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The Inflammatory Cells and Their Respiratory Burst

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In the last 20 years, a great number of investigations have been performed in many laboratories on the manifold functions of granulocytes and monocytes in the inflammatory process and, mostly, on the mechanisms by which these cells exert a key role in host defense and in the evolution of the inflammation.

The term "respiratory burst" refers to the increase in the respiration and in the oxidation of glucose via the hexosemonophosphate shunt which occurs when white cells are exposed to appropriate stimuli. The first observation (Table 1) was made in 1933 when Baldridge and Gerald noted that granulocytes displayed a substantial increase in O_2 consumption during phagocytosis (4). For a long period of time, this finding has been ignored. The study of the phenomenon was resumed more than 20 years later and in the laboratory of Suter the important observation has been made that O_2 consumption stimulated by phagocytosis is accompanied by an increase of glucose catabolism through the HMP pathway (47). In 1961, the laboratory of Quastel discovered, by using an indirect method, that phagocytosing leucocytes generate H_2O_2 (25). In subsequent years, the generation of the peroxide was quantitated in our (55) and in other laboratories (39). This finding has been a milestone for understanding the relationship between the respiratory burst and the defensive mechanisms against invading organisms.

In 1973, Babior observed that the excitation of the respiratory metabolism in human granulocytes is also associated with the production of O_2^- (1). This observation was subsequently confirmed in other phagocytes and enlarged with the finding

TABLE 1. *Milestones of the respiratory burst*

Year	Event	Reference
1933:	Increase in O_2 consumption	(4)
1956:	Increase in glucose oxidation through HMP pathway	(47)
1961:	H_2O_2 production	(25)
1973:	O_2^- production	(1)

that during the burst, other species can be produced, in particular OH· and singlet oxygen (28,48).

FEATURES OF THE RESPIRATORY BURST

The respiratory burst, induced by particulate matter such as bacteria, fungi, viruses, aggregated proteins, etc., is not a postengulfment event, as was originally thought. We have shown since 1964 that the activation of the respiration starts after the adhesion of the particles to the plasma membrane of phagocytes, is associated with the formation of pseudopodia around the particles, and ceases when the phagocytic act is completed (40,54).

Evidence has been also provided in our and in other laboratories that leucocytes undergo a metabolic stimulation when they adhere to non-phagocytosable substrates. Two experimental findings worthwhile mentioning in this chapter, come to mind. The first one, obtained in our laboratory (38,50), shows that the binding of granulocytes and macrophages to sepharose beads coated with concanavalin A (Con A) or with C3b, induces an excitation of glucose metabolism (Table 2).

The second involves the stimulation of glucose oxidation and of exocytosis in leucocytes by immunocomplexes bound to collagen or to micropore filters obtained by Hawkins (20) and by Henson (23). These experiments are good models of the pathological events triggered by immunocomplexes, for example, in arthritic joints, in nephrotoxis nephrites, in alveolitis, and in other situations when immunocomplexes are deposited. These types of cell-substrate interaction are to be regarded as an equivalent of phagocytosis, the so-called frustrated phagocytosis of Weissmann (52). In fact, the adhesion of phagocytes to solid substrates is followed by membrane-movement along the surface that mimic pseudopodia emission.

A peculiarity of the leucocytes is that they undergo a stimulation of oxidative metabolism also when a variety of soluble agents interact with their surface membrane. Table 3 shows these stimulatory agents in list form.

Some of these agents interact in an unspecific way; others bind to specific receptors or binding sites. Most of them, such as immunocomplexes, chemotactic compounds, endotoxins, fatty acids, mediators, and anti-leucocyte antibodies, are

TABLE 2. *Stimulation of $^{14}CO_2$ production from ^{14}C-glucose by phagocytes bound to immobilized concanavalin A*

	$^{14}CO_2$ from 1- ^{14}C-glucose		$^{14}CO_2$ from 6- ^{14}C-glucose	
	Resting	Adherent	Resting	Adherent
Guinea pig granulocytes	22,415	63,260	210	290
Rabbit alveolar macrophages	5,125	7,510	1,525	1,500

1×10^7 cells incubated in 2.5 ml of calcium-free KRP with 0.2 mM cold glucose containing 0.8 μCi of 1-^{14}C-glucose or 2.0 μCi of 6-^{14}C-glucose (high specific activity) in absence and in presence of Con A covalently bound to Sepharose 2 B (about 1.2×10^4 beads).

TABLE 3. *Stimulants of phagocyte oxidative metabolism*

Phagocytosable particles	Endotoxin
Surfactants	Lantanium ions
Anti-leucocyte antibodies	Chemotactic peptides
Phospholipase C	Divalent cation ionophores
Immune complexes	Valinomycin
Na F	Cytochalasins
Complement fragments (C5a, C567)	Phytoemagglutinin
	Leucocyte pyrogen
PMA	Hystamine-coated beads
Kallicrein	Protease inhibitors (TPCK)
Concanavalin A	Fatty acids

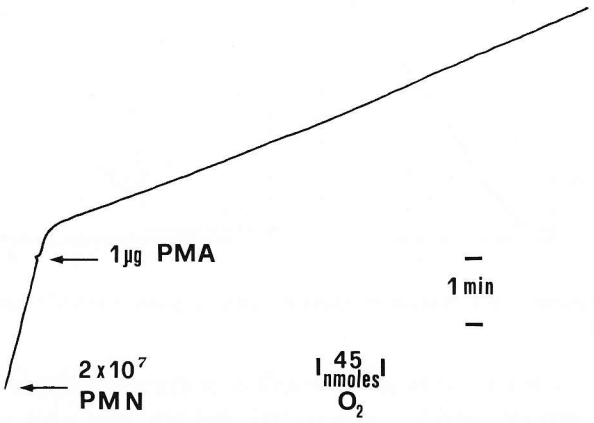

FIG. 1. Oxygen electrode trace of the respiratory burst induced by PMA in guinea pig granulocytes.

of great biological importance since they frequently come in contact with the phagocytic cells at the inflammatory sites or in the blood stream. It is worthy to point out that many of these stimuli induce other responses such as secretion, chemokinesis, and chemotaxis. Thus, it seems natural that the state of respiratory activation is a common and frequent feature of phagocytes engaged in different functions inside and outside the blood vessels, for example, during chemotaxis, during the adhesion to endothelial cells, cartilage, or collagen fibers through bound immunocomplexes or chemotactic factors, during phagocytosis of infective agents or cell debris, denaturated proteins, and aggregated immunocomplexes, and during the aggregation induced by complement factors, etc.

The respiratory burst is a very impressive phenomenon as far as the intensity and the rapidity of its occurrence are concerned. Figure 1 shows the O_2 electrode tracing of the respiration induced by phorbol myristate acetate (PMA) in guinea pig granulocytes and Fig. 2 shows the time course of the burst measured as O_2 consumption, as O_2^- and H_2O_2 release, and as $^{14}CO_2$ production from $1-{}^{14}C$ glucose.

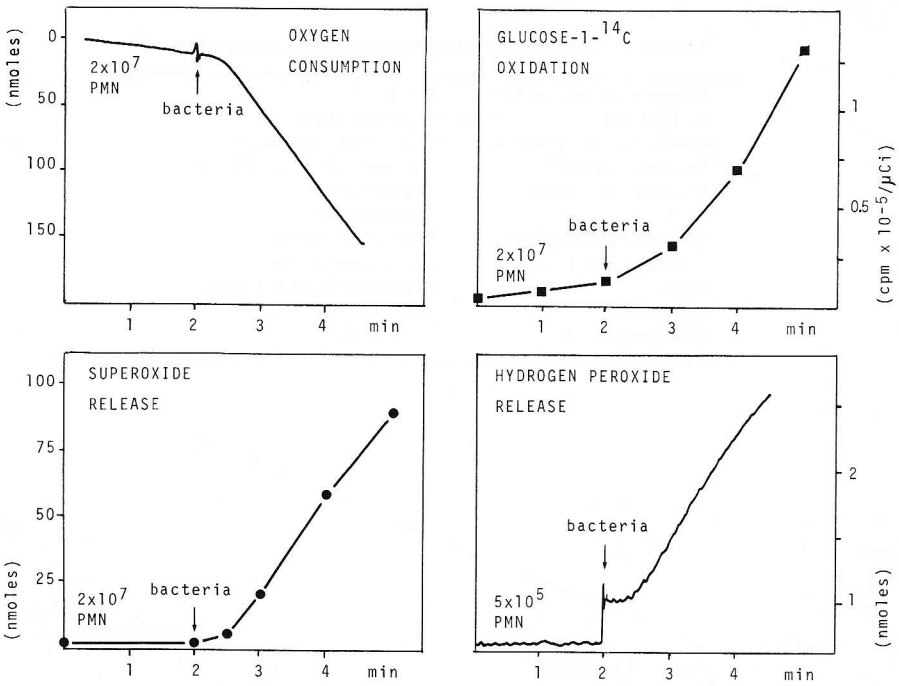

FIG. 2. Time course of the respiratory burst induced by phagocytosable bacteria in guinea pig granulocytes.

The respiratory burst occurs in the so-called professional phagocytes (granulocytes and macrophages) and also in mast cells and basophils when exposed to the degranulating agents 48/80 and anti IgE antibodies (22). The intensity of the respiratory burst is a direct function of the intensity of the stimulus (for example, of the number of bacteria) and varies depending on cell type, cell sources, animal species, and state of differentiation. The neutrophils and the eosinophils of mammalians are more responsive than the monocytes. The macrophages exhibit large variations between cells from different species, organs, and grade of differentiation (42).

The mechanism triggering the excitation of the oxidative metabolism is not completely understood. It is widely accepted that the plasma membrane exerts a key role in the function of the white cells (Fig. 3).

The triggering event includes the participation of a three-element system; *a recognition*, *a transduction*, and *a target*.

a) *The recognition* is the interaction between the stimulatory agents and the receptors or binding sites of cell surface, for example Fc receptors, C3B receptors for opsonized particles, glycoproteins for Con A, phospholipids for phospholipase C, cholesterol for saponine, etc. The recognition system is quite heterogeneous.

b) *The transduction* mechanism includes the molecular and functional modification of the plasma membrane triggered by the recognition events and is responsible for the subsequent responses. This is one of the more complex events, since the rec-

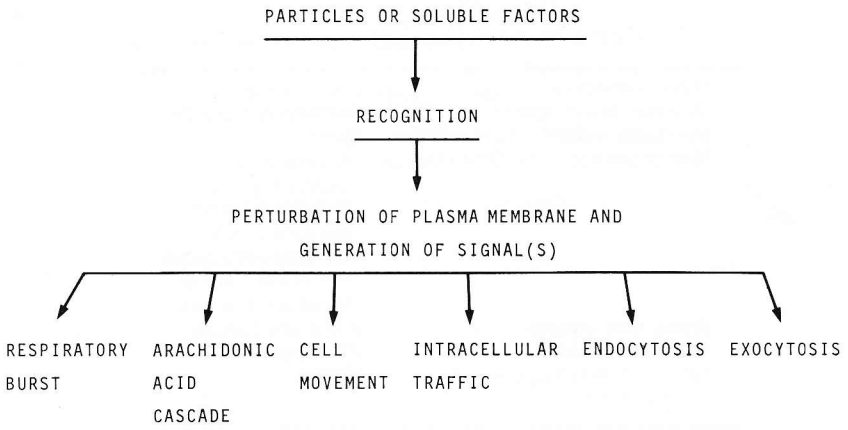

FIG. 3. Role of the plasma membrane in the regulation of phagocytic functions.

ognition systems are manifold and the responses are various. For example, the same recognition event, such as the interaction between C5a and cell membrane, triggers the respiratory burst, the exocytosis, and the chemotaxis; the interaction between opsonized particles and cell membrane triggers endocytosis, exocytosis, and respiratory burst. Evidence is now accumulating that the transduction mechanism is associated with or includes changes of ion fluxes, changes of the state of polarization, and changes of disposal of free calcium (5,16,53).

c) The third element is *the activation of the target system*. For the chemotaxis, it involves the contractile proteins; for the exocytosis, it involves the intracellular traffic and the fusion of the granule with the plasma membrane and for the respiratory burst, it involves the primary oxidase.

THE ENZYMATIC BASIS OF THE RESPIRATORY BURST

The recognition of the enzyme(s) involved in the stimulation of the respiration in phagocytes is one of the most controversial problems in the field of the biology of white cells (Table 4). Evidence has been obtained during the last 15 years in our (6,35,41,43,54) and in other laboratories (3,9,26,36) showing that the mechanism responsible for the respiratory burst is the activation of an oxidase which uses NADPH as substrate.

We have recently reinvestigated the matter by using more appropriate methods of assay of the enzyme, that is the O_2^- forming activity. The data of the Table 5 show the activity of the NADPH oxidase in cell-free particles of granulocytes and macrophages stimulated by PMA.

The localization of the oxidase is also a matter of controversy. Until 1974, it was almost a general belief that the enzyme was located in some intracellular organelles. In the last years, a new trend began to emerge which favoured a plasma membrane localization (17,49). The first direct demonstration of a localization of the NADPH oxidase on the plasma membrane has been obtained by Dewald et al.

TABLE 4. *Enzymes proposed as primary oxidase*

Myeloperoxidase	(Quastel's group)
"Soluble" NADH oxidase	(Karnovsky's group)
Membrane-bound NADH oxidase	(Segal)
Membrane-bound NADPH oxidase	(Rossi's group Sbarra's group Hohn and Lehrer Babior's group De Chatelet's group Kakinuma's group Minakami's group)
Amino acid oxidase	(Cline and Lehrer)
Ascorbate oxidase	(De Chatelet)
NAD(P)H dehydrogenase-cytochrome b	(Segal)

TABLE 5. *NADPH oxidase activity of cell-free particles from resting and PMA-stimulated peritoneal granulocytes and macrophages of guinea pig*[a]

	Granulocytes		Macrophages	
	Resting	Stimulated	Resting	Stimulated
NADPH 0.15 mM	0–0.45 (5)	45.5 ± 8.4 (5)	0–2.3 (8)	55.7 ± 17.8 (10)
1.0 mM	0–0.69 (5)	65.2 ± 15.6 (5)	0–3.4 (4)	62.5 ± 27.0 (5)
Vmax	—	54.0 ± 18.0 (5)	—	46.7 ± 5.8 (3)
Km (mM)	—	0.057 ± 0.01 (5)	—	0.03 ± 0.005 (3)

[a]Results are expressed as nmoles of O_2^-/min/mg protein ± SD. The number of experiments is given in parentheses.

(12) by using human blood granulocytes stimulated with PMA. We have confirmed the results by fractionating homogenates of guinea pig granulocytes and macrophages in discontinuous and continuous sucrose gradient. Table 6 shows the results obtained in guinea pig-elicited macrophages treated with PMA. The results are expressed as relative specific activity and indicate that the NADPH oxidase follows the same pattern of enrichment of 5′ nucleotidase, a marker of the plasma membrane and of G-6-Pase, a marker of endoplasmic reticulum.

We have recently obtained a good separation of phagosomes by centrifuging in discontinuous sucrose gradient homogenates of guinea pig granulocytes and inflammatory macrophages phagocytosing latex particles. Table 7 shows the data on the enzymatic content of phagosomes of macrophages. It can be seen that the phagosomes, 3 min after phagocytosis, are enriched 5-fold in 5′-nucleotidase, the enzyme marker of plasma membrane, and not enriched in glucose-6-phosphatase, marker of endoplasmic reticulum, and in β-glucuronidase, marker of lysosomes. The NADPH oxidase follows the same pattern of 5′nucleotidase, confirming its

TABLE 6. *Relative specific activities of marker enzymes and of NADPH oxidase in subcellular fractions obtained from guinea pig elicited peritoneal macrophages stimulated with PMA*[a]

Fraction	5′-nucleotidase	Glucose-6 phosphatase	Succinate dehydrogenase	β-glucuronidase	NADPH oxidase
20%/34%	3.6	4.1	0.6	0.4	4.3
34%/40%	1.4	2.6	1.7	1.3	2.5
40%/45%	0.8	1.5	0.9	2.1	1.6
45%/50%	0.8	0.8	0.1	0.9	0.8

[a]The data are means of four experiments of separation in discontinuous sucrose gradient of nuclei-free homogenates.

TABLE 7. *Relative specific activities of marker enzymes and of NADPH oxidase in phagosomes obtained from phagocytosing guinea pig peritoneal elicited macrophages*[a]

	5′-nucleotidase	Glucose-6 phosphatase	β-glucuronidase	NADPH oxidase
Phagosomes	4.6	1.7	1.2	7.1
Fraction 33%/46%	2.2	1.7	1.1	3.0
Pellet	1.8	1.7	0.9	2.1

[a]Means of 5 experiments.

association with the plasma membrane. Similar results have been obtained on phagosomes of granulocytes. It is worthy to point out that the enrichment of NADPH oxidase is higher than that of 5′nucleotidase. This finding indicates that the activated oxidase is associated only with the plasma membrane that forms the phagosomes. This is the best strategic position for the discharge of the intermediate products of oxygen reduction (O_2^- and H_2O_2) inside the vacuole, where the engulfed matter is processed.

The nature of the enzyme and the mechanism of its activation are not completely understood. The oxidase would be a flavoprotein, would require phospholipids for its activity, and would catalyze the reaction ($NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$) (2,15). According to others, the oxidase would be a system involving a new type of cytochrome b (32,46) and the activation would be a process of assembly of more components triggered by molecular changes of the plasma membrane.

Apart from this, the enzyme works on the plasma membrane. It is likely that it is activated in the whole membrane of the cell when the stimulatory agent is a

soluble factor and only in the invaginated portion of the membrane in the case of phagocytosis.

Owing to the localization of the activated enzyme, the first product of the oxidative reaction, that is O_2^-, is mainly discharged either in the phagocytic vacuole or outside the cell and dismutates to H_2O_2. The peroxide equilibrates with the intracellular compartment. Inside the vacuole, inside the cell, or outside the cell, H_2O_2 is used as substrate for peroxidatic or catalatic reactions. One of these reactions is catalyzed by the glutathione-peroxidase, and in this manner the HMP pathway for glucose oxidation is activated (41). This mechanism of activation of HMP pathway plays the role of protecting the cells from the toxic effect of H_2O_2. Its relevance greatly varies in different cells, depending on the variable activity of glutathione peroxidase. It is high in macrophages and lower in granulocytes (42).

The activation of HMP pathway is also linked to a decrease of the ratio $NADPH/NADP^+$, that is directly caused by the increased activity of the NADPH oxidase.

FUNCTION OF THE RESPIRATORY BURST

The function of the respiratory burst is linked to the production of the intermediates of oxygen reduction. The main species are O_2^- and H_2O_2, but other reactive species such as $OH^\cdot$ and singlet oxygen can be indirectly formed (28).

The O_2^- can act either as a reducant or as an oxidant. In the first case, O_2^- loses one electron and forms molecular oxygen. In the second case, O_2^- is reduced to H_2O_2. There are many indications that O_2^- interacts with proteins, lipids, polysaccharides, nucleic acids, and other biochemicals (14).

The hydroxyl radical is a very strong oxidant and can react with lipids, proteins, nucleic acids, etc. H_2O_2 either directly or indirectly can oxidize many reduced compounds through the catalysis of peroxidases.

Lipid peroxidation is one of the most common events associated with reactions forming oxygen radicals and H_2O_2 (31). The effect of the respiratory burst, however, is only partially understood. In an attempt to clarify the matter, it seems reasonable to distinguish the effects of the burst into beneficial and harmful and intracellular and extracellular.

The main beneficial effect is that of providing a potent battery of weapons for killing ingested organisms (28,45) in addition to other mechanisms such as the discharge of enzymes or of other factors into the phagosomes. The oxygen-killing mechanism is active mostly in the phagosomes and also in the extracellular environment. It may be mediated by MPO or linked to the direct effect of O_2^- of $OH^\cdot$ and of H_2O_2. The first mechanism uses the peroxidase secreted into the phagosomes or in the extracellular medium, H_2O_2 and a halide, usually chloride as oxidizable factor. The final mechanism is due to toxic agents produced by the reaction between peroxidase, H_2O_2 and chloride. It is likely that these include chlorinium ions, chloramine, aldehyde, and singlet oxygen (28,45).

The efficiency of this MPO-mediated mechanism is variable in different cells, depending on the intensity of the respiratory burst, on the amount of intermediate

discharged, on the amount of the intermediate degraded, on the content of MPO, and on its secretion from intracellular storage. The efficiency is high in granulocytes and greatly varies in macrophages. There is now growing evidence that also the cytostatic or cytocidal action of phagocytes against tumor cells is, at least partially, linked to the respiratory burst (27,33). This activity is not phagocytosis-dependent, but it is triggered by the contact of granulocytes and macrophages with target cells mediated by antibodies or by activating factors immunologically secreted by lymphocytes.

On the basis of what we know of free radical pathology, it is evident that the intermediates of oxygen reduction can produce a harmful effect on tissue components. Part of the degenerative processes that occur in the inflammatory sites are likely due to a direct effect of free radicals of oxygen and of other toxic agents originated by chain reactions triggered by the radical or by H_2O_2. There is evidence concerning depolimerization of hyaluronic acid (10,30), structural modification of collagen (18), endothelial cell injury (44), and hemolysis (51).

Another relevant effect of the respiratory burst is the damage of cell structure of the phagocyte itself. This cell is usually protected against the toxic effect of the radicals and of H_2O_2 by the activity of enzymes that degrade these compounds and by the presence of scavengers such as glutathione and other thiols, ascorbic acid, metals, etc. These mechanisms may become insufficient and, as a consequence, the death of the cell occurs followed by a discharge of cellular components such as lysosomal and nonlysosomal enzymes that act on extracellular structures and amplify the tissue damage.

It is worthwhile to point out that the effects of the respiratory burst are more complex than those until now presented. There are data in the literature showing that the products of the respiratory burst would play a role in many events of the inflammatory reaction. Examples of these are the increase in vascular permeability (11), the inhibition of ADP-induced aggregation of platelets (29), the O_2^--induced secretion of serotonin and aggregation of platelets (19), the activation of histamine secretion by mast cells (34), the modulation of lymphocyte blastization (13), the stimulation of collagen synthesis (24), the stimulation of arachidonic acid cascade (21), the generation of chemotactic factor in the plasma (37), the direct and non-enzymatic formation of a chemotactic lipid by arachidonic acid (53), the increase in adhesive interaction between endothelia and granulocytes (11), the inactivation of chemotactic factors (8), and the regulation of protease and antiprotease activities at the inflammatory sites through the inactivation of α_1-proteinase inhibitor (7).

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