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The neutrophil: structure and defence function

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1. INTRODUCTION

Neutrophilic granulocytes, also called polymorphonuclear leukocytes, are mobile arsenals capable of seeking and destroying a variety of biological targets. They are one of the most important components of the body defence system and of the acute inflammatory reactions in the lung and other tissues. An outline of the various biological events by which neutrophils exert their defence functions is reported in Figure 1.
When pathogenic agents are formed and/or deposited in a tissue, circulating neutrophils may be recruited from the intravascular compartment into the tissue by the local generation of a variety of soluble mediators. These mediators are derived from numerous sources, including the invading parasite, cell debris, activated plasma components and other cell types such as endothelial cells and macrophages. The acute inflammatory response may be rapidly amplified by the neutrophils themselves and by the production of mediators that diffuse in the bloodstream (interleukin-1, colony-stimulating factors, tumor necrosis factor, etc.) leading to a systemic body response with synthesis of acute phase proteins by the liver, neuro-endocrine modifications, demargination of neutrophils from normal microvasculature, accelerated myeloid proliferation in the marrow, etc. These events lead to marked leukocytosis and priming of circulating neutrophils to potentiate their activity in the subsequent cellular functions.
Circulating neutrophils and endothelial cells in the area of the phlogistic reaction express membrane anchoring molecules and receptors that facilitate the adhesion and the initial migration of the leukocyte through the vessel wall. Directed cell migration of neutrophils, that are able to orientate the movement in a gradient of chemotactic factors, causes the accumulation of highly responsive phagocytes in the centre of the inflammation, where they react with the etiopathogenic agent. These activated cells have increased number of receptors such as for example those for formylated bacterial peptides, for the Fc portion of immunoglobulins and for complement factors, that enable them to actively engulf the foreign particles. In this phase, opsonization and initial damage to the invading microbes by components of the extravasated plasma effectively cooperate with the neutrophil defence function. The foreign particle is internalized within cytoplasmic vacuoles which fuse with lysosomal granules, forming phagolysosomes. Hydrolytic enzymes and other bactericidal components are concomitantly released into the phagolysosome during the process known as "degranulation". A substantial amount of these factors may excape from the forming endocytic vesicle ("regurgitation during feeding") or may be directly released in the external milieu due to the membrane perturbation caused by soluble mediators ("exocytosis"), thus causing damage to the connective tissue and nearby cells.

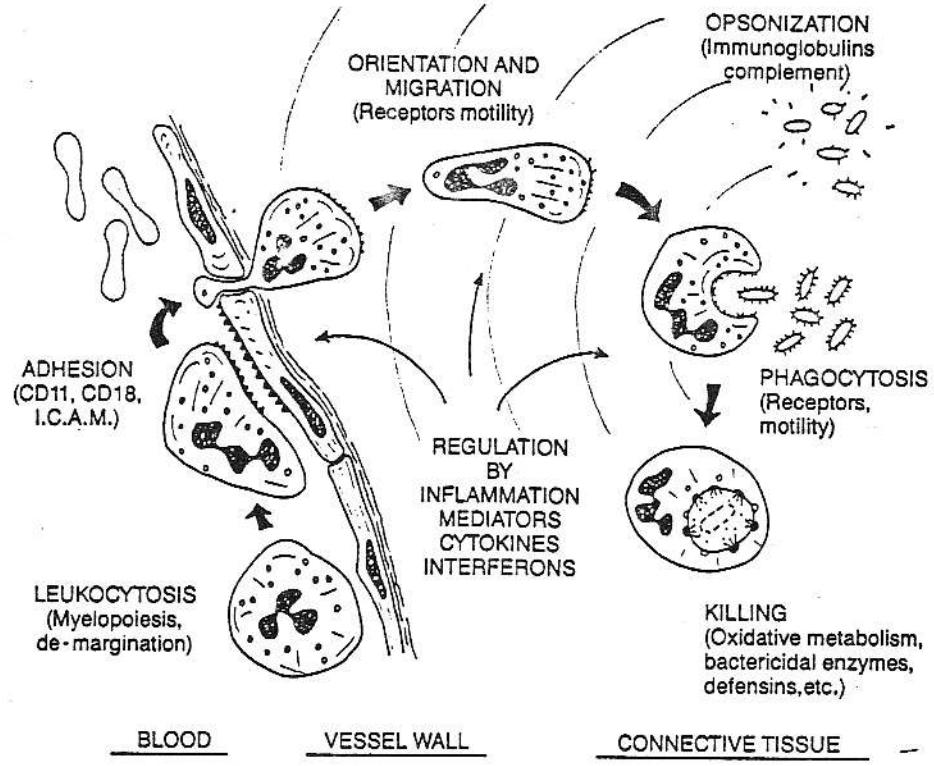

Fig. 1. *The main functions of neutrophils in host defence.*

At the same time, a dramatic increase of oxygen consumption occurs, with activation of the hexose monophosphate shunt and generation of oxygen-derived free radicals and other toxic derivatives (see section 2.3) that are released into the phagolysosome or eventually outside the cell. The intravacuolar pH changes first by rapid alkalinization, due both to protonation of superoxide and consumption of hydrogen ions in the dismutation reaction, followed by slow and progressive acidification, due to activation of an Na/H^+ antiporter.

All the above mentioned events are necessary and essential for the microbicidal activity and for the digestion of the engulfed particles, but the same mechanisms are involved in other effects, i.e. tumoricidal activity, cytotoxicity, tissue matrix injury, amplification of the inflammatory reaction, priming of the tissue healing processes.

The purpose of this report is to briefly describe some of the structural properties of neutrophils (Figure 2) and specifically to relate these properties to their physiological function. In particular, the main neutrophil structures that will be considered are the plasma membrane, the cytoskeleton and the various lysosomal granules. Other cell components such as nuclei, endoplasmic reticulum and mitochondria are of minor importance in mature neutrophils, which do not divide, have very low protein synthesis and derive most of their energy supply from glycolysis. Due to the large body of knowledge

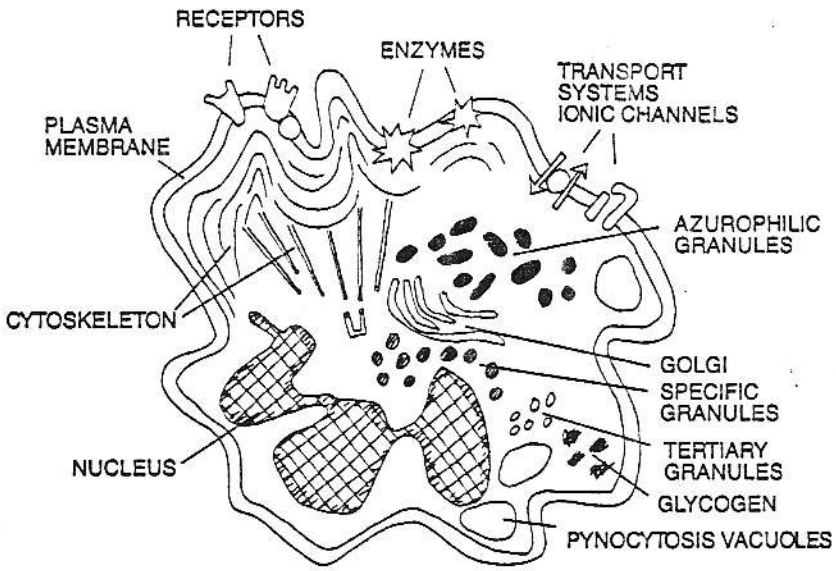

Fig. 2. *Schematic representation of the ultrastructure of a neutrophil granulocyte.*

that has been reviewed, many informations will be tabulated. A detailed discussion of each of these is not possible in the space available, but other references can be consulted for further reading [1-17].

2. The plasmamembrane

The external membrane of neutrophils is a highly sophysticated structure by which the cell communicates with the external milieu and by which many of its effector functions are realized. Both the membrane lipid bilayer and the proteins that are there embedded have important and specific functions for this cell type.

2.1 The recognition apparatus

The recognition apparatus of neutrophils is able of specific binding with a large series of ligands, some of which are listed in table I. Considering the various molecules that interact with neutrophil membrane, it can be realized that the receptors are involved in the regulation of cell growth, in targeting the cells to endothelium of inflammed areas, in chemotactic orientation and movement, in

Table I. *Ligands binding to defined neutrophil receptors*

Regulatory proteins:
Interleukin–1
Interferon–α
Interferon–γ
Tumor Necrosis Factor
GM–CSF and G–CSF
Interleukin–8 (NAP)
Immunoglobulins and complement:
Monomeric Ig (to Fc receptor–I)
Immunocomplexes and aggregates (to Fc rec. II and III)
CR 1 (C3b)
CR 3 (C3bi) = Mac 1 (CD 11b)
C5a
Complement decay accelerating factor
Glycoproteins and carbohydrates:
Mannose/fucose glycoproteins
Mannose 6–phosphate
Glucan
Concanavalin A
Lipopolysaccharide
Muramyl dipeptide
Lipids:
Leukotriene C_4, D_4, B_4
Platelet Activating Factor
Phorbol–myristate acetate (PK C)
Peptides:
f–Met–Leu–Phe
Substance P
Additional significant ligands:
Transferrin
Fibronectin
Laminin
Plasminogen
I.C.A.M.–1 and –2 (to CD11)
ATP
Adenosine
Beta adrenergic agonists
Opioids

particle uptake and ingestion and also in the modulation of the cell activity. The LFA-1 molecule is broadly expressed on the surface of leukocytes; it consists of two glycoprotein transmembrane units: α (CD 11a) and β (CS 18) of 170 and 95 KDa respectively. During the cell-cell contact LFA-1 binds to molecules called intercellular adhesion molecules (I.C.A.M.-1 and -2). Defective expression of CD 18 in some individuals causes severe disfunction of leukocyte adhesivity and of resistance to infections.

2.2 Transduction systems

The membrane is also the site of most of the components of the transduction systems. These are the biochemical mechanisms which perform the transmission of biological information between the membrane receptors and the various effector machineries involved in the functions such as movement, degranulation, metabolic activation, etc. The transduction systems are mainly, but not exclusively, located in the plasma membrane and are composed by a series of proteins with enzymatic activity (adenylate cyclase, kinases, proteases, phospholipases and other enzymes of lipid metabolism) or regulatory function (channel proteins, G-protein subunits, anchoring proteins) and by several other factors (messengers: calcium, inositol phosphates, diacylglycerol, phosphatidate, cAMP, etc.) generated by these enzymatic mechanisms. It is not our purpose to analyse in detail the complexity of these systems and their interrelationships, but some aspects of these merit particular attention.

First of all, many receptors (for chemotactic peptides, leukotrienes, PAF) are coupled to cell responses through a guanine nucleotide binding protein (G-protein) similar but not identical to the one of the adenylate cyclase system. Studies using pertussis toxin have proven instrumental in the understanding of G-protein involvement in the stimulus-response coupling pathways. Pertussis toxin ADP-ribosylates the α-subunit of some G-proteins and by this mechanism inhibits a number of responses of neutrophils. This biochemical effect could be a factor of the elevated pathogenicity of *Bordetella pertussis* infection.

Second, a series of marked variations in the physicochemical state of the lipids takes place in the membrane of stimulated neutrophils. Among these, a membrane-associated phosphoinositide-specific phospholipase is activated upon exposure to chemotactic stimulants. In particular, phospholipase C hydrolyzes phosphatidylinositol-4,5 biphosphate (PiP_2) to the second messenger products inositol 1,4,5 triphosphate (IP_3) and 1,2 diacylglycerol. IP_3 is thought to be the main intracellular messenger for release of Ca^{2+} from intracellular stores, while diacylglycerol is responsible for the activation of protein kinase C. It should be pointed out that the stimulus-induced rise in intracellular Ca^{2+} also results from an increase in plasma membrane permeability to extracellular Ca^{2+}. The elevation of intracellular Ca^{2+} is important for various subsequent events such as: a) triggering of phospholipase A_2, another membrane enzyme, that plays a pivotal role in the activation of arachidonic acid cascade with generation of other messengers such as arachidonic acid, thromboxanes, hydroxy acids and leukotrienes, b) activation of calmodulin-dependent protein kinases and also of protein kinase C, c) modulation of cytoskeleton function by activating gelsolin (see below). On the other hand, evidence from various laboratories suggest that phagocytosis and metabolic activation may also occur in experimental conditions where the intracellular Ca^{2+} is completely buffered, suggesting that calcium-independent activation pathways

may exist [18, 19].
Other stimulus-response coupling events that are located in the plasmamembrane are: a) opening of ionic channels for Na^+, with subsequent membrane depolarization; b) cyclic nucleotide metabolism variations: exposure of neutrophils to phagocytosable particles results in a rapid doubling of cAMP levels. The precise role of the generation of this second messenger in neutrophils is unknown. Besides its possible effect in glycogen metabolism, like in other cells, it may represent a negative regulatory factor. In fact, agents increasing intracellular cAMP such as PGE, and dibutiryl-cAMP cause inhibition of cellular responses, possibly due to inhibition of phospholipase C by cAMP-dependent protein kinase A; c) protein phosphorylation, due to the activity of kinases which are strictly associated with receptors, such as those for growth factors, or that are translocated from the cytosol following cell stimulation, such as protein kinase C. The intracellular targets of stimulus associated phosphorylation processes in neutrophils are at least 20 protein species and they include myosin light chain, factors necessary for NADPH oxidase activity or activation, including a membrane protein of 32 KDa that we have described in pig neutrophils [20], cytochrome b_{550}. However, most of neutrophil phosphoproteins have not been identified yet.

2.3 NADPH oxidase

Another peculiar enzymatic system that is located in the plasmamembrane of neutrophils is the NADPH oxidase. This is the enzyme that generates the oxygen-derived free radicals and is present in the membrane of all the phagocytes. The structure and the activation mechanism of NADPH oxidase have been the object of many investigations in several laboratories, including ours, in the past ten years [21-34]. These problems have not been fully clarified yet, but there is now evidence that this enzyme consists of an electron transport chain where a flavoprotein, a cytochrome and probably other proteins are assembled in a functional complex. The assembly of the various components could be promoted by phosphorylation of particular proteins, by changes of the lipid milieu of the membrane, by G-proteins or by other unidentified transduction pathways [35].
Figure 3 shows a tentative model of the active NADPH oxidase, which accounts for the present state of knowledge. The first protein to be considered is the protein bearing the NADPH binding site. This protein has been identified by tracing with radioactive nonhydrolysable NADPH analogues and has the molecular weight of 66 KDa. According to most authors, this protein is a FAD-containing flavoprotein, with the fundamental role of accepting two electrons from NADPH on the internal site of the membrane and of carrying out one-electron reduction of cytochrome b_{558}. However, there is not definite evidence that NADPH binding site and FAD belong to the same polypeptide.
A fundamental factor of the electron transport system is cytochrome b_{558}, a

peculiar b-type cytochrome with a number of unusual properties, including a very low oxido-reduction mid point potential (-245 mV). Its molecular and functional features have been defined in a better detail compared to the flavoprotein. Purification studies showed that human cytochrome b_{550} is a heterodimer with two subunits of 22 KDa (n. 3 in Fig. 3) and of 68-92 KDa (n. 4 in Fig. 3). The former is probably the heme-bearing subunits, while the latter is a glycopro-

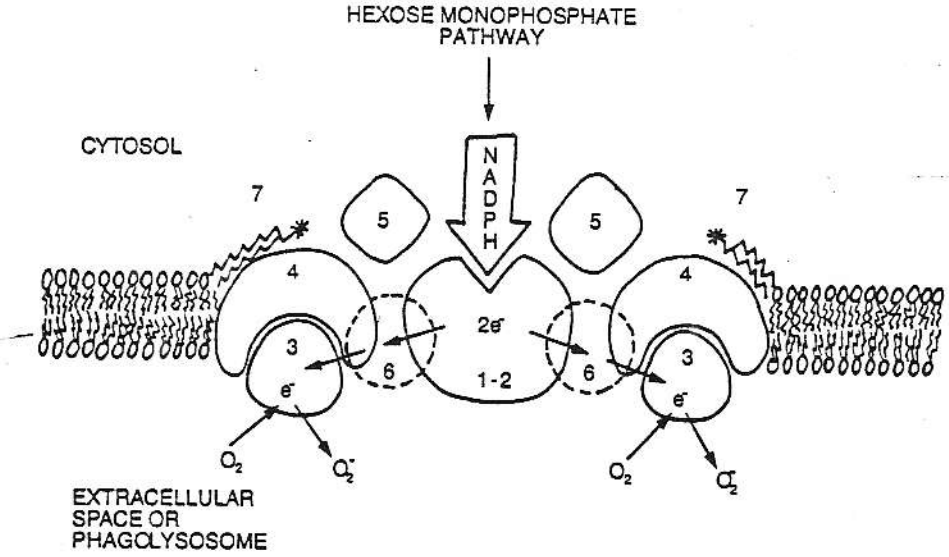

Fig. 3. *A working model of the enzymatic system NADPH oxidase. 1–2: NADPH binding protein and flavoprotein (possibly the same polypeptide); 3: Cytochrome b_{550} (low-molecular weight subunit); 4: Cytochrome b_{550} (high-molecular weight subunit); 5: Cytosolic factors that are translocated to the membrane during activation of the enzyme; 6: Other putative protein components that are involved in the enzymatic activity; 7: Lipids (e.g.: Phosphatidylserine, phosphatidic acid, arachidonic acid).*

tein whose function is probably to anchore the cytochrome to the membrane and to connect it with other components. In fact, genetic absence of the 68-92 KDa protein, as it occurs in the X-linked form of chronic granulomatous disease (CGD) causes lack of expression also of the low-molecular weight subunit, of cytochrome spectrum and lack of binding of regulatory cytosolic components to NADPH oxidase complex. We have observed that in partially purified preparations the cytochrome b_{558} is present in large molar excess with respect to the flavoprotein [36, 37].

Other components of the system have been recently identified on the basis of various experimental approaches: a) Studies in cell-free models of oxidase activation have shown that at least two proteins (n. 5 in Fig. 3) with molecular

weight of 47 and 65 KDa present in the cytosol of resting cells participate in the activation process of NADPH oxidase. Genetic absence of one of these two proteins, as it occurs in the autosomal recessive forms of CGD, causes lack or marked deficiency of superoxide production by neutrophils from these patients [38, 39]. Recently a G-protein that is associated with cytochrome b_{550} has been described [40]. b) Monoclonal antibodies raised against the active oxidase complex of pig neutrophils and possessing strong inhibitory capacity over NADPH dependent superoxide production bind to small polypeptides of 14/18 KDa, that have therefore to be considered putative components of the system (n. 6 in Fig. 3) [41]. c) The important role of lipid factors (n. 7 in Fig. 3) for the enzymatic activity or for mantaining the optimal conformation of the system is demonstrated by the requirement of phospholipids exhibited by the solubilized oxidase in order to function [42] and by the fact that phosphatidic acid is able to activate the dormant oxidase in membranes isolated from unstimulated pig neutrophils [43]. The participation of quinones that may shuttle electrons between the various components of the system in the lipid environment has been also proposed by some authors [44, 45].

The NADPH oxidase is activated only in that region of the membrane that is in contact with the target [46]. It generates O_2^- as the first oxygen reduction product, but subsequent rapid reactions lead to spontaneous and/or enzyme-catalysed formation of several other toxic derivatives (Fig. 4). The physiologic function of the free radical production is the destruction of invading microorganisms and tumor cells. The range of microbial targets is very wide and includes all kinds of bacteria, fungi and viruses. The most powerful oxidizing agent is hydroxyl radical, but other very toxic products are also generated by the H_2O_2/peroxidase/halide system (see section 3). The neutrophil is also susceptible to damage by these reactive oxidants so that it risks to be killed along with the target. However, the phagocytes are able to defend themselves against oxidants, at least to a limited extent. The antioxidant systems of the phagocytes include superoxide dismutase, catalase and glutathione peroxidase, α-tocopherol and ascorbic acid [47].

3. The cytoskeleton

The neutrophil cytoplasm contains a highly filamentous tridimensional network composed of microfilaments and microtubules. These structures have been implicated in pseudopod formation, cell movement and intracellular traffic of other organelles.

The microfilaments are mainly composed of the contractile proteins actin and myosin, which comprise about 20% of cytoplasmic proteins. Actin exists either as a globular monomer (G-actin) or as a double-helical filamentous polymer

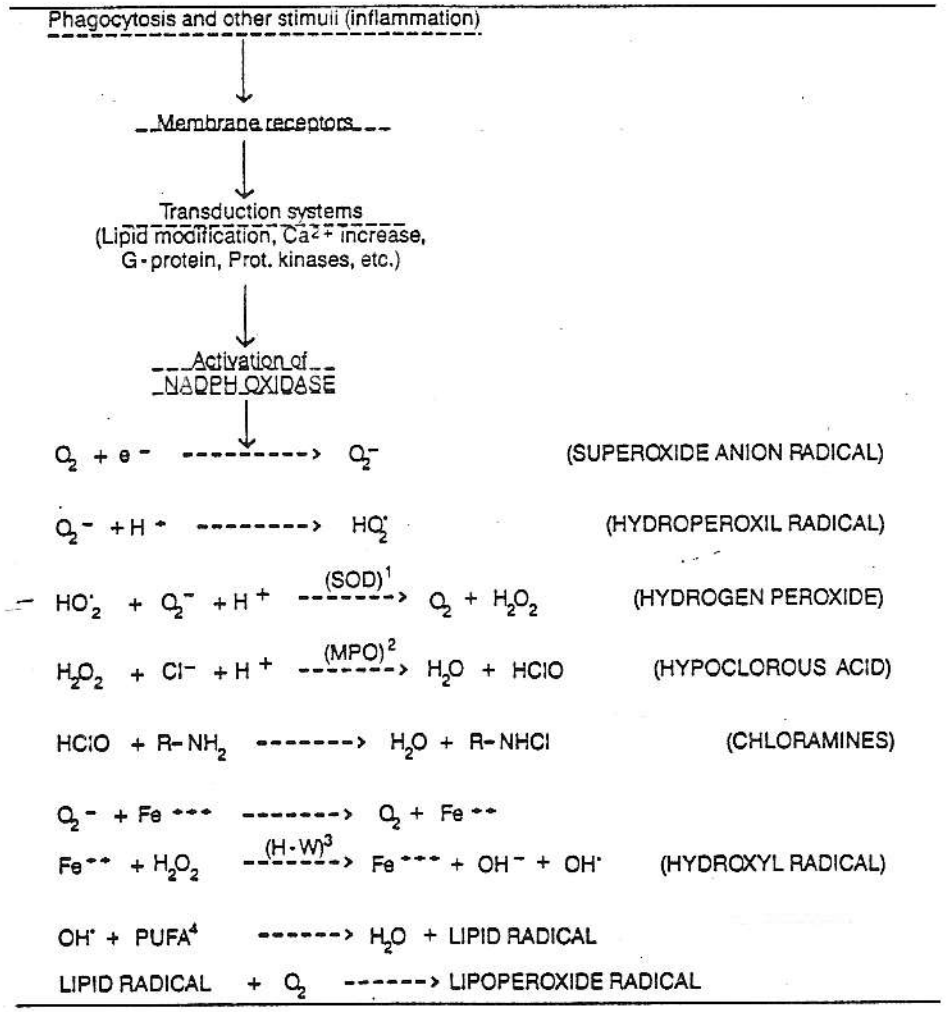

Fig. 4. *Generation of superoxide anion radical by stimulated neutrophils and some subsequent reactions that produce toxic derivatives. 1: SOD: superoxide dismutase; 2: MPO: myeloperoxidase; 3: H–W: Haber–Weiss reactions; 4: PUFA: polyunsaturated fatty acids.*

(F-actin). The distribution between these two actin states shifts dramatically after cell activation, namely the amount of G-actin decreases with an accompanying increase in F-actin. Moreover there is a local redistribution of the two forms in the extending pseudopods during phagocytosis, with rapid polymerization/depolimerization events associated with the movement of the membrane. The cytochalasins, fungal metabolites known to react with actin filaments and to inhibit their assembly, powerfully inhibit phagocytosis and locomotion, while enhancing exocytosis.

Myosin is an hexamer composed of two heavy polypeptide chains and four light

polypeptide chains. The heavy chains contain globular heads which bind actin and also possess ATPase activity. Myosin is concentrated in the pseudopods of migrating and phagocytosing neutrophils and contracts the actin gel by hydrolysis of ATP which provides energy for contraction. This phenomenon may be modulated by phosphorylation of myosin light chains.
In addition to actin and myosin, neutrophils contain several actin-binding proteins which regulate the polymerization state of actin in the cell and its binding to the plasmamembrane. Calcium concentrations greater than 2×10^{-7} M dissolve the actin gel by activating the protein gelsolin which inhibits polymerization. Additional information on the mechanical responses of neutrophils may be found in other recent papers [48-51].
Other important cytoskeletal structures in neutrophils are the microtubules, which are hollow fibers, 24 nm in diameter. They assemble as cylindrical polymers of the protein tubulin, which is found as a heterodimer of α and β subunits. Soluble tubulin is in equilibrium with the assembled form, allowing rapid growth and dissolution of these structures by shifting of the equilibrium. Microtubule assembly and disassembly can be controlled in a variety of ways, i.e. by cyclic nucleotides, by Ca^{2+} and by the intracellular concentration of S-D-lactoylglutathione. We have demonstrated that in stimulated neutrophils the concentration of S-D-lactoylglutathione increases approximately 100% of the resting concentration during the first few minutes of the activation period and that this may be related to stimulation of microtubule assembly [52, 53].
The modulation of microtubule functions is probably carried out by a series of microtubule-associated proteins. The fact that the microtubule-disrupting agent colchicine inhibits phagocytosis, chemotaxis, surface adhesion and degranulation indicates that these structures are important in all these phenomena. It is also possible that microtubules participate in sensory perception of the cell's external environments: it has been suggested that these cytoskeletal polymers may be capable of capturing and utilizing ambient or biologically generated electromagnetic energy [54].

4. Cytoplasmic granules

Neutrophils are endowed with a powerful armamentarium of enzymes and other toxic substances that are accumulated in particular granules, that have been distinguished in at least three groups according to separation in density gradients and composition (Table II). The content of these granules is released following cell stimulation, so that neutrophils in the inflammed areas are often completely degranulated. Besides the release of pre-packaged constituents, activated neutrophils also release products that are synthetized "de novo", such as platelet activating factor (PAF), arachidonic acid and its metabolites produced by cyclo-oxygenase and lipoxygenase pathways, proteins such as

interleukin-1 and granulocyte- and macrophage-colony-stimulating factors. Some of the components of specific granules (receptors, cytochrome b_{550}, flavoproteins) are not released outside the cell during degranulation, but they are incorporated in the plasmamembrane, where they contribute to the neutrophil function and particularly to adhesion, phagocytosis and assembly of NADPH oxidase active complex.

Table II. *Molecular constituents of granules of human neutrophils*

Azurophil granules	Specific granules	Other granules
Myeloperoxidase	Cytochrome b	Gelatinase
Cathepsins D and G	Flavoproteins	Acid proteinase
Elastase	Collagenase	β-glucosaminidase
β-glucuronidase	Histaminase	Glycosaminoglycans
Lysozyme	Lysozyme	Tetranectin
Cationic proteins	Laminin receptor	Laminin receptor
Bactericidal/permeability /inducing protein (BPI)	C3bi receptor	
Defensins	fMLP receptor	
Acid phosphatase	Lactoferrin	
Arylsulfatase	Vitamin B_{12} binding protein	
Glycosaminoglycans		
Chondroitin sulfate		
Heparan sulfate		
α-mannosidase		
β-galactosidase		
β-glycerophosphatase		

Most of the granule constituents, which are listed in table II, possess a definite toxicity to microorganisms. First of all, myeloperoxidase (MPO) which is present in high concentration in neutrophils (2-5% of dry weight) and confers a greenish color to neutrophil-rich exudates. MPO is a hemoprotein with a molecular weight of about 140 KDa. Its antimicrobial properties are linked to the ability of utilizing H_2O_2 and halides (iodide, bromide and chloride) to generate highly toxic agents such as hypohalous acids, long-lived oxidants such as chloramines or aldehydes, and possibly hydroxyl radicals and singlet oxygen (see also Fig. 4).

The BPI, a protein of 59 KDa contained in azurophilic granules, exerts a noxious effect on capsulated gram-negative rods by binding to the outer membrane of the bacteria and causing an increase in the permeability of the membrane and activation of bacterial phospholipases.

The defensins are a family of related small molecular weight (< 4,000 Da) bactericidal proteins that have been recently identified. The peptides, 32-34 aminoacid residues in lenght, are rich in cysteine and have a broad antibiotic spectrum. How the defensins exert their cytotoxic effect on target cells remains to be clarified.

Among the cathepsins, cathepsin G is a chymotrypsin-like protease with molecular weight of about 27 KDa, and optimum neutral pH. It has been shown to have marked antimicrobial activity against S aureus and other gram-positive bacterial species.

Lactoferrin is an 80 KDa iron-binding glycoprotein that in neutrophils is found in specific granules but it is present also in most secretory fluids (it has been actually firstly identified in milk). The role of lactoferrin as antimicrobial agent may be related to its iron-binding capacity and hence to its ability to compete with iron-requiring bacteria for an essential growth factor. Another possibility is that lactoferrin participates as iron donor (when the metal is released from the protein by reducing agents and by the superoxide anion itself) in the reaction that generates the hydroxyl radical (Fig. 4).

Lysozyme is a small (14.5 KDa) neutrophil enzyme, that is also widely distributed in tissues and body fluids, since it is also constitutively released by tissue macrophages. It is strongly bactericidal for some gram positive bacteria and exerts its action by cleaving the beta (1-4) glycosidic link between N-acetylglucosamine and N-acetylmuramic, resulting in the disassembly of bacterial cell wall.

Other possible factors that may contribute to the non-oxidative antimicrobial effects are: a) other proteases such as elastase and acid hydrolases (β-glucuronidase, β-glycerophosphatase, α-mannosidase, cathepsins B and G), that can digest some of the outer membrane proteins and polysaccharides of the bacteria; b) lipid hydrolases, in particular the phospholipid-degrading enzymes, that may perturb the bacterial envelope; c) nucleases, that may degradate chromosomal DNA of engulfed bacteria.

It should be pointed out that these and other granule constituents may be also released extracellularly when the neutrophils are stimulated by soluble membrane-perturbing agents or when they adhere to objects that are too large to be phagocytosed ("frustrated phagocytosis") or to surfaces that have been coated by immune complexes or complement factors. Enzymes and other factors with potentially cytotoxic properties are then complexed in serum with protease inhibitors and by other proteins such as albumin and lipoproteins. However, when the protective capacity of these systems is saturated, or too low due for example to hepatic failure or inactivation by oxidative stress, a local or systemic neutrophil-mediated damage may result. We have recently carried out a study of the intravascular release of elastase in patients affected by burns of various degrees of severity. The results of a typical and representative patient with severe burns (UBS score = 104, 17.5% of profound lesions) are reported in Fig. 5. It can be seen that ten days after the burn, a dramatic increase in serum elastase (measured as complex between elastase and α-1 antitrypsin) occurs. The fact that this increase follows the first febrile episode, but does not correlate with leukocyte count may be interpreted as an evidence that in this patient (and in several others with similar behaviour) the leukocytes

are released in the bloodstream in the first postburn period, then they are stimulated to adhere to endothelia, to migrate in the exudate and to release their constituents. It is conceivable that these events are mediated by interleukin-1 and by tumor-necrosis factor. We have also found enormous amounts of elastase in the blister fluid of most of burned patients (unpublished results). Moni-

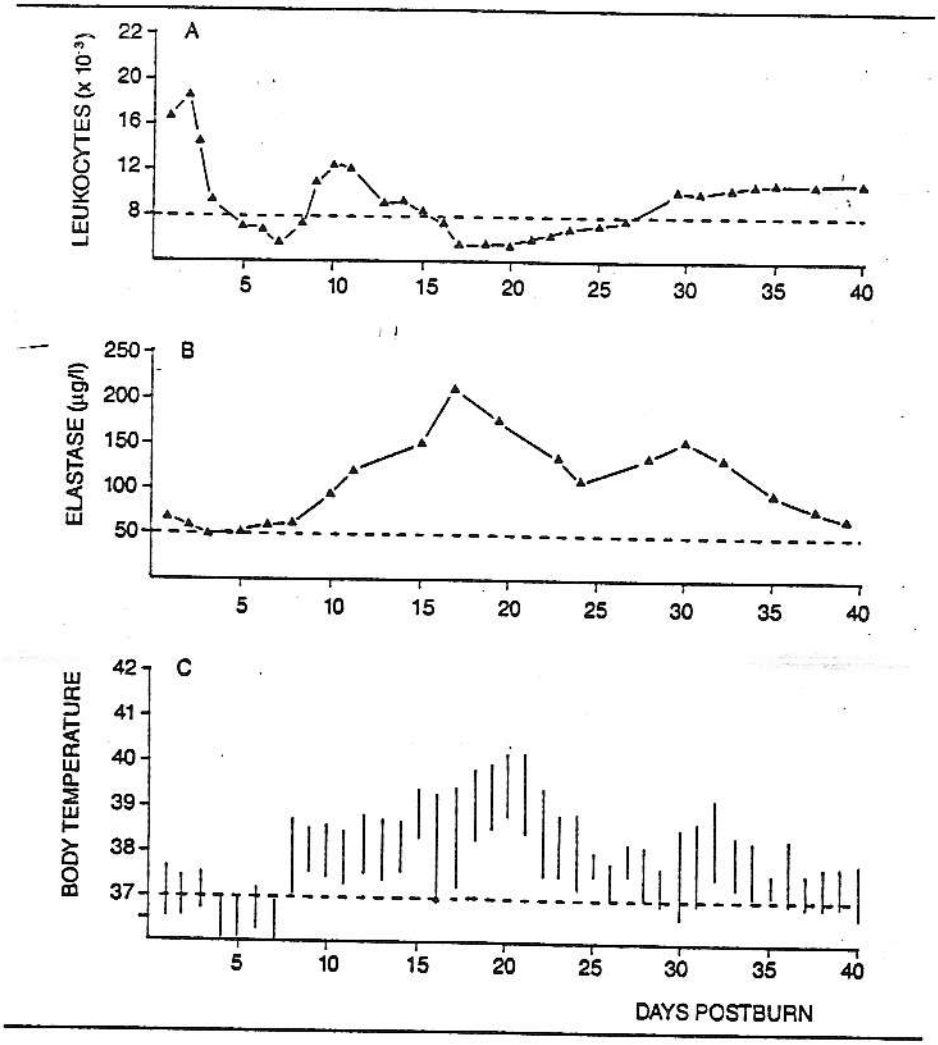

Fig. 5. *Monitoring of blood leukocytes (a), of serum elastase (b), and of body temperature (daily maximum and minimum) (c) in a representative patient after a serious burn injury. Elastase was measured by immunoassay as elastase/elastase inhibitor complex.*

toring of serum elastase levels may be a simple and informative index of systemic activation of leukocytes in severe burns, trauma and infections.
The recognition of neutrophils as having major secretory functions as well as active phagocytic capacities is in accord with their dual role in the inflammatory process and underlines the importance of studies of the mechanisms that may control the function of neutrophils "in vivo". One of the most ambitious goals of this kind of research is the possibility of biological or farmacological enhancement of phagocyte function in the immunocompromised patients and, on the other hand, the production of selective and non toxic inhibitors of the unnecessary damaging effects of neutrophil activation.

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