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Free Radicals Generation by the Inflammatory Cells

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Abstract

One of the most impressive property of the leucocytes is that of changing the oxidative metabolism during various functions. When challenged with phagocytosable particles or with membrane perturbing agents such as chemotactic factors, detergents, lectins and other ligands, granulocytes and mononuclear phagocytes undergo a dramatic increase of oxygen consumption which is associated with the production of superoxide anion (O_2^-), of hydrogen peroxide (H_2O_2) and of hydroxyl radical (OH·).

These events are referred to as 'respiratory burst'. Most of the functions of the inflammatory cells (killing of micro-organisms, tissue damage, amplification of the inflammatory process) are linked to the production, to the fate and to the chemical reactivity of these highly reactive compounds.

The authors examine the following aspects: (i) the mechanism responsible for the respiratory burst; (ii) the conditions present in the inflammatory site that induces the metabolic activation of leucocytes; (iii) the variability of the respiratory burst in different types of leucocytes; (iv) the fate, the interrelationships and the reactivity of the intermediate products of oxygen reduction; (v) the relationships between the inflammatory process and the production of free radicals by the inflammatory cells.

1. The respiratory burst of phagocytes

Professional phagocytes (polymorphs, monocytes, macrophages) have the distinctive feature of possessing an oxidative metabolism which can be excited by a variety of environmental stimuli. Ingestion of particulate objects or interaction with some membrane perturbing agents causes a dramatic enhancement of oxygen consumption, of hexose monophosphate pathway activity and of generation and release of superoxide anion and hydrogen peroxide [1–13]. These events are referred to as the 'respiratory burst' of phagocytes. Figure 1 shows the time course of the various events of the respiratory burst in phagocytosing guinea-pig polymorphs. It is worthy of note that the onset of these events takes place a few seconds after the addition of heat-killed bacteria.

The stimuli which induce the metabolic excitation of phagocytes are various and differ in both chemical nature and mode of action [14–16] but can be easily grouped into two main categories, (a) phagocytosable stimulants and (b) soluble stimulants. The soluble stimulants are listed in Table 1. Some of these compounds, for example concanavalin A [17] and divalent cation ionophores [18, 19], are interesting since they provide a laboratory model to study the stimulus–response coupling [17–19]. Other agents, for example immune complexes [20, 21], complement fragments [22–24], kallikrein [25] and endotoxins [26], are important since they may play a role 'in vivo', in modulating the inflammatory process due to their presence at the inflammatory site.

2. Enzymatic basis of the respiratory burst

Direct and indirect evidence indicate that the respiratory burst is due to the activation of an oxidase that uses NADPH as

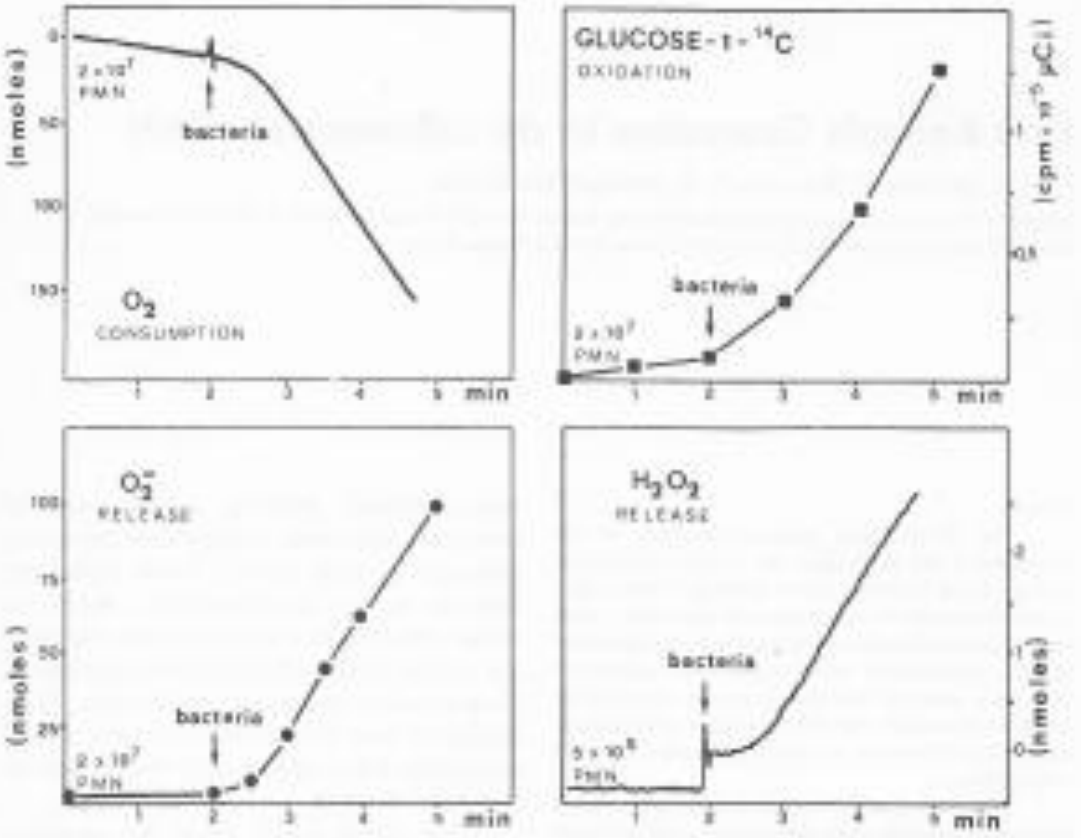

Figure 1
Time course of the respiratory burst in guinea-pig granulocytes.

substrate and generates superoxide anion and hydrogen peroxide [2, 7, 11, 13–15, 27–31]. This enzyme is practically inactive in resting cells but increases its rate and its affinity for NADPH upon stimulation of the phagocytes by particles or soluble stimulants [7, 15, 30]. Table 2 shows the specific activity of NADPH oxidase of the 100,000 *g* pellet of postnuclear supernatant of guinea-pig polymorphs at rest and stimulated with phorbol myristate acetate (PMA). It can be seen that the enzymatic activity, measured either as oxygen consumption or as O_2^- generation, is very low in resting cells and increases several times in stimulated cells.

2.1. Subcellular localization of the NADPH oxidase

The subcellular localization of the NADPH oxidase is still unclear. Some authors think that it is localized at the plasmamembrane level [32, 33], others that it is associated to the granules [31, 34]. Recently we have obtained evidence that in guinea-pig granulocytes stimulated by PMA the oxidase is located in subcellular fractions of lower

Table I
Soluble agents that stimulate the oxidative metabolism of phagocytes.

Surfactants	Cytochalasin E, D
Anti-leucocyte antibodies	Lanthanum ions
Phospholipase C	Endotoxin
Immune complexes	N-formyl methionyl peptides
Fatty acids	
Phorbol myristate acetate	
Complement fragments	
Kallikrein	
Concanavalin A	
Divalent cation ionophores	

Table 2
Specific activity of NADPH oxidase in the 100,000 *g* pellets of the postnuclear supernatant of resting and PMA-treated leucocytes.

	Resting cells	PMA-treated cells
O_2 consumption (nmoles/min)	61.3 ± 16.5	693.6 ± 140
O_2^- production (nmoles/min)	1.2 ± 0.2	43.2 ± 8.4

Oxygen consumption was assayed with a Clark oxygen electrode connected with a plastic vessel under continuous stirring. Final volume 2 ml. The assay medium contained: 0.065 *M* Na^+/K^+ phosphate buffer pH 5.5, 0.17 *M* sucrose, 1 m*M* NADPH, 2 m*M* NaN_3, $T = 37°C$.

O_2^- production was assayed by monitoring the reduction of cytochrome *c* at 550 nm. The assay medium contained: 0.065 *M* Na^+/K^+ phosphate buffer pH 7.0, 0.17 *M* sucrose, 0.15 m*M* NADPH, 2 m*M* NaN_3, 100 μ*M* cytochrome *c* and when required, 30 μg of SOD. Final volume 1 ml, $T = 37°C$. The O_2^- produced was calculated by the difference between the reduction of cytochrome *c* in absence and in presence of SOD. The values reported are the means of four experiments ± S.E.M.

density than those containing myeloperoxidase (MPO). Table 3 shows the average percent distribution of acid paranitrophenyl phosphatase (pNPPase) taken as a membrane marker, of MPO a marker of azurophilic granules, and of NADPH oxidase among four groups of fractions obtained by rate zonal sedimentation of the postnuclear supernatant of guinea-pig polymorphs stimulated by PMA through a discontinuous sucrose gradient. From these data it can be seen that MPO activity is found in the heavier part of the gradient (D) whereas acid pNPPase and NADPH oxidase are predominantly distributed in the lighter zones of the gradient (A and B). The A and B zones of the gradient were predominantly made up of vesicles as judged from electron microscopic examination. It remains to be determined whether the membrane fractions to which the NADPH oxidase is associated originate from the plasmamembrane or from other membranes.

Table 3
Average percent distribution of acid pNPPase, MPO and NADPH oxidase after rate zonal centrifugation of PMA-treated leucocytes.

Gradient	Density range (g/cm³)	Acid pNPPase	MPO	NADPH oxidase	
				O_2 consumption	O_2^- production
A	1.04–1.06	31	5	28	25
B	1.06–1.08	23	8	35	43
C	1.08–1.12	16	18	16	18
D	1.12–1.27	30	68	25	14
Recovery (%)		87	65	91	70

Rate zonal centrifugation of the postnuclear supernatants was accomplished in a A1-14 rotor operated by a Spinco L50 ultracentrifuge. 40 ml of postnuclear supernatant were sandwiched between a 200 ml overlayer of 1 m*M* $NaHCO_3$ and a gradient made of 35-ml portions of sucrose solutions of density 1.059, 1.063, 1.072, 1.076, 1.083, 1.089 and 45-ml portions of density 1.089 and 1.103. All sucrose solutions contained 1 m*M* $NaHCO_3$. The gradient rested on 100 ml sucrose of density 1.286. After 15 min at 13,500 rpm, the gradient was displaced from the rotor with a sucrose solution of density 1.304 and 10-ml fractions were collected. Each fraction was assayed for glucose-6-phosphate dehydrogenase, MPO, acid pNPPase and protein. The sucrose concentration of each fraction was also determined. On the basis of the distribution of protein and enzymes, the fractions were pooled in four groups (A–D). These groups were then diluted with 1 m*M* $NaHCO_3$ to a final density of 1.04 and then centrifuged at 100,000 *g* for 45 min. The resulting pellets were resuspended in 0.34 *M* sucrose and assayed for acid pNPPase, MPO and NADPH oxidase. The recovery was calculated with respect to the 100,000 *g* pellet of an aliquot of the postnuclear supernatant. The means of two experiments are reported.

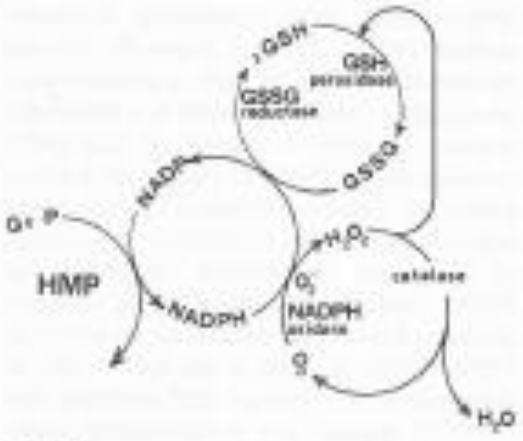

Figure 2
Link between stimulation of NADPH oxidase and activation of the hexose monophosphate pathway. GSH = reduced glutathione, GSSG = oxidized glutathione, G-6-P = glucose-6-phosphate, HMP = hexose monophosphate pathway.

2.2. Link between stimulation of the NADPH oxidase and activation of the hexose monophosphate pathway (HMP)

Stimulation of the NADPH oxidase leads to increased oxidation of glucose through the HMP by two mechanisms (Fig. 2). (1) The oxidation of NADPH by the stimulated oxidase modifies the radio NADP/NADPH which is the trigger factor which controls the activity of the first two enzymes of the HMP that is glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, (2) in some types of phagocyte hydrogen peroxide generated by the stimulated oxidase is reduced to H_2O by glutathione peroxidase with formation of oxidized glutathione (GSSG). GSSG in turn is reduced to GSH by a NADPH dependent glutathione reductase with formation of NADP.

Table 4
Oxygen consumption and release of O_2^- and H_2O_2 by different types of phagocytes during phagocytosis.

		nmoles/4 min/1.5 × 10^7 cells		
		O_2	H_2O_2	O_2
Granulocytes				
Human blood	(6)	171.2 ± 22.0	35.4 ± 9.0	70.1 ± 15.8
Bovine blood	(3)	100.8 ± 8.0	–	31.7 ± 7.5
Rabbit peritoneal exudate*	(4)	163.8 ± 24.7	22.2 ± 3.8	40.8 ± 6.0
Guinea-pig peritoneal elicited*	(4)	173.0 ± 35.1	41.1 ± 10.5	89.9 ± 10.4
Macrophages				
Guinea-pig peritoneal elicited*	(5)	229.5 ± 28.1	81.3 ± 14.7	163.3 ± 25.1
Rabbit peritoneal elicited*	(5)	75.5 ± 18.0	17.1 ± 2.2	6.1 ± 0.9
Rabbit alveolar resident	(4)	88.1 ± 12.6	0	0.4 [0–1.4]
Rabbit alveolar BCG-activated	(4)	97.7 ± 14.1	6.5 ± 1.2	5.0 ± 1.4

The data are expressed as means of the differences between phagocytosing and resting cells ±S.E.M. The number of experiments is shown in parenthesis. Assay medium: 1–2 × 10^7 granulocytes or macrophages in 2 ml of Krebs Ringer Phosphate containing 0.5 mM $CaCl_2$ and 5 mM glucose. Serum-opsonized *Bacillus mycoides* was used as phagocytosable particle (ratio cell/bacteria 1:100). Oxygen consumption was assayed with a Clark oxygen electrode connected with a plastic vessel under continuous stirring at 37°C. Hydrogen peroxide and superoxide anion were measured as previously described [51].

* Casein.

Table 5
Enzyme activities of different phagocytes.

	Polymorphs					Macrophages			
	Guinea-pig peritoneal exudate (3)	Rabbit peritoneal exudate (6)	Human blood (5)	Mouse[a] peritoneal exudate	Rat[a] peritoneal exudate	Guinea-pig peritoneal elicited (2)	Rabbit peritoneal elicited (4)	Rabbit alveolar resident (3)	Rabbit alveolar BCG-activated (6)
GSH peroxidase[b]	8.6 ± 0.2	8.6 ± 0.2	1.8 ± 0.4	7.2	13.9	5.0	12.4 ± 4.0	21.6 ± 3.6	75.3 ± 13.7
GSSG reductase[c]	1.6 ± 0.7	8.3 ± 0.87	2.0 ± 0.5	6.1	3.9	7.0	3.7 ± 1.3	3.8 ± 0.3	5.3 ± 0.4
Catalase[d]	6.7 ± 3.1	4.0 ± 0.3	14.5 ± 3.2	0.1	0.1	20.8	4.8 ± 2.6	31.1 ± 1.3	98.2 ± 5.1
Peroxidase[e]	115.0 ± 28.0	52.5 ± 13.5	365.0 ± 69	37.1	218.0	19.0	6.8 ± 3.0	6.3 ± 1.4	2.0 ± 0.1
SOD[f]	0.18 ± 0.01	0.15 ± 0.05	0.44 ± 0.05			0.69	0.56 ± 0.06	1.63 ± 0.24	0.84 ± 0.17

The enzymatic activities are expressed as follows: GSH peroxidase and GSSG reductase (12) as nmoles NADPH oxidized/min/10^6 cells; catalase (31) as nmoles H_2O_2 degraded/min/10^6 cells; peroxidase (34) as nmoles tetraguaiacol formed/min/10^6 cells and SOD (33) as units/10^6 cells. One unit of SOD is the amount of enzyme that causes an inhibition of cytochrome *c* reduction corresponding to 0.0125 OD/min at 550 nm. The values are the means ± S.E.M. The number of experiments is given in parentheses.

[a] From Higgins et al. Proc. Soc. Exp. Biol. Med. 159, 478–481 (1978).
[b] Measured on 100,000 *g* supernatant of the whole homogenate.
[c] Measured on 100,000 *g* pellet of the whole homogenate.
[d] Measured on the whole homogenate.

3. Quantitative aspects of the respiratory burst among various types of phagocytic cell

The oxidative metabolism of activated phagocytes is qualitatively similar in all the cells so far examined. However marked quantitative differences exist among the various cell types as shown in Table 4. The main difference regards the extracellular release of superoxide anion and of hydrogen peroxide, as compared to the oxygen consumption. For example granulocytes and guinea-pig elicited peritoneal macrophages release considerable amounts of both superoxide anion and hydrogen peroxide, while this release is hardly detectable in resident alveolar macrophages of rabbit and is very low in BCG-activated alveolar macrophages of rabbit.

Both superoxide anion and hydrogen peroxide are toxic products and therefore the need arises for the presence of cell detoxifying mechanisms which may protect the cellular structures from the dangerous effects of these molecules. The detoxifying mechanisms may be either chemical or enzymatic. Ascorbate for example, which is abundant in phagocytes [35], may act as a scavenger of superoxide anion. The enzymatic degradation of superoxide anion is carried out by superoxide dismutase (SOD) and that of H_2O_2 by catalase, peroxidase and glutathione peroxidase. We have assayed these enzymes in different types of phagocyte and have found that their activities vary considerably. Table 5 shows in fact that all the granulocytes studied are rich in peroxidase, while this activity is low in macrophages. Macrophages on the other hand have a higher content of catalase (with the exception of rabbit peritoneal elicited macrophages) than polymorphs. Rabbit alveolar macrophages have the highest GSH-peroxidase activity among the phagocytes studied. These data suggest different patterns of hydrogen peroxide degradation in the two types of phagocyte. The SOD content appears to be higher in macrophages than in polymorphs, being the highest in rabbit resident alveolar macrophages, which release no superoxide anion and the lowest in rabbit granulocytes, which release considerable amounts of superoxide. Therefore the different activities of the enzymes which degrade O_2^- and H_2O_2 together with a different rate of production of these molecules may, at least in part, explain the heterogeneity of phagocytes with respect to O_2^- and H_2O_2 release.

4. Biologic role of the respiratory burst of phagocytes

The functional meaning of the respiratory burst is linked to the reactivity of the products of oxygen reduction. These are mainly superoxide anion and hydrogen peroxide but other molecules of biological interest can derive from them such as hydroxyl radical [36] and singlet oxygen [37, 38]. These products can exert both beneficial and harmful effects. The main beneficial effect is their participation in the oxygen dependent microbicidal and cytocidal activities of phagocytes, either inside or outside the cell [6, 36–40]. The harmful effects can be exerted both inside the cell, where a damage to the phagosome membrane or to the plasma membrane can result with, possibly, cell death [41] and outside with damage to endothelial cells [42] and to the surrounding connective tissue [43, 44]. Finally a modulation of the inflammatory process could result from an increase in microvascular permeability [45], the stimulation of histamine secretion [46], the generation of chemotactic factors [47], the influence on platelet aggregation [48], the lymphocyte stimulation [49]. Thus we can say that the respiratory burst has a dual central relevance in the inflammatory process since it gives origin to highly reactive molecules that kill the foreign invaders and, at the same time, damage the tissues.

The role of O_2^- and H_2O_2 in the host–parasite interaction is clearly documented by the occurrence of diseases caused by a defect in production and/or utilization of superoxide anion and hydrogen peroxide (see Ref. [50]). Chronic granulomatous disease of childhood, which is characterized by repeated infections is a typical example of a disease due to a defective production of O_2^- and H_2O_2. The molecular basis of this disease is in fact a lack of NADPH oxidase, the enzyme which produces O_2^-, or alternatively a defective activation of this enzyme during phagocytosis [28].

The MPO deficiency syndrome may be taken as an example of a disease in which the products of the oxygen metabolism cannot be utilized. In this case in fact both H_2O_2 and O_2^- are released from the defective cells in higher quantities than from the normal cells. While the reason for this high release of O_2^- is not clear, it is conceivable that the hydrogen peroxide escapes from the cells since it cannot be utilized in the H_2O_2–halide–MPO system described by KLEBANOFF [6] and SBARRA [39].

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