

The activated neutrophil — formidable forces unleashed

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The human neutrophil is the small, aggressive, front-line, circulating phagocyte. The enormous intrinsic destructive potential of neutrophil is captured in the following quotation, which dramatically emphasises the critical requirement for latency and self-limiting activation of these cells:

'Unlike cytotoxic lymphocytes and the complement system, which destroy their targets with a drop of poison, the professional phagocytes kill like Attila the Hun, deploying a battery of weapons that lay waste to both the target and the nearby landscape with the subtlety of an artillery barrage.'¹

In this review recent advances in neutrophil physiology and function, as well as mechanisms of inflammation-related tissue injury and carcinogenesis, are highlighted.

Neutrophil production

About 55 - 60% of bone marrow is dedicated to the production of one cell type — the neutrophil, the most abundant professional phagocyte.² During maturation in the marrow the neutrophils undergo progressive differentiation and loss of biosynthetic activity. After the myelocyte stage these cells become end-cells and enter a large storage pool from which they are released into the circulation after about 5 days, where they have a half-life of about 6 hours.² Although they are end-cells, mature neutrophils retain some residual biosynthetic capacity which is activated on exposure to leuco-attractants or the cytokines GM-CSF and TNF,³ enabling limited adaptation to a changing micro-environment. The steady state production of mature neutrophils is about 1×10^9 /kg body weight per day, but this is dramatically increased by co-operative interactions between leucocytosis-promoting cytokines (TNF- α , IL-1, IL-3, IL-6, IL-8, G-CSF and GM-CSF) and leuco-attractants (C5a, PAF and LTB₂).⁴

Neutrophil cytoplasmic granules

The most notable structural features of the neutrophils are the abundant, heterogeneous cytoplasmic granules and the highly dynamic plasma membrane; these make this cell ideally suited to the performance of its primary function, viz. adherence to locally activated vascular endothelium, extravasation, rapid migration to sites of infection and engulfment and intracellular destruction of invasive microbial pathogens.

With regard to function, the plasma membrane is equipped with adhesion molecules, receptors for leuco-attractants, opsonins and cytokines, as well as the unique

cytochrome, cytochrome b_{245} . There are two major types of cytoplasmic granule, which differ in respect of size, number, constituents and functions. These are the primary and secondary granules. The major constituents of the primary granules are MPO, an abundant component comprising about 5% of the total cellular protein, the antimicrobial enzyme, lysozyme, and at least 3 neutral serine proteinases, elastase, cathepsin G and the recently described proteinase 3.⁵ These granules also contain several cationic antimicrobial polypeptides which have been reviewed in detail elsewhere.⁶ Briefly, the defensins, also known as human neutrophil peptides 1, 2, 3 and 4 (HNP 1 - 4), are a group of 4 small, strikingly similar peptides which are active against Gram-negative and Gram-positive bacteria, yeasts, Herpesviridae and tumour cells, and account for 5 - 8% of total cellular protein. Cationic antimicrobial protein of MW 37 kD (CAP37) and cationic antimicrobial protein of MW 57 kD (CAP57), also known as bactericidal protein and bactericidal/permeability increasing protein, are active against Gram-negative bacteria.⁶ Cathepsin G is also microbicidal for Gram-negative bacteria, a property unrelated to its proteolytic activity.⁶

Secondary granules outnumber primary granules by about 2:1 and are also smaller (200 nm v. 500 nm in diameter). They are rapidly mobilised during cell migration and in addition to intragranule constituents their membranes act as a reservoir for adhesion molecules, leuco-attractant receptors and cytochrome b_{245} , thereby sustaining the adhesive, migratory, phagocytic and antimicrobial activities of the cell.^{7,8} About 90% of the total cellular lysozyme, vitamin B_{12} -binding protein, the bacteriostatic iron-binding protein lactoferrin, as well as the latent metallo-enzymes, collagenase and gelatinase, are located in the secondary granules.⁹ Collagenase degrades types I, II and III collagen at a single specific locus, while gelatinase attacks types IV, V and possibly VI collagen.⁹

Adhesion and migration

Localised adhesion of circulating neutrophils to vascular endothelium is an early and critical step in the acute inflammatory response. However, only recently have the requisite molecular events involved in neutrophil adhesion been unravelled. These are the subject of several reviews.^{10,11} Several families of adhesion molecules are sequentially involved. The initial interaction is mediated by the binding of constitutively functional neutrophil adhesion molecules to inducible counter-receptors on endothelial cells. The best characterised example involves the selectins, a family of 3 distinct glycoprotein adhesion molecules (L, E and P selectins), each of which consists of a short cytoplasmic tail, a transmembrane domain and an extracellular lectin (carbohydrate-binding) region which is critically involved in adhesion. L-selectin is constitutively expressed on circulating neutrophils while P and E selectins are inducibly expressed on endothelial cells following exposure to histamine/thrombin and cytokines (IL-1 and TNF) respectively.^{10,11} The selectins bind to the Sialyl-Lewis^x-bearing oligosaccharide regions of poorly characterised counter-receptors on neutrophils (P- and E-selectins) or endothelial cells (L-selectin).^{10,11}

Upregulation of P-selectin occurs within seconds, does not require *de novo* protein synthesis and is accomplished by translocation of this AM from endothelial cells' secretory

granules to the plasma membrane following exposure of these cells to thrombin or histamine. It is then rapidly reinternalised resulting in transient surface expression that parallels neutrophil adhesion to the activated endothelial cell. Upregulation of E-selectin on endothelial cells requires *de novo* protein synthesis and is expressed on the cell surface over a period of 2 - 6 hours after cytokine stimulation. L-selectin-mediated adhesion is characterised by rapid binding of low affinity; this leads to transient and reversible loose binding ('tethering') to, and rolling of neutrophils along segments of activated endothelium.^{10,11} Rolling greatly retards the transit of neutrophils through inflamed venules, allowing these cells to sample the local micro-environment for activating or leuco-attractant signals and is followed by release of neutrophils back into the bloodstream or by firm adhesion (termed 'sticking') and subsequent extravasation.

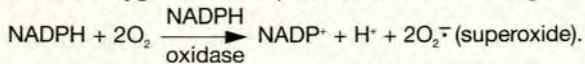
Sticking has been shown to be mediated by different families of adhesion molecules, the heterodimeric β_2 -integrins on neutrophils and their counter-receptors intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, both of which are expressed constitutively and inducibly (by leuco-attractant-activated neutrophils or cytokine-activated endothelial cells). Although the β_2 -integrins (predominantly CR3) are constitutively expressed on the surface of neutrophils, they are normally in a functionally inactive state. Conversion of inactive to active forms occurs rapidly in response to leuco-attractants, possibly as a result of a critical conformational change mediated by phosphorylation of the β -subunit. The function of the neutrophil integrins therefore requires the interaction of the cell with specific activating signals to ensure that integrin-mediated firm adhesion to endothelial cells occurs predominantly at sites of tissue insult. This firm adhesion is followed by transvasation of neutrophils in response to leuco-attractants.

Antimicrobial systems

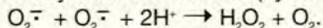
While granule-located, preformed cationic polypeptides and enzymes contribute to the antimicrobial potential of the neutrophil, the major contributors are undoubtedly the reactive oxidants generated after activation of the oxygen-dependent antimicrobial systems. The oxidant-generating enzyme of the neutrophil is a membrane-associated, electron-transporting NADPH-oxidase, consisting of three cytosolic proteins and a unique, low-potential cytochrome, cytochrome b_{245} .¹² The cytosolic proteins which translocate to the plasma membrane during activation of the oxidase probably initiate electron transport through a direct physical interaction with cytochrome b_{245} , producing a critical conformational change in this molecule.¹² The membrane requirement appears to be entirely satisfied by cytochrome b_{245} , since the β -subunit of this molecule has recently been reported to function as a NADPH-binding, FAD-containing flavocytochrome b .¹³

Receptor-mediated (leuco-attractants, cytokines, opsonised particles) activation of the dormant enzyme is achieved by several different transmembrane signalling systems involving phospholipase A_2 ,¹⁴ phospholipase C ¹⁵ and phospholipase D ¹⁶ acting individually or in concert, depending on the nature of the stimulus or membrane-associated oxidative metabolism. The probable point of convergence of the various second messengers generated

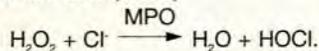
by these diverse signalling systems is protein kinase C¹⁷ which in turn promotes phosphorylation-dependent interaction of the cytosolic components of the oxidase with cytochrome b₂₄₅. The primary consequence of assembly of the NADPH-oxidase complex is the univalent reduction of molecular oxygen to the superoxide anion radical (O₂⁻):



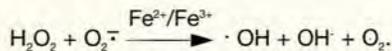
Superoxide (O₂⁻), a weak and unstable antimicrobial oxidant, is the precursor of a series of potent microbicidal oxidants. Hydrogen peroxide (H₂O₂) is formed by spontaneous or enzymatic dismutation (by SOD) of O₂⁻:



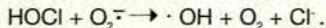
The antimicrobial potential of H₂O₂ is dramatically potentiated by the granule enzyme MPO, which utilises this oxidant to oxidise chloride to the extremely potent oxidising agent hypochlorous acid (HOCl):



It has also been proposed that neutrophils transform H₂O₂ by the iron-catalysed Haber-Weiss reaction to hydroxyl radical (·OH), the most potent oxidant known in biological systems:

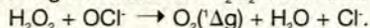


However, production of ·OH by neutrophils via this pathway has only been demonstrated *in vitro* in the presence of added iron, and appears to be of little or no biological relevance given that under physiological conditions, iron in and around neutrophils is tightly complexed to binding proteins.¹⁸ Recently, however, a novel transition metal-independent pathway of ·OH has been demonstrated in neutrophils. This pathway is MPO-dependent and involves the interaction of HOCl and O₂⁻:



The significance of ·OH formation through this MPO-dependent pathway in microbicidal activity and neutrophil-mediated tissue injury has not yet been established.

The ability of neutrophils to generate significant amounts of singlet oxygen (¹Δg), a highly reactive, diffusing and long-lived electronically excited state of molecular oxygen, has also been uncertain. However, it has recently been reported that phagocytosing neutrophils transform up to 20% of oxygen consumed to singlet oxygen by a MPO-dependent pathway involving interaction of H₂O₂ and HOCl:¹⁹



These various neutrophil-derived reactive oxidants (O₂⁻, H₂O₂, HOCl, ·OH and O₂(¹Δg)) are devastatingly effective antimicrobial agents. They are, however, indiscriminate, and if released extracellularly pose the potential threat of oxygen toxicity to bystander host cells and tissues in the vicinity of inflammatory reactions.

Oxidant-mediated tissue damage

Phagocyte activation and generation of reactive oxidants are clearly crucial events in host defences, and oxidant-inflicted injury to bystander tissue cells is usually minimal because of the transient, self-limiting nature of the inflammatory response and the protection provided by innate biological anti-oxidant defence systems which neutralise wayward oxidants. If, however, the inflammatory response is ineffectively down-regulated and hyper-acute or chronic activation of neutrophils results, then anti-oxidant defences

may be overwhelmed, which would predispose host tissues to oxidative damage. These neutrophil-derived oxidants are capable of a range of harmful activities and have been reported to be cytotoxic for a wide variety of eukaryotic cells. These are also immunosuppressive, carcinogenic, pro-lytic and pro-adhesive. The primary mediators of cytotoxic activity are H₂O₂ and HOCl which penetrate bystander cells and oxidatively inhibit glycolysis and the Krebs cycle by mechanisms which have been described in detail elsewhere.²⁰ The consequence is ATP depletion with consequent cellular dysfunction and cytotoxicity. These permeant oxidants (H₂O₂ and HOCl) also inhibit the proliferative activity and functions of bystander B lymphocytes, T lymphocytes and natural killer cells by interfering with ATP generation.²¹

Neutrophils as carcinogens

The association between chronic inflammation and development of epithelial cancers is well recognised.²² Activated phagocytes have been identified as potential carcinogens because they oxidatively damage DNA and promote malignant transformation in bystander cells in tissue culture.²³ The oxidant responsible for neutrophil-induced DNA damage in neighbouring cells is predominantly permeant H₂O₂ which interacts with intracellular transition metals²³ to generate ·OH in close proximity to DNA; this leads to oxidative damage to adenine, guanine, thymine and cytosine, and also causes DNA strand breaks.²⁴ Although DNA damage in living cells is subject to cellular repair, it may occasionally escape repair, or repair may be incorrect. In such cases unrepaired or mis-repaired DNA could have deleterious consequences, e.g. gene modifications that could ultimately promote cellular transformation. Moreover, it has recently been reported that permeant HOCl oxidatively inactivates the DNA repair enzyme poly (ADP-ribose) polymerase in bystander cells exposed to activated neutrophils;²⁵ this indicates that neutrophil-derived oxidants not only damage DNA directly, but also compromise DNA repair mechanisms.

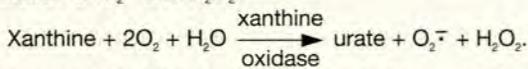
Pro-lytic activities of neutrophils

Neutrophil granules contain a large family of over 20 enzymes, but 4 proteolytic enzymes, the neutral serine proteases, elastase and proteinase 3, and the 2 metalloproteinases, collagenase and gelatinase, seem to have the greatest potential to act as mediators of tissue injury.⁹ These proteases are released extracellularly during neutrophil activation and each cleaves key components of the extracellular matrix, which is composed of a complex mix of collagens, elastin, proteoglycans and glycoproteins; these lie under epithelia and surround connective tissue cells.⁹ Extracellular release of proteases and generation of reactive oxidants by activated neutrophils are parallel and inter-related events. Reactive oxidants, especially HOCl, dramatically potentiate the proteolytic activity of these neutrophil proteases by direct and indirect mechanisms. In the case of elastase and proteinase 3, HOCl promotes the oxidative inactivation of API (alpha-1-antitrypsin), the major plasma and tissue inhibitor of these enzymes.^{5,9} This oxidant also inactivates the elastase inhibitory activity of secretory leukoprotease inhibitor.²⁶ This protease inhibitor, which is found only in mucous secretions, is particularly effective in

neutralising elastase secreted by adherent neutrophils, but does not inhibit proteinase 3.^{26,27} Collagenase and gelatinase are secreted in a latent form by neutrophils and undergo oxidative activation on exposure to HOCl.^{9,27} Moreover, when oxidatively activated, collagenase and gelatinase potentiate the activity of elastase and protease 3 by cleaving API within its active site loop, causing irreversible inactivation of this protease inhibitor.²⁷ The extracellular matrix, essential for the maintenance of tissue integrity, function and repair, is extremely vulnerable to the combined assault of neutrophil-derived oxidants and proteases.

Pro-adhesive activity of oxidants and post-ischaemic vascular injury

Exposure of vascular endothelium to O_2^- or H_2O_2 induces prolonged expression of P- and E-selectins on endothelial cells, with resultant adherence of neutrophils to these cells.^{28,29} Oxidant-mediated activation of latent vascular endothelium and resultant adherence of neutrophils to endothelial cells is probably closely involved in the pathogenesis of post-ischaemic vascular injury. In this setting however, the pro-adhesive oxidants originate primarily from the endothelial cells. Ischaemia and hypoxia promote the proteolytic conversion of xanthine dehydrogenase to the superoxide-generating enzyme, xanthine oxidase, in endothelial cells. Subsequent activation of this enzyme during reperfusion/reoxygenation leads to generation of O_2^- and H_2O_2 :



These oxidants (O_2^- and H_2O_2) not only cause direct vascular injury, but also up-regulate expression of P- and E-selectins on endothelial cells; this leads to adherence and activation of neutrophils which potentiate vascular damage by pro-oxidative mechanisms.

Circulating neutrophils, cardiovascular disease and cancer

The relationship between the circulating leucocyte count and immediate prognosis in acute illness is beyond dispute. However, there is also abundant evidence from numerous epidemiological studies that the circulating leucocyte count, and the neutrophil count in particular, measured well before the development of manifest clinical disease is a consistent and *independent* predictor of several cardiovascular conditions, including myocardial infarction, sudden cardiac death, all coronary heart disease combined, stroke and essential hypertension, as well as lung cancer incidence and mortality, possibly cancer at all sites and death from all causes.³⁰⁻³² The precise molecular/biochemical mechanisms by which chronic, albeit relatively modest, increases in the circulating leucocyte count predispose to future development of cardiovascular disease and cancer remain to be established, but a sustained, increased level of neutrophil-mediated oxidative stress is a likely contributor.

Biological anti-oxidant defence mechanisms

Innate, biological anti-oxidant defence systems protect host tissues against wayward, neutrophil-derived oxidants.

Detoxification of reactive oxidants is mediated by enzymes, by low- and high-molecular-weight scavengers, or by inhibition of NADPH-oxidase. Intracellular protection of cytoplasmic and nuclear components is achieved predominantly by inducible anti-oxidant enzymes; SODs neutralise O_2^- , while catalase and glutathione peroxidases detoxify H_2O_2 . Extracellular neutralisation of neutrophil-derived oxidants is accomplished largely by non-enzymatic, scavenging mechanisms. Ascorbate is the major anti-oxidant in blood and interstitial fluids, assisted by plasma proteins, carotenes and alpha-tocopherol (vitamin E).³³ Ascorbate scavenges (O_2^- , $\cdot OH$, O_2 (Δg) and HOCl). Beta-carotene is also an efficient scavenger of reactive oxidants, especially in the hydrophobic milieu at low oxygen tensions.³⁴ Importantly, neither of these agents (ascorbate and beta-carotene) interferes with NADPH-oxidase, nor do they neutralise H_2O_2 .²⁰ Alpha-tocopherol is the classic lipid-soluble oxidant scavenger, but is an ineffective scavenger of phagocyte-derived O_2^- and H_2O_2 .³⁵ However, alpha-tocopherol has recently been described as a potent *inhibitor* of the activity of NADPH-oxidase; it interferes with transductional mechanisms involved in the activation of this enzyme.³⁵ This property of the vitamin, which is unrelated to its classic oxidant-scavenging activity, suggests that vitamin E may be of particular importance in regulating the toxic potential of hyperactive phagocytes because it blocks the production of the complete spectrum of phagocyte-derived oxidants, including H_2O_2 , at source. In addition, several acute-phase reactants such as alpha-1-acid glycoprotein, haptoglobin and API, as well as peptides and polypeptides generated during proteolytic cleavage of fibrinogen and C-reactive protein,^{36,37} have been reported to inhibit the activity of NADPH-oxidase. A number of clinically available pharmacological agents have also been reported to inhibit the generation and/or reactivity of phagocyte-derived oxidants. These have been reviewed elsewhere.²⁰

Conclusion

Phagocyte-derived reactive oxidants are closely involved in the pathogenesis of many diseases including pulmonary emphysema, chronic bronchitis, atherosclerosis, rheumatoid arthritis, adult respiratory distress syndrome, vasculitis, glomerulonephritis, malignancy, myocardial infarction and cerebral infarction. The molecular/biochemical mechanisms by which reactive oxidants promote tissue injury have been well characterised and may be amenable to relatively simple preventive strategies such as administration of the dietary anti-oxidants, vitamins C and E and beta-carotene. Alternatively, pharmacological agents designed to potentiate the activity of anti-oxidant enzymes, or which function as oxidant-scavengers or as inhibitors of NADPH-oxidase, may lead to new anti-oxidative chemoprophylactic and therapeutic strategies.

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Accepted 15 Apr 1993.

Abbreviations. GM-CSF — granulocyte-macrophage colony-stimulating factor; TNF — tumour necrosis factor; G-CSF — granulocyte colony-stimulating factor; IL — interleukin; PAF — platelet-activating factor; LTB₄ — leukotriene B₄; MPO — myeloperoxidase; CR — complement receptor; API — alpha-1-protease inhibitor; NADPH — nicotinamide-adenine-dinucleotide phosphate (reduced form); FAD — flavine adenine dinucleotide; SOD — superoxide dismutase; ATP — adenosine triphosphate.