

NEUTROPHILS generate potent microbicidal molecules via the oxygen-dependent pathway, leading to the generation of reactive oxygen intermediates (ROI), and via the non-oxygen dependent pathway, consisting in the release of serine proteinases and metalloproteinases stored in granules. Over the past years, the concept has emerged that both ROI and proteinases can be viewed as mediators able to modulate neutrophil responses as well as the whole inflammatory process. This is well illustrated by the oxidative regulation of proteinase activity showing that oxidants and proteinases acts in concert to optimize the microbicidal activity and to damage host tissues. ROI and proteinases can modify the activity of several proteins involved in the control of inflammatory process. Among them, tumour necrosis factor- α and interleukin-8, are elective targets for such a modulation. Moreover, ROI and proteinases are also able to modulate the adhesion process of neutrophils to endothelial cells, which is a critical step in the inflammatory process.

Key words: Adhesion molecules, IL-8, Myeloperoxidase, Neutrophil, Oxidants, Oxidative burst, Phagocyte, Proteinases, TNF α .

Neutrophil-derived oxidants and proteinases as immunomodulatory mediators in inflammation

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Introduction

The aim of this report is to review our current understanding of radical oxygen intermediates (ROI) and proteinases in the context of a modulatory role of neutrophils in the inflammatory process. We will also review studies showing that besides their usual microbicidal role these molecules have important regulatory functions. The notion that inflammation is the net result of pro- and contra-inflammatory pathways¹ is well illustrated by ROI and proteinases which either alone, or in concert, may interact in up or down regulating the major inflammatory process. Added to the intrinsic complexity of the subject is the diversity of effects that can be mediated by ROI and proteinases, either on the phagocyte itself or on the target cells.

Neutrophil-derived oxidants and proteinases

Neutrophils react to invading microorganisms and inflammatory mediators with a variety of coordinated responses, such as motility in response to chemotactic agents, cytoskeletal rearrangement, phagocytosis, and production of toxic mediators to allow the neutrophil to destroy pathogens. Although critical to host defence, neutrophils can damage normal cells and dissolve connective tissues by the

release of a complex assortment of deleterious agents, including ROI and proteinases, leading to inflammatory disorders.^{2,3}

Conventionally, two microbicidal pathways are defined, depending on whether or not they require oxygen. The oxygen dependent pathway depends on ROI whose production follows the activation of NADPH oxidase; the non-oxygen-dependent pathway reflects the actions of preformed enzymes and antimicrobial proteins stored in the neutrophil cytoplasmic granules and released upon activation as illustrated in Fig. 1.

Oxidative metabolism activation, known as the respiratory burst, first involves NADPH oxidase, which is an enzymatic complex composed of cytosolic and membrane proteins which ultimately translocate to the plasma membrane, leading to the generation of superoxide anion (O_2^-). The dismutation of O_2^- can generate hydrogen peroxide (H_2O_2). Myeloperoxidase (MPO), an enzyme contained in azurophilic granules, in the presence of chloride and H_2O_2 , catalyses the formation of potent chlorinated oxidants such as hypochlorous acid (HOCl) and chloramines, the so-called long-lived oxidants.⁴⁻⁸ Much of what is known about the NADPH oxidase has come from studies of patients deficient in the system, who have chronic granulomatous disease (CGD). Owing to a genetic defect in the major component of the oxidase,

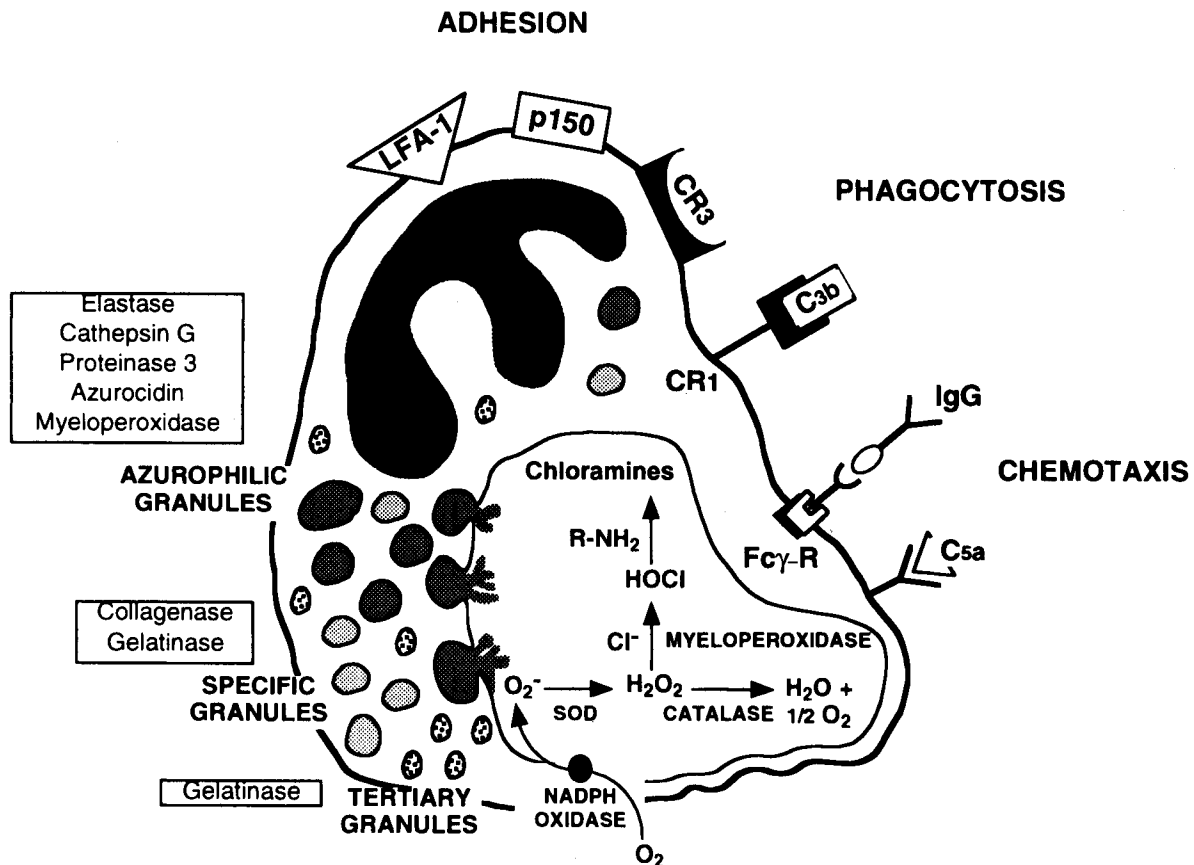


FIG 1. Neutrophil effector mechanisms involved in the defence against pathogens and in the modulation of inflammatory process. Neutrophils respond to inflammatory agents by expressing membrane receptors appropriate to the stimulation and by developing coordinated responses such as spreading, diapedesis, phagocytosis, production and release of toxic effector molecules. The selectins and the leukocyte integrins of the CD18 family, including LFA-1 (CD11a/CD18), CR3 (CD11b/CD18), p150 (CD11c/CD18), are necessary for proper adhesion to endothelium. The complement opsonins C3b and C4b are recognized by CR1. IgG opsonins are recognized via the immunoglobulin receptors (FcγR). The stimulus dependent expression of these receptors is required for phagocytosis, degranulation and respiratory burst. The first microbicidal pathway is the oxidative response which consists of the production of ROI following NADPH-oxidase complex activation, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and via myeloperoxidase, hypochlorous acid (HOCl) and chloramines. The second microbicidal pathway is non-oxygen dependent and consists in the release in the phagolysosome or in the extracellular medium, of preformed proteins stored in granules. The serproteins (serine proteases with antibiotic activity including elastase, cathepsin G, proteinase 3 and azurocidin) as well as myeloperoxidase are contained in the azurophilic granules. The metalloproteases (collagenase and gelatinase) are contained in specific and tertiary granules (for gelatinase only).

namely cytochrome b-245, or in cytosolic factors, phagocytes of CGD patients fail to mount a respiratory burst. Although their phagocytic capacity is normal, CGD phagocytes are incapable of producing ROI and of subsequently killing ingested pathogens.^{9,10}

Neutrophils contain several thousand cytoplasmic granules which act as storage compartments for macromolecules destined for secretion, stored in specific granules, or for fusion with phagosomes, stored in azurophilic granules.¹¹⁻¹³ Neutrophils must degrade the connective tissues to extravasate and migrate to the site of inflammation. For this, they contain metalloproteinases such as collagenase, stored in the specific granules and gelatinase, stored in tertiary granules. The azurophilic granules contain the majority of the antibiotic proteins. Among the ten identified so far, two are thought to be unique in primary structure—lysosyme and bactericidal/perme-

ability increasing protein (BPI).^{14,15} The remaining eight fall into two families, each with four members: the defensins¹⁶ on the one hand, and the serproteins (serine proteinases with microbicidal activity) including elastase, cathepsin G, proteinase 3 and their enzymatically inactive homologue azurocidin, on the other.¹⁷

In neutrophils, ROI have indeed traditionally been viewed primarily as potent microbicidal agents. We would like to focus on the two paradigms that (1) despite the clearcut distinction between oxygen-dependent or non-dependent toxic mechanisms, the neutrophil is constructed to use both the NADPH oxidase system and the granule constituents in a cooperative and concerted manner and to realize its ultimate destructive potential; and (2) besides their toxic effects, ROI and proteases at lower concentrations may cooperate with other immunomodulatory molecules such as cytokines and

adhesion molecules, and thus function as intercellular signalling molecules and to regulate phagocyte functions and modulate the overall inflammatory process.

Neutrophil-derived reactive oxygen intermediates:

Biochemical basis of ROI-mediated effects. An extensive report on mechanisms of cell injury by ROI will not be given; the reader can easily find several reviews on this subject in the literature.^{18,19} Nevertheless, a brief outline of ROI biochemistry will be presented. There are three intermediates in the reduction of O₂ to H₂O₂. As a result, O₂⁻, H₂O₂ and the hydroxyl radical (OH[•]) are formed by successive one electron additions. Despite numerous studies, the formation of OH[•] in phagocytes is still controversial.²⁰⁻²³ So is the formation of singlet oxygen.^{24,25} However, in this review, the term ROI will include O₂⁻, H₂O₂, OH[•], singlet oxygen and chlorinated oxidants such as HOCl and chloramines, and the defined oxygen species will be specified when possible.

Although the hallmark of the phagocyte remains the major source of ROI following NADPH oxidase activation, other cells can generate ROI through different pathways. Perhaps the most significant source of non-leukocyte generated ROI is the production of superoxide anion by xanthine oxidase, observed in ischaemia/reperfusion injury.^{26,27} The cellular alterations following hypoxia promote the conversion of xanthine dehydrogenase to xanthine oxidase. Many other potential mechanisms derived from normal cellular metabolism could result in ROI generation, including mitochondrial transport, peroxisomal reactions and arachidonate metabolism.²⁸

Free radicals and related oxidants have long been studied as agents of tissue damage. Lipid peroxidation is a well documented free radical chain reaction which can be initiated by OH[•] or transition metal complexes and remains an archetype of ROI-mediated toxicity.²⁹⁻³¹ Yet few studies have focused on its involvement in neutrophil oxidative reactions. Likewise, numerous studies have characterized the mechanisms underlying oxidative damage to RNA and DNA.³²⁻³⁴

In contrast, the reactions of proteins with various radicals/oxidants have not been so extensively studied although it is now clear that amino acids, peptides and proteins are, indeed, vulnerable to attack by a variety of ROI. Oxidation of particularly sensitive amino acid residues, aggregation or cross-linking, fragmentation and loss of enzymatic or other functional properties are but a few of the documented examples.³⁵⁻⁴¹

Sulfhydryl groups can play an essential role in the function of several proteins, such as catalytic activity of enzymes and receptor binding capacity. These sulfhydryl groups are especially vulnerable to

oxidative attack, with conversion to S-S bonds, particularly in the presence of metal ions. ROI, and particularly HOCl and chloramines, are all able to oxidize sulfhydryl groups resulting in the formation of a disulfide bridge. This oxidation could be corrected by thiol-containing molecules such as glutathione (GSH).^{42,43}

Ultimately, oxidatively damaged proteins which exhibit higher proteolytic susceptibilities than other damaged proteins serve no useful role, and will be removed by specific proteolytic degradation. It is now clear that proteasome, a 670 kDa ATP-independent proteinase complex (also called macroxyproteinase) is responsible for most of the selective degradation of oxidatively modified proteins. This enzymatic complex exhibits a serine proteinase activity, a sulfhydryl proteinase activity and a metallo-peptidase activity.⁴⁴⁻⁴⁶ The primary, secondary or tertiary structure of proteins may be altered depending on the oxidant and the overall result may be denaturation and increased hydrophobicity.⁴⁷

Besides drastic oxidative attack on a protein, ROI may affect protein function by the oxidation of a critical residue in its active site with minor modification in the secondary or tertiary structure of the protein. This type of reaction could be defined more as 'oxidative regulation'⁴⁸ than 'oxidative attack'.

Many proteins may be oxidatively inactivated because of the sensitivity of methionine residues to oxidation. Much evidence suggests that methionine oxidation and subsequent loss of protein activity not only occur widely in living systems but are physiologic, homeostatic processes.⁴⁹ In some cases, methionine oxidation may occur without changes in physical or immunochemical properties and without loss of biologic activity, as is the case for the bovine growth hormone and the human α 2-plasmin inhibitor.^{50,51} Among the oxidants produced by activated neutrophils following the respiratory burst, H₂O₂ and the MPO-derived oxidants, namely HOCl and chloramines appear to be of biological relevance. By virtue of its strong oxidant potential, HOCl seems to be the toxic species used by neutrophils to mediate cell injury. Chloramines (*N*-chloroamine) arise from the chlorination of a wide spectrum of amine-containing compounds by HOCl.^{6,52} Although these species differ in their reactivity with susceptible target molecules, they are able to mediate diverse biologic effects, ranging from the modulation of cellular functions to the induction of cell lysis. Chloramines have a half-life greater than radical oxygen species, ranging from 5 h at 37°C to more than 100 h at 4°C, depending on the surrounding environmental concentration of anti-oxidant. As a result, they can persist at sites of inflammation long after the disappearance of other oxygen metabolites, and have been termed long-lived oxidants. Generated chloramines

are a mixture of chlorinated endogenous and exogenous amines of molecular weight ranging from 150 to 50 000.⁵³ The presence of exogenous amine-containing compounds can influence both the quantity and the characteristics of the long-lived oxidants generated. For example, the plasma protein albumin can be chlorinated and retain long-lived oxidant activity. The presence of appropriate nitrogenous compounds in the extracellular medium can result in the generation of lipophilic N-Cl oxidants of a strong cytotoxic potential. However, only hydrophilic chloramines are detected in supernatants from stimulated cells. Of primary physiologic importance among the hydrophilic generated chloramines is *N*-chlorotaurine formed by chlorination of the sulfur-containing amino acid taurine, which is plentiful in neutrophils.⁵⁴ Taurine is the major amine released into the supernatants of triggered neutrophils,⁵² and thus its chloramine derivative is the most abundant.⁵³ Taurine chloramine is not a potent toxin but does oxidize proteins by specific attack at thioether moieties. The notion that methionine oxidation may be a relevant mechanism by which many proteins are regulated is corroborated by the fact that this oxidation can be reversed by methionine sulfoxide reductase, an enzyme which reduces methionine sulfoxide to methionine, using thioredoxin as cofactor.⁵⁵ The ability of this enzyme to reduce methionine sulfoxide in a variety of proteins suggests that it has broad specificity. It has been cloned and expressed in *Escherichia coli*.⁵⁶ Interestingly enough, it is widely distributed in different organs and in particular has been found at high concentration in neutrophil extracts. The ability of methionine sulfoxide reductase to reduce methionine sulfoxide residues suggests that it may be a repair enzyme for proteins inactivated by oxidation, or may participate in a novel type of post-translational regulation of proteins involved in the inflammatory process.

Antioxidant-mediated cell protection. Several antioxidant systems or ROI scavengers can protect tissue from oxidative damage.⁵⁷ Antioxidant defences appear to be an elaborate arsenal of antioxidants which function in concert to scavenge and detoxify ROI (superoxide dismutase (SOD), catalase, glutathione peroxidase), block free radical chain reactions (tocopherol, carotenoids and ascorbic acid), or sequester transition metals which can serve as a ready source of free electrons (lactoferrin, ceruloplasmin and transferrin).⁵⁸

Owing to the chemical nature of ROI, especially radical species, it was difficult to measure these species directly. Thus, besides their physiologic role, ROI scavengers provide useful pharmacologic tools to probe cellular reactions in order to demonstrate the involvement of ROI in the underlying mechanisms.

Oxidants have regulatory effects by modulating antioxidant defences. Glutathione (GSH), the major non-protein thiol, is present in virtually all cell types and is involved in numerous biological functions.⁵⁹ It can act as a co-enzyme, an antioxidant by preserving intracellular reducing conditions, a regulatory molecule in cell cycle initiation and progression⁶⁰ and in microtubule formation.⁶¹

Regulation of intracellular content of GSH in lymphocytes has been shown to modify immune response capacity. The sensitivity of lymphocytes to oxidants is well established. For example, H₂O₂ impairs the proliferative capacity of human blood lymphocytes.^{62,63} Several lymphocyte functions, such as mitogen-induced proliferation, natural killer activity, generation of immunoglobulins, are sensitive to the myeloperoxidase-H₂O₂-chloride system.^{64,65} Thiol compounds, including GSH, are of critical significance.⁶⁶ The proliferative response is directly related to GSH availability.⁶⁷ Neutrophil derived chloramines markedly inhibit lymphocyte mitogen-induced proliferative response by decreasing GSH content.⁶⁸ GSH is involved in cytokine metabolism. It regulates interleukin-2 and interleukin-4 activity on cytotoxic T cells.^{69,70} Interestingly, in an *in vitro* model of chronic HIV infection, GSH inhibits the induction of HIV expression suggesting that antioxidant therapy may be effective in limiting progression of the disease process.⁷¹

The importance of oxidant-mediated injury has prompted research on new antioxidant therapy by gene therapy. *In vitro* evaluation of the regulation of catalase gene expression in human airway epithelial cells has shown that the expression of the human catalase gene is not upregulated in these cells in response to hyperoxia, because the 5'-flanking region of the gene has characteristics of a non-regulated 'housekeeping' gene and does not respond to a hyperoxic stimulus. However, when human airway epithelial cells are infected with an adenoviral vector containing human catalase cDNA, catalase activity increases, as does the survival of cells subjected to oxidant stress.⁷² A similar antioxidant strategy is efficient in protecting endothelial cells against H₂O₂-mediated injury.⁷³

Neutrophil-derived metalloproteinases and serine proteinases: Neutrophil-derived proteinases are packed in granules which are released upon cell activation. Granule biogenesis follows the granulocyte differentiation pathway. The azurophilic granules first emerge at the stage of promyelocytes and contain neutral serine proteinases. Later in differentiation, at the stage of metamyelocytes, specific granules containing collagenase and gelatinase emerge.^{74,75} However, a tertiary granule population containing gelatinase has been identified. The mechanisms underlying the secretion of the three

morphologically distinct populations of granules may be under separate control. The order of exocytosis observed after ionophore-induced elevation of cytosolic calcium was gelatinase granules, specific granules, and lastly azurophilic granules.⁷⁶

Metalloproteinases. Matrix metalloproteinases (MMPs) constitute a family of closely related enzymes which play important roles in a variety of physiological and pathological processes of matrix degradation. Human neutrophils contain both a collagenase and a gelatinase. One of the most intriguing aspects of the physicochemical properties of these neutrophil metalloproteinase is the fact that the enzyme can be isolated from the cell in a latent, inactive form. Neutrophil collagenase has been isolated as a 91 kDa latent enzyme that could be activated to yield active collagenase of 64 kDa which can degrade native interstitial collagens. Neutrophil gelatinase has also been isolated in its latent form, with reported molecular weights of 92, 130 and 225 kDa. The molecular basis underlying the latency of gelatinase has not been studied as extensively as that for collagenase. Collagenase attacks native interstitial collagen whereas gelatinase only degrades denatured collagen as well as native type IV or type V collagen.⁷⁷ Human neutrophil collagenase and gelatinase have been cloned.^{78,79} Comparison of the primary structures of MMPs shows that they are structurally homologous with defined functional domains.^{80,81} All these enzymes contain an essential catalytic zinc-binding domain, an NH₂-terminal domain which preserves the latent state of the enzyme and a COOH-terminal domain which plays a major role in substrate specificity. In the case of neutrophil collagenase, mutagenesis analysis has shown that substrate specificity is determined by a 16 amino-acid sequence in the COOH-terminal domain and is influenced by the integrity of a disulfide defined loop at the COOH-terminal for maximal activity. In addition, mutation of a critical aspartic residue at position 253 within the presumptive zinc-binding locus resulted in complete loss of proteolytic activity, suggesting that this aspartic residue might function as one of the ligands for divalent cations, which are essential for enzymatic activity.⁸²

Metalloproteinase inhibitors. Secretion of metalloproteinase in an inactive precursor form is an important feature which regulates their activity in extracellular milieu. Organomercurials activate the proenzyme *in vitro* by inducing a conformational change. This reaction removes the amino terminal pro-segment, permanently converting the enzyme to the active form. Other mechanisms of activation may involve oxidants or serine proteinase. Further regulation of the activity of metalloproteinases in the extracellular milieu is achieved by specific inhibitors interacting with the activated enzymes.

Metalloproteinases can be inhibited by α 2-macroglobulin, a 725 kDa plasma proteinase inhibitor whose inhibitory properties are not specific and markedly different from all other known inhibitors.⁷⁷ Two tissue inhibitors of metalloproteinases (TIMP) have been characterized and cloned. TIMP-1 is a glycoprotein present in many tissues and biological fluids. It is secreted by several mammalian cell types, including fibroblasts, endothelial cells, smooth muscle cells and chondrocytes.⁸³⁻⁸⁵ The isolation and cloning of a second metalloproteinase inhibitor, also called MI or TIMP-2, raises the question of the existence of a family of TIMP-like proteins. TIMP-2 displays 40% amino acid sequence homology with TIMP-1, with conservation of all twelve cysteine residues.⁸⁶⁻⁸⁸ Both TIMP interact only with the activated enzyme and it has been shown that TIMP-2 has another regulation role which involves blocking the autoproteolytic activation of procollagenase.⁸⁹ The activity of TIMP is inhibited by different serine proteinases, including human neutrophil elastase, trypsin and α -chymotrypsin.⁹⁰ In addition, it seems that TIMP can be oxidatively regulated.

Serine proteinases. Serine proteinases are a large family of enzymes whose active site comprises the so-called 'catalytic triad' of histidine, aspartic acid and serine, in which a charge relay system allows the histidine and the aspartic acid to transiently bind a proton from the serine which can then attack the peptide bond in the target protein. In the azurophilic granules of the neutrophil, the group of the neutral serine proteinase homologues includes cathepsin G, elastase, proteinase 3 and azurocidin. Because they also possess microbicidal activity distinct from their proteolytic capacity, they will be referred to as serprocidins. Members of this family are cationic glycoproteins of similar size (25-29 kDa). They have all been cloned.⁹⁴⁻⁹⁷ The serprocidins exhibit sequence homology between each other and with T cell proteinases, human lymphocyte proteinase, granzyme B, and rat mast cell proteinase.^{13,95,98} Cloning of the genomic segment that contains the functional genes for neutrophil elastase, proteinase 3 (PR3), also called p29b or myeloblastin, and azurocidin, also called CAP37, has revealed that these genes form a cluster of genes, located in the terminal region of the short arm of chromosome 19 and are coordinately down-regulated in the promonocytic cell line U937 during induced terminal differentiation.⁹⁹ The gene of cathepsin G belongs to another cluster of genes encoding haematopoietic serine proteinases together with granzyme H and granzyme B genes on chromosome 14q11.2.^{100,101}

Two well studied serprocidins include neutrophil elastase and cathepsin G. The remaining two, PR3 and azurocidin, more recently isolated and cloned,

were identified as a result of a general screening for neutrophil-derived antibiotic proteins.^{102,103} Neutrophil elastase and PR3 display very similar patterns of proteolytic activities.¹⁰⁴ They are both capable of cleaving insoluble elastin and a variety of matrix proteins, including fibronectin, laminin, vitronectin and collagen type IV. They show minimal activity against interstitial collagens, type I and III.^{105,106} As a result, neutrophil elastase and PR3 have been shown to induce experimental pulmonary emphysema.¹⁰⁷⁻¹⁰⁹

Although the azurocidin sequence reveals extensive homology with serine proteinase, and in particular with neutrophil elastase, two amino acid substitutions, His→Ser and Ser→Gly, in the catalytic triad nullify its enzymatic activity.⁹⁵

Cathepsin G shows little enzymatic activity compared with elastase. Nonetheless, specific substrates have been used to probe its proteolytic activity. Thanks to their proteolytic activity, serprocidins have been shown to be involved in platelet aggregation. Purified cathepsin G activates platelets in terms of aggregation, serotonin release, calcium movements and thromboxane B₂ formation.^{110,111} Although neutrophil elastase or PR3 alone is unable to trigger platelet activation, each enhances cathepsin G induced platelet activation when added in combination with cathepsin G.¹¹²

Serine proteinase inhibitors. Neutrophil elastase, cathepsin G and PR3 are typical serine proteinases, which are rapidly inactivated by di-isopropyl fluorophosphate, synthetic acylating inhibitors¹¹³ and specific serine proteinase inhibitors. The use of inhibitors seems to be the only way to regulate their activity inasmuch as these proteinases are probably functional in their packaged forms. Most likely the dipeptide activation fragment acts to process the enzyme for localization in the azurophilic granules; indeed, the nature of the enzyme responsible for the proteolytic cleavage is unknown.¹¹⁴ Several human proteins function as potent inhibitors of elastase. Each has a characteristic physiological location, likely equivalent to its principal site of inhibitory action. The host's primary defence against uncontrolled elastase-mediated damage is α 1-proteinase inhibitor (α 1-PI, formerly termed α 1-antitrypsin). Synthesized by hepatocytes, α 1-PI is a 52 kDa glycoprotein, present at highest concentrations in plasma, but also found in human azurophilic granules.¹¹⁵ Its high plasma concentration accounts for more than 90% of the elastase inhibitory capacity of human plasma.¹¹⁶ α 1-PI belongs to the serpin family composed of structurally homologous serine proteinase inhibitors. Interestingly, this typical tertiary structure is found in ovalbumin, one of the several members of the family with no known inhibitory function.¹¹⁷ Members of this family have been identified in plants, insects and

throughout the animal kingdom.¹¹⁸ Most serpins are found in extracellular fluids but cytosolic serpins have been found in a wide variety of mammalian PMNs.^{119,120}

When serpins interact with their cognate enzymes, a covalently stabilized serpin-enzyme complex is formed. The interaction results in a change in the conformation of the inhibitor. It has been shown that a pentapeptide domain in the carboxy-terminal fragment of α 1-antitrypsin, which is highly conserved among the serpin family, is recognized by a specific cell surface receptor, the serpin enzyme complex (SEC), and through this SEC receptor is linked to several important physiological processes well characterized in the case of α 1-PI, such as upregulation of the synthesis of α 1-PI itself, internalization and endosomal/lysosomal degradation of SEC.¹²¹⁻¹²³

Current concepts on the pathogenesis of emphysema largely emphasize the role of unrestrained proteolytic activity in the lung extracellular matrix. Since α 1-PI provides almost all the protective screen of the lower respiratory tract against neutrophil elastase, emphysema might result from inactive α 1-PI unable to inhibit neutrophil elastase in the lung.^{124,125} Of particular interest is α 1-PI deficiency, an autosomal hereditary disorder characterized by reduced levels of α 1-PI in plasma and lung fluids, thereby leading to unopposed proteinase activity and culminating in pulmonary emphysema. The deficiency of α 1-PI results from various mutations in five α 1-PI coding exons.¹²⁶ Moreover, this absence of a normal anti-neutrophil elastase screen permits free elastase to bind to alveolar macrophages,^{127,128} resulting in the release of leukotriene B₄ (LTB₄), a process which attracts neutrophils to the alveoli of α 1-PI-deficient individuals, thus accelerating the lung destruction that characterizes this disorder.¹²⁹ Several strategies of α 1-PI augmentation therapy for α 1-PI deficiency can be used in order to restore a normal level of α 1-PI.¹³⁰ An original approach consists in using endothelial cells as target for gene therapy of α 1-PI deficiency, since the modified endothelial cell would secrete human α 1-PI directly to the circulation, where it would diffuse into the alveolar interstitium, providing protection against neutrophil elastase. A modified adenovirus has been shown to transfer α 1-PI cDNA into human endothelial cells, thus conferring upon the cells the ability to synthesize and secrete α 1-PI capable of combining with neutrophil elastase.¹³¹

As for metalloproteinases, α 2-macroglobulin inhibits elastase activity.¹¹⁶ Another cell protein that inhibits leukocyte elastase is the secretory leukoproteinase inhibitor (SLPI), a 12 kDa non-glycosylated protein which is produced by cells of mucosal surfaces and found in the corresponding epithelial fluids, including in the lung. The molar concentrations of SLPI in total respiratory tract

epithelial lining fluid (ELF) are $56 \pm 10\%$ that of $\alpha 1$ -PI, suggesting that SLPI may be important for local anti-elastase protection. However, despite its relatively high concentration, functional studies have shown that only one-third is operative. Moreover, *in vitro* studies showed that exposure to oxidants combined to elastase causes the molecule to be cleaved from a 12 to an 8 kDa inactive fragment. It is conceivable that SLPI plays a first-line role, particularly in the upper airway where its concentration is highest. SLPI might be useful therapeutically in helping to augment the anti-elastase defences of the lung.¹³² In an experimental rat model of immune complex-induced alveolitis, recombinant SLPI intra-tracheally instilled provided significant protection against pulmonary damage.¹³³

A small acid-stable polypeptide of 7 kDa, elafin, is found in skin and appears to be an elastase inhibitor.

Human monocyte/neutrophil elastase inhibitor, a 42 kDa glycoprotein, and member of the serpin family, has recently been isolated and cloned.^{119,120,134} A study of the reactive centre residues indicates that this new serpin seems to inhibit more than one proteinase and is likely to be oxidation sensitive. The latter feature would limit the sphere of action of EI to the immediate vicinity of carrier cells, thus regulating the proteolytic action of the neutrophil itself.

The substrate specificities of PR3 being so close to those of elastase, the pattern of inhibition with serine proteinase inhibitors are almost the same. PR3 can be inhibited by $\alpha 1$ -PI, $\alpha 2$ -macroglobulin or elafin, but not by SLPI. In addition, PR3 is able to selectively degrade both native and oxidized SLPI.¹³⁵ Its sensitivity to monocyte/neutrophil EI is unknown.

Cathepsin G displays a different pattern of inhibition, inasmuch as the serpin which accounted for the greatest inhibition is $\alpha 1$ -antichymotrypsin ($\alpha 1$ -AC). Moreover, elastase- $\alpha 1$ -PI and cathepsin G- $\alpha 1$ -antichymotrypsin complexes are chemotactic for neutrophils, this effect being mediated by the SEC receptor.¹³⁶⁻¹³⁸ As for oxidant scavengers, synthetic proteinase inhibitors of narrow specificity have been developed to probe the involvement of proteinases in biological processes.

Microbicidal and antibiotic activities of serprocidins. Among purified neutrophil-derived proteinases, those that so far appear to have significant antibacterial potential independent of their enzymatic action are cathepsin G^{139,140} and PR3.¹⁰⁶

The antimicrobial potential of the proteinases could be expressed through an indirect mechanism involving antibiotic peptides synthesized as proforms, such as defensin.^{141,142}

It is unknown how these antibiotic proteins stored in the granules as inactive proproteins undergo proteolytic cleavage for conversion to active cytotoxins, or where and when in the course of

granulopoiesis this happens. However proteinases could be good candidates able to process and activate these antibiotic proteins, thus revealing an important role for these enzymes in antimicrobial events.¹³

Role of proteinases in healing and wound repair. Phagocytes, predominantly neutrophils and macrophages, play a critical role in early control of wound repair.¹⁴³ The movement of these cells is directed by the numerous chemoattractant substances that result from degradation of bacteria and autologous proteins.^{144,145} The predominant action of the neutrophil in the wound is to express degradative enzymes and to provide an antibiotic shield through production of ROI and the release of antibiotic peptides. In the skin, neutrophils are responsible for the release of a variant form of collagenase, gelatinase and elastase. Proteinases participate both in destruction of invading micro-organisms and in removal of cellular and matrix debris. In addition, at least at some inflammatory sites, neutrophils can release other soluble signals such as IL-8, IL-1 and TGF-beta.¹⁴⁶

Serprocidins as target antigens for antineutrophil cytoplasmic antibodies. Serprocidins are now recognized to be target antigens for antineutrophil cytoplasmic antibodies (ANCA) found in sera of patients with vasculitis, glomerulonephritis or other systemic inflammatory syndromes. When observed by indirect immunofluorescence microscopy on alcohol fixed PMN, ANCA can be divided into a group displaying a cytoplasmic staining pattern (C-ANCA) and a second group displaying perinuclear staining (P-ANCA).¹⁴⁷ Investigations of antigen specificity have been aimed at identifying the proteins recognized by these ANCA.¹⁴⁸ It appears that the majority of C-ANCA react with PR3, although in a few cases C-ANCA could be directed against BPI, elastase¹⁴⁹ or cathepsin G.¹⁵⁰ The major target antigen of P-ANCA is myeloperoxidase.¹⁵¹

Despite difficulties in classifying vasculitic syndromes, the correlation between clinical expression of Wegener's granulomatosis and ANCA reactivity has now well established that PR3 is the target autoantigen.^{152,153} The pathogenesis of this form of immune necrotizing vasculitis that involves neither antibody directed against basement membrane nor immune complex deposition is only beginning to be understood.¹⁵⁴ Whether ANCA are serologic epiphenomena or play a pathogenic role in the course of the disease is still a matter of debate.

Many studies have focused on *in vitro* ANCA-induced activation of neutrophils and consequent damage to endothelial cells. It is possible that ANCA target antigens such as PR3 translocate to the cell surface to bind ANCA and then trigger the res-

piratory burst and subsequent release of ROI.^{155,156} Other studies have been directed at characterization of these autoantigens, thus pointing out that there are different species of C-ANCA that bind different epitopes of PR3 thus increasing the difficulty of assessing the pathophysiological meaning of these ANCAs.^{157,158}

ROI and proteinases in the modulation of inflammation

Cooperation between ROI and proteinases in host tissue damage: Modulation of the proteolytic capacity of neutrophils by their chlorinated oxidants relies on their ability to inactivate serine proteinase inhibitors by oxidation as well as to activate latent metalloproteinases such as collagenase or gelatinase, thus potentiating the resulting deleterious effect of the proteinases.¹⁵⁹

The α 1-PI contains a methionine residue critical to its activity at position 358¹⁶⁰ which can be oxidized and thus inactivated by hypochlorous acid or chloramines from activated neutrophils. Oxidation of Met-358 causes a 2 000-fold decrease in the rate of association between neutrophil elastase and the modified antiproteinase. Recombinant α 1-PI resistant to oxidation does not undergo inactivation and efficiently protects connective tissue from neutrophil damage.^{161,162} Some nonsteroidal anti-inflammatory drugs appear to rescue α 1-PI from oxidative inactivation by efficiently limiting the extracellular availability of HOCl in the neutrophil surroundings.¹⁶³ Inasmuch as both α 2-macroglobulin and SLPI appear to be sensitive to oxidation, the complete anti-elastase defence is regulated by neutrophil-derived chlorinated oxidants. The direct effect of oxidant attack on elastase using free radicals produced in a Fenton reaction (H_2O_2 in the presence of copper) shows that elastase, as well as three of its inhibitors, eglin c, α 1-PI and SLPI, are efficiently inactivated.¹⁶⁴ Study of the effect of chloramines on α -chymotrypsin, another serine proteinase, has shown that oxidation barely modifies its catalytic properties, whereas sensitivity to specific proteinase inhibitors is decreased.¹⁶⁵ Study of the synergy between myeloperoxidase- H_2O_2 -chloride system and elastase in degrading endothelial cell matrix heparan sulfate proteoglycan has shown that elastase alone, and the myeloperoxidase system alone, cause degradation, and when a 4 h exposure to elastase was followed by 15 min of the myeloperoxidase system, the effect was greater than additive. No such effect was seen when both systems were added together, or when elastase followed the myeloperoxidase system.¹⁶⁶ Studies of the interactions of elastase with its insoluble substrate, elastin, have shown that elastase forms a stable complex with elastin-derived peptides during elastinolysis and, as a result, elastin derived peptides could con-

tribute to modulation of the proteolytic activity of elastase.¹⁶⁷

Metalloproteinases are stored in latent form within granules. When neutrophils are stimulated to release their lysosomal enzymes, these latent enzymes must be activated before they can attack their substrate. The mechanisms by which neutrophil-derived oxidants activate latent metalloproteinases is still not completely understood, but latent collagenase can be activated by HOCl whereas progelatinase seems to require both oxidant- and serine proteinase-dependent pathways.¹⁶⁸⁻¹⁷⁰ Some reports indicate that cathepsin G is a key mediator in neutrophil collagenase activation, and that HOCl under certain conditions leads to activation of collagenase or the stimulation of cathepsin G ability to activate neutrophil collagenase.¹⁷¹ Moreover, the activity of metalloproteinases is also regulated via their specific inhibitors, the TIMPs, which can be inactivated by oxidation or proteolytic cleavage.^{90,172} The modulatory effect of ROI on neutrophil-derived serine proteinases and matrix metalloproteinases appears to be of crucial importance in the inflammatory lung process where these enzymes play a key role in acute lung damage. The co-administration of the serine proteinase inhibitor SLPI and the metalloproteinase inhibitor TIMP significantly prevents pulmonary damage in a rat model of immune complex-induced alveolitis.¹³³ The inactivation of human α 1-PI can occur by interaction with bacterial proteinases such as seryl-cysteiny- and metalloproteinases from *Staphylococcus aureus*. This process of inactivation of α 1-PI by pathogenic proteinases could amplify dysregulation of elastase activity during septicaemia.¹⁷³

Of special relevance in the study of proteinases/antiproteinases and oxidant/antioxidant balances is the case of cystic fibrosis. Cystic fibrosis is a hereditary disorder caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) the product of which is a membrane protein thought to function as a chloride channel. The lethal clinical manifestations are clearly related to the thick, infected mucus and chronic neutrophil-dominated airway inflammation.¹⁷⁴⁻¹⁷⁶ In these patients, chronic airway infection, especially by *Pseudomonas aeruginosa*, is of critical importance in prognosis. This pathogen can never be permanently eradicated despite intensive antibiotic treatment and leads invariably to respiratory failure, which is the cause of death in most patients with cystic fibrosis. It is now well established that neutrophil-derived oxidants inactivate antiproteinases such as α 1-PI, thus potentiating the deleterious effects of elastase. Moreover, antiproteinase imbalance has been recognized as a factor favouring the persistence of *Pseudomonas aeruginosa*.¹⁷⁷⁻¹⁷⁹ In order to increase local antiproteinase defence, different aerosol treatments have

been tested in cystic fibrosis patients. Aerosol administration of α 1-PI efficiently suppresses the burden of active elastase on the respiratory epithelial surface.¹⁸⁰ Likewise aerosolized recombinant SLPI reduces elastase levels and lung inflammatory state.¹⁸¹

Interactions of ROI and proteinases with pro-inflammatory cytokines. There is now evidence for several possible functional points of contact between ROI and proteinases, on the one hand, and cytokines, on the other. Cytokines can be regulated at different levels, e.g. the molecule itself, its inhibitor or its receptor, and at different stages such as transcription, synthesis or activity. An additional level of regulation is possible inasmuch as the effector mechanisms of cytokine activities could involve ROI interacting with transcription factors.

Given the wide diversity of cytokines involved in the control of the inflammatory process and the immune response,¹⁸² we will focus only on tumour necrosis factor- α (TNF α) and the chemokine IL-8, both of which are able to activate neutrophils and trigger ROI generation. They will be used as representative examples in our discussion.

Tumour necrosis factor- α . TNF α is a cytokine which has important functions in coordinating immunological and inflammatory responses. TNF α is released primarily by activated macrophages and induces changes in cell shape, differentiation and proliferation of a variety of cell types, including T and B cells, neutrophils, macrophages, thymocytes, endothelial cells, keratinocytes, glial cells and fibroblasts.¹⁸³ The interaction between TNF α and ROI is two-way since TNF α induces neutrophils to produce ROI,¹⁸⁴⁻¹⁸⁶ which in turn acts on TNF α expression and release. Lastly, ROI seem to be involved in the transduction pathway of TNF α as well as in its transcription control through the nuclear factor NF kappa B.¹⁸⁷

Evidence suggests that ROI might provide a positive signal for the release of TNF α and, in various cells, TNF α -mediated cell lysis can be inhibited by thioredoxin¹⁸⁸ or *N*-acetylcysteine, a thiol antioxidant and GSH precursor.¹⁸⁹⁻¹⁹¹ TNF α induces oxidative stress in isolated mouse hepatocytes. Hepatocytes exposed to a TNF α concentration that does not induce cell toxicity exhibited intracellular GSH depletion and GSSG efflux during the first 2 h of exposure, followed by a decrease in cellular ATP content. The antioxidants mannitol and benzoate, as well as the iron chelator desferoxamine, reduce the extent of TNF α induced oxidative stress, suggesting involvement of the hydroxyl radical.¹⁹² In mesangial cells, TNF α , as well as aggregated IgG, increase mRNA levels for the monocyte chemoattractant protein, JE/MCP-1, and the colony-stimulating factor, CSF-1. Superoxide anion generated by the xanthine oxidase system, but not H₂O₂ increases mRNA levels

for both JE/MCP-1 and CSF-1. Addition of NADPH to activate membrane bound NADPH-oxidase dependent superoxide generating system increases mRNA levels and further enhances stimulation by TNF α . It appears that generation of ROI, possibly via a NADPH-oxidase pathway, is involved in the induction of JE/MCP-1 and CSF-1 genes by TNF α and aggregated IgG complexes.¹⁹³ The finding that pretreatment of sensitive targets with TNF α induces resistance to TNF α was attributed to the synthesis of the mitochondrial form of superoxide dismutase, MnSOD.¹⁹⁴ Moreover, cellular sensitivity and resistance to TNF α are correlated, respectively, with decreased and increased levels of superoxide dismutase. The effect of TNF α on the expression of antioxidant enzymes has been studied in TNF α sensitive myeloid cell lines and in normal peripheral blood lymphocytes and monocytes. Exposure to TNF α induces a striking increase in manganese superoxide dismutase (MnSOD) RNA levels whereas no changes are observed in the case of copper-zinc superoxide dismutase (Cu/ZnSOD) or glutathione peroxidase. Furthermore, TNF α resistant leukaemic cell lines have higher constitutive levels of MnSOD RNA and activity, and these levels do not change in the presence of TNF α .¹⁹⁵ The effects of IL-1, TNF α and LPS treatment on the expression of superoxide dismutases have been studied in both rat pulmonary artery and microvascular endothelial cells. Similar results were obtained insofar as these mediators produced an increase in MnSOD but not Cu/ZnSOD expression in both tested cell lines.¹⁹⁶ In contrast, another study showed that two cell lines in which mutants have been selected to be resistant to cytolysis by TNF α behave differently. The SK-MEL-109 variants had relatively low levels of MnSOD that were inducible by TNF α ; the HeLa variants had very low levels of MnSOD that were poorly inducible by TNF α . It was thus concluded that an elevated level of MnSOD was not required to protect these cells from TNF α mediated cytolysis.¹⁹⁷

Glutathione appears to be of critical importance in TNF α -mediated effects. In bovine pulmonary microvascular endothelial cells, stimulation with TNF α increases the permeability induced by H₂O₂, at a concentration which by itself has no effect on the permeability of endothelial cells. Pretreatment with TNF α for 6 h had no direct effect on transendothelial permeability. However, this TNF α pretreatment enhanced the susceptibility of endothelial cells to H₂O₂. Measurement of intracellular antioxidant content following exposure to TNF α revealed a decrease in reduced GSH and an increase in the oxidized form (GSSG), whereas no change in catalase content could be detected. The decrease in oxidant buffering capacity secondary to TNF α -induced reduction in intracellular GSH content mediates the increased susceptibility of endothelial cells to H₂O₂.¹⁹⁸ The

same type of study has been performed with phorbol myristate acetate (PMA)-activated neutrophils as ROI generating system. Challenge of neutrophils layered on TNF α -pretreated endothelial cells with PMA increases permeability. In contrast, challenge of CGD-neutrophils fails to induce any change in endothelial cell permeability.¹⁹⁹

It is still unclear whether ROI are implicated in TNF α -induced injury derived from the phagocyte respiratory burst or from the action of xanthine oxidase, and further studies are needed to address this issue. However, it is possible that intracellular ROI may result in the activation of genes responsible for apoptosis, conceivably through an oxidative stress-responsive nuclear transcription factor such as NF-kappa B.²⁰⁰

The regulatory effect of H₂O₂ on both the cytotoxic activity and the specific binding of TNF α was studied in TNF α -sensitized murine L929 cells. Half-hour pretreatment with H₂O₂ altered cell sensitivity to TNF α , whereas H₂O₂ pretreatment did not modify TNF α activity. The inhibitory effect of H₂O₂ was suppressed by catalase, but was unaffected by the scavengers of OH⁻ and HOCl, suggesting that H₂O₂, and not one of its metabolites, was responsible for this inhibition. The H₂O₂ effect was associated with an approximately 50% decrease in density of cell membrane TNF α receptors, without any change in their affinity. Moreover H₂O₂ did not affect the rate of degradation of TNF α receptors. It appears that H₂O₂ can down-regulate the cellular response to TNF α , possibly by reducing its binding capacity.²⁰¹

A growing body of evidence has emerged supporting the implication of proteinases in the modulation of cellular responses to cytokines,²⁰² and especially to TNF α . Specific proteolytic cleavage of the molecules themselves can lead to release of membrane anchored precursors, facilitate secretion and abolish or generate biological activities. As a result, proteolysis can affect specific cell surface receptors and free their extracellular domains to circulate as soluble inhibitors of cytokine activity.

Two types of TNF α receptors have been isolated in the human and their genes have been cloned and structure activity studies carried out.²⁰³⁻²⁰⁶ The type I receptor is a 55 kDa molecule which is expressed preferentially in cells of epithelial origin, whereas the 75 kDa type II receptor is more abundant in myeloid cells, human neutrophils and monocytes, expressing similar amounts of each receptor at their surface. The mechanisms of TNF α receptor shedding are still not completely understood. The release of either type I or type II receptor seems to involve different proteolytic pathways. A first TNF α releasing activity has been localized in azurophilic granules and identified by its inhibitory pattern as elastase. This enzyme preferentially acts on the p75 TNF α receptor of neutrophils and mononuclear cells, releasing a solu-

ble fragment of 32 kDa that retains its ability to bind TNF α . A second TNF α releasing activity insensitive to serine-proteinase inhibitor is operative in intact fMLP-stimulated neutrophils, shedding a 42 kDa fragment from the p75 TNF α receptor and a 28 kDa fragment from the p55 TNF α receptor.²⁰⁷ It appears that the mechanisms underlying TNF α receptor shedding involve proteinases other than serine proteinases contained in azurophilic granules, and further studies are needed to localize and identify these proteinases.

The pathophysiological importance of the interactions between oxidants, proteinases and pro-inflammatory cytokines has been extensively studied by our own group in patients with end-stage renal disease receiving haemodialysis therapy.²⁰⁸ It is now clear that each dialysis session triggers neutrophil activation, mainly through the generation of activated complement components following the contact of blood with bioincompatible dialysis membranes.²⁰⁹ This is evidenced by massive generation of ROI²¹⁰ and the presence of high levels of neutrophil elastase and α 1-PI.²¹¹ This peridialytic activation contrasts with an overall depression in the expected responses of these cells to pathogens, e.g. chemotactic, phagocytic and oxidative response capacities.²¹² Among the numerous side effects of ROI that could account for dialysis related complications, the possibility that they could contribute to β 2 microglobulin amyloidosis arthropathy, by inducing fragmentation, polymerization and thus favouring subsequent intra-articular deposit of β 2 microglobulin, has recently been raised by our study of the effect of defined radiolytically generated oxygen species on β 2 microglobulin structure.⁴¹

Acting in concert with neutrophil-derived ROI and proteinases, monocyte-derived circulating pro-inflammatory cytokines could also play a critical role in dialysis related chronic inflammatory process and contribute to the underlying immune system dysregulation associated with uraemia.²¹³

Recently, high levels of both TNF-sR55 and TNF-sR75 and an imbalance between TNF α and its soluble receptors, acting as naturally occurring inhibitors,²¹⁴ have been reported in dialysis patients.^{215,216} It thus seems that as with the oxidant/anti-oxidant balance the cytokine/anti-cytokine balance could play a critical role in complications observed in long-term dialysis patients.

Interleukin-8. IL-8 belongs to a novel class of small cytokines, now called chemokines, which are widely studied because of their ability to activate leukocytes and their potential role as mediators of inflammation. IL-8 has been described as a potent neutrophil chemoattractant and activator.²¹⁷ Studies using whole blood as a model of cytokine production have shown that the OH⁻ scavenging agent dimethyl

sulfoxide (DMSO) drastically inhibits IL-8 production in blood stimulated with lipopolysaccharide (LPS) or other agents such as TNF α , IL-1 β , phytohaemagglutinin A (PHA) and aggregated immune complexes.²¹⁸ However, using the same whole blood model, in both normal individuals and CGD patients, DMSO inhibitable production of IL-8 could be triggered in response to LPS, PHA or aggregated immune complexes. Direct exposure to H₂O₂ stimulates IL-8 production in a concentration-dependent manner in a hepatoma cell line, Hep-G2, a pulmonary type II epithelial cell line, A549, and human skin fibroblasts.²¹⁹ It seems that ROI are important regulators of IL-8 expression whether or not they are derived from NADPH oxidase activation.

Oxidative regulation of IL-8 activity could be mediated through its receptor. Studies in neutrophils have shown that two sulfhydryl groups participate in the binding of the ligand to the receptor and consequently regulate receptor-mediated cell functions.²²⁰

There is evidence that neutrophil-derived proteinases are involved in the control of IL-8 production. The fluid lining the respiratory epithelium in cystic fibrosis contains large numbers of neutrophils and, consequently, active neutrophil elastase. Neutrophil elastase appears to be the mediator responsible for inducing bronchial epithelial cells to express IL-8 gene and release neutrophil chemotactic activity. In addition, neutrophil elastase inhibitors prevent IL-8 gene expression.^{221,222} Aerosolization of recombinant SLPI in patients with cystic fibrosis suppresses the neutrophil elastase burden on the respiratory epithelial surface and at the same time greatly reduces IL-8 levels, thus decreasing the number of PMN in the epithelial lining fluid.¹⁸¹ The role of IL-8 in lung inflammatory reactions has been demonstrated by the protection afforded by treatment with an anti-IL-8 monoclonal antibody in a model of E-selectin-dependent lung injury.²²³

Studies of the modulation of IL-8 production have revealed the critical role of ROI or proteinases alone or in combination. These new findings could form the basis of new therapeutic practice in the management of inflammation.

Interactions of ROI and proteinases with adhesion molecules: The interaction of neutrophils with endothelial cells, one of the first events in the acute inflammatory response, induces profound changes in the biosynthesis of potent endothelial modulators. Although it has been known for more than a decade that endothelial cells can be damaged *in vitro* by neutrophil derived ROI,^{224,225} it is now apparent that sub-injurious concentrations of ROI could alter the physiologic status of endothelial cells and modify their response to inflammatory mediators.

Mechanisms of endothelial cell killing by neutrophil-derived ROI can be blocked by

superoxide dismutase, catalase, iron chelators or scavengers of the hydroxyl radical. There is evidence that products from xanthine oxidase of endothelial cells are necessary for the toxic effects of hydrogen peroxide or phorbol ester-activated neutrophils. Conversion of xanthine dehydrogenase to xanthine oxidase in endothelial cells occurs during contact of endothelial cells with neutrophils. This conversion is not related to neutrophil-derived ROI.²²⁶ The glutathione redox cycle has been shown to protect cultured endothelial cells against lysis by extracellularly generated H₂O₂²²⁷ and appears to be critical for the survival of tumour cells exposed to H₂O₂.²²⁸

Adhesion of circulating PMN to endothelial cells is a critical step in the inflammatory process. This complex cell/cell interaction requires increased expression of surface adhesive molecules either on neutrophils or on endothelial cells. The adherence of human neutrophils to human endothelial cells *in vitro* is increased by chemotactic stimulation of the neutrophils,^{229,230} or by stimulation of endothelial cells with endotoxin²³¹ or cytokines such as TNF α or IL-1 β .²³²⁻²³⁵ It has been recognized that within the post-capillary venules of inflamed tissue, emigrating leukocytes leave the central stream of the circulation, roll more slowly along the vessel wall, then stop by adhering tightly to the endothelial surface before squeezing between adjacent endothelial cells into the sub-endothelial tissue.²³⁶ More recently, it has been appreciated that the rolling phenomenon is mediated by the selectin class of adhesion molecules, while the leukocyte β 2 integrins (CD11/CD18) and their endothelial counterreceptors, (intercellular adhesion molecule or ICAM) are involved in the tight adhesion.²³⁷⁻²³⁹ CD11/CD18 receptors on neutrophils are known to modulate chemotaxis, neutrophil/neutrophil aggregation, neutrophil adhesion and transvascular migration.²⁴⁰ In addition, other adhesion molecules are involved in transendothelial migration of leukocytes such as the platelet/endothelial cell adhesion molecule 1 (PECAM-1).^{241,242} Recent evidence indicates that ROI and proteinases modulate adhesion molecule functions. In a model of cat mesenteric venule, H₂O₂ and chloramines but not HOCl, promote leukocyte adhesion to venular endothelium. H₂O₂-induced CD18-mediated leukocyte adherence appears to be elicited by PAF and by a direct effect of the oxidant on CD11/CD18 expression.

Treatment with low concentrations of H₂O₂ (50 to 100 μ M) selectively increases the surface expression and mRNA levels of ICAM-1 and major histocompatibility complex class I, but not endothelial E-selectin, vascular cell adhesion molecule-1 (VCAM-1), or gp96, a constitutively expressed endothelial cell surface protein. H₂O₂ does not activate the transcription nuclear factor kappa B,

an important mediator of TNF α -induced gene expression. It seems that subinjurious doses of H₂O₂ can activate endothelium, the molecular mechanisms underlying this phenomenon differing from those of inflammatory cytokines.²⁴³ After exposure to the hypoxanthine/xanthine oxidase ROI producing system, human umbilical vein endothelial cells display increased adherence to resting neutrophils, compared with untreated endothelial cells. ICAM-1, constitutively present on the surface of resting endothelial cells, is involved in neutrophil adherence to ROI treated cells, since this adherence is inhibited by anti-ICAM1, anti-CD11a, anti-CD11b and anti-CD18 monoclonal antibodies.²⁴⁴ Another study showed that endothelial cells exposed to H₂O₂ express the granule membrane protein-40 (GMP-40), which can be translocated from its intracellular storage pool to the surface of endothelial cells where it acts as a ligand for neutrophil adhesion.²⁴⁵ Similar results were obtained in an *in vitro* model of hypoxia/reoxygenation induced endothelial free-radical generation and adhesion of resting PMN. Oxidative stress induced an increase in the expression of GMP-40 as well as *de novo* synthesis of endothelial adhesion molecule 1 (ELAM-1) on the endothelial surface, these effects being blocked by free radical scavengers.²⁴⁶

One possible interaction between proteinases and membrane proteins such as adhesion molecules, is proteolytic cleavage, leading to soluble factors, as has been described for TNF α receptors. It is well documented that the circulating neutrophils respond to inflammatory products by expressing surface receptors appropriate to the inflammatory signal.²⁴⁷ Typically, this expression involves exposure or functional activation of opsonin receptors including receptors for Fc fragment of IgG (Fc γ R) and receptors for complement components. Neutrophils possess two classes of Fc γ R which have different functions, as well as different sensitivity to proteolytic cleavage by neutrophil elastase. Fc γ RII (CD32) is made up of an external opsonin-binding portion, a membrane domain and a cytoplasmic tail, whereas Fc γ RIII (CD16) is anchored to the phagocyte membrane through a phosphatidylinositol linkage. Fc γ RII, which is resistant to elastase, is primarily responsible for IgG mediated activation of human PMN since Fc γ RIII, which is sensitive to elastase, binds immune complexes without directly activating neutrophils.²⁴⁸

Complement receptors include the opsonin receptor CR1 (CD35) which binds C3b and C4b, the chemotactic receptor for C5a and CR3, a member of the integrin family (CD11b/CD18 or Mac-1) which recognizes C3bi and ICAM as its endothelial counterpart receptor. Expression of both CR1 and CR3 is increased upon neutrophil activation²⁴⁹ and in the presence of cytokines.²⁵⁰

Interestingly enough an upregulation of CR3 on both monocytes and neutrophils has been reported in dialysis patients during the session²⁵¹ providing a molecular mechanism for dialysis induced neutrophil sequestration in the pulmonary vascular bed.

The expression of both CR1 and CR3 has also been studied in circulating or bronchoalveolar lavage (BAL) neutrophils in the case of cystic fibrosis patients compared with controls. In circulating neutrophils, CR1 and CR3 expression was similar both in cystic fibrosis patients and controls. In BAL, CR3 expression was also similar to the controls, whereas CR1 expression was significantly decreased and neutrophil elastase was, at least in part, responsible for the proteolytic cleavage of CR1.²⁵²

Leukosialin is the major sialoprotein of human leukocytes.^{253,254} Due to the net negative charge of leukosialin, its function may be to prevent nonspecific cell/cell interactions through negative charge repulsions. Phorbol myristate acetate (PMA) or fMLP activation of neutrophils reduces the expression of leukosialin via a shedding mechanism, blocked by both metalloproteinase and serine proteinase inhibitors.²⁵⁵⁻²⁵⁷ Nonetheless, both exogenous and endogenous elastase have been reported to be responsible for this shedding. Moreover, in participating in the shedding of such a strongly anionic membrane protein which drastically modifies the neutrophil surface charge, neutrophil-derived proteinases participate in the regulation of the cell adhesion process.

In order to assess the *in vivo* role of elastase in neutrophil adhesion and emigration, intravital microscopy has been used to study PMN/endothelial cell interactions in single inflamed post-capillary venules in a model of platelet activating factor (PAF) superfusion onto the mesentery. *In vivo*, elastase significantly contributes to neutrophil infiltration, aggregation, adhesion and emigration, these effects being blocked by elastase inhibitors. Moreover, the direct action of elastase on neutrophils assessed *in vitro* indicates that neutrophil elastase directly upregulates the β subunit of the CD11/CD18 glycoprotein complex without any change in L-selectin levels. Moreover, the increase in CD18 expression appears to be independent of degranulation, superoxide anion production and cell lysis. By selectively enhancing and regulating CD18 dependent chemotaxis, a rate-limiting step for other neutrophil functions *in vivo*,²⁵⁸ elastase may modulate neutrophil infiltration and consequently the overall migration process.²⁵⁹

Conclusions

A combination of reductionist and integrationist approaches is rapidly expanding and revising our understanding of the role of phagocyte-derived ROI and proteinases. For nearly two decades, investiga-

of the toxic potential of ROI prompted numerous studies of different cell types aimed at describing and characterizing the mechanisms of cell lysis, the target cell involved and the defences used to counteract ROI. Because of the new concept that ROI could have a role as signalling molecules, investigation has focused on the effect of sub-lethal and non-injurious concentrations of ROI on known biological functions of given cell types. Studies of the involvement of ROI and proteinases in different regulatory processes suggest an overall system of cooperation. A growing body of evidence supports this notion of cooperation in the field of cytokines, as illustrated by TNF α and IL-8, and in the field of adhesion molecules, both fields having several points in common with ROI and proteinases.

The neutrophil itself appears to be well equipped with this unusual combination of contrasting molecules: ROI, small, ubiquitous and short-lived molecules; and proteinases, macromolecules with specific long-lasting activity. The neutrophil safely keeps them apart through separate intracellular compartmentalization under basal conditions. Nevertheless, in the defence of the host against pathogens, their cooperation maximizes neutrophil microbicidal potential as well as damage to host tissue. However, the cooperation appears to be involved at another level, where they act more as immunomodulatory mediators than toxic weapons, in the whole inflammatory process. The overall picture is of a classical alliance, for better and for worse.

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