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Phagocytes use oxygen to kill bacteria

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The blood phagocytes, neutrophils, eosinophils and monocytes, have a main function in common: they attack and kill invading microbes and parasites. The prey is usually phagocytosed by these cells, i.e. fully enclosed in a vacuole where killing and digestion take place without harm to the surrounding tissues. On interaction with the microorganisms and other particles which are recognized as phagocytosable, the phagocytes suddenly increase their oxygen consumption. This phenomenon is called respiratory burst. It was discovered in the 1930's and believed to provide energy for phagocytosis[9]. Its role as a source of microbicidal oxidants became apparent much later, through studies from the laboratories of M.J. Karnovsky[45,38] and J.H. Quastel[26]. The demonstration that oxygen-derived products are of primary importance for microbial killing was then provided by experiments with neutrophils from chronic granulomatous disease patients, a condition characterized by the inherited inability of phagocytes to mount a respiratory burst[25].

The blood phagocytes

Neutrophils, eosinophils and monocytes have several common features[8]. They are formed in the bone marrow, are equipped with storage granules which (in part at least) contain peroxidase and lytic enzymes, are able actively to move from the blood stream to the tissues in response to chemotactic stimuli, and – as their name indicates – are able to phagocytose. Neutrophils and eosinophils are end-cells, suited for brisk but short-lasting interventions. They have abundant deposits of export enzymes (in their granules) and fuel in the form of glycogen, but are unable to replenish these stores. The monocytes, by contrast, are long-lived. Early in their life cycle, they discharge their granules[8], and – unlike the other phagocytes – then continue to function as granule-free phagocytic cells which are called macrophages. Most of what is known about the microbicidal activity of phagocytes has been elaborated in studies with neutrophils, and this cell will be the main object of my review.

Phagocyte activation

Bacteria usually colonize the interstitial space of a tissue. Neutrophils and other phagocytes are attracted to these sites by chemotactic signals which the microorganisms emit or induce[39]. The intervention of the phagocytes takes place in 2 steps. The cells are first activated by the chemotaxins in the micro-vessels which irrigate the affected tissue. As a consequence, they marginate and migrate through the walls of post-capillary venules towards the source of the chemotactic signal. Later, at the site of infection, the phagocytes engulf and kill the bacteria. Diapedesis, migration and phagocytosis are the physical consequences of activation. On the biochemical level, this process is characterized by the release of products necessary to the ultimate goal of phagocyte mobilization, i.e. the killing and disposal of the microorganism. The full extent of the response is displayed during phagocytosis. Experiments in the test tube, in which opsonized particles are added to a suspension of neutrophils and the products are determined in the incubation medium, permit the analysis of the whole release repertoire. Two classes of products are distinguished: those which are pre-formed and already present in the storage organelles of the resting cells; and those which are synthetized following stimulation. The pre-formed products are enzymes and other macromolecules; they are liberated by exocytosis following fusion of the membrane of the storage organelles with the

plasma membrane[7]. The newly-formed species are small molecules, superoxide and H_2O_2[5,26], arachidonic acid oxidation products[19,23] and platelet activating factors[42]. In the latter cases, release depends on the activation of key enzymes: the respiratory burst oxidase[2], a phospholipase, most likely phospholipase A_2 which delivers arachidonic acid[10], and a transferase which acetylates the lyso-procursors of platelet activating factors[1].

The respiratory burst enzyme

The respiratory burst is brought about by an enzyme or enzyme system which oxidizes NADPH and reduces molecular oxygen to superoxide. The oxidase is inactive in resting cells and is turned on upon phagocytosis by a mechanism which is apparently initiated by receptor-mediated interaction between particles and phagocytes. This concept, which is now generally accepted, satisfies most of the experimental evidence which has been gathered over more than 20 years. Sbarra and Karnovsky showed that the respiratory burst was cyanide-insentitive and accompanied by increased glucose oxidation through the hexose monophosphate shunt[45]. H_2O_2 was originally described as the product[26] and superoxide later identified as its precursor, using cytochrome c as reactant to prevent dismutation[5]. The amounts of superoxide and H_2O_2 released by phagocytosing neutrophils account in fact for most of the oxygen consumed by the burst[36]. The activated oxidase accepts both pyridine nucleotides as substrates but has higher affinity for NADPH[4,37]. This and other evidence rule out a direct involvement of NADH[2]. The primary reaction involved in the respiratory burst is therefore as follows:

$$2\ O_2 + NADPH \longrightarrow 2\ O_2^- + NADP^+ + H^+$$

At least in neutrophils where it was studied, the oxidase is located in the plasma membrane[17]. Its binding site for NADPH is exposed on the cytosolic surface, while the oxygen binding site is buried in the membrane[6]. This configuration ensures the intracellular access of the electron donor and the binding of extracellular oxygen, which is lipophilic enough to enter the lipid bilayer. Superoxide is initially delivered on the extracellular surface of the phagocyte membrane and then into the phagocytic vacuoles that form around the triggering particles (fig. 1).

The nature of the oxidase is still unknown. Since the enzyme is membrane bound and rapidly loses its activity upon cell disruption, purification is extremely difficult. Optimum activity has been shown to require the presence of FAD[4], suggesting the involvement of a flavoprotein. This would appear reasonable for one-electron reduction of oxygen by the two-electron donor, NADPH. More recently a b-type cytochrome was proposed as part of the respiratory burst oxidase[41]. The association of this cytochrome, apparently characteristic for phagocytes, with neutrophils that are able to mount a respiratory burst and its absence from defective cells has been documented in a number of cases[40]. This does not necessarily mean, however, that the cytochrome belongs to the electron transport system producing superoxide[20]. Ubiquinone was also recently proposed as an additional component[44]. A subsequent study, however, showed that this redox substance is largely associated with the mitochondria, making its participation in the respiratory burst appear unlikely[15]. The isolation of the components and the reconstitution of the active enzyme in vitro appears to be the only way to elucidate the biochemical mechanism of the respiratory burst.

Subcellular fractionation experiments with human neutrophils had suggested that cytochrome b was localized in the plasma membrane and the specific granules[41]. More recent work proposed its exclusive localization in the specific granules[11]. Although the latter results were subsequently disproved[21], the existence of the granular pool of cytochrome b suggested that exocytosis was required for the activation or the maintenance of the respiratory burst[11,21].

Oxygen-dependent killing

It is well established that superoxide formation is required for the killing of many bacteria by phagocytes[2]. Superoxide itself, however, does not appear to be the cidal agent. It has only low toxicity toward bacteria[29] and the killing by phagocytes is not impaired when the conversion of superoxide to H_2O_2 is accelerated by the addition of superoxide dismutase. Within a phagocytic vacuole, the site of its release, superoxide is most likely to react with itself (dismutation) and with H_2O_2. Vacuoles do not appear to contain superoxide dismutase, which was reported to be largely localized in the cytosol[16], but many bacteria, i.e. most aerobes, do[34]. Most superoxide is probably immediately converted to H_2O_2 non-enzymatically since spontaneous dismutation is most rapid at pH 4.8[35], a level of acidity which is soon reached within newly-formed phagosomes[27]. Superoxide and H_2O_2 react with each other giving rise to metabolites such as hydroxyl radical ($OH^\cdot$) and singlet oxygen ($^1O_2$) which are more reactive and presumably more toxic[31,46].

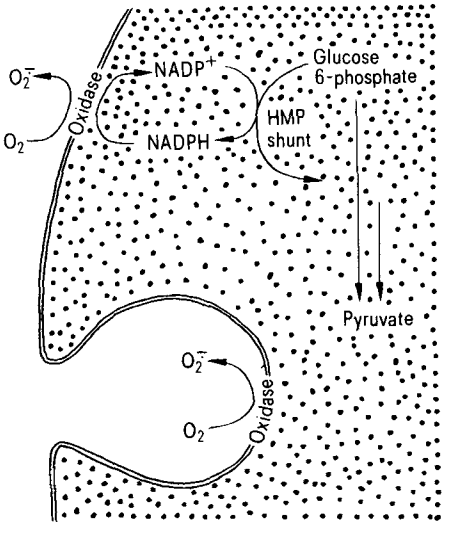

Figure 1. Scheme of the subcellular localization of the respiratory burst oxidase. The membrane-bound enzyme interacts with NADPH on the cytosolic and with oxygen on the outer face of the plasmalemma. Superoxide is delivered to the pericellular space or in the vacuolar space when the oxidase is activated by phagocytosable particles.

The cidal action of H_2O_2 is greatly amplified by myeloperoxidase, a major constituent of the azurophil granules[12], which is released in large quantity into phagocytic vacuoles[7]. Myeloperoxidase mediates the oxidation of a variety of substrates including halides through the reduction of H_2O_2. Halides, H_2O_2 and myeloperoxidase constitute a cidal system with very broad target specificity[28,30]. The major substrate in vivo is probably chloride because of its high concentration in body fluids. The anti-microbial mechanism proper is unknown, and a number of possibilities have been considered (see Klebanoff[30] for bibliography) *Halogenation* (e.g. chlorination or iodination) is the covalent linkage of the halogen to a molecular acceptor on the microorganisms. It only occurs in the presence of the complete reaction mixture and the target, suggesting that the reactive form of the halogen is a short-lived intermediate. In addition, several microbicidal species are formed by the peroxidase system, i.e. *halogens, hypohalous acids and derivatives* (OCl^-, HOCl, Cl^+ and Cl_2O as possible products of the oxidation of chloride), *chloramines, aldehydes* arising from the peroxidase-dependent de-amination and de-carboxylation of amino acids, and *singlet oxygen* resulting from the reaction of H_2O_2 with hypochlorite.

In view of the versatility of these reactions, one is puzzled to realize that the myeloperoxidase-dependent antimicrobial systems are not essential. Myeloperoxidase deficiency is a relatively common condition[14] and most individuals affected have little problems with clinically relevant infections. In addition, macrophages, which do not possess peroxidase-containing granules kill microorganisms with remarkable efficiency. This suggests an essential role for the metabolites arising from the primary reaction sequences involving superoxide and H_2O_2. It is not possible, however, to name a single molecule as the mediator of microbial killing. In view of the multiplicity of reactions occurring in a phagocytic vacuole, it is reasonable to assume that the overall anti-microbial performance of a phagocyte is the result of a number of processes, a few essential and several others only supportive in nature.

Because of their toxic potential, the products of the respiratory burst must be inactivated when they evade the vacuolar space. Superoxide and H_2O_2 diffuse easily through the phagosome membrane into the cytosol. Superoxide dismutase, catalase* and glutathione peroxidase, which are all present in the cytosol, detoxify these products to water (fig. 2). The other oxygen metabolites are unlikely to travel. They probably react with acceptors in the immediate vicinity of their site of formation, i.e. within the phagocytic vacuoles.

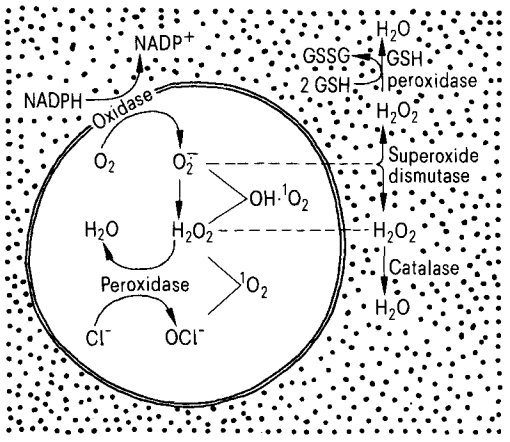

Figure 2. Some pathways for the generation of microbicidal agents from superoxide and H_2O_2 within a phagocytic vacuole, and mechanisms for the scavenging of superoxide and H_2O_2 that escape into the cytosol.

Non-oxidative killing

For a long time phagocytes were believed to kill microorganisms by means of pre-formed anti-microbial agents. Factors with suggestive names like leukin, phagocytin and others[24,43], lysozyme[13], and – more generally – cationic granule proteins[32,48] were indeed reported to kill a variety of bacteria. Antibacterial activity was also suggested for lactoferrin[33]. The interest in such agents diminished with increasing knowledge of the respiratory burst-dependent mechanisms. Without challenging the overwhelming importance of the latter, I would like to point to 2 recent developments which indicate that oxygen-independent killing mechanisms should not be totally disregarded. In a series of reports, Elsbach and his colleagues[18,47] described granule-associated, permeability-increasing proteins which selectively and very rapidly kill *E. coli*. The cidal effect involves a disturbance of the permeability of the bacterial membrane. It is conceivable that other agents of this kind will be identified in the future. The other development relates to a study which is still being persued in my laboratory. Subcellular fractionation[22] and ultrastructural analysis demonstrated that the neutrophils of ruminants contain a novel, major population of granules in addition to the common azurophil and specific granules. These novel organelles lack peroxidase and the neutral or acid hydrolases normally stored in neutrophils, but contain instead powerful bactericidal agents which probably correspond to a characteristic group of highly cationic proteins revealed by electrophoretic analysis of the constituents of the novel granules[22]. Although the biological role of these factors still has to be studied, the identification of a major organelle as their apparently exclusive site of storage suggests that such oxygen-independent defense systems are biologically relevant.

*Subcellular fractionations of rabbit neutrophils showed that catalase is a soluble enzyme (Baggiolini, unpublished).

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