Endogenous Formation of Novel Halogenated 2’-Deoxycytidine

HYPOHALOUS ACID-MEDIATED DNA MODIFICATION AT THE SITE OF INFLAMMATION*1

Received for publication, July 20, 2004, and in revised form, August 18, 2004
Published, JBC Papers in Press, September 13, 2004, DOI 10.1074/jbc.M408210200

Yoshichika Kawai‡, Hiroshi Morinaga, Hajime Kondo, Noriyuki Miyoshi, Yoshimasa Nakamura, Koji Uchida, and Yoshikiko Osawa
From the Laboratory of Food and Biodynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

A potential role of DNA damage by leukocyte-derived reactive species in carcinogenesis has been suggested. Leukocyte-derived peroxidases, such as myeloperoxidase and eosinophil peroxidase, use hydrogen peroxide and halides (Cl⁻ and Br⁻) to generate hypohalous acids (HOCI and HOBr), halogenating intermediates. It has been suggested that these oxidants lead to the formation of halogenated products upon reaction with nucleobases. To verify the consequences of phagocyte-mediated DNA damage at the site of inflammation, we developed a novel monoclonal antibody (mAb2D3) that recognizes the hypohalous acid-modified DNA and found that the antibody most significantly recognizes HOCl/HOBr-modified 2’-deoxycytidine residues. The immunoreactivity of HOCl-treated oligonucleotide was attenuated by excess methionine, suggesting that chloramine-like species may be the plausible epitopes of the antibody. On the basis of further characterization combined with mass spectrometric analysis, the epitopes of mAb2D3 were determined to be novel N⁴,5-dihalogenated 2’-deoxycytidine residues. The formation of the dihalogenated 2’-deoxycytidine in vivo was immunohistochemically demonstrated in the lung and liver nuclei of mice treated with lipopolysaccharides, an experimental inflammatory model. These results strongly suggest that phagocyte-derived oxidants, hypohalous acids, endogenously generate the halogenated DNA bases such as a novel dihalogenated 2’-deoxycytidine in vivo. Halogenation (chlorination and/or bromination) of DNA therefore may constitute one mechanism for oxidative DNA damage at the site of inflammation.

Reactive oxidants generated by phagocytic white blood cells are critical to host defense because they kill invading pathogens. However, they are also potentially dangerous because they may damage tissues at sites of inflammation. The heme enzyme myeloperoxidase (MPO), synthesized and secreted by neutrophils and monocytes, is an important source of oxidants. It uses H₂O₂ generated by the phagocyte NADPH oxidase and chloride ion to produce the potent cytotoxic hypochlorous acid (HOCl). HOCl can oxidize sulphydryl groups (1), halogenate and oxygenate unsaturated lipids (2, 3), and halogenate aromatic compounds (4–6). MPO-derived chlorinating agents also generate secondary oxidants such as monochloramines, dichloramines (7–9), and amino acid-derived aldehydes (10, 11). It has been demonstrated previously that HOCl generated by MPO is in equilibrium with molecular chlorine (Cl₂) through a reaction that requires chloride (Cl⁻) and H⁺ (5, 12). Cl₂ generated by this pathway has been implicated in the production of 3-chlorotyrosine by activated neutrophils (5). Elevated levels of protein-bound 3-chlorotyrosine and MPO are found in human atherosclerotic tissue, strongly suggesting that oxidative reactions involving HOCl damage proteins in this chronic inflammatory disorder (6). A structurally related heme protein, eosinophil peroxidase (EPO), is released by activated eosinophils and helps kill invading parasites. At plasma concentrations of halide (100 mM Cl⁻, 20–100 μM Br⁻, and <1 μM iodide; Ref. 13), EPO preferentially oxidizes Br⁻ to produce the brominating agent HOBr (14). HOBr also oxidizes biomolecules at the site of eosinophilic inflammation (15).

Chronic inflammation increases the risk of cancer, raising the possibility that reactive intermediates generated by neutrophils, eosinophils, monocytes, and macrophages might damage nucleic acids and compromise the integrity of the genome (16–18). Genetic epidemiological studies have found a relationship between cancer risk and a polymorphism in the promoter region of the MPO gene. Individuals with -463G → A MPO polymorphism that lowers MPO expression were at decreased risk for lung and laryngeal cancers (19–22). In addition, schistosomiasis, characterized by an intense eosinophilic granulomatous reaction to the eggs of the blood fluke Schistosoma, greatly increases the risk for cancer (23). These results suggest that MPO- and EPO-catalyzed reactions may play important roles in carcinogenesis.

It has been suggested that reactive intermediates generated by phagocytes might damage nucleic acids in cells at the site of inflammation. In vitro studies have demonstrated that various modified nucleic acids and/or DNA strand breaks were formed in the reaction with reactive oxygen/nitrogen species (24–27). 8-Oxo- and 8-nitroguanine were recognized as potential markers for DNA damage. Glutathione, a major endogenous antioxidant, protects DNA from oxidative damage by both products of the MPO and EPO oxidative systems (28). It has been reported that HOCl/HOBr-modified bases can be cleaved by the endonuclease APE1/Ref1 from mammalian cells (29). However, it is still unknown whether these modified bases are recognized by cellular repair enzymes.
ers for the oxidative DNA damage derived from reactive oxygen and nitrogen species, respectively. Hypohalous acid can halogenate/oxidize pyrimidines and purines. Henderson et al. (12) has reported that HOCl-derived Cl₂ chlorinates 2'-deoxycytidine to generate 5-chloro-2'-deoxycytidine (5-ClCdC) as the major product. 8-Chlorinated products of 2'-deoxyadenosine (28, 29) and 2'-deoxyguanosine (30) have also been identified in the reaction with HOCl. Alternatively, in contrast to these stable carbon-chlorinated products, the formation of semistable or unstable nitrogen-chlorinated products, chloramines (RNHCl and RR’NCI), was also observed (31–34). Unstable nucleoside chloramines, such as thymidine chloramine, are suggested to initiate DNA single and double strand breaks via nitrogen-centered radicals and to transfer their chlorine atoms to other nucleosides (35, 36). The formation of EPO-catalyzed brominated nucleobases has also been demonstrated (37, 38).

Although these in vitro studies suggest that hypohalous acid-derived halogenation of DNA bases may provide one pathway for mutagenesis and cytotoxicity at sites of inflammation, the in vivo formation and localization of halogenated nucleobases and the precise mechanisms have not yet been elucidated. In the current study, to investigate the mechanisms for the halogenation of nucleobases in vivo, we raised a monoclonal antibody directed to hypohalous acid-derived halogenated 2'-deoxycytidine residues and determined its production in the inflammation tissues in a mouse model. Our results showed that both carbon and nitrogen halogenation of nucleobases endogenously occurred in vivo and that the halogenated nucleobases may be implicated in the mutagenesis and cytotoxicity of target cells at sites of inflammation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sodium hypochlorite was obtained from Wakoh (Osaka, Japan). Calf thymus DNA, polyoxyethyleneamines, 2'-deoxynucleosides, DNase I, nucleases P₁, and 8-oxo-2'-deoxyguanosine (8-oxo-G) were obtained from Sigma. Phosphodiesterases I and II were purchased from Worthington Biochemical Corp. Alkaline phosphatase was obtained from Stratagene (La Jolla, CA). MPO from human sputum was purchased from Elastin Products Co. Inc. (Owensville, MO). Oligonucleotides were obtained from Hokkaido System Science (Hokkaido, Japan). The 4C 50-mer (4C) and 15-mer (15C) oligonucleotides (5-BrCdC) were obtained from Sigma-Genosys Japan (Hokkaido, Japan). The oligonucleotide sequence of 5-BrCdC₁₅CdC₁₅ was 5'-C₅X₆C₆X₇C₅X₇C₅X₇X₇-3' (X = 5-BrCdC). Methylated BSA (mBSA) was prepared as reported previously (39). HOBr was prepared as reported elsewhere (40).

**Methods**

**General Procedures**—Liquid chromatography-mass spectrometry was performed with a Micromass VG Platform II (Micromass, Manchester, UK) in electrospray ionization-positive mode. The concentration of H₂O₂ was determined spectrophotometrically (ε₂₄₅ = 43.6 m⁻¹ cm⁻¹). Ref. 12. 2'-Deoxynucleoside oxidation products were analyzed by reverse-phase high performance liquid chromatography (HPLC) at a flow rate of 0.8 ml/min using a C₁₈ column (Develosil ODS-HG-5; 5-μm resin, 8.0 x 250 mm; Nomura Chemicals, Aichi, Japan) with monitoring of absorbance at 280 nm. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The column was equilibrated with 97% solvent A. Compounds were eluted with a linear gradient of 50% solvent B containing 6% methanol at a flow rate of 0.8 ml/min. Peroxynitrite was prepared as described previously (41). DNA (0.5 mg/ml) was treated with peroxynitrite (1 mM) at 37 °C for 1 h.

**Preparation of a Monoclonal Antibody to HOCl-modified DNA**—Calf thymus DNA (0.5 mg/ml) was incubated with 1 μM HOCl in 50 mM sodium phosphate buffer (pH 4.5) containing 100 μM sodium chloride at 37 °C for 1 h. The reaction was terminated by adding 4 μM methionine; the DNA was ethanol-precipitated, washed with 70% ethanol, and quantitated by fluorescence measurement as described previously (42). The peroxi-treated calf thymus DNA (0.5 mg/ml) was electrosynthetically complexed with an equal volume of mBSA (0.5 mg/ml). The complex was emulsified with an equal volume of adjuvant. Six-week-old female BALB/c mice were intraperitoneally immunized with this emulsion (100 μl). After 2 weeks, the mice were boosted with the HOCl-treated DNA/mBSA emulsified with an equal volume of adjuvant. In the final boost, 100 μl of HOCl-treated DNA/mBSA in phosphate-buffered saline (PBS) were mixed with an equal volume of the antibody (1:100 dilution) and subcutaneously injected. Three days after the final boost, one of the mice was sacrificed, and the spleen was removed for fusion with P3U1 myeloma cells. The fusion was carried out by polyethylene glycol. The cells were cultured in the hypoxanthine/aminopterin/thymidine selection medium. After a week, culture supernatants of the hybridomas were screened by enzyme-linked immunosorbent assay (ELISA) using HOCl-modified DNA and untreated DNA as the coating agents. Among them, a clone (named mAb2D3) was used in the following experiments due to its specificity and high ability to cell death. ELISA—The indirect noncompetitive ELISA procedure has been described previously (43). Briefly 100 μl of antibodies in PBS were coated in wells and kept at 4 °C overnight. After washing and blocking with PBS containing 5% dry skim milk, 100 μl of the antibody (1:100 dilution) was added, and the wells were incubated at 37 °C for 2 h. After washing, 100 μl of peroxidase-labeled anti-mouse IgG goat antibody (1:5000 in TPBS) was added, and the wells were incubated at 37 °C for 1 h. After washing, 100 μl of o-phenylenediamine solution (0.5 mg/ml containing 0.03% H₂O₂ in citrate-phosphate buffer) was added. The color development was stopped by the addition of 50 μl of 2 N H₂SO₄. The binding of the antibody to the antigen was evaluated by measuring the optical density at 490 nm.

**In Vitro Modification of 2'-Deoxycytidine in Vivo**

In vitro modification of 2'-deoxycytidine was performed using a photodiode array detector (Jasco) was equilibrated with 97% solvent A. Compounds were eluted with a linear gradient of 50% solvent B containing 6% methanol at a flow rate of 0.8 ml/min. Peroxynitrite was prepared as described previously (41). DNA (0.5 mg/ml) was treated with peroxynitrite (1 mM) at 37 °C for 1 h.

**Detection of Halogenated 8C by HPLC-Tandem Mass Spectrometry**

HPLC-MS/MS analyses were carried out on the API 2000 triple quadrupole mass spectrometer (Applied Biosystems) through a TurbolonSpray source. Chromatography was carried out on a Develosil ODS-HG-3 column (2.0 x 50 mm) using an Agilent 1100 HPLC system. The chromatographic separation was performed by a gradient elution as follows: 0–20 min, linear gradient from 0.5% aqueous acetonitrile containing 0.01% formic acid to 20% aqueous acetonitrile containing 0.01% formic acid; 20–25 min, linear gradient to 50% aqueous acetonitrile containing 0.01% formic acid; 25–30 min, linear gradient to 0.5% aqueous acetonitrile containing 0.01% formic acid; flow rate = 0.2 ml/min. The instrument response was optimized by infusion experiments of the standard compounds using a syringe pump at a flow rate of 0.2 ml/min. Halogeneration of DNA bases were detected using electrospray ionization tandem mass spectrometry in the multiple reaction monitoring mode. Specific transitions used to detect products in the positive ionization mode were those between the molecular cation of the products and the characteristic daughter ion formed from the loss of the 2'-deoxyribose moiety.

**Halogenated 8C Analysis in DNA Samples**—Enzymatic digestion of DNA with DNase I, nuclease P₁, alkaline phosphatase, and phosphodiesterase of (5-BrdC)₃-(dC)₄₇ was obtained from Sigma-Genosys Japan (Hokkaido, Japan). The oligonucleotide sequence of (dC)₅₀ containing three 5-BrdC residues, (5-BrdC)₃-(dC)₄₇, was obtained from Biochemical Corp. Alkaline phosphatase was obtained from Stratagene (La Jolla, CA). MPO from human sputum was purchased from Elastin Products Co. Inc. (Owensville, MO). Oligonucleotides were obtained from Hokkaido System Science (Hokkaido, Japan). The 4C 50-mer (4C) and 15-mer (15C) oligonucleotides (5-BrCdC) were obtained from Sigma-Genosys Japan (Hokkaido, Japan). The oligonucleotide sequence of 5-BrCdC₁₅CdC₁₅ was 5'-C₅X₆C₆X₇C₅X₇C₅X₇X₇-3' (X = 5-BrCdC). Methylated BSA (mBSA) was prepared as reported previously (39). HOBr was prepared as reported elsewhere (40).
estates I/II was performed reported previously (44). Oligonucleotides were hydrolyzed with alkaline phosphatase and phosphodiesterase I. Before digestion, DNA was purified by ethanol precipitation (for calf thymus DNA) or C<sub>18</sub> solid phase extraction column (for oligonucleotides) to eliminate low molecular compounds. After digestion, the reaction mixtures were filtered through Ultrafree-MC membrane (nominal molecular weight limit 5000; Millipore) by centrifugation (10,000 rpm, HIMAC centrifuge, Hitachi, Japan) to remove enzymes; 10 µl of filtrates were injected for HPLC/MS/MS.

Animal Experiment and Immunohistochemistry—Female C57BL/6J mice (7 weeks old) were given an intraperitoneal injection of lipopolysaccharide (LPS, from Escherichia coli, Sigma) in PBS at a dose of 3 mg/kg of body weight. They were sacrificed at 24 h after administration. Livers and lungs were quickly removed and frozen in liquid nitrogen and immediately used for the MPO and GSH assays. MPO activity in tissues was monitored at 512 nm using H<sub>2</sub>O<sub>2</sub> and 4-aminoantipyrine as substrates (45). Measurement of GSH in the cells was performed fluorometrically according to the method of Hissin and Hilf (46). In brief, tissues were homogenized and extracted with 25% (w/v) metaphosphoric acid solution containing 5 mM EDTA. After ultracentrifugation (105,000 × g, 30 min, 4 °C), 0.9 ml of 0.1 M phosphate solution (pH 8.0) containing 5 mM EDTA and 50 µl of the o-phenanthroline solution (1 mg/ml) were added to the resulting supernatant (50 µl). Then the fluorescence intensity at 420 nm was determined with excitation at 350 nm. For the immunohistochemical studies, tissues were fixed with 20% formalin neutral buffer solution (Wako), embedded in paraffin, and cut at 3-µm thickness. Immunostaining was performed using the avidin-biotin complex method with the Vectastain ABC-AP (alkaline phosphatase) kit and Vector Alkaline Phosphatase Substrate kit 1 (Vector Laboratories, Inc., Burlingame, CA). Before the immunostaining, to provide maximum assurance of mAb2D3 to DNA, sections were boiled in a microwave oven for 5 min in 10 mM citrate buffer (pH 6.0). After cooling at room temperature, sections were treated with 0.1% trypsin solution (in 50 mM Tris-HCl, 150 mM NaCl, 0.1% CaCl<sub>2</sub>, pH 7.8) for 15 min at room temperature. A competitive experiment was performed by staining with mAb2D3 preincubated with an excess of HOCl-modified calf thymus DNA (100 µg/ml) at 37 °C for 1 h. Sections were also counterstained with hematoxylin.

Immunocytochemical Detection of Halogenated dC—HL-60 cells in PBS were treated with HOCl at 37 °C for 15 min. Residual HOCl was inactivated by adding 4 mM methionine. Cells were washed with PBS three times and then fixed overnight in phosphate buffer (pH 7.4) containing 4% parafomaldehyde at 4 °C for 1 h. To prevent nonspecific antibody binding, the cells were washed two times in PBS and blocked for 1 h at room temperature with 2% BSA in TBPS. Membranes were permeabilized by exposing the fixed cells to PBS containing 0.3% Triton X-100. The cells were then incubated in the primary antibody (mAb2D3) for 1 h at room temperature. The cells were then incubated for 1 h in the presence of fluorescein isothiocyanate-labeled anti-mouse IgG (Dako Japan Co., Ltd., Kyoto, Japan), rinsed with PBS containing 0.3% Triton X-100, and mounted on glass slides using 50% glycerol in PBS. Images of cellular immunofluorescence were acquired using a Zeiss LSM5 PASCAL confocal laser scanning microscope with a ×40 objective (488 nm excitation and 518 nm emission). The DNA was stained with propidium iodide, while damage was co-localized using a fluorescein isothiocyanate-labeled secondary antibody.

RESULTS

Monoclonal Antibody to HOCl-modified DNA—Several lines of evidence suggest the possibility that leukocyte-derived oxidant hypohalous acids (HOCl and HOBr) can covalently modify the nucleobases in vivo. To examine the modifications of DNA bases by hypohalous acid in vivo, we prepared a monoclonal antibody to the hypohalous acid-modified DNA. The immunizing antigen was prepared by electrostatically coupling HOCl-treated calf thymus DNA with methylated BSA. We finally obtained a clone (clone no. 2D3), named mAb2D3, that showed the most distinctive recognition of the HOCl-modified DNA over unmodified DNA. The specificity of the obtained antibody was then characterized by ELISA. As shown in Fig. 1A, mAb2D3 could recognize the DNA treated with the MPO/H<sub>2</sub>O<sub>2</sub>-Cl<sup>−</sup> system. The formation of immunoreactive materials required MPO, H<sub>2</sub>O<sub>2</sub>, and Cl<sup>−</sup> and was blocked by catalase, a scavenger of H<sub>2</sub>O<sub>2</sub> (data not shown). Heme enzyme inhibitor, sodium azide, also blocked the formation of epitopes. In addition, the formation of immunoreactive materials was also inhibited by methionine, which is a scavenger for HOCl, suggesting that MPO-catalyzed generation of HOCl may be implicated
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Fig. 2. Specific recognition of HOCl-modified dC by mAb2D3 (A) and characterization of dC chlorinated products (B–D). A, oligonucleotide 50-mer (dA)50, (dT)50, (dG)50, or (dC)50 (0.02 mM; base concentration = 1 mM) was exposed to HOCl (1 mM) for 60 min at 37 °C. Reactions were terminated by adding 4 mM l-methionine. Oligomers were diluted with PBS and coated on a microtiter plate. The immunoreactivity of HOCl-modified oligomers with mAb2D3 was then estimated by ELISA. B, reverse-phase HPLC analysis of dC (2 mM) exposed to HOCl (1 mM) for 60 min at 37 °C. Three peaks were detected as the major products (peaks a–c). C, infusion mass spectrometric analysis of peak c (upper, Q1 scan mode; lower, product ion scan of m/z 296). D, chemical structures of the compounds that produced peaks a–c: a, 5-CldC; b, N4-CldC; c, N4,5-diCldC.

in the increase of immunoreactivity. Indeed the immunoreactivity of mAb2D3 with reagent HOCl-treated DNA was confirmed, and the formation of immunoreactivity was inhibited in the presence of HOCl scavengers, such as thiol compounds or taurine (Fig. 1B). It has been reported that Br−, NO2−, and tyrosine are also potent substrates of MPO and generate reactive species with DNA (47). The MPO/H2O2-catalyzed oxidation of DNA in the presence of plasma levels of NO2− or tyrosine did not generate the antigenic structures (Fig. 1C). In the presence of Br−, a weak immunoreactivity was observed, suggesting that HOBr may also be a potential oxidant that generates the antigenic materials. Indeed reagent HOBr-modified DNA was significantly recognized by mAb2D3 (Fig. 1D). It has been shown that the reaction of hypohalous acid with superoxide generates hydroxyl radical (·OH), a reactive oxidant species that generates 8-oxo-dG (a typical marker for DNA damage) (27); however, the mAb2D3 could not recognize Fe2+/H2O2-treated DNA in which elevated levels of 8-oxo-dG were observed (Fig. 1D). In addition, peroxynitrite, another inflammatory oxidant, also failed to generate the epitope of this antibody in DNA (data not shown). These results strongly suggest that the epitope of the antibody may be hypohalous acid-specific DNA lesions, but not ·OH-mediated oxidation products, such as 8-oxo-dG. Furthermore mAb2D3 recognized neither HOCl-modified BSA nor HOBr-modified BSA, showing that protein halogenation products could not be the epitope of the antibody (data not shown).

Characterization of the Epitope. We examined the cross-reactivity of mAb2D3 with various 2’-deoxynucleoside modification products (8-oxo-dG, 8-CldG, 5-CldC, N4-CldC, 5-methyl-dC, and thymidine glycol) using competitive ELISA; however, no compound exhibited an inhibitory effect (data not shown). In addition, the 2’-deoxynucleoside mixture (containing dA, dT, dC, and dG; 0.5 mM each) exposed to HOCl was not recognized by mAb2D3. Furthermore the enzymatic hydrolysate of HOCl-treated DNA resulted in the disappearance of the immunoreactivity (data not shown). These results suggest that this antibody weakly recognizes low molecular (monomeric) nucleosides. Then we characterized the epitope of mAb2D3 using HOCl-modified oligomeric nucleotides and found that the antibody specifically recognizes HOCl-modified dC residues (Fig. 2A). To characterize the reaction products upon reaction of HOCl with dC residues, HOCl-treated monomeric dC was then analyzed using an HPLC-UV system, and three peaks (a–c) were detected as the major products (Fig. 2B). Liquid chromatography-mass spectrometry analysis of the reaction mixture showed that both peaks a and b exhibited their molecular ions at m/z 262 and m/z 264 (data not shown), suggesting that they might be the monochlorinated (35Cl or 37Cl)dC. It has been reported that the N-4 or C-5 position of dC was the target of chlorination during the reaction with HOCl (12, 32). Compared with authentic compounds, peaks a and b were identified to be 5-CldC and N4-CldC, respectively. On the other hand, mass spectrometric analysis of peak c showed the molecular ions at m/z 296, m/z 298, and m/z 300. The intensities of the ions exhibited approximately an expected isotopic ratio (9:6:1) for a dichlorinated compound containing 35Cl and/or 37Cl (Fig. 2C, upper). The formation of a dichlorinated compound was also confirmed by the tandem mass spectrum of peak c (Fig. 2C, lower). In addition, the treatment of authentic 5-CldC with HOCl generated a single major product that co-migrated with peak c in the HPLC analysis (data not shown). Based on these observations, peak c was identified to be N4,5-dichloro-2’-deoxycytidine (N4,5-diCldC), a novel chlorinated nucleobase. The structures of the compounds that produced peaks a–c are illustrated in Fig. 2D. No other detectable compounds were observed in the reaction mixture.

As shown in Fig. 3A, the treatment of the reaction mixture (HOCl/dC) with excess methionine resulted in the disappearance of N4,5-dCdC and N4,5-diCldC that contain a chloramine group) with increased concentrations of dC (data not shown) and 5-CldC (Fig. 3, A and B), respectively. Significant increase of 5-CldC concentration in HOCl-treated DNA after treatment with methionine was also observed (30). Our result suggests that the for-
formation of \( N^4,5\)-diCldC represents an alternative pathway for the formation of 5-CldC in the presence of thiol compounds such as methionine. As shown in Fig. 3, the treatment of HOCl-modified oligonucleotide with methionine significantly decreased the immunoreactivity with mAb2D3, suggesting that the epitope of the antibody may be a chloramine-like species but not 5-CldC. The negative immunoreactivity of the antibody to 5-CldC was also supported by the result that both the addition of plasma concentration of chloride ion (100 mM) and acidic condition (pH 4.5) in the reaction of HOCl with DNA (optimal reaction condition for the formation of 5-CldC, Ref. 12) failed to enhance the immunoreactivity (data not shown).

HOCl can chlorinate both the N-4 and C-5 positions of dC; therefore, determination of the precise epitope of mAb2D3 in the HOCl-modified oligonucleotides might be difficult. It has been reported that thymidine is the major initial site of chloramine formation in DNA and that the formed unstable thymidine-derived chloramine can transfer its chlorine atom to other nucleobases including cytosine residues (35, 36). As shown in Fig. 4A, chlorine atom transfer to dC with thymidine chloramine (N-CldT) resulted in the formation of a single chlorination product, \( N^4\)-CldC. In the reaction mixture, the formation of neither 5-CldC nor \( N^4,5\)-diCldC was observed, suggesting that N-CldT can transfer chlorine atom to the N-4 position of dC but not to the C-5 position of dC. The similar reactivity of N-CldT was also confirmed in the oligonucleotide 50-mer. As shown in Fig. 4B, \( N^4\)-CldC was detected in the enzymatic hydrolysate of \( N^4\)-CldT-modified (dC)50, whereas 5-CldC and \( N^4,5\)-diCldC could not be detected. When the immunoreactivity of N-CldT-modified oligonucleotides with mAb2D3 was estimated by competitive ELISA, the \( N^4\)-CldT-modified (dC)50 could not be recognized by the antibody. As mentioned above, 5-halogenated dC was suggested not to be the epitope of mAb2D3 (Fig. 3). This was further confirmed by the result that the dC oligonucleotide 50-mer that contains three 5-BrdC residues was not recognized by the antibody. However, it was found that the treatment of this 5-BrdC-containing oligomer with N-CldT resulted in the generation of significant immunoreactivity. HPLC analysis clearly showed that the treatment of 5-BrdC with N-CldT generated a major single product, \( N^4\)-Cl-5-Br-dC (data not shown).

In addition, compared with authentic \( N^4\)-Cl-5-Br-dC standard, all of the expected isotope peaks of \( N^4\)-Cl-5-Br-dC at m/z 340 → 224 (\( ^{35}\)Cl\(^{79}\)Br), m/z 342 → 226 (\( ^{37}\)Cl\(^{79}\)Br and \( ^{35}\)Cl\(^{81}\)Br), and m/z 344 → 228 (\( ^{37}\)Cl\(^{81}\)Br) were also detected in the hydrolysate of N-CldT-modified 5-BrdC-containing oligomer (Fig. 5B).
These results strongly suggested that N^4,5-dihalogenated dCs may be the plausible epitopes of mAb2D3. The possibility that the antibody recognizes the broad sequence containing both 5-halo-dC and N^4-halo-dC was excluded by the result that mAb2D3 recognized HOCl-treated oligonucleotide 50-mer containing a single dC residue (Supplemental Fig. 1). In addition, the N-CldT-treated oligomer that contains 5-methyl-dC, an endogenous 5-substituted dC, was not recognized by mAb2D3 (data not shown), suggesting the specificity of this antibody to the halogenated dC residues.

**Endogenous Generation of Dihalogenated dC in Nuclear DNA**—Formation of antigenic materials with mAb2D3 in vivo was immunohistochemically assessed in an inflammatory animal model with LPS. LPS is a component of the cell wall of Gram-negative bacteria. LPS activates alveolar macrophages and causes neutrophils to infiltrate and damage the lung and liver (48, 49). The lungs and livers of mice intraperitoneally treated with 3 mg/kg of body weight LPS were excised after sacrifice and then fixed with formalin. In this model, leukocyte infiltration and increase in MPO activity were observed (Supplemental Fig. 2). In the lung and liver of control mice, negative immunoreactivity with mAb2D3 was observed (Fig. 6, A and D). The intense immunoreactivities appeared in the nuclei of liver 24 h after the LPS treatment (Fig. 6, B and E). As shown in Fig. 6, C and F, preabsorption of the antibody with HOCl-treated DNA completely abolished the immunostainings, indicating the specificity of the antibody with the epitopes. These data suggest that the epitopes of mAb2D3, novel dihalogenated dC residues, are indeed generated in the nuclear DNA of target tissues at sites of inflammation. In
addition, extracellular treatment of cultured cells with HOCl reproduced the formation of epitopes in the nuclei (Supplemental Fig. 3), supporting the in vivo situation that leukocyte-derived diffusible hypohalous acid could halogenate the nuclear DNA in the target tissues. This is the first demonstration that immunochemically showed the presence and localization for the leukocyte-mediated halogenation of DNA bases in vivo.
**Halogenation of 2'-Deoxycytidine in Vivo**

Several lines of evidence have suggested that hypohalous acid can halogenate DNA bases in *vivo*. It has been shown that reaction of HOCl with NH or NH₂ groups of nucleosides resulted in the formation of semistable chloramines (31, 32). On the other hand, stable carbon-chlorinated nucleosides including 5-Cl-dC (12, 32), 5-Cl-uracil (33), 8-Cl-dA (29), and 8-Cl-dG (30) have been detected in the reaction of HOCl with nucleosides. It has been suggested that these chlorinated (or brominated) nucleobases are endogenously generated *in vivo* (50–52). However, a detailed mechanism for the formation of halogenated nucleobases *in vivo* has not yet been fully established. Immunological detection is a powerful tool that can be used to evaluate the presence and localization of target molecules *in vivo*. The detailed characterization of an antibody combined with chemical analysis may enable us to understand not only the chemical nature of the epitope(s) but also the precise reaction mechanism for the formation *in vivo*. In this study, we obtained a monoclonal antibody, mAb2D3, that specifically recognized hypohalous acid (HOCl and HOBr)-modified DNA. Upon characterization of antigenic structure with mAb2D3, we identified novel halogenated nucleosides, N⁴,5-dihalogenated dCs, as the major epitopes of the antibody. *In vivo* formation of the dihalogenated dC lesions was demonstrated in the liver and lung sections of mice intraperitoneally treated with LPS, an inflammatory animal model (Fig. 6). In this model, leukocyte infiltration and subsequent increase in MPO activity were observed (Supplemental Fig. 2). As far as we know, *in vivo* formation of nucleoside haloamines in DNA has not yet been experimentally demonstrated. Our current results strongly suggested that both N- and C-halogenation of nuclear DNA bases could be endogenously generated in cells at sites of inflammation (Fig. 7).

**DISCUSSION**

HOCl is thought to be the major product in the MPO-H₂O₂-halide system. In contrast, a similar peroxidase of eosinophil (called EPO) preferentially converts Br to HOBr. The mAb2D3 obtained in this study recognized dihalogenated dC epitopes formed in the both HOCl- and HOBr-modified DNA (Fig. 1). It has been shown that, in addition to the chlorination, HOCl also brominates dC in the presence of plasma concentration of Br⁻ (100 μM) by generating BrCl as the potential intermediate (53). Therefore, we hypothesized that antigenic lesions with mAb2D3 observed in the tissues of LPS-treated mice (Fig. 6) might contain multiple dihalogenated dCs, such as dichloro-dC, dibromo-dC, and bromo-chloro-dC, but the antibody could not distinguish these lesions.

The immunogen for the preparation of mAb2D3 was obtained upon reaction of HOCl with calf thymus DNA followed by terminating the reaction with excess methionine (see “Experimental Procedures”). In addition to the ability to scavenge hypohalous acid, methionine can reduce the nucleoside haloamines: N⁴-Cl-dC and N⁴,5-dCl-dC were reduced to dC and 5-Cl-dC, respectively (Figs. 3 and 7). However, the epitope of the obtained mAb2D3 was identified to be haloamine-type compounds, N⁴,5-dihalo-dCs (Fig. 4), suggesting that the addition of an excess methionine failed to completely reduce the haloamines in the HOCl-modified DNA. The immunoreactivities of HOCl-modified dC (monomer) and HOCl-modified (dC)_2⁵ were sensitively attenuated by the addition of methionine (Fig. 3), whereas immunoreactivities with methionine addition were scarcely attenuated in DNA of increased chain lengths ((dC)_2⁵, poly(dC), and calf thymus DNA). In addition, cytotoxic N⁴-haloamines (N⁴-Cl-dC and N⁴-Cl-5-Br-dC) were indeed detected using liquid chromatography-MS/MS in the hydrolysates of halogenated (dC)_2⁵ with methionine treatment (Figs. 4 and 5). These observations suggest that a part of the cytotoxic N⁴-chloramines formed in high molecular DNA may be relatively stable even in the presence of reducing agents such as thiols and thioethers (Fig. 7). It has been reported that the formation of N⁴-Br-dC could not be observed in the reaction of brominating agent (HOBr or several bromamines) with dC, showing the instability of N⁴-Br-dC (38). However, our current results that the mAb2D3 recognized HOBr-modified DNA suggest that a part of dC bromamines might also be stably present in DNA. The detailed mechanism for the stabilization of chloramines/bromamines, such as the effect of tertiary structures of DNA molecules, has not yet been elucidated.

Antigenic materials with mAb2D3 were localized in the nuclei of target cells at sites of inflammation (Fig. 6). Generally two possible mechanisms for oxidant-induced mutagenesis are thought to occur in cells: direct damage to DNA and incorporation of oxidized/modified nucleotide precursors into DNA. Incorporation of several halogenated nucleotides, such as 5-bromodeoxycytidine, into the genomic DNA has been reported (38). However, experimental evidence whether hypohalous acid can directly oxidize nuclear DNA has not yet been demonstrated. Based on our current results that the formation of haloamine (chloramines and bromamines) epitopes was immunochromically revealed in the nuclei, we hypothesized that hypohalous acid might directly, at least in part, halogenate nuclear DNA *in vivo* because the haloamine formation in monomeric nucleoside(tide)s upon reaction with hypohalous acid was scarcely observed in the presence of intracellular concentration (millimolar range) of thiols such as GSH.

Chronic inflammation is a risk factor for cancer, and DNA damage by halogenating (chlorinating and/or brominating) intermediates generated by leukocyte-derived peroxidases has been implicated in the process. Here we showed immunochromically the endogenous halogenation of nuclear DNA. These observations strongly suggest that leukocyte-derived hypohalous acids play an important role in the nuclear DNA damage of target tissues at sites of inflammation.

**Acknowledgment**—We thank Harue Kumon (Nagoya University) for technical support in the preparation of mBSA.

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