Generation of Nitrogen-Chlorine Oxidants by Human Phagocytes

Samuel T. Test, Mark B. Lampert, Peter J. Ossanna, Jess G. Thoene, and Stephen J. Weiss
Department of Internal Medicine, Division of Hematology and Oncology, Simpson Memorial Research Institute, and the Department of Pediatrics, University of Michigan, Ann Arbor, Michigan 48109

Abstract. Human phagocytes can be triggered to generate large quantities of long-lived nitrogen-chlorine derivatives. This class of oxidants can be detected as early as 5 min after the addition of phorbol myristate acetate or opsonized zymosan particles. Unlike all other oxygen metabolites known to be generated by phagocytes, the nitrogen-chlorine compounds can be readily detected in cell supernatants 90 min after stimulation. The generation of these oxidants is linear with neutrophil concentration, favored at alkaline pH, and inhibited by supraphysiologic concentrations of iodide or bromide. The oxidants are hydrophilic in nature and have a half-life ranging from 5 h at 37°C to >100 h at 4°C. Gel filtration chromatography of the accumulated nitrogen-chlorine derivatives revealed that the oxidants generated by neutrophils or monocytes are a complex mixture of products whose M_r range from 150–5,000. One-half of the nitrogen chlorine derivatives migrate as a single peak with an M_r of ~150. Amino acid analysis of this fraction identified the β-amino acid, taurine, as the single nitrogenous compound present. Neutrophils triggered in the presence of serum albumin accumulated increased amounts of the nitrogen-chlorine derivatives while continuing to generate their endogenous low M_r oxidants. Quantitative analysis of the ^{36}Cl incorporation revealed that the albumin molecule was chlorinated with the formation of both nitrogen-chlorine and carbon-chlorine bonds. We conclude that human phagocytes can chlorinate both endogenous and exogenous nitrogenous compounds at inflammatory sites to generate a heterogeneous mixture of nitrogen-chlorine derivatives. The ability of phagocytes to generate this class of long-lived oxidants whose hydrophilic characteristics restrict their localization to the extracellular space suggests that these species play an important role in modulating the inflammatory response.

Introduction

In vivo, immune-triggered phagocytes have the potential to generate and use a family of reactive oxygen metabolites to mediate a wide range of extracellular inflammatory effects (1–3). Phagocyte-generated oxidants including superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH·), and hypohalous acids (HOX) have been implicated in the regulation of the activity of soluble inflammatory mediators and inflammatory cells (1, 2), mutagenesis (4, 5), and tissue injury or destruction (1–3). Based on either their chemical reactivity or their rapid catabolism by host defense systems, oxygen metabolites have relatively short lifespans in complex biological systems (1). Thus, these species are expected to mediate their direct oxidant effects over relatively short distances and not to accumulate at inflammatory foci.

Recently, we described the ability of triggered human neutrophils to release a chemically distinct class of oxidants that can be detected in the extracellular milieu after the other oxygen metabolites have disappeared (6). Our preliminary studies indicated that these oxidants belong to a general family of nitrogen-chlorine (N-Cl) derivatives that are generated via the myeloperoxidase-mediated chlorination of a nitrogenous compound or set of compounds (N-compounds) (6). Thus,

$$H_2O_2 + Cl^- \xrightarrow[H^+]{\text{myeloperoxidase}} HOCl + H_2O \quad \text{and} \qquad (1)$$

$$HOCl + RR'NH \rightarrow RR'NCl + H_2O. \qquad (2)$$

At present, almost nothing is known about the generation or characteristics of the endogenous N-Cl compounds released

This work was presented in part at the annual meeting of the American Society of Hematology, San Francisco, California, 6 December 1983.

Address reprint requests to Dr. Weiss, Simpson Memorial Research Institute, 102 Observatory, Ann Arbor, MI 48109-0010.

Received for publication 17 April 1984 and in revised form 26 June 1984.

J. Clin. Invest.

0021-9738/84/10/1341/09 $1.00
Volume 74, October 1984, 1341–1349

by intact human phagocytes. In this study we have (*a*) examined the factors regulating N-Cl generation by human neutrophils; (*b*) assessed the physical characteristics of the accumulated oxidants; (*c*) demonstrated their heterogeneous composition; (*d*) identified the major endogenous N-chloro compound released; and (*e*) characterized the modulating effect of an exogenous serum protein on the formation of the long-lived oxidants.

Methods

Cell preparation. Neutrophils were obtained from the venous blood of volunteers and isolated by Ficoll-Hypaque density centrifugation followed by dextran sedimentation (7). The preparations contained >96% neutrophils and 2–3% eosinophils. Cells were suspended in Dulbecco's phosphate buffered saline (M.A. Bioproducts, Walkersville, MD), pH 7.4, supplemented with 1 mg/ml glucose.

Purified monocytes were obtained from the isolated mononuclear cell fraction by an adhesion step as previously described (7). The preparations contained >95% monocytes and 1–5% lymphocytes, and viability assessed by trypan blue exclusion was >95%.

Gel filtered platelets were prepared from venous blood according to the method of Lages et al. (8).

Assay conditions. Neutrophils or monocytes (2.5×10^6) were incubated in Dulbecco's buffer (pH 7.4, except as noted) in a final volume of 1 ml on a rocking platform for indicated periods at 37°C. Phagocytes were stimulated with phorbol myristate acetate (PMA)[1] (30 ng/ml; Consolidated Midland Corp., Brewster, NY) or opsonized zymosan particles (1.25 mg/ml; ICN Nutritional Biochemicals, Cleveland, OH) prepared as described (7).

Quantitation of endogenous N-Cl oxidants. At the end of the incubation period, bovine catalase (5 μg/ml, 88,000 U/mg; Worthington Biochemical Corp., Freehold, NJ) was added, the cells were pelleted by centrifugation (1,250 *g*, 10 min), and the supernatants were assayed for endogenous oxidant activity. The concentration of oxidants was determined by the oxidation of 5-thio-2-nitrobenzoic acid (Nbs) ($\epsilon_{412\ nm} = 1.36 \times 10^4\ M^{-1}cm^{-1}$) to its colorless disulfide 5-5′-dithiobis(2-nitrobenzoic acid) (reference 6).

Quantitation of HOCl generation. The generation of HOCl by phagocytes was quantitated by the taurine trapping method as previously described, except that incubations were performed on a rocking platform (9, 10).

Ethyl acetate extraction of endogenous N-Cl derivatives. The supernatant from stimulated phagocytes was extracted with an equal volume of ethyl acetate according to the method described by Thomas (11). The Nbs oxidation method was used to quantitate the amount of oxidant present in both the organic phase and the aqueous phase.

Fractionation of chlorinated oxidants by gel filtration chromatography. Neutrophils or monocytes ($10–12.5 \times 10^6$/ml) were stimulated as described above, and the supernatants were analyzed by gel filtration chromatography (12) on either Sephadex G-10 or G-25 (fine; Pharmacia Fine Chemicals, Piscataway, NJ). In order to determine potential recoveries of N-Cl derivatives after gel filtration, a simple N-Cl compound (N-chlorotaurine, 100 μM) was analyzed. In preliminary experiments, it was determined that only 30–40% of the oxidizing equivalents could be recovered after Sephadex G-10 chromatography. Washing of the column bed with a dilute solution of NaOCl (150 μM) resulted in >90% recovery of subsequently analyzed N-Cl-containing preparations. Sephadex G-25 required no pretreatment, as recoveries of oxidant activity were routinely >90%.

In experiments that used Sephadex G-10 (1.6 × 36 cm column), supernatant (1.5 ml) was applied and eluted with phosphate-buffered saline (pH 7.4) at a flow rate of 1 ml/min at 4°C, and 1.5-ml fractions were assayed for oxidant activity as described. In experiments that used Sephadex G-25 (1.5 × 80 cm), supernatant (2.0 ml) was applied and eluted with phosphate-buffered saline (pH 7.4) at a flow rate of 0.8 ml/min at 4°C, and 2.5-ml were fraction volumes assayed. Molecular weights were determined after standardization of the columns with sodium azide (J. T. Baker Chemical Co., Phillipsburgh, NJ); N-chlorotaurine; and reduced glutathione, bacitracin, and ferricytochrome c (Sigma Chemical Co., St. Louis, MO).

Amino acid analysis. Amino acid analyses of the Sephadex G-10 chromatographic fractions were performed on a Beckman amino acid analyzer (CL118) equipped with a spherical resin type W-3P column (Beckman Instruments, Inc., Fullerton, CA). A 200-μl aliquot was acidified to a pH of ≤1 with sulfosalicylic acid and analyzed as the ninhydrin derivative.

Quantitation of ^{36}Cl incorporation into albumin. Neutrophils ($10–12.5 \times 10^6$) were stimulated in the absence or presence of albumin (500 μg/ml fatty acid-free human serum albumin; Sigma Chemical Co.) in 1 ml of Dulbecco's phosphate-buffered saline, in which 15.6 μCi ^{36}Cl (as $Na^{36}Cl$, 9.7 mCi/g Cl; ICN Chemical and Radioisotope Div., Irvine, CA) had been substituted for the equivalent amount of unlabeled NaCl. A 1.5-ml sample of supernatant was then applied to a Sephadex G-25 column and eluted as described above. The 2.5-ml fractions were analyzed for radioactivity by liquid scintillation counting and for oxidant activity as described.

All results are expressed as the mean±1 SD of the indicated number of experiments.

Results

The generation of N-chloro compounds by human neutrophils. The time course of N-Cl generation by stimulated neutrophils (2.5×10^6/ml) is shown in Fig. 1 *A*. These long-lived oxidants could be detected in supernatants as early as 5 min after cells were triggered with either PMA or zymosan. The amount of oxidant activity detected reached a maximum at 60 min for both stimuli (27.8±3.2 nmol, $n = 6$ for PMA vs. 21.3±2.1 nmol, $n = 6$ for zymosan), and then showed a gradual decrease (Fig. 1 *A*). As expected, a similar time-dependent increase in the ultraviolet absorption maximum at 252 nm characteristic of N-Cl derivatives was observed (Fig. 1 *A*, and *B*). N-Cl generation by PMA- or zymosan-triggered neutrophils increased linearly with cell concentration, with yields >100 μM generated by 15×10^6 neutrophils/ml (data not shown). Resting cells did not generate detectable quantities of the N-Cl compounds.

The oxidizing equivalents of HOCl can be consumed in oxidation reactions or conserved in the formation of N-Cl derivatives (12–14). In order to quantitate the yield of N-Cl compounds relative to the amount of HOCl generated, the total chlorinating potential of the neutrophil was assessed (9). As shown in Fig. 1 *C*, PMA- or zymosan-triggered neutrophils

1. *Abbreviations used in this paper:* PMA, phorbol myristate acetate; Nbs, 5-thio-2-nitrobenzoic acid.

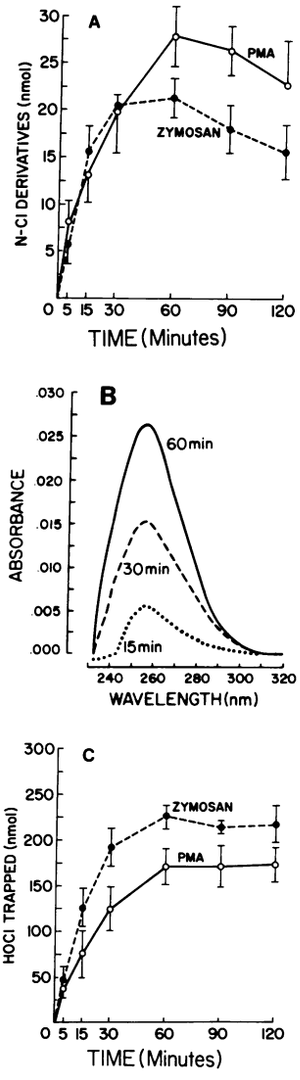

Figure 1. (*A*) N-Cl generation as a function of time. Neutrophils (2.5 × 10^6) were incubated in Dulbecco's phosphate-buffered saline, pH 7.4, on a rocking platform. Cells were stimulated with PMA (30 ng/ml) (——) or opsonized zymosan particles (1.25 mg/ml) (- - -). After various incubation times, catalase (1,000 U) was added, the cells were pelleted by centrifugation (1,250 *g*, 10 min), and supernatants were assayed for oxidizing activity as described. Results are expressed as the mean±1 SD of six experiments. (*B*) Neutrophils (10^7/ml) were stimulated with PMA and supernatants were prepared as in *A*. Difference spectra were obtained by comparison of the supernatant of PMA-stimulated neutrophils (sample cuvette) with an identical supernatant treated with 500 nmol methionine (reference cuvette) to reduce the N-Cl derivatives, at 15 (· · ·), 30 (- - -), and 60 min (——). (*C*) HOCl generation by triggered neutrophils as a function of time. Neutrophils were incubated as described in *A*, but in the presence of 15 mM taurine. Results are expressed as the mean±1 SD of four experiments.

generated HOCl with kinetics similar to those observed for N-Cl generation but in greater quantities. Under conditions identical to those described for peak N-Cl generation by 2.5 × 10^6 cells (i.e., 60 min incubation, pH 7.4), 173.0±18.5 nmol (n = 4) of HOCl was trapped with PMA-stimulated cells, and 225.0±12.5 nmol (n = 4) was trapped with zymosan-triggered neutrophils. Thus, triggered neutrophils use ~10–15% of the total amount of HOCl detected in our system to generate the N-Cl derivatives.

Quantitation of N-Cl compounds generated by neutrophils was routinely performed at pH 7.4, but an examination of oxidant formation as a function of pH revealed that the yields of the oxidants were consistently higher at more alkaline pH's (Fig. 2 *A*). Peak values for either stimulus were obtained at a pH of ~8.0.

Although Cl^- is probably the preferred substrate for the myeloperoxidase system in vivo, both I^- and Br^- can also be oxidized by the enzyme (1, 2). As shown in Fig. 2 *B*, the addition of supraphysiologic concentrations of KI or KBr interfered with either the generation or accumulation of the N-Cl derivatives. At a concentration of 10^{-5} M KI or 5 × 10^{-3} M KBr, the N-Cl yield was almost undetectable.

Do platelets play a role in the generation of N-Cl derivatives by neutrophils? Routine preparations of neutrophils obtained after a successive Ficoll-Hypaque density centrifugation and dextran sedimentation contained ~1 platelet/20 neutrophils. If neutrophils were prepared from defibrinated blood (initial platelet counts reduced from 240.3±59.0 × 10^3/mm^3 to 0.6±0.5 × 10^3/mm^3, n = 3) the platelet contamination fell to <1 platelet/100 neutrophils without significantly reducing N-Cl formation. In three paired experiments, neutrophils prepared from whole blood generated 27.2±1.1 nmol of N-Cl derivatives when triggered with PMA and 21.0±2.7 nmol with zymosan, whereas neutrophils prepared from defibrinated blood produced 25.4±0.6 and 18.4±2.6 nmol, respectively. Thus, the neutrophil is the source of both the nitrogenous compound(s) and the HOCl required for the generation of the N-Cl derivatives.

Although contaminating platelets do not appear to be necessary in the formation of N-Cl derivatives in our system, they may represent an exogenous source of nitrogenous compounds at inflammatory sites. However, the addition of gel-filtered platelets had no effect on oxidant generation at a platelet/neutrophil ratio of 1:1, modestly increased N-Cl formation at a 10:1 ratio (from 25.3±4.6 to 37.1±2.1 nmol for PMA-stimulated cells and from 14.2±3.2 to 16.2±9.4 nmol for zymosan-triggered neutrophils; n = 3), and almost completely inhibited oxidant generation or accumulation at a 100:1 ratio. Thus, platelets do not play a direct or accessory role in the generation of endogenous N-Cl derivatives by triggered neutrophils in our system.

Properties of endogenous N-Cl compounds generated by neutrophils: hydrophilicity and stability. Depending on the characteristics of the available nitrogenous compounds, the neutrophil could generate either hydrophilic or lipophilic N-Cl derivatives. In order to determine the lipid solubility of the oxidants generated in our system, N-Cl containing supernatants from triggered cells (2.5 × 10^6/ml, 90 min incubation) were extracted with ethyl acetate, and the partitioning of the oxidizing equivalents between the aqueous and organic phases was assessed. As controls, a highly polar N-chloroamine, N-chlorotaurine, and a relatively lipophilic oxidant NH_2Cl (15), were extracted under identical conditions. Under these conditions, ~90% of the oxidant activity found in the neutrophils' supernatant was recovered in the aqueous phase, whereas no oxidants were detected in the organic phase. Extractions performed with the control chloroamines revealed that 90% of the N-chlorotaurine activity could be recovered in the aqueous phase and none could be detected in the organic phase, whereas 62% of the oxidizing equivalents associated with NH_2Cl were extracted into the organic phase and 36% remained

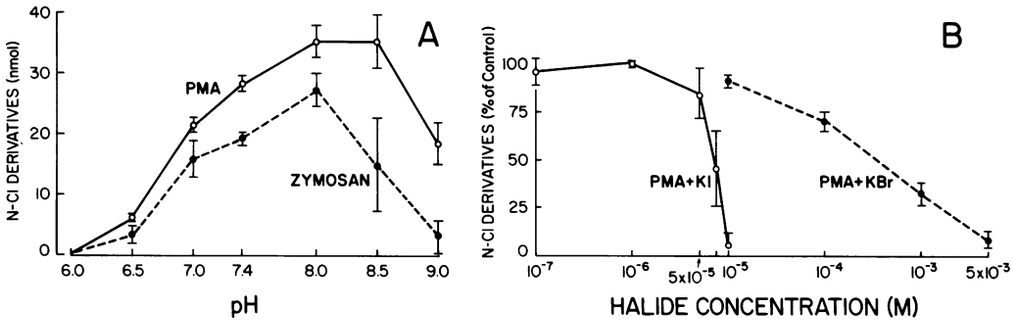

Figure 2. (*A*) The effect of pH on N-Cl generation by stimulated neutrophils. Neutrophils (2.5×10^6) were stimulated with PMA (——) or zymosan (- - -) as described in Fig. 1, except that the final pH of incubation mixtures was adjusted to various values from 6.0 to 9.0. Supernatants were assayed for N-Cl activity as described. Results are expressed as the mean±1 SD of three experiments. (*B*) The effect of exogenous halides on N-Cl generation by triggered neutrophils. Neutrophils (2.5×10^6) were stimulated with PMA as in Fig. 1 in either the absence or the presence of various concentrations of KI (——) or KBr (- - -), and supernatants were assayed for N-Cl activity. Results are expressed as the percentage of control (mean±1 SD of three experiments). Control preparations without exogenous halides generated 26.7±2.9 nmol of N-Cl derivatives (n = 3).

in the aqueous phase. We conclude that most, if not all, of the endogenous N-Cl derivatives that accumulate in supernatants obtained from triggered neutrophils are hydrophilic.

The hydrophilic characteristics of the cell-generated N-Cl compounds will allow them to accumulate in the extracellular space, but their lifespan will also be dictated by their intrinsic stability. N-Cl derivatives are known to vary widely in their stability depending on the characteristics of the nitrogen containing moiety (13, 16, 17). When the loss of oxidizing equivalents in the cell-free supernatants was monitored as a function of time at 4, 25, and 37°C, the rate followed apparent first order kinetics (Fig. 3). The calculated half-lives for the endogenous N-Cl derivatives generated by PMA- or zymosan-triggered cells were remarkably similar and ranged from 5.0±0.7 and 5.5±1.0 h (n = 5) at 37°C to 116.4±31.4 and 157.0±51.4 h, respectively, at 4°C. It should be stressed that these values represent apparent half-lives, because the N-Cl compounds could disappear not only as a result of their intrinsic instability but also by their reaction with reducing equivalents released from the triggered neutrophils. Indeed, the loss in oxidizing activity was more rapid when the N-Cl-containing supernatants were not separated from the neutrophils. For example, the half-life of PMA-generated N-Cl compounds at 37°C decreased from ~5.0 h in the cell-free supernatant to 1.9±0.7 h in the complete mixture (n = 4) and from ~116 to 27.6±8.8 h (n = 3) at 4°C. Nonetheless, the N-Cl compounds could be detected in these complex mixtures far longer than predicted for any of the other oxygen metabolites known to be generated by neutrophils.

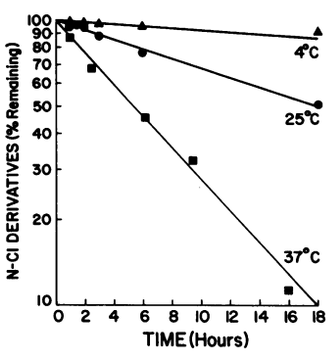

Figure 3. The stability of endogenous N-Cl derivatives generated by stimulated neutrophils. Neutrophils (2.5×10^6) were stimulated with PMA as in Fig. 1, and several preparations were pooled, centrifuged (1,250 *g*, 10 min) to remove cells, and divided into three equal portions which were incubated at 4, 25, and 37°C. Aliquots were removed at various intervals and assayed for oxidant activity. Results are expressed as the mean of a single representative experiment of three (4°C) or five (25, 37°C) performed.

Neutrophils and monocytes generate a complex mixture of endogenous N-Cl derivatives. The HOCl generated by triggered phagocytes has the potential to react with a wide range of endogenous nitrogenous compounds to form N-Cl derivatives (14, 17). To determine whether the N-Cl compounds detected in our system represent a single or multiple species, the oxidant-containing supernatants were fractionated by Sephadex G-10 gel filtration chromatography (Fig. 4 *A*). In 10 experiments with PMA-triggered cells, 55.2±3.3% of the applied oxidizing activity was recovered in a low M_r peak of ~150, and 27.0±3.8% was recovered in a peak that eluted at the void volume (M_r ~ 700).[2] When supernatants from zymosan-triggered cells were fractionated, almost identical elution patterns were obtained (see Fig. 4 *A*; 54.4±1.1% activity in low M_r peak of ~150 and 28.0±2.6% in the void volume; n = 4).

Chromatography of the triggered cells' supernatants on a Sephadex G-25 column again revealed the ~150 M_r peak, but the single peak that eluted at the void volume on the G-10 column clearly contained a complex mixture of N-Cl compounds ranging in M_r from ~400–5,000 (Fig. 4 *B*). Thus, either PMA- or zymosan-stimulated neutrophils generate a similar but heterogeneous group of N-Cl derivatives that range in M_r from ~150 to 5,000.

2. Note that the exceptionally high stability of the endogenous N-Cl compounds at 4°C ($t_{1/2}$ > 100 h) allowed for the recovery of 94–100% of the total oxidizing activity applied to the column.

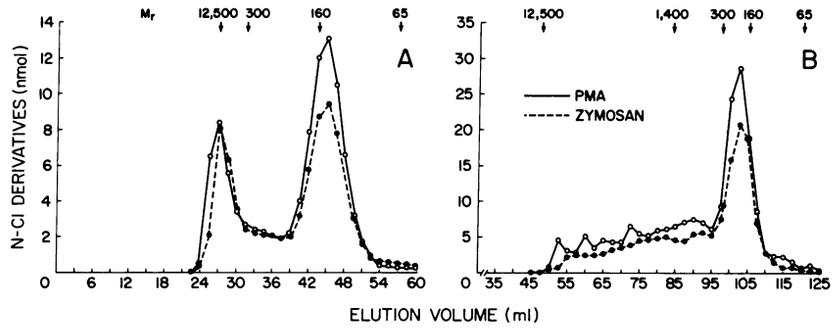

Figure 4. Gel filtration chromatography of N-Cl derivatives obtained from stimulated neutrophils. Neutrophils (10^7) were stimulated with PMA (30 ng/ml) (——) or zymosan (1.25 ng/ml) (- - -) in a final volume of 0.8 ml for 90 min. Catalase (1,000 U) was added, several preparations were pooled, and the supernatants were analyzed by gel filtration chromatography at 4°C on a column of Sephadex G-10 (*A*) or G-25 (*B*). In the experiments portrayed, the recovery of N-Cl derivatives in the eluted fractions was ≥94% of that applied to the column. Results are expressed as single representative experiments of 10 performed with PMA-stimulated cells and 4 performed with zymosan-treated cells.

We have recently demonstrated that triggered human monocytes can generate substantial quantities of HOCl (10). Thus, these cells might also have the potential to generate endogenous N-Cl compounds. Indeed, 2.5×10^6 PMA-stimulated monocytes generated 19.3±2.1 (n = 6) nmol of the N-Cl derivatives, which eluted in a fashion identical to that observed with the neutrophils on Sephadex G-25 (data not shown).

Identification of the low M_r N-chloroamine generated by neutrophils. The elution of a large fraction of the oxidant activity as a single, low M_r peak after gel filtration chromatography suggested that the nitrogenous compound(s) present in this fraction might be identified by amino acid analysis. In order to reduce the N-chloroamine(s) present in this fraction back to the parent amine(s) for analysis, the oxidant was first treated with an excess of methionine. Based on the ability of N-Cl derivatives to react with thioethers in a 1:1 stoichiometry (6, 14), amino acid analysis should reveal (in the simplest case) the formation of an equimolar amount of methionine sulfoxide along with the parent amine. Thus,

$$\text{R-NHCl} + \text{R-S-R} + \text{H}_2\text{O} \rightarrow \text{RNH}_2 + \text{R-}\overset{\overset{\text{O}}{\|}}{\text{S}}\text{-R} + \text{H}^+ + \text{Cl}^- . \quad (3)$$

In four experiments, amino acid analysis revealed that (*a*) the addition of methionine to the major fraction of the low M_r N-chloroamine peak led to the formation of an almost equimolar amount of the sulfoxide (N-chloroamine/methionine sulfoxide ≃ 0.9:1); and (*b*) the β-amino acid, taurine, was the only endogenous amine detectable in this fraction (Table I). Fractions analyzed from resting cell supernatants that coeluted with the peak fractions obtained from stimulated cells were devoid of oxidant activity, did not oxidize methionine to its sulfoxide, and contained only small amounts of taurine (2.8±1.0 nmol; n = 3).

Modulation of N-Cl derivatives generated by neutrophils in the presence of albumin. Neutrophils stimulated in vivo would be expected to release HOCl into a complex environment containing a multiplicity of oxidizable substrates. These exogenous substrates might effectively compete with neutrophil-derived N-compounds for HOCl and lower the concentration of N-Cl derivatives found in the extracellular fluid. Hence, we examined the influence of human albumin, the plasma protein present in the highest concentration in vivo, on N-Cl generation by the neutrophil. When neutrophils (10^7/ml) were stimulated with PMA or zymosan in the presence of 500 μg/ml albumin for 90 min, N-Cl generation increased 30.9±8.4 (n = 4) and 18.9±5.5% (n = 3), respectively. Gel filtration chromatography of the albumin-containing supernatant clearly demonstrated

Table I. Amino Acid Analysis of the Low Molecular Weight N-Chloro Derivatives

	Expt 1	Expt 2	Expt 3	Expt 4	Mean±1 SD
N-Cl content of fraction (*nmol*)	10.7	8.5	8.1	9.8	9.3±1.2
Methionine sulfoxide content of fraction (*nmol*)	12.4	10.3	10.1	10.1	10.7±1.1
Taurine content of fraction (*nmol*)	12.5	11.4	11.1	12.8	12.0±0.8

Neutrophils (10^7) were incubated with or without PMA (30 ng/ml) in a final volume of 0.8 ml for 90 min at 37°C on a rocking platform. Reactions were terminated by the addition of catalase (1,000 U), then cells were removed by centrifugation (1,250 *g*, 10 min), and supernatants were subjected to chromatography on Sephadex G-10 as described. The amount of N-Cl activity in each fraction was determined, and a 1-ml aliquot of the major fraction in the low molecular weight peak was removed, reduced by the addition of a fivefold excess of methionine, and subjected to amino acid analysis. Values for taurine and methionine sulfoxide were obtained by amino acid analysis, those for N-Cl derivatives by the Nbs oxidation technique. Expt, experiment.

the presence of a new peak of oxidant activity at the void volume (Fig. 5), whereas more than two-thirds of the oxidant activity in the low and intermediate M_r peaks was still present (93.2±15.1% for PMA-stimulated cells and 69.6±6.3% for zymosan-triggered cells, $n = 3$). In order to rule out the possibility that the endogenous N-Cl compounds generated by the neutrophil bound to albumin and then eluted at the void volume, oxidants were generated by PMA-stimulated cells in the absence of albumin, the supernatant was removed, and albumin (500 μg/ml) was then added. Gel-filtration of the N-Cl/albumin mixture did not reveal a change in the elution profiles of the oxidants (data not shown). Thus, the addition of a large M_r plasma protein resulted in an actual increase in the N-Cl content of cell supernatants and the formation of a new chlorinated oxidant.

The retention of oxidizing equivalents in the albumin molecule suggests that chlorination of amine or amide moieties has taken place. However, HOCl can also react with aromatic or aliphatic functions to yield "silent" C-Cl derivatives that do not act as oxidizing agents (12, 13, 18). In order to assess the degree to which neutrophil-mediated chlorination represents N-Cl vs. C-Cl formation, the net incorporation of ^{36}Cl into the albumin molecule was quantitated, and the distribution of chlorinated products was determined. Compounds known to reduce N-Cl derivatives should release bound ^{36}Cl as $^{36}Cl^-$ (see Eq. 3), whereas C-Cl derivatives remain unreactive. PMA-stimulated neutrophils were incubated with 500 μg of albumin in a $Na^{36}Cl$-containing buffer as described in Methods. Supernatants were then chromatographed on Sephadex G-25, and the oxidant activity and ^{36}Cl content were determined in each fraction. As shown in Fig. 6, the incorporation of ^{36}Cl into the albumin peak could readily be demonstrated, with 20.5×10^3 cpm or 83 nmol eluting at the void volume.[3] As expected, the incorporation of ^{36}Cl into the albumin molecule was completely inhibited by 1 mM azide (Fig. 6). If neutrophils were triggered in the absence of albumin, only 18 nmol of ^{36}Cl eluted at the void volume. The chlorinated albumin molecule oxidized 84.0 nmol of Nbs, corresponding to the presence of 42.0 nmol of N-Cl derivatives. In close agreement, treatment with methionine resulted in the complete loss of detectable oxidizing activity and the release of 51.5 nmol of ^{36}Cl. As shown in Fig. 6, the remainder of the incorporated ^{36}Cl, representing the C-Cl fraction, eluted at the void volume of the Sephadex column. In two experiments, 62.1 and 64.3% of the HOCl was incorporated into the N-Cl pool, and 37.9 and 35.7% formed C-Cl derivatives. Thus, neutrophils chlorinated albumin with ~60% of the HOCl ultimately distributed into the N-Cl pool, and the remainder was lost in the formation of C-Cl derivatives.

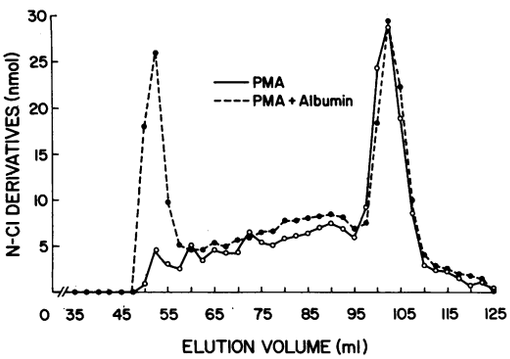

Figure 5. Modulation of N-Cl derivatives generated by neutrophils in the presence of albumin. Neutrophils (10^7/0.8 ml) were stimulated with PMA in either the absence (——) or the presence of 500 μg/ml fatty acid-free human serum albumin (– – –) and incubated as described in Fig. 4. Supernatants were collected and analyzed on Sephadex G-25 as described. N-Cl recoveries in the eluted fractions were 100% for both experiments illustrated. Results are expressed as a single representative experiment of three performed.

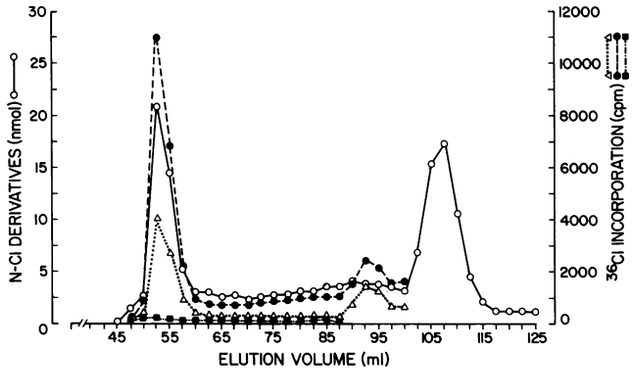

Figure 6. ^{36}Cl incorporation into albumin and endogenous amines by stimulated neutrophils. Neutrophils (10^7) were stimulated with PMA in 1 ml of Dulbecco's phosphate-buffered saline in which 15.6 μCi of ^{36}Cl as $Na^{36}Cl$ was substituted for an equivalent amount of unlabeled NaCl. Cells were incubated as described in Fig. 4. Untreated supernatants from triggered neutrophils were chromatographed on Sephadex G-25 as described in Fig. 4 and fractions were analyzed for oxidant activity (——) and ^{36}Cl incorporation (– – –). In addition, supernatants that had been reduced with an excess of methionine (· · ·) or supernatants from cells stimulated in the presence of 1 mM azide (–·–·–) were chromatographed and analyzed for ^{36}Cl incorporation. These supernatants contained no detectable oxidant activity as assessed by the Nbs assay. Results are expressed as a single representative experiment of two performed.

Discussion

Neutrophils and monocytes can use the myeloperoxidase system to generate N-Cl compounds, a class of oxidants whose unique physicochemical properties distinguish them from all the other

3. The amount of ^{36}Cl incorporated into the low M_r peak could not be assessed because the large hydration sphere of Cl^- causes the free halide to elute as an ~150 M_r molecule.

oxygen metabolites implicated in the inflammatory process (6, 15, 19, 20). The N-Cl compounds are generated rapidly and their kinetics of accumulation parallel those of HOCl generation. Quantitative assessment of the fraction of HOCl used in the formation of the stable N-Cl derivatives revealed an efficiency of ~10–15%. The bulk of the oxidizing equivalents associated with the HOCl disappears in a number of biologically relevant reactions. HOCl can react rapidly with (*a*) thiol-containing compounds to yield disulfides, or sulfenic or sulfonic acid derivatives; (*b*) thioethers to generate sulfoxides; (*c*) aliphatic or aromatic compounds to yield C-Cl derivatives; or (*d*) N-compounds that either undergo internal oxidation reactions or form highly reactive N-Cl compounds that rapidly react with cellular components (14, 17, 21, 22). Indeed, when the multiple pathways that result in the loss of HOCl oxidizing equivalents are considered, it is noteworthy that a relatively large fraction of the HOCl-derived oxidants succeed in accumulating in a cellular system.

Maximal accumulation of the N-Cl compounds was favored at an alkaline pH and by the presence of chloride as the donor halide. These requirements occur despite the acid pH optimum of the myeloperoxidase system and its ability to use bromide or iodide as oxidizable substrates (1, 2). Nonetheless, the myeloperoxidase system can function under alkaline conditions (23) and the higher pH favors both H_2O_2 generation (24) and the requirement that the nitrogenous substrate be chlorinated in its deprotonated form (12, 25). The inhibitory effect of supraphysiologic concentrations of iodide or bromide reflects either the instability of the corresponding haloamines (i.e., iodoamines or bromoamines) in a cell system or the ability of these halides to reduce directly N-Cl compounds (9, 13, 26). Taken together, the neutrophils' capacity to generate chlorinated oxidants at physiologic pH and halide concentrations underlines the in vivo potential for N-Cl production.

Our studies on the physicochemical characteristics of the endogenous N-Cl compounds revealed that they are hydrophilic, long lived, and heterogeneous in composition. The hydrophilicity of the detected N-Cl compounds is a prerequisite for their longevity because lipophilic oxidants would react directly with intramembranous compounds or diffuse into the cytosolic space (15, 27). Indeed, Thomas et al. demonstrated that neutrophils could chlorinate exogenous, uncharged, or nonpolar N-compounds to form liphophilic oxidants, but these products failed to accumulate in the complex cell mixtures (15). The hydrophilicity of the endogenous N-Cl compounds detected in our system resulted in their sequestration in the extracellular space, where their long half-lives allowed for the maintenance of an oxidizing environment long after the end of the respiratory burst.

Gel-filtration chromatography of the endogenous N-Cl derivatives generated by neutrophils or monocytes revealed that the products range in M_r from ~150 to 5,000. Despite the large number of *N*-compounds present in phagocytes (amines, amino acids, nucleic acids, polyamines, cationic proteins, etc.), at least one-half of all the accumulated N-Cl compounds eluted as an ~150 M_r peak. Amino acid analysis demonstrated that the β-amino acid, taurine, was the only amine detectable in this fraction and that its concentration was in a 1:1 molar ratio with the N-Cl content. These findings demonstrate that neutrophils produce N-chlorotaurine as the major hydrophilic, long-lived oxidant:

$$HOCl + H_2NCH_2CH_2SO_3^- \rightarrow ClNHCH_2CH_2SO_3^- + H_2O. \quad (4)$$

Taurine has the highest concentration among the free amino acids found in human leukocytes (~14 nmol/10^6 polymorphonuclear leukocytes), but its intracellular distribution and site of chlorination are currently unknown (28).

Neutrophils generate N-Cl compounds in the absence of exogenous substrates, but our data indicate that phagocytes continue to generate endogenous chloroamines even in the presence of an extracellular, oxidizable protein. Radiochromatography revealed that the increased yield of long-lived oxidants detected in the neutrophil-albumin mixtures was due to the chlorination of appropriate acceptors in the protein molecule via HOCl or endogenous N-Cl compounds (13, 17, 18, 29, 31). Although we assume that the albumin-associated oxidants are N-Cl derivatives (e.g., chlorinated ϵ-amino groups, imidazolium, guanidium, indole residues, or amide moieties; 13, 18, 29), sulfenyl chloride derivatives (RSCl) also retain two oxidizing equivalents (12, 30). However, these species rapidly hydrolyze under alkaline conditions, lose their oxidizing activity, and yield the corresponding sulfenic acids (12, 30). Finally, chlorination reactions were not limited to nitrogen- or sulfur-containing compounds because a portion of the incorporated halide was not released by reducing agents, indicating the formation of C-Cl bonds (26). Thus, the albumin molecule is subjected to dual chlorination reactions, resulting in either the retention or the loss of oxidizing equivalents, depending on the targeted molecules. During the inflammatory process serum proteins may act as innocent bystanders and undergo HOCl-dependent alterations in structure and function or participate directly by providing additional substrates for N-Cl formation.

In conclusion, neutrophils can generate at least three groups of long-lived oxidants: endogenous N-chlorotaurine, endogenous high M_r N-Cl compounds, and exogenous N-Cl products derived from extracellular substrates. These species are reactive enough to oxidize sulfhydryls to disulfides or thioethers to sulfoxides (6, 15, 20), mediate chlorination reactions (17, 31), and catalyze the incorporation of amines into proteins (32, 33). It is clear that a variety of bioactive amines, peptides, proteins, and arachidonate metabolites containing critical, oxidizable moieties are susceptible to N-Cl mediated attack (1, 2). Indeed, in a recent report we demonstrated that the endogenous N-Cl compounds released from triggered neutrophils could inactivate the chemotactic factor N-formyl-methionyl-leucyl-phenylalanine or the plasma antiproteinase, α-1-proteinase inhibitor (6). In addition to their reactivity, the sequestration of the hydrophilic, long-lived oxidants to extracellular sites provides them with the unique potential to either accumulate or diffuse far from their site of generation. It is

significant that evidence already exists demonstrating the ability of N-Cl compounds to traverse large distances in vivo to oxidize susceptible targets. Abrams et al. have shown that the oral administration of a synthetic N-Cl compound (chloramine T) to dogs almost completely depleted their plasma pool of α-1-proteinase inhibitor without exerting overt toxicity (34). The N-Cl derivatives generated by phagocytes have a similar reactivity and stability, which reinforces their potential impact in vivo. Thus, by preserving the oxidizing equivalents present in HOCl as a complex spectrum of N-chloro derivatives, the phagocyte can broaden and extend the effects of the H_2O_2-myeloperoxidase-chloride system far beyond the confines of the inflammatory site and the termination of the respiratory burst.

Acknowledgments

We thank Gertrude Maglott and Candace Irvine for expert technical assistance in the platelet studies and Rosemary Lemons for performing the amino acid analysis. We also thank E. Thomas (St. Judes Hospital, Memphis, TN) and R. Lawton (Department of Chemistry, University of Michigan) for helpful discussions.

This work was supported in part by National Institutes of Health grants 5 R01 HL-28024-03 and R01 AI-21301 and was done during Dr. Test's tenure as a Research Fellow of the American Heart Association of Michigan.

References

1. Weiss, S. J. 1983. Oxygen as a weapon in the phagocyte armamentarium. *In* Handbook of Inflammation. Vol. 4, Immunology of Inflammation. P. A. Ward, editor. Elsevier, New York. 37–87.

2. Clark, R. A. 1983. Extracellular effects of the myeloperoxidase-hydrogen peroxide-halide system. *Adv. Inflammation Res.* 5:107–146.

3. Fantone, J. C., and P. A. Ward. 1983. Mechanisms of neutrophil-dependent lung injury. *In* Handbook of Inflammation. Vol. 4. P. A. Ward, editor. Elsevier Science Publishing Co. Inc., New York. 89–119.

4. Weitzman, S. A., and T. P. Stossel. 1981. Mutation caused by human phagocytes. *Science (Wash. DC).* 212:546–547.

5. Weitberg, A. B., S. A. Weitzman, M. Destrempes, S. A. Latt, and T. P. Stossel. 1983. Stimulated human phagocytes produce cytogenetic changes in cultured mammalian cells. *N. Engl. J. Med.* 308:26–30.

6. Weiss, S. J., M. B. Lampert, and S. T. Test. 1983. Long-lived oxidants generated by human neutrophils: characterization and bioactivity. *Science (Wash. DC).* 222:625–628.

7. Weiss, S. J., and A. Slivka. 1982. Monocyte and granulocyte-mediated tumor cell destruction: a role for the hydrogen peroxide-myeloperoxidase-chloride system. *J. Clin. Invest.* 69:255–262.

8. Lages, B., M. C. Scrutton, and H. Holmsen. 1977. Secretion by gel filtered human platelets: response of platelet Ca^{++}, Mg^{++}, and K^+ to secretory agents. *J. Lab. Clin. Med.* 90:873–882.

9. Weiss, S. J., R. Klein, A. Slivka, and M. Wei. 1982. Chlorination of taurine by human neutrophils. *J. Clin. Invest.* 70:598–607.

10. Lampert, M., and S. J. Weiss. 1983. The chlorinating potential of the human monocyte. *Blood.* 62:645–651.

11. Thomas, E. L. 1981. Lactoperoxidase-catalyzed oxidation of thiocyanate: equilibria between oxidized forms of thiocyanate. *Biochemistry.* 20:3273–3280.

12. Thomas, E. L. 1979. Myeloperoxidase, hydrogen peroxide, chloride antimicrobial system: nitrogen-chlorine derivatives of bacterial components in bactericidal action against *Escherichia coli. Infect. Immun.* 23:522–531.

13. Stelmaszynska, T., and J. M. Zgliczynski. 1978. N-(2-oxoacyl)amino acids and nitriles as final products of dipeptide chlorination mediated by the myeloperoxidase/H_2O_2/Cl^- system. *Eur. J. Biochem.* 92:301–308.

14. Campbell, M. M., and G. Johnson. 1978. Chloramine T and related N-halogeno-N-metallo reagents. *Chem. Rev.* 78:65–79.

15. Thomas, E. L., M. B. Grisham, and M. M. Jefferson. 1983. Myeloperoxidase-dependent effect of amines on functions of isolated neutrophils. *J. Clin. Invest.* 72:441–454.

16. Zgliczynski, J. M., T. Stelmaszynska, J. Domanski, and W. Ostrowski. 1971. Chloramines as intermediates of oxidation reaction on amino acids by myeloperoxidase. *Biochim. Biophys. Acta.* 235:419–424.

17. Nelson, G. D. 1979. Chloramines and bromamines. *In* Kirk-Othmer Encyclopedia of Chemical Technology. Third ed. John Wiley & Sons, New York. 5:565–580.

18. Zgliczynski, J. M., and T. Stelmaszynska. 1975. Chlorinating ability of human phagocytosing leucocytes. *Eur. J. Biochem.* 565:157–162.

19. Agner, K. 1972. Biological effects of hypochlorous acid formed by "MPO"-peroxidation in the presence of chloride ions. *In* Structure and Function of Oxidation-Reduction Enzymes. A. Akeson and A. Ehrenberg, editors. Pergamon Press Ltd., Oxford. 329–335.

20. Sagone, A. L., Jr., R. M. Husney, M. S. O'Dorisio, and E. N. Metz. 1984. Mechanisms for the oxidation of reduced glutathione by stimulated granulocytes. *Blood.* 63:96–104.

21. Albrich, J. M., C. A. McCarthy, and J. K. Hurst. 1981. Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. *Proc. Natl. Acad. Sci. USA.* 78:210–214.

22. Downs, A. J., and C. J. Adams. 1973. *In* Comprehensive Inorganic Chemistry. J. C. Bailar, H. J. Emeleus, R. Nyholm, and A. F. Trotman-Dickenson, editors. Pergamon Press Ltd., Oxford. 1399–1412.

23. Sbarra, A. J., R. J. Selvaraj, B. B. Paul, P. K. F. Poskitt, G. W. Mitchell, F. Louis, and M. A. Asbell. 1977. Granulocyte biochemistry and a hydrogen peroxide-dependent microbicidal system. *In* The Granulocyte: Functional and Clinical Utilization. T. Greenwalt and G. A. Jamieson, editors. Alan R. Liss Inc., New York. 29–35.

24. Gabig, T. G., S. I. Bearman, and B. M. Babior. 1979. Effect of oxygen tension and pH on the respiratory burst of human neutrophils. *Blood.* 53:1133–1139.

25. Morris, J. C. 1967. Kinetics of reactions between aqueous chlorine and nitrogen compounds. *In* Principles and Applications of Water Chemistry. S. D. Faust and J. V. Hunter, editors. John Wiley & Sons, New York. 23–53.

26. Zgliczynski, J. M. 1980. Characteristics of myeloperoxidase from neutrophils and other peroxidases from different cell types. *In*

The Reticuloendothelial System. A Comprehensive Treatise. Vol. 2, Biochemistry and Metabolism. A. J. Sbarra and R. R. Strauss, editors. Plenum Publishing Corp., New York. 255–278.

27. Thomas, E. L. 1979. Myeloperoxidase-hydrogen peroxide-chloride antimicrobial system: effect of exogenous amines on antibacterial action against Escherichia coli. *Infect. Immun.* 25:110–116.

28. Wakayama, K., E. C. Besa, and S. I. Baskin. 1983. Changes in intracellular taurine content of human leukemic cells. *Nagoya J. Med. Sci.* 45:89–96.

29. Wright, N. C. 1926. The action of hypochlorites on amino acids and proteins. *Biochem. J.* 20:524–532.

30. Silverstein, R. M., and L. P. Hager. 1974. The chloroperoxidase-catalyzed oxidation of thiols and disulfides to sulfenyl chlorides. *Biochemistry.* 13:5069–5073.

31. Rudie, N. G., D. J. T. Porter, and H. J. Bright. 1980. Chlorination of an active site tyrosyl residue in D-amino acid oxidase by N-chloro-D-leucine. *J. Biol. Chem.* 255:498–506.

32. Bearman, S. I., G. A. Schwarting, E. H. Kolodny, and B. M. Babior. 1980. Incorporation of glucosamine by activated human neutrophils. A myeloperoxidase-mediated process. *J. Lab. Clin. Med.* 96:893–902.

33. Thomas, E. L., M. M. Jefferson, and M. B. Grisham. 1982. Myeloperoxidase-catalyzed incorporation of amines into proteins: role of hypochlorous acid and dichloramines. *Biochemistry.* 21:6299–6308.

34. Abrams, W. R., A. B. Cohen, V. V. Damiano, A. Eliraz, P. Kimbel, D. R. Meranze, and G. Weinbaum. 1981. A model of decreased functional α-1-proteinase inhibitor. Pulmonary pathology of dogs exposed to chloramine T. *J. Clin. Invest.* 68:1132–1139.