridine, a known mutagen (59–63). Moreover, myeloperoxidase converts deoxycytidine to 5-bromodeoxycytidine, and we have shown that 5-bromodeoxycytidine generated by eosinophil peroxidase is incorporated into the genomic DNA of cultured mammalian cells as 5-bromodeoxyuridine.

Mutagenesis by oxidants is generally thought to occur via direct damage to DNA. However, our results suggest an additional mechanism: halogenation of nucleobases within a precursor pool. In this scenario, activated phagocytes generate halogenating intermediates, which react with nucleotides or nucleotide precursors in the extracellular and intracellular milieu. Consequently, potentially cytotoxic and mutagenic deoxyribonucleotide derivatives become incorporated into the genomes of daughter cells. If they find their way into tumor suppressor genes, genes for DNA repair, or potential oncogenes, they might increase the risk for cancer. A similar incorporational mechanism of mutagenesis has been suggested for the MutT system in bacteria, which cleanses the deoxyribonucleotide pool of 8-oxodGTP (64). When MutT is genetically inactivated, the spontaneous mutation rate increases 100–1,000-fold (65–67).

We found that two primary haloamines, N-bromotaurine and N-chlorotaurine, also brominate deoxycytidine at plasma concentrations of halide. However, the reaction with N-chlorotaurine required more acidic conditions than bromination by HOBr or HOCl; bromination by N-bromotaurine and N-chlorotaurine were half-maximal at pH 5.8 and 4.5, respectively. Also, the pyrimidine ring of cytosine contains an exocyclic amino group and two nitrogens, raising the possibility that N-chlorocytose is an intermediate in the bromination reaction (20, 68). However, we found that N-chlorodeoxycytidine was unable to generate 5-bromodeoxycytidine under our standard experimental conditions. These observations indicate that primary chloramines and bromamines, but not N-chlorocytosine, can be intermediates in the bromination of deoxycytidine. Haloamines are relatively stable compounds that can diffuse long distances and cross plasma membranes. Because hypohalous acids rapidly react with primary amino groups and extracellular fluids contain high concentrations of low molecular weight amines, it is possible that haloamines represent one mechanism for brominating nucleobases under acidic conditions.

Chronic inflammation is a risk factor for cancer, and reactive chlorinating, brominating, and nitrating intermediates generated by myeloperoxidase damage nucleobases in vitro (20, 69). These observations suggest that phagocytes may constitute a physiologically important pathway for oxidative damage to DNA. This hypothesis would be strongly supported by detection of brominated or chlorinated pyrimidines in vivo, with important implications for the pathogenesis of tissue injury and perhaps mutagenesis during inflammation.

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