Molecular Chlorine Generated by the Myeloperoxidase-Hydrogen Peroxide-Chloride System of Phagocytes Produces 5-Chlorocytosine in Bacterial RNA*

(Received for publication, May 7, 1999, and in revised form, September 14, 1999)

Jeffrey P. Henderson‡§, Jaeman Byun‡, and Jay W. Heinecke¶¶

From the Departments of ‡‡ Medicine and ¶¶ Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Myeloperoxidase, a heme enzyme secreted by activated phagocytes, uses H₂O₂ and Cl⁻ to generate the chlorinating intermediate hypochlorous acid (HOCl). This potent cytotoxic oxidant plays a critical role in host defenses against invading pathogens. In this study, we explore the possibility that myeloperoxidase-derived HOCl might oxidize nucleic acids. When we exposed 2'-deoxycytidine to the myeloperoxidase-H₂O₂-Cl⁻ system, we obtained a single major product that was identified as 5-chloro-2'-deoxycytidine using mass spectrometry, high performance liquid chromatography, UV-visible spectroscopy, and NMR spectroscopy. 5-Chloro-2'-deoxycytidine production by myeloperoxidase required H₂O₂ and Cl⁻, suggesting that HOCl is an intermediate in the reaction. However, reagent HOCl failed to generate 5-chloro-2'-deoxycytidine in the absence of Cl⁻. Moreover, chlorination of 2'-deoxycytidine was optimal under acidic conditions in the presence of Cl⁻. These results implicate molecular chlorine (Cl₂), which is in equilibrium with HOCl through a reaction requiring Cl⁻ and H⁺, in the generation of 5-chloro-2'-deoxycytidine. Activated human neutrophils were able to generate 5-chloro-2'-deoxycytidine. Cellular chlorination was blocked by catalase and heme poisons, consistent with a myeloperoxidase-catalyzed reaction. The myeloperoxidase-H₂O₂-Cl⁻ system generated similar levels of 5-chlorocytosine in RNA and DNA in vitro. In striking contrast, only cell-associated RNA acquired detectable levels of 5-chlorocytosine when intact Escherichia coli was exposed to the myeloperoxidase system. This observation suggests that oxidizing intermediates generated by myeloperoxidase selectively target intracellular RNA for chlorination. Collectively, these results indicate that Cl₂ derived from HOCl generates 5-chloro-2'-deoxycytidine during the myeloperoxidase-catalyzed oxidation of 2'-deoxycytidine. Phagocytic generation of Cl₂ therefore may constitute one mechanism for oxidizing nucleic acids at sites of inflammation.

Oxidants generated by activated white blood cells play a central role in host antimicrobial defenses but may also damage host tissues (1–5). Oxidant production begins with a membrane-associated NADPH oxidase, which reduces molecular oxygen to superoxide. Dismutation of superoxide then yields H₂O₂. Phagocytes can use the oxidizing capacity of this H₂O₂ to generate potent cytotoxic oxidants because they also secrete the heme enzyme myeloperoxidase. At plasma concentrations of chloride (Cl⁻), the major action of the enzyme is to convert Cl⁻ to hypochlorous acid (HOCl) (6, 7).

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

HOCl undergoes numerous reactions with biomolecules, including aromatic chlorination (8, 9), double bond addition (10, 11), chloramine formation (12–16), aldehyde generation (12, 17, 18), and oxidation of thiols (19). Myeloperoxidase also converts free tyrosine to tyrosyl radical, a reactive intermediate that cross-links proteins and initiates lipid peroxidation (20–23). Recent studies indicate that myeloperoxidase catalyzes the nitrite-dependent nitration of tyrosine (24–26). Mass spectrometric analyses of tissue proteins have detected elevated levels of oxidized amino acids characteristic of these reactions at sites of inflammation, implicating myeloperoxidase as one pathway for oxidative damage in vivo (8, 27–29).

We have previously demonstrated that HOCl generated by myeloperoxidase is in equilibrium with molecular chlorine (Cl₂) through a reaction that requires Cl⁻ and H⁺ (30).

\[ \text{HOCl} + \text{H}^+ + \text{Cl}^- \rightarrow \text{Cl}_2 + \text{H}_2\text{O} \] (REACTION 2)

In vitro studies suggested that the production of 3-chlorotyrosine by reagent HOCl involves molecular chlorine rather than HOCl itself. Moreover, Cl₂ derived from HOCl converted cholesterol into a battery of chlorinated and oxygenated sterols (9). We recently demonstrated that 3-chlorotyrosine levels are selectively elevated in human atherosclerotic tissue, strongly suggesting that oxidative reactions involving HOCl or its derivatives are physiologically relevant to tissue damage at sites of inflammation (8).

Chronic inflammation is associated with an increased risk of cancer, raising the possibility that reactive intermediates generated by phagocytes might damage nucleic acids in living cells, compromising the integrity of the genome or altering cellular functions (31). Moreover, in vitro studies have identified numerous modified nucleic acids as products of oxidative reactions. Bases in DNA, for example, are hydroxylated by hydroxyl radical and singlet oxygen (32–34). Reactive nitrogen species generate nitrated nucleobases, such as 8-nitroguanine, and deaminated nucleobases, such as xanthine (35–39). Single-strand breaks in DNA have been proposed as a major effect of...
superoxide damage (40). Halogenated and oxygenated nucleo-
bases are synthesized when high concentrations of hypohalous
acid are reacted with free pyrimidines and purines in dilute aci-
d (14–16, 41). 5-Chlorouracil has been detected in acid-
hydroyzed DNA exposed to reagent HOCl at neutral pH (42),
although DNA does not contain uracil. Nucleobases also can be
modified by aldehydes, which result from lipid peroxidation
(43). Oxidized nucleic acids have been detected in DNA ex-
tracted from cells exposed to oxidizing conditions and in human
urine (44–46). However, the pathways that promote the pro-
duction of oxidized nucleobases in vivo and their relevance to
human pathology are incompletely understood.
We examined the ability of the myeloperoxidase-H2O2-Cl–
 system to oxidize nucleic acids by using 2-deoxycytidine as a
target. Using mass spectrometry, chromatography, UV-visible
spectroscopy, and NMR spectroscopy, we identified 5-chloro-2`
-deoxycytidine as the major stable product. Activated neutro-
phils were also observed to generate 5-chloro-2-deoxycytidine in
a reaction that was inhibited by heme poisons and catalase.
Reactions with reagent HOCl implicated molecular chlorine as
the oxidizing intermediate in the reaction pathway. Unexpect-
edly, 5-chlorocytosine was generated in the RNA but not the
DNA of Escherichia coli exposed to the myeloperoxidase-H2O2-
Cl– system. These observations raise the possibility that chlo-
rinating intermediates generated by myeloperoxidase modify
nucleic acids of pathogens that are attacked by phagocytes.
Such intermediates also might modify nucleic acids in host
cells at sites of inflammation, setting the scene for carcinogen-
esis or other deleterious changes.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sodium hypochlorite, H2O2, organic solvents, and sodium phosphate
were obtained from Fisher. Methionine and nuclease P1 were from
Calbiochem (San Diego, CA). *bis-(trimethylsilyl)trifluoroacetamide*
(BSTFA)1 + 1% trimethylchlorosilane, *N*-methyl-(*-butyldimeth-
ylsilyl)trifluoroacetamide (*TMSTFA* + 1% tert-butyldimethylchlorosilane),
silicate grade pyridine, and acetonitrile were from Regis Technologies, Inc. (Morton Grove, IL).
Porcine eosinophil peroxidase was provided by Dr. M. L. McCormick
(Department of Medicine, University of Iowa). Unless otherwise indi-
cated, all other materials were purchased from Sigma.

**Methods**

**Isolation of Myeloperoxidase (Donor: Hydrogen Peroxide, Oxidoreduc-
tase, EC 1.11.1.7)—**HL-60 cells were used as starting material for
preparation of myeloperoxidase. The enzyme was isolated by sequential
lectin affinity and size exclusion chromatographies (20, 47). Purified
myeloperoxidase (NADHp$_{max}$ ratio of 0.6) was dialyzed against water
and stored in 50% glycerol at −20 °C. Enzyme concentration was deter-
mined spectrophotometrically ($ε_{430}$ = 178 mM$^{-1}$ cm$^{-1}$) (48).

**Human Neutrophils—**Neutrophils were prepared by density gradi-
et centrifugation and suspended in Hanks’ balanced salt solution, pH 7.0
(magnesium-, calcium-, phenol red-, and bicarbonate-free; Life
Technologies, Inc.) supplemented with 100 μM diethylenetriaminepenta-
çetic acid (DTPA) (49). The cells (≤98% neutrophils; ≤4% eosino-
phils) were incubated at 37 °C for 60 min and maintained in suspension
with intermittent inversion. The reaction was terminated by addition
of methionine to 6 mM and centrifugation of the cells at 400
×$g$ for 10 min. The supernatant was concentrated to dryness under vacuum, dissolved
in 0.3 ml of water, centrifuged at 14,000 ×$g$ for 10 min, and subjected
to HPLC fractionation.

**Preparation of Chloride-free Sodium Hypochlorite—**Chloride-free so-
dium hypochlorite (NaOCl) was prepared by a modification of previ-
sously described methods (12). Reagent NaOCl (100 mM) mixed with ethyl
cetate (100 ml) was protonated by dropwise addition of concentrated

1 The abbreviations used are: BSTFA, *bis-(trimethylsilyl)trifluoroacet-
amide; DTPA, diethylenetriaminepentaacetic acid; GC, gas chroma-
tography; MS, mass spectrometry; MtBSTFA, *N*-methyl-(*-butyldimeth-
ylsilyl)-trifluoroacetamide; HPLC, high performance liquid
chromatography; DMTBS5, dimethyl, tert-butyldimethyl.  

Nucleic Acid Chlorination by Myeloperoxidase 33441

**Nucleic Acid Chlorination by Myeloperoxidase**

33441

$^1$ Nucleic acids were precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of ice-cold ethanol. For RNA analysis DNA was removed by DNase I and 500 μg/ml proteinase K for 90 min at 37 °C. Cell lysates were extracted twice with phenol/chloroform/isomyl alcohol (25:24:1 v/v/v) and once with chloroform/isomyl alcohol (24:1 v/v). Nucleic acids were precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of ice-cold ethanol. For RNA analysis DNA was removed by DNase I digestion in the presence of placental ribonuclease inhibitor, whereas for DNA analysis RNA was removed with a combination of RNase T1 and RNase A (52). Nucleases and cell debris were extracted with phe-
nol/chloroform, and nucleic acid polymers were isolated by ethanol precipitation.

**Gas Chromatography-Mass Spectrometry (GC/MS)**—For GC/MS analysis, ethanol-precipitated nucleic acids were suspended in 99% formic acid and hydrolyzed for 45 min at 140 °C under a helium or argon atmosphere. For HPLC fractions of cell media, 3 pmol 5-fluoro-2'-deoxycytidine internal standard was added to each sample. Uniform derivatization efficiency between samples was confirmed by monitoring the m/z 300 ion [M+] – C,H,N of derivatized 5-fluorocytosine. After drying under vacuum, residual water was removed from the samples by forming an azeotrope with 50 μl of pyridine and again drying the suspension under vacuum. DNA bases were converted to dimethyl, tert-butyldimethylsilyl (DMTBS) derivatives with excess N-methyl-N-(2-butyldimethylsilyl)-trifluoroacetamide containing 1% t-butyldimethylchlorosilane (MtBSTFA + 1% tert-butyldimethylchlorosilane) in acetonitrile (3:1 v/v) at 100 °C for 60 min. 1-μl aliquots of the reaction solution were analyzed on a Varian Star 3400 CX gas chromatograph equipped with a 12-m DB-1 capillary column (0.2-mm inner diameter, 0.33-μm film thickness; J&W Scientific) interfaced with a Finnigan SSQ 7000 mass spectrometer operated in the positive electron ionization mode. Injector and interface temperatures were 250 and 280 °C, respectively. The initial GC oven temperature was 80 °C for 2 min, followed by a 60 °C/min increasing ramp to 180 °C and a final 10 °C/min ramp to 220 °C. Samples were analyzed in order of increasing concentration, and derivatizing reagent injections were analyzed between samples to ensure that traces of analyte were not adhering to the injector. GC retention times were established for each analysis session by injection of authentic compounds.

**Electrospray Ionization-Mass Spectrometry**—HPLC fractions were subjected to solid phase extraction on a C18 column (Supelclean LC-18 SPE tubes, 3 ml; Supelco, Inc., Bellefonte, PA) equilibrated with H2O to remove TFA. The column was washed with 2 ml of H2O and eluted with 3 ml of 50% methanol, and the recovered product was dried under vacuum. Full mass scanning, zoom scanning, and low energy collisionally activated dissociation were carried out on a Finnigan LCQ. A 5-μl portion of sample was injected into the electrospray source at a flow rate of 3 μl/min. The electrospray needle was held at 4500 V, and the counter electrode was held at ground potential. Methanol/water/acetic acid (50:49:1 v/v/v) was used to dissolve samples and as carrier solvent. Helium was used as a damping gas and collision activation partner. The flow of gas (1 ml/min) into the mass analyzer cavity was regulated by a pressure regulator and a capillary restrictor. Flow rates were matched to adjust the partial pressure of helium in the mass analyzer cavity to approximately 10−3 Torr. The temperature of the heated capillary was 200 °C. The collision energy was varied by changing the resonance excitation RF voltage. In the full scan mode (m/z 150 to m/z 300), injection time and microscans were 300 ms and 3 s for acquiring each scan, respectively. For each full scan mass spectrum, 10 scans were signal averaged, and the background from the same number of scans was subtracted.

**RESULTS**

The Myeloperoxidase System Oxidizes 2'-Deoxycytidine to 5-Chloro-2'-deoxycytidine—In preliminary experiments, we exposed adenine, cytosine, guanine, and thymine 2'-deoxynucleosides to the myeloperoxidase-H2O2-Cl− system in buffer A (100 mM NaCl, 100 μM DTPA, 50 mM sodium phosphate, pH 4.5). After terminating the reaction with methionine (which scavenges HOCI, chloramines, and H2O2), we analyzed the reaction mixture by HPLC, monitoring absorbance at 254 nm for purines and 280 nm for pyrimidines. 2'-Deoxycytidine generated the highest yield of product (Fig. 1).

The oxidation product generated by myeloperoxidase eluted from a reverse phase column at a higher methanol concentration than 2'-deoxycytidine; it also absorbed ultraviolet light at a longer wavelength (Fig. 1, inset). The product cochromatographed with authentic 5-chloro-2'-deoxycytidine (Fig. 1). Moreover, its absorption spectrum and that of authentic 5-chloro-2'-deoxycytidine were indistinguishable. When reagent HOCI was substituted for the enzymatic system, we obtained a product from 2'-deoxycytidine whose chromatographic behavior was identical to that of 5-chloro-2'-deoxycytidine. These observations suggest that the HOCI generated by myeloperoxidase converts 2'-deoxycytidine into 5-chloro-2'-deoxycytidine.

![FIG. 1. Reverse phase HPLC chromatogram of 2'-deoxycytidine oxidized by myeloperoxidase. 2'-Deoxycytidine (1 mM) was incubated with (-MPO/H2O2) or without (-MPO/H2O2) myeloperoxidase (20 nM) and H2O2 (500 μM) in buffer A (50 mM sodium phosphate, 100 mM sodium chloride, 100 μM DTPA, pH 4.5) for 60 min at 37 °C. Reactions were initiated by the addition of H2O2 and terminated with 6 mM t-methionine. Where indicated, authentic 5-chloro-2'-deoxycytidine was added alone (5-Cl-dC) or added to the complete myeloperoxidase reaction mixture (+MPO/H2O2 + 5-Cl-dC). Inset, normalized UV-visible absorption spectra of 2'-deoxycytidine (dC) and the 2'-deoxycytidine oxidation product generated by myeloperoxidase (5-Cl-dC) (retention time, 12.1 min).](http://www.jbc.org/content/10.1074/jbc.M117.868685)

To further investigate the oxidation product structure, we isolated the compounds by HPLC and subjected them to electrospray ionization tandem mass spectrometry. The positive ion mass spectra of the myeloperoxidase product (Fig. 2A), the hypochlorite reaction product, and authentic 5-chloro-2'-deoxycytidine yielded the same major [M+H]+ ion at m/z 262. All three compounds also exhibited a prominent ion at m/z 264. The relative abundances of the ions at m/z 262 and 264 reflected that of the natural isotopic abundance of 35Cl and 37Cl, strongly suggesting that the 2'-deoxycytidine oxidation product was monochlorinated. The collisionally activated dissociation tandem mass spectrum of the m/z 262 ion generated a product ion at m/z 146, which is consistent with cleavage of the N-glycoside bond of 5-chloro-2'-deoxycytidine to yield 35Cl-substituted cytosine (Fig. 2B). The collisionally activated dissociation tandem mass spectrum of the m/z 264 ion likewise generated a product ion at m/z 148, consistent with cleavage of the chlorinated nucleoside to yield 37Cl-substituted cytosine (Fig. 2C). The electrospray ionization MS/MS spectrum of the myeloperoxidase product indicates that the chlorine substitution site is likely to reside on the cytosine base of 2'-deoxycytidine.

Further structural characterization of the modified nucleobase generated from 2'-deoxycytidine by myeloperoxidase was achieved using GC/MS to obtain informative electron ionization mass spectrum and GC retention time from material isolated by HPLC. This procedure yields information only about the nucleobase because the N-glycoside bond of the nucleoside is hydrolyzed during the derivatization reactions. After establishing retention times and mass spectra for the trimethylsilyl and DMTBS derivatives of authentic 5-chloro-2'-deoxycytidine, we analyzed the oxidation product generated by
Nucleic Acid Chlorination by Myeloperoxidase

Reaction Requirements for Chlorination of 2′-Deoxycytidine by Myeloperoxidase—We used reverse phase HPLC to characterize the chlorination of 2′-deoxycytidine by myeloperoxidase. Generation of 5-chloro-2′-deoxycytidine required myeloperoxidase, Cl−, and H2O2; it was blocked by catalase, a scavenger of H2O2 (Table I). The overall yield of the reaction was 15% relative to H2O2. Two heme enzyme inhibitors, cyanide and azide, also blocked product formation. Chlorination also was inhibited by methionine, which scavenges HOCI, chloramines, and H2O2. These results demonstrate that chlorination of 2′-deoxycytidine by myeloperoxidase requires active enzyme, Cl−, and H2O2. Eosinophil peroxidase, but not lactoperoxidase and horseradish peroxidase, was also capable of chlorinating 2′-deoxycytidine, although with much less efficiency than myeloperoxidase (Table I). Unlike lactoperoxidase and horseradish peroxidase, eosinophil peroxidase can generate low levels of HOCI from H2O2 and Cl− (54). This property might account for its chlorinating ability.

Fig. 4 illustrates the effect of varying reaction conditions on 5-chloro-2′-deoxycytidine generation by myeloperoxidase. Chlorination was maximal at plasma concentrations of Cl− (100 mM) and under acidic conditions. The reaction was proportional to H2O2 concentration up to 400 μM, with little increase in product yield at higher peroxide concentrations. It’s
TABLE I

Requirements for the conversion of 2'-deoxycytidine into 5-chloro-2'-deoxycytidine by the myeloperoxidase-H$_2$O$_2$-Cl$^-$ system

<table>
<thead>
<tr>
<th>Condition</th>
<th>5-chloro-2'-deoxycytidine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete peroxidase system</td>
<td></td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>45.1</td>
</tr>
<tr>
<td>Eosinophil Peroxidase</td>
<td>11.3</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Herosderash Peroxidase</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Complete myeloperoxidase system minus</td>
<td></td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Complete myeloperoxidase system plus</td>
<td></td>
</tr>
<tr>
<td>Azide (10 mM)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Cyanide (10 mM)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Methionine (6 mM)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Catalase (200 mM)</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Fig. 4. Reaction requirements for 5-chloro-2'-deoxycytidine generation by myeloperoxidase. 2'-Deoxycytidine (1 mM) was modified by incubation for 60 min at 37°C with myeloperoxidase (20 nM) and H$_2$O$_2$ (300 µM) in buffer A. The reaction was terminated by the addition of 6 mM l-methionine and 80 nM catalase. Where indicated, catalase was omitted by reverse phase HPLC. Its structure was confirmed in three ways: (i) by UV-visible spectroscopy, which revealed absorbance maxima at 227 and 273 nm, which are similar to previously observed values for N-chloro-deoxycytosine (14); (ii) by electrospray ionization tandem mass spectrometry; the collisionally activated tandem mass analysis of the ion demonstrated that the molecular ion at m/z 262 was converted to an ion at m/z 146, which is consistent with the loss of deoxyribose, and the collisionally activated tandem mass analysis of the ion at m/z 146 showed that a single major ion formed at m/z 111, which is consistent with the loss of chlorine radical from the precursor ion; this ion was not observed with 5-chloro-2'-deoxycytidine; and (iii) by using methionine to convert the product to 2'-deoxycytidine and detecting 2'-deoxycytidine through UV-visible spectroscopy and HPLC.

At plasma concentrations of Cl$^-$ (100 mM NaCl), there was no detectable conversion of N-chloro-2'-deoxycytosine to 5-chloro-2'-deoxycytosine at either pH 7 or pH 4.5 (Fig. 6). In contrast, 5-chloro-2'-deoxycytosine was generated under strongly acidic conditions (0.1 M HCl), with a yield of 51%. Collectively, these observations provide strong evidence that, under our experimental conditions, 5-chloro-2'-deoxycytidine generation competes with chloramine formation and that N-chloro-2'-deoxycytidine is not an intermediate in the reaction.

Myeloperoxidase Produces 5-Chloro-deoxycytosine in Double-stranded DNA and RNA—To determine whether myeloperoxidase could form 5-chloro-deoxycytosine in double-stranded DNA and RNA, we determined whether isolated 5-chloro-2'-deoxycytidine was synthesized. 5-Chloro-2'-deoxycytidine was synthesized (14) and isolated by reverse phase HPLC. Its structure was confirmed in three ways: (i) by UV-visible spectroscopy, which revealed absorbance maxima at 227 and 273 nm, which are similar to previously observed values for N-chloro-deoxycytosine (14); (ii) by electrospray ionization tandem mass spectrometry; the collisionally activated tandem mass analysis of the ion demonstrated that the molecular ion at m/z 262 was converted to an ion at m/z 146, which is consistent with the loss of deoxyribose, and the collisionally activated tandem mass analysis of the ion at m/z 146 showed that a single major ion formed at m/z 111, which is consistent with the loss of chlorine radical from the precursor ion; this ion was not observed with 5-chloro-2'-deoxycytidine; and (iii) by using methionine to convert the product to 2'-deoxycytidine and detecting 2'-deoxycytidine through UV-visible spectroscopy and HPLC.

Fig. 5. Reaction requirements for 5-chloro-2'-deoxycytidine generation by reagent HOCl. 2'-Deoxycytidine (1 mM) was exposed to HOCl for 60 min at 37°C to chloride-free HOCl (300 µM) in buffer B (50 mM sodium phosphate, 100 mM sodium chloride, pH 4.5). The reaction was terminated by the addition of 6 mM l-methionine and 140 nM catalase. Where indicated, catalase was omitted by reverse phase HPLC.
Nucleic Acid Chlorination by Myeloperoxidase

Table II
Effect of order of addition of chloride ion on the generation of 5-chloro-2'-deoxycytidine by HOCl

<table>
<thead>
<tr>
<th>Order of addition</th>
<th>5-Chloro-2'-deoxycytidine</th>
<th>Relative yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>dC, buffer, chloride, HOCl</td>
<td>25.8</td>
<td>100</td>
</tr>
<tr>
<td>dC, buffer, HOCl, chloride</td>
<td>0.7</td>
<td>3</td>
</tr>
</tbody>
</table>

![Image of a chromatogram showing the generation of 5-chloro-2'-deoxycytidine](image)

Both myeloperoxidase and H₂O₂ were required for generation of the compound. These results demonstrate that the myeloperoxidase-H₂O₂-Cl⁻ system can chlorinate cell-associated nucleic acids, we suspended E. coli in buffer B (50 mM sodium phosphate, 100 mM sodium chloride, pH 4.5) containing 50 nM myeloperoxidase. We then added H₂O₂ to a final concentration of 300 μM and incubated the suspension for 30 min at 37 °C. The reaction was terminated by adding 6 mM L-methionine. To isolate RNA, total cellular nucleic acid was exposed to DNase, and the polymer that remained was isolated by precipitating it with ice-cold ethanol. We used RNase to isolate DNA in a similar manner. Isolated RNA and DNA then were subjected to acid hydrolysis.

When the acid hydrolysate of the E. coli RNA was derivatized with DMTBS and subjected to electron ionization GC/MS, we detected a peak of material with a GC retention time and mass spectrum identical to those of the bis-DMTBS derivative of authentic 5-chlorocytosine. The compound contained the characteristic ³⁵Cl and ³⁷Cl peaks of the [M⁺ – C₄H₉] ion of 5-chlorocytosine at m/z 316 and 318, respectively (Fig. 8). This signal was not observed when H₂O₂ and myeloperoxidase were omitted from the incubation medium.

Selected ion monitoring and comparison with a standard curve suggested that 2% of the cytosine in RNA had been
converted to 5-chlorocytosine in E. coli exposed to the complete myeloperoxidase system. In contrast, 5-chlorocytosine was undetectable in the bacterial DNA samples. The limit of detection for 5-chlorocytosine was <0.4% of DNA cytosine, suggesting that myeloperoxidase generates reactive intermediates that selectively chlorinate RNA within bacterial cells.

Activated Human Neutrophils Employ the Myeloperoxidase-H2O2-Cl System to Generate 5-Chloro-2'-deoxycytidine—To determine whether HOCl generated by human neutrophils might chlorinate nucleic acids, we activated human neutrophils with phorbol myristate acetate in physiological salt solution supplemented with 2'-deoxycytidine. Detectable amounts of 5-chloro-2'-deoxycytidine were observed by GC/MS analysis of the cell-conditioned medium when cells were stimulated with phorbol ester or phorbol ester plus superoxide dismutase. 5-Chloro-2'-deoxycytidine formation required activation of the cells with phorbol ester and was inhibited by catalase and heme poisons, implicating myeloperoxidase and H2O2 in the reaction (Fig. 9). Superoxide dismutase enhanced the yield of the reaction, perhaps by increasing H2O2 availability or preventing inactivation of myeloperoxidase by superoxide (55).

**DISCUSSION**

Our results demonstrate that the myeloperoxidase-H2O2-Cl system can oxidize nucleobases, particularly 2'-deoxycytidine. Multiple lines of evidence indicate that the major product of 2'-deoxycytidine oxidation is 5-chloro-2'-deoxycytidine. First, the HPLC retention time and absorption spectrum of the single major oxidation product that results from exposing 2'-deoxycytidine to myeloperoxidase was identical to that of authentic 5-chloro-2'-deoxycytidine. Second, the electrospray positive ion mass spectrum and collisionally activated tandem mass spectra of the reaction product that contained 35Cl and 37Cl were indistinguishable from those of authentic 5-chloro-2'-deoxycytidine. Third, the retention times on gas chromatography and the mass spectra of two different derivatives of the oxidation product were essentially identical to those of 5-chloro-2'-deoxycytidine. Fourth, the 1H NMR spectrum of the reaction product is identical to that of authentic 5-chloro-2'-deoxycytidine. Fifth, the optimal reaction conditions for synthesis of the product were similar to those previously reported for HOCl synthesis. Sixth, scavengers of HOCl inhibited the generation of 5-chloro-2'-deoxycytidine by myeloperoxidase, whereas reagent HOCl could replace the enzymatic system. Collectively, these results indicate that reactive species generated by myeloperoxidase chlorinate 2'-deoxycytidine at the 5-position.

HOCl or its conjugate base, hypochlorite, is generally thought to be the chlorinating intermediate generated by myeloperoxidase. However, HOCl is in equilibrium with Cl2 through a reaction that requires Cl− and H+ (Reaction 2). Two lines of evidence strongly suggest that a Cl2-like species is the chlorinating intermediate in 5-chloro-2'-deoxycytidine production. First, chlorination of 2'-deoxycytidine by reagent HOCl was optimal at acidic pH. Second, HOCl failed to chlorinate 2'-deoxycytidine in the absence of Cl− (Scheme 1). These observations implicate a Cl2-like species in the conversion of 2'-deoxycytidine into 5-chloro-2'-deoxycytidine.

Patton et al. (14) synthesized halogenated cytosine by the addition of high concentrations of reagent hypohalous acid to cytosine in dilute acid; they proposed that 5-chlorocytosine is generated by Cl2 when N-chlorocytosine is oxidized (3, 14). To address the relative role of Cl2 generated directly from HOCl (Reaction 2) versus Cl2 generated indirectly from chloramine, we first determined whether the yield of 5-chloro-2'-deoxycytidine is affected by the order in which Cl− is added. If the Cl2 that mediates 5-chloro-2'-deoxycytidine formation is derived from chloramine, the product yield should be the same or greater when Cl− is added to the reaction mixture last rather...
than first. However, we observed that the yield declined dramatically when Cl\(^-\) was added last. This implies that reaction conditions that favor chloramine formation actually inhibit 5-chloro-2'-deoxyctydine generation. We then directly examined the ability of N-chloro-2'-deoxyctydine to generate 5-chloro-2'-deoxyctydine, but we detected no product under our standard reaction conditions. In contrast, when the chloramine was subjected to strongly acidic conditions, we obtained a high yield of 5-chloro-2'-deoxyctydine, as previously reported (14). Taken together, these observations provide strong evidence that N-chloro-2'-deoxyctydine is not an intermediate in the reaction under our experimental conditions. Instead, generation of 5-chloro-2'-deoxyctydine from HOCI competes with chloramine formation over a physiologically plausible pH range.

We detected 5-chlorocytosine in the formic acid hydrolysates of calf liver RNA, calf thymus DNA and RNA isolated from E. coli exposed to the myeloperoxidase-H\(_2\)O\(_2\)-Cl\(^-\) system. These observations indicate that the chlorinating intermediate(s) generated by myeloperoxidase is capable of reacting with biological targets remote from the active site of the enzyme. We were surprised to discover, however, that 5-chlorocytosine appeared only in the RNA and not the DNA of bacteria exposed to myeloperoxidase. This selective halogenation suggests that cytosine bases of RNA are vulnerable to oxidation by the chlorinating intermediate, whereas those of intracellular DNA are not, perhaps because of the structure of DNA, the location in the cell, or the interactions with protective proteins. We were unable to detect 5-chlorocytosine in bacteria exposed to activated neutrophils. However, the limit of detection in this analysis was \(-1\) in 10\(^8\) nucleobases, and studies of other DNA oxidation products suggest that the physiologically relevant level will be on the order of 1 in 10\(^7\)–10\(^8\) nucleobases (56). In future studies it will clearly be important to use more sensitive methods to determine whether phagocytes halogenate nucleotides and polymeric nucleic acids of intact bacterial and mammalian cells.

Experiments with cultured neutrophils demonstrated that when activated by phorbol myristate acetate, these cells could convert extracellular 2'-deoxyctydine to 5-chloro-2'-deoxyctydine. Cellular deoxyctydine chlorination was enhanced by superoxide dismutase but blocked in the presence of heme enzyme inhibitors and catalase, consistent with a role for hydrogen peroxide and myeloperoxidase in the reaction.

One important question is whether the acidic pH optimum for 5-chlorocytosine generation by myeloperoxidase is likely to exist in vivo. It is relevant, therefore, that cultured activated macrophages form phagocytic compartments that achieve a pH of less than 4 (57). Also, tissue hypoxia at sites of inflammation or infection results in acidic conditions that would enhance the generation of Cl\(_2\) by myeloperoxidase (58, 59). It is possible that myeloperoxidase-derived chloramines could generate 5-chlorocytosine when exposed to strongly acidic conditions such as those in phagocyte organelles or gastric lesions. Furthermore, human neutrophils use a Cl\(_2\)-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 (8, 30).

Detecting 5-chlorocytosine in vivo would strongly support a role for Cl\(_2\) as a physiologic oxidant generated by activated phagocytes. Moreover, chlorinated pyrimidines such as 5-chlorocytosine are attractive candidates for exploring the role of phagocytes in oxidizing nucleic acids because myeloperoxidase is the only known human enzyme that generates HOCI at plasma halide ion concentrations. One strategy for addressing this issue would be to analyze inflammatory tissues for 5-chloro-2'-deoxyctydine. The mass spectrometric methods we have developed should provide powerful tools for investigating the role of myeloperoxidase in damaging nucleic acids in vivo.

If chlorinated pyrimidines are generated in vivo, they could have profound effects on the cells in which they form and even on neighboring cells (60-64). Thus, cultured mammalian cells take up and phosphorylate 5-chloro-2'-deoxyctydine, which then can be incorporated into genomic DNA. Moreover, cellular enzymes deaminate 5-chloro-2'-deoxyctydine to the thymidine analog, 5-chloro-2'-deoxyuridyline (60). Such chlorinated pyrimidines exhibit antiviral activity, alter cellular pyrimidine metabolism, induce sister chromatid exchange, and mutate genes (61–64). These observations raise the possibility that myeloperoxidase-catalyzed halogenation of extracellular or intracellular nucleosides and nucleotides may yield products that can be incorporated into nucleic acids, where they would exert mutagenic and cytotoxic effects. Potential substrates for halogenation include plasma nucleosides and nucleobases, intracellular RNA and DNA, deoxyctydine released by T and B cells, and intracellular pools of cytidine and deoxyctydine nucleotides (65, 66).

It is interesting to note that another halogenated pyrimidine, 5-fluorouracil, is known to be incorporated into RNA (67). Many of the cytotoxic effects of this chemotherapeutic agent are thought to be mediated by RNA, suggesting that oxidative damage to RNA may also be biologically relevant. Oxidatively damaged bases also can be incorporated into DNA by DNA polymerase. Organisms have enzymes that cleanse the nucleotide pool of such potentially dangerous species. The bacterial MutT protein, for example, is a nucleoside-triphosphate pyrophosphohydrolase that destroys a mutagenic form of deoxyguanosine triphosphate, 8-oxo-dGTP (68). Mutations in mutT increase the spontaneous mutation rate in the E. coli genome 100–10,000-fold (69–71).

The suggestion that phagocyte-derived toxins could contribute to carcinogenesis by oxidizing cellular nucleic acids is consistent with the association between chronic inflammation and malignancy. Our demonstration that myeloperoxidase can generate reactive chlorinating and nitrating intermediates suggests that nucleic acids may indeed be a target for damage. Detecting chlorinated pyrimidines at sites of inflammation in vivo would strongly support this hypothesis, with important implications for tissue injury and tumor development at chronically inflamed sites.

Acknowledgments—We thank Dr. A. d’Avignon (Department of Chemistry, Washington University) for assistance with NMR studies and Dr. M. L. McCormick (Department of Medicine, University of Iowa) for helpful discussions.
for providing eosinophil peroxidase. Mass spectrometry experiments were performed at the Washington University School of Medicine Mass Spectrometry Resource.

REFERENCES

Molecular Chlorine Generated by the Myeloperoxidase-Hydrogen Peroxide-Chloride System of Phagocytes Produces 5-Chlorocytosine in Bacterial RNA

Jeffrey P. Henderson, Jaeman Byun and Jay W. Heinecke

doi: 10.1074/jbc.274.47.33440

Access the most updated version of this article at http://www.jbc.org/content/274/47/33440

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 67 references, 27 of which can be accessed free at http://www.jbc.org/content/274/47/33440.full.html#ref-list-1