absence of added heparin. One explanation for this observation might be that crude ECGF preparations contain a heparin-like substance present in sufficient quantity to promote growth when high ECGF concentrations are used. Consistent with this possibility, the stimulatory activity of ECGF at 200 μg/ml was completely blocked by addition of a high dose of protamine (1200 μg/ml); cell proliferation was restored by addition of excess heparin (990 μg/ml). In addition to heparin, the glycosaminoglycans chondroitin sulfate, hyaluronic acid, and keratin sulfate were tested for growth-promoting capabilities and had no effect on endothelial cell growth (*8*). The sulfated polysaccharide, dextran sulfate, significantly enhanced proliferation, although dextran itself was inactive (*8*).

The mechanism by which heparin promotes human endothelial cell proliferation is unknown. Azizkhan *et al.* (*10*) showed that heparin (and dextran sulfate) increased bovine capillary endothelial cell migration but had no effect on proliferation, a finding that is consistent with reported differences between large vessel and capillary endothelium (*11*). Other reports on the effects of heparin on various cell types have yielded conflicting results (*12–14*). Several investigators have shown that heparin binds to the cell surface (*14, 15*) and thus may influence intercellular communication (*16*) and membrane receptor accessibility (*17*). Morphologic changes (*18*) and modifications of cellular behavior (*10, 19*) consistent with cell membrane–heparin interactions have been reported. In vivo, the extracellular matrix of vascular tissue contains high concentrations of glycosaminoglycans (*20*). In vitro, heparin-like molecules are secreted by endothelial cells (*21*) and inhibit smooth muscle cell growth (*13*). Our demonstration that heparin enhances endothelial cell proliferation suggests that heparin-like substances may play an important role in cell growth regulation in normal and injured vessels.

In the past, existing culture techniques permitted only restricted proliferation of human endothelial cells, and therefore many basic and applied studies had to be performed on endothelial cells from other animal species. Theoretically, the procedures described above for serial subcultivation can increase the yield of HUVE cells by 10^8-fold and of adult vessel endothelial cells by 10^{12}-fold over previously published methods (*4, 5*). This will permit minimal amounts of human vascular tissue to be used for the generation of large numbers of cultured endothelial cells, and thus problems of human pathology involving the endothelium now can be approached directly by means of a human endothelial cell model. In addition, this cell system should prove valuable for various clinical applications, such as in vitro testing of vasoactive agents and the coating of artificial graft materials.

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Long-Lived Oxidants Generated by Human Neutrophils: Characterization and Bioactivity

Abstract. *Human neutrophils were found to generate an unusual class of oxidants with a half-life of approximately 18 hours and with characteristics similar to, if not identical with, those of* N*-chloroamines. These neutrophil-derived* N*-chloroamines have sufficient oxidizing potential to attack sulfhydryl- or thioether-containing compounds and can react with both a methionine-containing chemotactic peptide and a plasma protease inhibitor. As judged by their stability and selective reactivity, the* N*-chloroamines generated by stimulated neutrophils may play an important role in the local and systemic regulation of inflammatory events in vivo.*

Human neutrophils can generate reactive oxygen metabolites and use them to destroy microorganisms and normal or neoplastic mammalian cells and to modulate the inflammatory response (*1*). After specific membrane perturbation, the neutrophil produces superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hypohalous acids (HOX), and possibly the hydroxyl radical ($OH^{\cdot}$). In vitro, the generation of these oxygen metabolites by neutrophils is self-limited and is nearly complete within 60 minutes after the addition of a stimulus (*1*). Under physiological conditions, $O_2^{\cdot -}$ rapidly dismutates to H_2O_2 spontaneously or enzymically via superoxide dismutase, and H_2O_2 is reduced by endogenous catalase, glutathione peroxidase, or myeloperoxidase (*1*). In addition, highly reactive oxidants like HOX or $OH^{\cdot}$ disappear rapidly as they react with multiple oxidizable substrates in the complex cell systems (*1*). Thus, the concentration of oxygen metabolites is expected to fall to undetectable levels shortly after the neutrophil terminates its production of these species. We have now shown (i) that human neutrophils can generate large quantities of a long-lived oxidant, with characteristics similar to, if not identical with, those of *N*-chloroamines and (ii) that this oxidant is powerful enough to attack thioether-containing peptides and proteins thought to be important in the inflammatory response.

Human neutrophils were isolated from venous blood by a Ficoll-Hypaque sepa-

Table 1. Characterization of mechanism involved in oxidant generation. Neutrophils (2.5×10^6) were incubated with either PMA (30 ng/ml) or opsonized zymosan (1.25 mg/ml) in a final volume of 1 ml for 90 minutes at 37°C. TNB oxidation was determined as described (*2*). Results are expressed as the means ± S.D.

Substance added to neutrophils	Amount of TNB-oxidized (nmole)		*N*
	PMA-stimulated	Zymosan-stimulated	
Neutrophils only	50.0 ± 9.8	28.8 ± 9.2	19
Superoxide dismutase (10 μg/ml)	68.9 ± 12.6	31.0 ± 12.0	7
Heat-inactivated superoxide dismutase (10 μg/ml)	50.4 ± 7.0	23.9 ± 8.0	5
Catalase (10 μg/ml)	3.6 ± 4.0	1.2 ± 1.8	10
Heat-inactivated catalase (10 μg/ml)	48.6 ± 5.6	28.0 ± 9.0	5
Ethanol (40 m*M*)	53.9 ± 10.6	27.8 ± 4.4	5
Mannitol (40 m*M*)	49.7 ± 8.4	27.7 ± 10.8	5
Azide (0.1 m*M*)	0.7 ± 1.8	0.1 ± 0.2	12
Cyanide (0.5 m*M*)	0.0 ± 0.0	0.0 ± 0.0	5

ration followed by dextran sedimentation (*2*). Neutrophils (2.5×10^6 per milliliter) were suspended in Dulbecco's buffer (Gibco, 1 mg per milliliter of glucose) at *p*H 7.4. The neutrophils were stimulated to generate oxygen metabolites and release lysosomal enzymes by the addition of serum-opsonized zymosan particles (Sigma) or phorbol myristate acetate (PMA) (Consolidated Midland) (*2*). After the mixtures were incubated for 90 minutes at 37°C, catalase (1000 U) was added (*3*), the neutrophils were sedimented (1250*g* for 10 minutes), and the supernatant was removed. As expected, the supernatant from either resting or stimulated neutrophils did not contain detectable amounts of $O_2^{\cdot -}$ or H_2O_2. However, the supernatant from PMA- or zymosan-stimulated cells contained a factor capable of oxidizing the sulfhydryl dye 5-thio-2-nitrobenzoic acid (TNB; maximum wavelength, 412 nm) to its disulfide, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; maximum wavelength, ~ 320 nm) (*2*). Quantitation of TNB oxidation by the factors present in the supernatants revealed their ability to react with substantial amounts of the sulfhydryl dye. The oxidizing activity found in the supernatant was very slowly lost at 25°C, with an apparent half-life of 18 hours. Supernatants from resting neutrophils did not oxidize significant amounts of TNB [mean ± standard deviation (S.D.), 0.4 ± 0.6 nmole; $N = 9$]. Thus, it appears that stimulated (but not resting) neutrophils can release large amounts of a long-lived oxidant capable of oxidizing TNB.

To determine the processes involved in the generation of this oxidant, we stimulated neutrophils with PMA or zymosan in the presence of superoxide dismutase or catalase (Table 1). Superoxide dismutase had no inhibitory effect on the formation of the oxidant, whereas catalase (but not heat-inactivated catalase) almost completely blocked its production. Although H_2O_2 can play a role in the generation of $OH^{\cdot}$ (*1*), the $OH^{\cdot}$ scavengers ethanol and mannitol did not inhibit oxidant formation (Table 1). The heme-lysosomal enzyme myeloperoxidase may use H_2O_2 to peroxidize Cl^- to hypochlorous acid (HOCl) (*1*). Almost no oxidant was detected if neutrophils were stimulated in the presence of the myeloperoxidase inhibitors azide or cyanide (Table 1). These results suggest that both H_2O_2 and myeloperoxidase play a role in the formation of the stable oxidant. However, this interpretation implies that catalase, azide, or cyanide does not react with the oxidant itself. Indeed, if the oxidant is generated and then incubated with catalase or azide there is no loss of its oxidizing activity, but in the presence of cyanide, no oxidant was detected after a 30-minute incubation at 37°C. Because cyanide is an excellent nucleophile, it appears that cyanide can block oxidant formation either by inhibiting myeloperoxidase or by reacting with the oxidant after it is formed.

Although H_2O_2 and myeloperoxidase may participate in the generation of the long-lived oxidant, it is unlikely that the species is HOCl. The latter can react with various biological substrates at rapid rates (*1, 4*) and would not be expected to accumulate in a cell system. In addition, HOCl is reduced by H_2O_2 (*2, 4*), whereas the stable oxidant generated by the neutrophil does not disappear in the presence of equimolar concentrations of H_2O_2 (data not shown). We have recently demonstrated that neutrophils can utilize the H_2O_2-myeloperoxidase-Cl^- system to chlorinate an exogenous amine to form an *N*-chloro derivative (*2*). If stimulated neutrophils chlorinate endogenous amines they would be expected to generate the respective *N*-chloroamines (RNHCl) (*2, 4*). *N*-Chloroamines are semistable oxidants that do not react

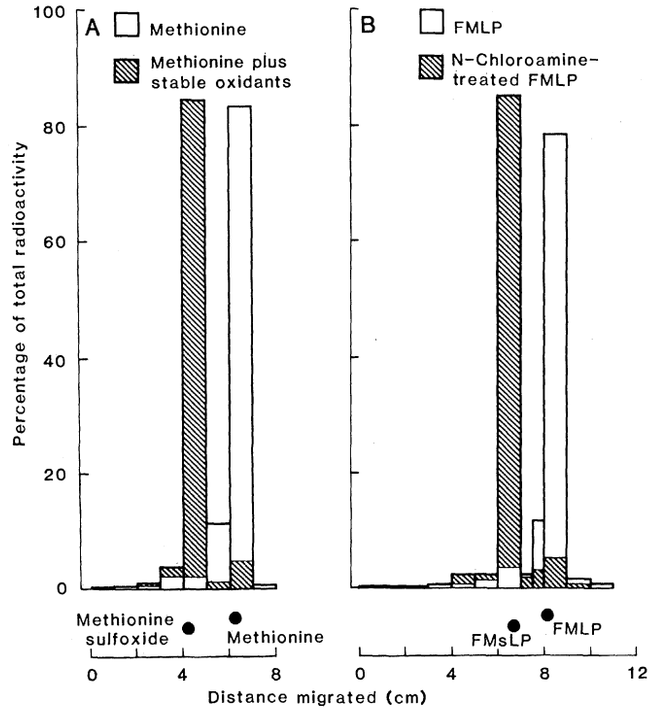

Fig. 1. (A) Oxidation of methionine by neutrophil-generated *N*-chloroamines. *N*-chloroamines (10 nmole) from PMA-stimulated neutrophils were incubated with 1 nmole of [^{14}C]methionine (2.0 μCi) for 60 minutes at 37°C. At the end of the incubation period, chloroamines were reduced with methionine, and [^{14}C]methionine oxidation products were assessed by radiochromatography in a solvent system containing *tert*-butanol, 2-butanone, H_2O, and NH_4OH (4:3:2:1) (*11*). Radioactive material was located by autoradiography, and the plate was cut into strips for liquid scintillation counting. The oxidized methionine product [relative mobility (R_F) = 0.26 ± 0.10)] migrated with methionine sulfoxide prepared with *N*-chlorosuccinimide (R_F = 0.24 ± 0.01; $N = 4$). (B) Oxidation of FMLP by neutrophil-generated *N*-chloroamines. *N*-chloroamines (10 nmole) were incubated with 1 nmole of [^{3}H]FMLP (20 μCi) in a final volume of 1 ml, and oxidized products were analyzed as described above. The oxidized FMLP product (R_F = 0.57 ± 0.01) migrated with FMsLP prepared with *N*-chlorosuccinimide (R_F = 0.58 ± 0.02; $N = 3$).

with H_2O_2 and can oxidize sulfhydryls to disulfides or thioethers to sulfoxides (*2, 4, 5*). Radiochromatography revealed that the oxidant generated by PMA- or zymosan-stimulated cells could oxidize the thioether-containing amino acid methionine to a material that migrated with methionine sulfoxide (Fig. 1A). Supernatants from resting cells did not oxidize methionine.

If the oxidants detected in our system are *N*-chloroamines they should be identifiable by their characteristic ultraviolet absorption peak at 252 nm (*2, 5*). Difference spectra obtained by reducing the oxidant with an excess of methionine resulted in the rapid formation of a peak with an absorption maximum at 252 to 255 nm (Fig. 2). If methionine (but not an equimolar amount of methionine sulfoxide) was added to this sample, the absorption peak disappeared. From calculations based on (i) an extinction coefficient of $429M^{-1}$ cm^{-1} at 252 nm for *N*-chloroamines (*5*) and (ii) the ability of 1 mole of RNHCl to oxidize 2 moles of TNB (*2, 4*), we found that all of the oxidant detected in the supernatant could be accounted for by the species absorbing at 252 to 255 nm. Therefore, from the data presented in Table 1, we conclude that 2.5×10^6 neutrophils release 25.0 ± 4.9 nmole of *N*-chloroamines when stimulated with PMA and 14.4 ± 4.6 nmole of *N*-chloroamines when stimulated with zymosan (*6*).

Various inflammatory regulators lose biological activity upon oxidation of thioether moieties (*1*), and the course of the inflammatory response may be controlled by the oxidation and reduction of critical methionine residues in bioactive molecules. We therefore examined the ability of the neutrophil-generated *N*-chloroamines to react with both a methionine-containing chemotactic peptide and a high molecular weight serum protease inhibitor.

N-Formylmethionylleucylphenylalanine (FMLP) is a synthetic oligopeptide that resembles naturally occurring bacterial products and is capable of stimulating neutrophil chemotaxis and metabolism at nanomolar concentrations (*7*). The methionyl residue is essential for the activity of the peptide, whereas the sulfoxide derivative (FMsLP) does not stimulate cellular activity (*1*). As shown in Fig. 1B, 10 nmole of the *N*-chloroamine transformed a $10^{-6}M$ solution of FMLP to a product that migrated with FMsLP. In five experiments, *N*-chloroamines generated by PMA- or zymosan-stimulated neutrophils oxidized 96.5 ± 2.5 percent (mean ± S.D.) of the peptide (*8*). If the chloroamine was first reduced with methionine, no oxidation of FMLP was observed. When 10 nmole of the *N*-chloroamine (from either PMA- or zymosan-stimulated neutrophils) was incubated with an excess of FMLP ($3 \times 10^{-5}M$; final volume, 1 ml) for 60 minutes at 37°C, 9.8 ± 0.2 nmole (mean ± S.D.; $N = 7$) of the oxidant had disappeared while 8.9 ± 1.4 nmole of the FMLP was oxidized. The observed stoichiometry is therefore close to 1:1 as would be expected if a two-electron oxidant (that is, the *N*-chloroamine) reacts with a thioether to form the sulfoxide.

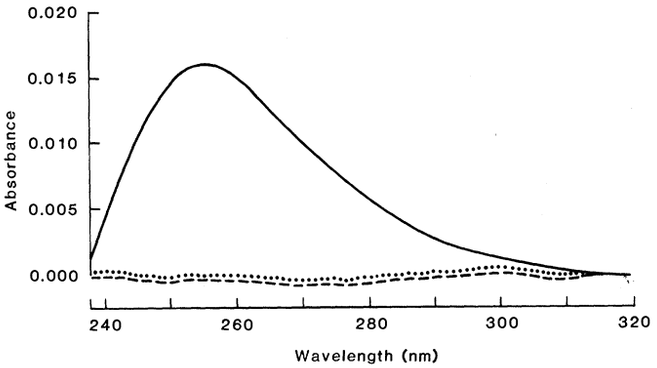

Fig. 2. Difference spectrum of oxidant generated by PMA-stimulated neutrophils. Supernatants were prepared from PMA-stimulated neutrophils (5×10^6 per milliliter) as described. Oxidants in the supernatants were reduced by adding methionine (500 nmole). (------) Resting cells with added methionine versus resting cells; (.....) PMA-stimulated cells versus PMA-stimulated cells; (——) PMA-stimulated cells with added methionine versus PMA-stimulated cells. After the 252- to 255-nm peak was obtained, the addition of methionine, but not methionine sulfoxide (500 nmole), to the sample cuvette returned the values to baseline.

After neutrophils migrate into an inflammatory site they can discharge proteases capable of degrading connective tissue. The major serum protein responsible for regulating the proteolytic activity of leukocyte elastase is α_1-protease inhibitor (α_1-PI), a glycoprotein with a molecular weight of 53,000 (*9*). Oxidation of a critical methionine residue yields a sulfoxide derivative that no longer exhibits elastase inhibitory capacity (*1, 9*). The ability of the cell-derived *N*-chloroamine to inactivate α_1-PI was determined by incubating chromatographically purified inhibitor (*10*) (Sigma) with the oxidant for 60 minutes at 37°C. The reaction was terminated with an excess of methionine, and the elastase inhibitory capacity of the α_1-PI was determined (*9*). In five experiments, supernatants from PMA- or zymosan-stimulated neutrophils containing 25 nmole of *N*-chloroamine inactivated 84.6 ± 5.1 and 77.2 ± 5.3 percent, respectively (mean ± S.D.) of the elastase inhibitory capacity in a 1-mg preparation of α_1-PI. If the *N*-chloroamines in the supernatants were reduced with methionine before α_1-PI was added, no loss of activity was observed. Although the disappearance of elastase inhibitory capacity might indicate the formation of an α_1-PI inhibitor rather than true inactivation, untreated α_1-PI added to the chloroamine-treated α_1-PI at the end of the incubation period retained full activity. Thus, the *N*-chloroamines generated by the neutrophil can mediate the inactivation of the elastase inhibitory capacity of human α_1-PI.

We have shown that human neutrophils can generate long-lived oxidants with characteristics similar to, if not identical to, those of *N*-chloroamines. Although we have not yet identified the amines that are chlorinated, it is clear that this class of oxidants has the potential to interact with important inflammatory regulators. Thus, *N*-chloroamines appear to be a class of neutrophil-derived oxidants with a balance of strong oxidizing potential, selective reactivity, and stability that allows their accumulation at inflammatory sites or diffusion over large distances before they oxidize susceptible target molecules. We postulate that phagocyte-derived *N*-chloroamines play an important role in modulating the activity of sulfhydryl- or thioether-containing molecules and in regulating the inflammatory process in vivo.

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Specific Endocrine Tissue Marker Defined by a Monoclonal Antibody

Abstract. *One of two mouse monoclonal antibodies (LK2H10) produced by hybridoma technology against a human endocrine tumor (pheochromocytoma) demonstrated specific immunoreactivity for 69 normal and neoplastic endocrine cells and tissues known to contain secretory granules. This immunoreactivity was specific, since other normal tissues, tumors from endocrine cells without granules, and tumors from other nonendocrine tissues were negative when tested with antibody LK2H10. The antibody reacted with human fetal adrenal medulla and human pancreatic endocrine cells and with adrenal medullary cells from monkeys and pigs. The antigen detected by antibody LK2H10 is associated with cytoplasmic secretory granules, has an estimated molecular weight of 68,000, and may be related to human chromogranin.*

Immunohistochemical techniques in which polyclonal antisera are used to identify various peptide hormones and amines have been indispensable in characterizing cells and tissues of the amine precursor uptake and decarboxylation (APUD), or diffuse neuroendocrine (DNE), system and tumors derived from these tissues (*1*). Conventional techniques used to identify neurosecretory granules in these cells, such as electron microscopy and the various silver stains (*2*), are relatively nonspecific compared to immunohistochemical methods with polyclonal antisera. With hybridoma technology (*3*), one may now generate virtually unlimited supplies of monoclonal antibodies to previously undetected hormones and proteins associated with the complex secretory granules in cells of the DNE system, which may help to further characterize this complex system of cells. Thus, monoclonal antibodies produced against purified synaptic junctions (*4*) or neural plasma membranes (*5*) have been found to cross-react extensively with cells of the DNE system. In addition, reovirus-induced polyendocrinopathy of SVL/J mice enabled the isolation of a variety of mouse monoclonal autoantibodies reactive with a number of polypeptide endocrine hormones (*6*). In this report we describe a monoclonal antibody that reacts with a 68,000-dalton protein associated with endocrine secretory granules. The availability of such a specific monoclonal antibody will allow for a simple and highly reproducible method to visualize cells with neurosecretory granules in endocrine tissues of human and certain vertebrate species.

It was our intention to use the hybridoma technique for developing monoclonal antibodies that would identify new endocrine markers in tissue specimens (Formalin-fixed, paraffin-embedded) routinely prepared and processed by the pathology laboratory. We immunized BALB/c mice with 1-mm^3 pieces of a human pheochromocytoma that had been minced with a razor blade and mixed with Freund's adjuvant before intraperitoneal injection. After five biweekly injections, a mouse was killed and its spleen cells were fused with NS-1 mouse myeloma cells (*7*). After 14 days in medium containing hypoxanthine, aminopterin, and thymidine, culture supernatant fluids from growing clones were screened for antibody activity against cryostat sections of the original tumor by indirect immunofluorescence. After this step, 50 clones were selected and 2 weeks later retested for reactivity with cryostat sections and Formalin-fixed, paraffin-embedded sections of the same tumor by avidin-biotin complex (ABC) (*8*) peroxidase staining (Vector Laboratories).

Table 1. Immunohistochemical reactivity of monoclonal antibody LK2H10 with normal human adult and fetal endocrine tissue. Numbers in parentheses are numbers of cases examined.

Tissue	Reactivity
Adult	
Adrenal medulla (5)	Yes
Adrenal cortex (5)	No
Gastrointestinal-endocrine (8)	Yes
Exocrine pancreas (5)	No
Endocrine pancreas (5)	Yes
Parathyroid (2)	Yes
Anterior pituitary (3)	Yes
Posterior pituitary (5)	No
Thyroid C cells (5)	Yes
Thyroid follicular cells (5)	No
Fetal (13 to 18 weeks)	
Adrenal medulla (4)	Yes
Endocrine pancreas (4)	Yes

Two hybridomas, designated LK2H10 and LK6D10, were selected for additional testing when their antibodies were found to react with tumor cells, but not connective tissue, in cryostat and Formalin-paraffin sections. Although both antibodies reacted exclusively in initial studies with endocrine tissues, only clone LK2H10 was successfully subcloned and thus available for more extensive testing in Formalin-paraffin sections. Reactivity of antibody LK2H10 with endocrine tissues could be detected by ABC peroxidase staining with up to a 100-fold dilution of hybridoma culture fluid. Thus a single large batch of LK2H10 culture fluid was collected and used throughout this study at a tenfold dilution. As a negative control for staining, we used culture fluids containing a monoclonal antibody to an antigenic determinant of human I-region-associated antigens that is denatured in Formalin-paraffin sections. No background staining was observed with hybridoma culture fluids containing our control antibody even when it was tested undiluted by the ABC peroxidase method.

Normal endocrine tissues that were reactive with LK2H10 are listed in Table 1. Immunostaining was done with the ABC peroxidase method (*8*) using a one- to tenfold dilution of the monoclonal antibody for 1 hour at 37°C. The intensity of immunostaining with the normal pancreatic islet was variable. Glucagon-producing cells showed the greatest immunoreactivity, while insulin-producing cells showed the least. Endocrine tumors that showed positive immunoreactivity included pheochromocytomas (five cases), pituitary adenomas (five), medullary thyroid carcinomas (five), pancreatic endocrine neoplasms (five), parathyroid adenomas (two), carcinoids (five), and paragangliomas (two). Endocrine tissues and tumors that were negative included normal adrenal cortex (five cases) (Fig. 1A), placenta (four), pineal gland (one), melanomas (four), follicular thyroid tumors (six), and papillary thyroid tumors (two). Endocrine tumors with few gran-