Advances in Inflammation Research,
Vol. 1, edited by G. Weissmann et al.
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Oxidative Metabolism of Inflammatory Cells

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RESPIRATORY BURST

Among the properties shared by granulocytes and macrophages, the most impressive is that of changing the oxidative metabolism during various functions. When challenged with bacteria, granulocytes and macrophages exhibit a dramatic increase in the oxygen consumption, in O_2^- and H_2O_2 production and in glucose catabolism through the hexose monophosphate pathway (HMP) (5,9,11,16,19,20,34,41,45,46,47,50). This group of events is referred to as the respiratory burst of phagocytes. This respiratory burst has the following characteristics: (a) It is insensitive to inhibitors of mitochondrial respiration. (b) All the events take place a few seconds after addition of the particles to the cells. Figure 1 shows that, the increase in oxygen consumption, in CO_2 production from 1-^{14}C-glucose and in O_2^- and H_2O_2 production is already evident 10 to 20 sec after the addition of bacteria to the phagocytes. These results strongly suggest that the respiratory changes are not postengulfment events, but are associated with the engulfing act. (c) By examining the relationship between the particle uptake and the time course of the increment of oxygen consumption, we have shown that the respiration falls back to the resting level when the engulfing act is completed (47). Thus, the duration of the respiratory burst is very short. It lasts until the ingestion is going on and ceases when it has been completed. (d) Although all the professional phagocytes are able to increase their oxidative metabolism during phagocytosis, the intensity of the various events of the respiratory burst greatly varies depending on the cell type, on the animal species, on the source of the cells, and for macrophages, on the state of activation (19,30,42,46). (e) A respiratory burst similar to that occurring during phagocytosis is induced when the phagocytic cells are challenged with a variety of membrane perturbing agents, such as endotoxin, complement fragments, kallikrein, phospholipase C, concanavalin A, etc. (44).

MECHANISMS RESPONSIBLE FOR THE RESPIRATORY BURST

The respiratory response involves a three element system: (a) a recognition at the level of the cell surface; (b) a transduction mechanism, that is a modification of the plasmamembrane whereby signals are generated; and (c) a target system,

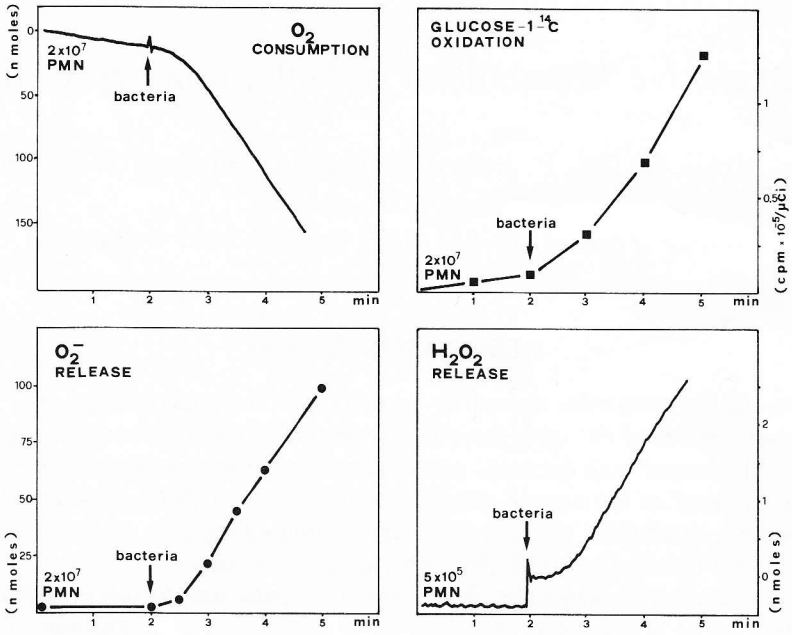

FIG. 1. Time course of the respiratory burst in guinea pig granulocytes.

that is the activation of the enzyme responsible for the increase of respiration.

The input signals, which induce the respiratory response, are various and interact in many ways with the cell surface (40,44). In some cases the recognition is nonspecific, as for example, when the stimulant is represented by detergents, by fatty acids, and by nonopsonized particles. In other cases the recognition is specific, that is, involves specific molecules, such as receptors for activated fragments of complement, chemotactic tetrapeptides, concanavalin A, Fc fragments of antibodies in immunocomplexes, etc. Many of these agents induce in the phagocytic cell, besides an activation of respiratory metabolism, other responses such as chemotaxis, chemokinesis, and exocytosis.

Many investigations have been performed in many laboratories on the modification of the plasmamembrane and on the signals that trigger the various responses. In spite of this, we are now faced more with problems than with facts (for a review on this matter see refs. 43,44).

The problem to be clarified is how stimuli of different nature involving different systems of recognition elicit a common response; that is, the activation of the respiratory metabolism, and vice versa how one stimulus can elicit different responses.

Enzymatic Basis of the Respiratory Burst

The enzymatic basis of the respiratory stimulation has been the subject of many controversies with regard to the substrate specificity: reduced nicotinamide-

TABLE 1. *Properties of NADPH-oxidase activity*

1. The V_{max} increases and the oxygen consumed in the oxidation of NADPH by granule fraction accounts for the respiration of the corresponding stimulated cells.
2. The affinity for NADPH increases. Its apparent K_m decreases 10 times, thus reaching a value which is very closed to the physiological concentration of NADPH in intact cells.
3. The activity is insensitive to KCN (in keeping with the insensitivity of the respiratory burst).
4. The oxidation of NADPH is associated with the formation of O_2^- and of H_2O_2.
5. The activation of the enzyme starts off in a matter of a few seconds.
6. The activation is reversible.
7. The maximal activity is performed at pH 5.5 to 6.0

adenine dinucleotide phosphate (NADPH) or of nicotinamide-adenine dinucleotide (NADH) and to the localization of the enzyme (intracellular or plasmamembrane) (18,45). Direct and indirect evidences clearly indicate that the respiratory burst is due to an activation of an oxidase that preferentially uses NADPH as substrate (3,10,13,14,16,35,36,45,47,54). This enzyme is practically inactive in resting cells, but increases its V_{max} and its affinity for NADPH on stimulation of the phagocytes with particles or with membrane perturbing agents. The properties of the enzyme, measured as oxygen consumption on 20,000 g pellet of postnuclear supernatants of activated cell homogenates, fit very well for the characteristics requested for a main role in the respiratory burst (45). These properties are reported in Table 1.

As regards the localization of the enzyme, different results have been presented by various authors by using different cell types and methods of assay. Direct evidence that granulocyte NADPH-oxidase is localized in a granule fraction analogous to lysosomes has been provided in our (32,44) and in other (14,15) laboratories by using subcellular fractions separated from human, guinea pig, and rabbit granulocyte homogenates.

According to others, an oxidase would be localized either in the cytosol or in the plasmamembrane of granulocytes. However, it is worthy to stress that most of the evidence supporting these views is indirect and that the direct ones either concern an enzyme which oxidizes NADH or have been obtained by measuring an activity that does not account for the oxygen consumption of the intact cells (for a review on this topic see ref. 31). Andrew and coworkers (2) showed that in alveolar macrophages of rabbit the oxidase is localized in the endoplasmic reticulum. (See note added in proof, page 154.)

It is likely that these discrepancies are due to different methods of assay (O_2 consumption, O_2^- production, NADPH-disappearance). In summary, the problem of NADPH-oxidase subcellular localization is still open. It is likely, however, that the rationale concerning the characterization of the enzyme responsible for the respiratory burst is based on noncorrect alternatives, that is NADH or NADPH oxidase, localization in subcellular structures or in the plasmamembrane. A participation of different enzymes with different localization and with different relevance and/or significance, could be involved in the respiratory burst. In this view and on the basis of our experimental results, the more effective enzyme would be the NADPH-oxidase located in the granule fraction; other

oxidases, active on NADPH or NADH, could either represent a supplementary source of O_2^- and/or act as a sparking mechanism. In this context a participation of a peroxidase in the oxidation of NADPH, as suggested by Roberts and Quastel (39) and discussed by Patriarca et al. (31), might be acceptable.

FORMATION OF THE INTERMEDIATE PRODUCTS OF OXYGEN REDUCTION

The basic assumption, which is widely accepted, is that all oxygen consumption involves an univalent reduction of oxygen, followed by the formation of H_2O_2. The activation of NADPH oxidase activity leads to the formation of O_2^-. The mechanism of this enzymatic reaction is not completely elucidated. According to Babior and Kipnes (4) the enzyme is a flavoprotein. On the basis of the inhibitory effect of superoxide dismutase (SOD) and of redogenic substances, such as ascorbate and hydroquinone, and of the stimulatory effect of manganese ions, we have proposed a multistep mechanism. This would include an enzymatic reaction producing a free radical followed by a nonenzymatic chain reaction (33).

The O_2^- formed can react with many compounds. Its main fate is the intracellular dismutation to O_2 and H_2O_2, either spontaneously or by SOD catalysis. Part of O_2^- escapes the intracellular dismutation and is released outside the cells, where it can react with other molecules or dismutate to O_2 and H_2O_2 (extracellular dismutation). Hydrogen peroxide derived from O_2^- dismutation can react with a variety of molecules inside the cell. Its main fate is the enzymatic degradation catalyzed by catalase, peroxidase, and glutathione peroxidase.

It is widely accepted that part of H_2O_2 formed inside the cell escapes the degradation and is released outside the cell (6,21,41). In this chapter we present data which clearly show that, in the absence of peroxidase and catalase inhibitors, all H_2O_2 molecules are degraded inside the cell.

OXYGEN CONSUMPTION, O_2^-, AND H_2O_2 RELEASE IN DIFFERENT PHAGOCYTES

By examining the correlation between oxygen consumption and extracellular recovery of O_2^- and H_2O_2 in various phagocytic cells of mammalian species, we have found a great variability. The results reported in Table 2 show that: (a) The stimulation of oxygen consumption during phagocytosis occurs in all the phagocytic cells. (b) In granulocytes and in guinea pig peritoneal macrophages, the respiratory increment is associated with a substantial recovery of O_2^- and H_2O_2 in the extracellular medium. (c) In alveolar macrophages of rabbit, both resident and activated by bacillus Calmette-Guérin (BCG), this recovery is negligible. (d) Peritoneal macrophages of rabbit show an intermediate situation between granulocytes and alveolar macrophages.

Two explanations would account for these differences: (a) in some cells, such

TABLE 2. *O_2 consumption, O_2^-, and H_2O_2 release during phagocytosis*[a]

	nmoles/4 min/1.5 × 10^7 cells		
	O_2	H_2O_2	O_2^-
Granulocytes			
human blood	151.1 ± 12.1 (4)	23.0 (2)	50.0 ± 9.2 (4)
guinea pig peritoneal exudate	188.7 ± 44.4 (3)	47.2 ± 12.0 (3)	96.1 ± 23.2 (3)
rabbit peritoneal exudate	183.2 ± 21.5 (3)	24.9 ± 3.7 (3)	45.4 ± 5.4 (3)
Macrophages			
guinea pig peritoneal elicited	218.9 ± 33.5 (4)	77.3 (2)	135.2 ± 27.4 (3)
rabbit peritoneal elicited	75.5 ± 18.0 (5)	17.1 ± 2.2 (3)	6.1 ± 0.9 (3)
rabbit alveolar resident	66.1 ± 12.6 (4)	0 (4)	0.4[0–1.4](4)
rabbit alveolar BCG-activated	97.7 ± 14.1 (9)	6.5 ± 1.2 (4)	5.0 ± 1.4 (4)

[a] The differences between phagocytosing and resting cells are reported, as mean ± SEM of the number of experiments indicated in parentheses. Assay medium: 1 − 2 × 10^7 granulocytes or macrophages in 2 ml of KRP containing 0.5 mM $CaCl_2$ 5 mM glucose. Opsonized B mycoides were used as stimulatory agent (ratio cell/bacteria, 1/100). Oxygen consumption was measured polarographically with a Clark-type oxygen electrode. Hydrogen peroxide was measured fluorimetrically by the conversion of the nonfluorescent compound homovanillic acid (HVA) to the highly fluorescent 2,2′-dihydroxy-3,3′-dimetoxydiphenil-5,5′-diacetic acid, catalyzed by horseradish peroxidase (HRP) in the presence of H_2O_2 (42). HVA (0.8 mM) and HRP (20 μg/ml) were included in the incubation mixture where oxygen consumption was being continuously recorded and the fluorescence developed was measured on supernatants of samples withdrawn from the electrode vessel. Superoxide anion was measured by the superoxide inhibitable reduction of cytochrome *c* (5,42), on samples withdrawn from the electrode vessel. Human leukocytes were obtained by dextran sedimentation. Rabbit and guinea pig granulocytes were collected from acute peritoneal exudates induced by casein. Macrophages were obtained as described elsewhere (46).

as alveolar macrophages, the oxygen consumption is not associated with O_2^- and H_2O_2 formation; and (b) the mechanisms and the rate of intracellular degradation of O_2^- and H_2O_2 have different efficiencies in different cell types.

As regards the first point, we have shown that under appropriate experimental conditions (42) also in alveolar macrophages, the respiratory burst is associated with production of O_2^- and H_2O_2. In fact, as shown in Table 3, the increase in oxygen consumption, induced by phagocytosis in cytochalasin B-treated alveolar macrophages of rabbit, is associated with an appreciable recovery of O_2^- and H_2O_2 in the extracellular medium. As regards the second point, we have measured the activity of the enzymes that are involved in the degradation of O_2^- and H_2O_2. The results, reported in Table 4, show that: (a) The SOD activity is higher in macrophages than in granulocytes. (b) The main reaction for H_2O_2 degradation is represented by peroxidase in granulocytes and by glutathione-peroxidase in macrophages.

TABLE 3. *O_2 consumption O_2^- and H_2O_2 release by phagocytosing BCG-activated alveolar macrophages from rabbit in the absence and in the presence of cytochalasin B*[a]

	nmoles/4 min/1.5 × 10^7 cells	
	+ bacteria	+ bacteria + CB
O_2	71.3 ± 12.8 (6)	38.1 ± 5.7 (4)
O_2^-	6.0 ± 1.5 (6)	24.5 ± 9.1 (4)
H_2O_2	7.1 ± 1.9 (6)	9.6 ± 1.7 (4)
$\%O_2$ recovered as H_2O_2	10	25

[a] The differences between phagocytosing and resting cells are reported, as mean ± SEM of the number of experiments indicated in parentheses. For assay conditions see Table 2. CB, 5 μg/ml. NaN_3, 2 mM.

The problem arises as to whether these patterns of enzymatic activities among various cells reflect an actual difference in the relative capacity of different mechanisms in the intracellular degradation of H_2O_2. In order to clarify this problem, we have investigated the fate of H_2O_2 in cells treated with NaN_3, a compound that inhibits catalase and peroxidase and does not influence the activity of glutathione peroxidase. Thus, in cells where H_2O_2 is exclusively or mainly degraded by glutathione-peroxidase, the expectation is that NaN_3 does not modify the accumulation of hydrogen peroxide. As shown in Table 5, this is, in fact, the case for rabbit alveolar macrophages and for peritoneal macrophages of guinea pig. Conversely, in the cells where H_2O_2 is mainly degraded by catalase and peroxidase, the expectation is that the presence of NaN_3 induces an increase of H_2O_2 accumulation. As shown in Table 5, this is the case for granulocytes and partially for peritoneal macrophages of rabbit. It is worthy to point out that the H_2O_2 accumulated in the extracellular medium of phagocytosing granulocytes in the presence of NaN_3 corresponds to the total amount that is formed, as shown in Table 6. The data of Table 6 also show that the activation of the HMP occurs independently of H_2O_2 degradation through the glutathione cycle. This fact is an indirect, but strong, evidence that at least in granulocytes the HMP activity is stimulated by a direct change of the NADPH/$NADP^+$ ratio via NADPH oxidase.

THE FATE OF O_2^- AND OF H_2O_2

By considering the activities of catalase, peroxidase, and glutathione peroxidase (Table 4), it seems that all the phagocytic cells are on the whole equipped with a very efficient machinery for H_2O_2 degradation. However, the fact that in the absence of inhibitors, H_2O_2 is recovered in the extracellular medium during the respiratory burst of many cells, seems to indicate that the actual efficiency of the mechanisms of its degradation is not so high and that part of the H_2O_2 formed is released. The analysis of the amount of O_2^- and of H_2O_2 that are measurable in the extracellular medium during the respiratory burst

TABLE 4. *Enzyme activities of different phagocytes*[a]

	Granulocytes			Macrophages			
	Human blood (5)	Rabbit peritoneal exudate (6)	Guinea pig peritoneal exudate (3)	Guinea pig peritoneal elicited (2)	Rabbit peritoneal elicited (4)	Rabbit alveolar resident (5)	Rabbit alveolar BCG-activated (8)
GSH-peroxidase[b]	1.8 ± 0.4	0.6 ± 0.2	0.6 ± 0.2	5.0	12.4 ± 4.0	25.4 ± 3.6	75.3 ± 13.7
GSSG-reductase[b]	2.0 ± 0.5	0.5 ± 0.07	1.6 ± 0.7	7.0	3.7 ± 1.3	3.8 ± 0.5	5.3 ± 0.4
Catalase[b]	14.5 ± 3.2	4.0 ± 0.3	6.7 ± 3.1	20.8	4.6 ± 2.6	31.3 ± 1.2	26.2 ± 3.1
Peroxidase[c]	365.0 ± 65.0	32.3 ± 12.5	115.0 ± 28.0	19.0	6.0 ± 3.0	6.3 ± 1.9	2.0 ± 0.3
SOD[d]	0.44 ± 0.05	0.13 ± 0.03	0.18 ± 0.01	0.69	0.56 ± 0.06	1.63 ± 0.34	0.94 ± 0.17

[a] The mean (Units/10^6 cells) ± SEM is reported. The number of experiments is given in parentheses. GSH-peroxidase, GSSG-reductase: nmoles NADPH/ min. Catalase: μmoles H_2O_2/min. Peroxidase: nmoles tetraguaiacol/min. One unit of superoxide dismutase (SOD) is the amount of enzyme that causes a decrease in the reduction of cytochrome *c* of 0.0125 OD/min.

[b] Measured on 100,000 g supernatant.

[c] Measured on 100,000 g pellet.

[d] Measured on total homogenate.

TABLE 5. *H_2O_2 measurable in the extracellular medium during phagocytosis in the absence and in the presence of NaN_3*[a]

	—	+ NaN_3
Granulocytes		
human blood	23 (2)	140.1 (2)
guinea pig peritoneal exudate	47.2 ± 12.0 (3)	234.6 ± 68.1 (3)
rabbit peritoneal exudate	24.9 ± 3.7 (3)	230.0 ± 14.1 (3)
Macrophages		
guinea pig peritoneal elicited	77.3 (2)	82.8 (2)
rabbit peritoneal elicited	17.1 ± 2.1 (3)	48.0 ± 7.4 (3)
rabbit alveolar resident	0 (4)	0.3[0–0.6](4)
rabbit alveolar BCG-activated	6.5 ± 1.2 (4)	7.2 ± 1.1 (9)

[a] The differences between phagocytosing and resting cells are reported. The values are expressed as nmoles/4 min/1.5 × 10^7 cells ± SEM. Number of experiments in parentheses. For assay conditions, see Table 2. NaN_3, 2 mM.

of granulocytes indicates that the only species released is O_2^-. In fact, the data of Table 7, show that the stoichiometric relationship between the amount of O_2^- and of H_2O_2 measured outside the cell is near to a ratio of 2:1, which corresponds to the stoichiometry of the reaction of dismutation. Thus, during the respiratory burst of granulocytes, (a) all the hydrogen peroxide formed inside the cells is degraded and (b) only O_2^- is released and the hydrogen peroxide found outside the cells derives from the dismutation of such O_2^-.

On the basis of this result the main problem regarding the extracellular disposability of the intermediates of oxygen reduction concerns the mechanism(s) that control(s) the release of superoxide anion, that is: (a) how it gets through the plasmamembrane, (b) how it escapes the activity of SOD, and (c) how much of the total O_2^- generated is released from the cell.

TABLE 6. *Hexose monophosphate pathway stimulation independent on H_2O_2 degradation in rabbit polymorphonuclear leukocytes*

	nmoles/4 min/1.5 × 10^7 cells	
	Resting + NaN_3	Phagocytosing + NaN_3
O_2	18.4	240.5
H_2O_2	2.3	230.1
$^{14}CO_2$ from 1-^{14}C glucose	6.3	45.9

The values are mean of three separate experiments.

TABLE 7. *O_2^- and H_2O_2 measured in the extracellular medium during the respiratory burst induced by phagocytosis in polymorphonuclear leukocytes*[a]

	nmoles/4 min/1.5 × 10^7 cells		
	O_2^-	Theoretical H_2O_2 formed by dismutation of O_2^-[b]	H_2O_2
Human blood	50.0	25.0	23.0
Guinea pig	96.1	48.05	47.2
Rabbit	45.4	22.7	24.9

[a] From the data of Table 2.
[b] $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

Lynch and Fridovich (24) by using either 4-acetamido-4′ isothiocyano-2-2′ disulfonic acid stilbene (SITS) or 4-4′ diisothiocyano-2-2′ disulfonic acid stilbene (DIDS), have shown that O_2^- crosses the membrane of erythrocytes through anionic channels. The existence of anionic channels in the phagocytic cells and their differences or modifications could explain some differences found in the release of O_2^- in various phagocytes.

The possibility that O_2^- escapes the intracellular dismutation depends on the rate of its formation, on the activity level, and on the subcellular localization of SOD. As regards the activity level of SOD, our data (reported in Table 8) show that an inverse correlation exists between the total activity of the enzyme and the amount of O_2^- released, except for guinea pig elicited peritoneal macrophages. As regards the subcellular localization of SOD, the data reported by various authors are controversial. According to Rister and Baehner (38), the SOD activity is low in the cytosol of guinea pig granulocytes and peritoneal macrophages, while it is high in the cytosol of alveolar macrophages. From the data presented by others (17,37,49), human granulocytes would have a high content of Cu-Zn SOD in cytosol. On this basis, it has been suggested that O_2^- might escape the dismutation and diffuse in the external medium, since in these cells the oxidase forming the radical would be located on the plasmamembrane (17,41,49). This suggestion seems rather weak. First of all we have shown that in some cells O_2^- is not released (alveolar macrophages—Table 2), while in other cells only a small aliquot of the total O_2^- generated is released and the main fraction dismutates to O_2 and H_2O_2 inside the cell. Thus one wonders why not all the O_2^- generated at the level of the plasmamembrane is released. Second, experimental evidence has been provided that O_2^- can escape the activity of SOD. Lynch and Fridovich (23) have recently shown that O_2^- produced by xanthine-oxidase, sealed in vesicles prepared from erythrocytes stroma, crosses the membrane and is recovered outside the vesicles. This release is only partially diminished when SOD is also sealed in the vesicles, that is in the site where O_2^- is formed. These results indicate that O_2^- can escape from a cell when it is generated in a site where also SOD is present, and that in order to explain its

TABLE 8. *Correlation between SOD activity and O_2^- release*[a]

	Granulocytes			Macrophages			
	Human blood	Rabbit peritoneal exudate	Guinea pig peritoneal exudate	Guinea pig peritoneal elicited	Rabbit peritoneal elicited	Rabbit alveolar resident	Rabbit alveolar BCG-activated
SOD units/1.5×10^7 cells	6.6	1.9	2.7	10.3	8.4	24.4	14.1
O_2^- nmoles/4 min/ 1.5×10^7 cells	50.0	45.4	96.1	135.2	6.1	0.4	5.0

[a] From the data of Tables 2 and 4.

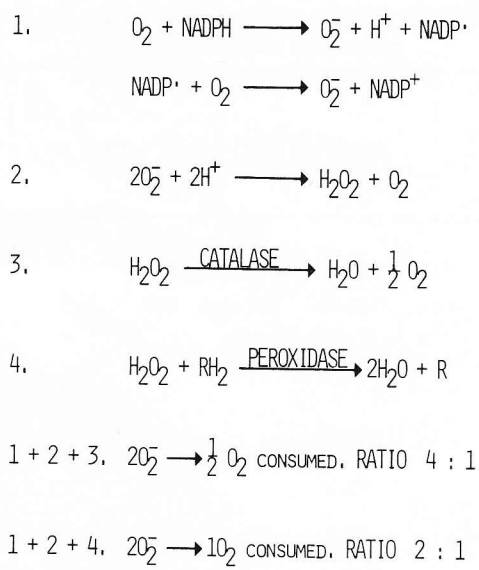

FIG. 2. Reactions involved in O_2 consumption and O_2^- and H_2O_2 degradation. For explanation see text.

secretion from the cells it is not necessary to postulate a generation in the plasmamembrane.

Finally, it has been recently shown (12) that in stimulated human granulocytes the activity of SOD drastically decreases, thus allowing more superoxide anion to escape the enzymatic degradation and to diffuse in the extracellular environment.

The last aspect to be considered is the amount of O_2^- that is released with respect to the total amount that is formed. In order to clarify this problem, we have to know how many nanomoles of O_2^- are formed for each nanomole of O_2 actually consumed. Assuming that the main fate of O_2^- formed is the dismutation to O_2 and H_2O_2, the ratio between the O_2 actually consumed and the O_2^- formed depends on the mechanism of H_2O_2 degradation (Fig. 2).

If the sequence of reaction involves the catalatic degradation of H_2O_2 (reactions $1 + 2 + 3$), for 2 nmoles of O_2^- formed 0.5 nmoles of O_2 are actually consumed (ratio $O_2^-/O_2 = 4$). If H_2O_2 is degraded by a peroxidatic mechanism (reaction $1 + 2 + 4$), for 2 nmoles of O_2^- formed 1 nmole of O_2 is actually consumed (ratio $O_2^-/O_2 = 2$). With this relationship in mind, we can calculate the amount of O_2^- released in relation to the amount of radical actually formed. Table 9 reports the values of the oxygen actually consumed and of O_2^- measured outside the cells in different phagocytes during phagocytosis. The data of columns A and B are theoretical and are calculated assuming that all the hydrogen peroxide is degraded by catalase (A) or by peroxidase (B). In different phagocytic cells the actual value of the percentage of O_2^- generated, which is released,

TABLE 9. *Values of O_2 actually consumed and O_2^- measured outside the cells in different phagocytes during phagocytosis*[a]

	nmoles/4 min/1.5 × 10^7 cells		O_2^- released percentage of O_2^- generated	
	O_2	O_2^- released	A[b]	B[c]
Granulocytes				
human blood	151.1	50.0	8.2	16.4
guinea pig peritoneal exudate	188.7	96.1	13.0	26.0
rabbit peritoneal exudate	183.2	45.4	6.0	12.0
Macrophages				
guinea pig peritoneal elicited	218.9	135.2	15.5	31.0
rabbit peritoneal elicited	75.5	6.1	2.0	4.0
rabbit alveolar resident	66.1	0.4	0.15	0.3
rabbit alveolar BCG-activated	97.7	5.0	1.3	2.6

[a] From the data of Table 2. For explanations see text.
[b] A = H_2O_2 degraded by catalase; theoretical ratio O_2^-/O_2 = 4:1.
[c] B = H_2O_2 degraded by peroxidatic reactions; theoretical ratio O_2^-/O_2 = 4:2.

lies between the figures of columns A and B, depending on the relative importance of the catalase and peroxidase mechanisms for H_2O_2 degradation. It is worthy to point out that the percentage of O_2^- which is released is rather low in every cell type we have examined.

The results so far presented can be summarized as follows: (a) the respiratory burst is associated with the formation of O_2^- and H_2O_2 in all the phagocytic cells; (b) the mechanism and the rate of the degradation of the intermediates are different among the phagocytic cells; (c) in physiological conditions all the H_2O_2 formed inside the cells is degraded; (d) the only molecular species released is O_2^-; (e) the amount of O_2^- released is always low with respect to the total amount generated by the oxidase; and (f) the amount of O_2^- released is extremely variable among the phagocytic cells. In general terms it can be said that the release is high in granulocytes, the cells of acute inflammation. In this respect the mononuclear phagocytes are heterogeneous. In some of these cells the release of O_2^- is negligible.

FUNCTION OF THE RESPIRATORY BURST

Before discussing this matter, some points have to be outlined. The first one is that the functional meaning of the respiratory burst is linked to the reactivity of the intermediate products of oxygen reduction. The main species are superox-

ide anion and hydrogen peroxide, but other reactive compounds, such as hydroxyl radical ($OH^{\cdot}$) and singlet oxygen, can be formed. Hydroxyl radical derives from the reaction between superoxide anion and hydrogen peroxide ($O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$) and singlet oxygen can be formed by MPO system or by decomposition of superoxide anion.

The second point is that the respiratory burst occurs in the phagocytes not only during phagocytosis, but also when the cells receive chemical signals of different nature, as we have seen before. Thus, it seems natural that the state of respiratory perturbation is a common feature of the cells engaged in different functions during the inflammatory response, such as during chemotactic migration, during the sticking to endothelial cells, when they stay in the inflammatory site and during phagocytosis.

It is also worthy to point out that the respiratory burst is a reversible phenomenon. The burst induced by phagocytosis is very short, being triggered by the engulfing act and ceasing when the engulfment is completed. In other cases, the activation of respiration is linked to a continuous interaction between the stimulatory factors and the target molecules of the cell surface (46).

In an attempt to simplify the matter it seems reasonable to distinguish the effect of the respiratory burst in beneficial and harmful, intracellular and extracellular.

The main beneficial effect is that of providing a very powerful system for killing ingested organisms, in addition to the other mechanisms such as the discharge of enzymes or other factors into the phagosome. The oxygen-dependent bactericidal system is operative mostly in the phagocytic vacuoles and also in extracellular environment, and involves both the participation of peroxidase and a direct effect of H_2O_2, O_2^-, $OH^{\cdot}$ and singlet oxygen (1,17). The peroxidase-mediated system uses the peroxidase secreted into the phagosome or in the extracellular medium, H_2O_2 derived from O_2^- released, and a halide as oxidable factor. The final mechanism is due to toxic agents produced by the reaction between peroxidase, H_2O_2, and halide. It is likely that the toxic agents include chlorinium ions, chloramine, aldehyde, and singlet oxygen (20,51).

The whole efficiency of the killing mechanism linked to the respiratory burst is dependent on the amount of the intermediates produced, on their release, on the activity, on the secretion of peroxidase from intracellular storage, and on the length of the respiratory burst. Since these events are variable, this mechanism plays a different role as bactericidal system in different phagocytes. The efficiency is high in granulocytes owing to a high generation and release of O_2^-. Mononuclear phagocytes are heterogeneous, as regards the intensity of the respiratory burst, the content of peroxidase, the amount of the release of the intermediates and, hence, the efficiency of the oxygen-dependent mechanism of killing. On this basis, it seems that this mechanism is operative in blood monocytes and in tissue macrophages recently migrated from blood stream (21), while it seems to be inefficient in resident and recently migrated alveolar macrophages.

Another important factor in relation to the efficiency of the oxygen-dependent system for killing microorganisms is the length of the respiratory burst. Since this event is triggered by the engulfing act (40,47) and ceases when the engulfment is completed, as we have shown *in vitro* (47), the efficiency of the system is very short. This is a theoretical situation that can occur when a phagocyte engulfs one or two bacteria. A helping effect can take place when a phagocyte engulfs many bacteria, since in this case, the subsequent acts of engulfment protract the length of the respiratory burst. If this is the case, among the various mechanisms for killing bacteria, the phagocytes use the oxygen-dependent one only in the first stage.

It is likely that an increased efficiency of this killing mechanism is performed by other mechanisms. In the inflammatory site, the phagocytic cells are continuously challenged by other stimulatory agents (chemotactic factors, endotoxins, material deriving from tissue damage, etc.) and these agents sustain the respiratory burst also when the engulfing act is completed. This would be a sort of subsidiary system that maintains and enhances the discharge of free radicals in the phagocytic vacuole. Figure 3B schematically shows this helping effect.

Granulocytes and macrophages can exert a cytotoxic and cytostatic activity against a variety of target cells, besides bacteria, including tumor cells, indepen-

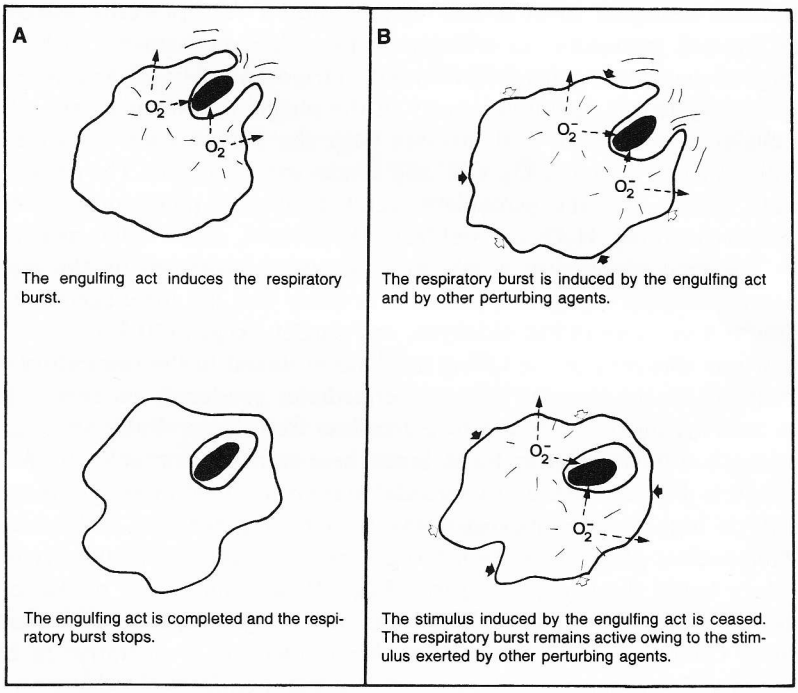

FIG. 3. A postulated helping effect **(B)** on oxygen-dependent killing exerted by membrane perturbing agents. For explanation see text.

dently on phagocytosis. This activity is performed by contact between phagocytic cells and target cells mediated by antibodies (antibody mediated cytotoxicity) or by contact between immunological armed or activated macrophages and tumor cells. Evidence has been provided that in this event the respiratory burst is essential for cytotoxicity (8).

On the basis of what we know on the free-radical pathology, it is evident that O_2^-, H_2O_2, $OH^{\cdot}$, and singlet oxygen can produce harmful effects on the tissue components. Direct evidence on harmful effects of free radicals produced during the respiratory burst of leukocytes on external structure during the inflammatory process (27) and on endothelial cells (48) has been presented.

Another important effect of the respiratory burst is a damage of some cell structure of the phagocyte itself. This event can be produced by intermediates discharged outside the cell (28) and by their intracellular reactivity. As a consequence, the death of the cell occurs and the discharge of cellular components can amplify the tissue damage.

It is worthy to point out, however, that to limit the extracellular effects to a damage is an oversimplification and that a number of problems and facts remain to be reinvestigated and settled. It is likely that indirectly the respiratory burst plays a role in the modulation and in the amplification of the inflammatory response. Some examples can be mentioned. The discharge of leukocyte components following a self-direct cytotoxicity can contribute to the activation of complement with the generation of chemotactic fragments (53); the depolymerization of jaluronic acid produced by hydroxyl radical (27) can facilitate the movement of the inflammatory cells in the tissue and in the passage through the vessels; the endothelial damage might be involved in the increased permeability in the delayed phase of the inflammatory process and in the attachment of the inflammatory cells to modified internal surface of the vessels; the influence on the aggregation of platelets by H_2O_2 (22) or by other compounds can modulate the participation of these elements both in hemostatic and in inflammatory response.

The last problem to be considered concerns the significance of the respiratory burst in cell function, that is the intracellular effect of the intermediates of oxygen reduction. At present, this matter is practically unexplored and we can hint at this problem only in perspective. The rationale of this problem is the great disproportion between the enormous amount of H_2O_2 and of free radicals that the phagocytes produce in very short time and the amount that they release. This release in some cells is practically absent. In general terms the problem arises whether or not the respiratory burst is instrumental in the secretory process, in the movement, in the process of fusion, in the modification of the fluidity of the membranes, in the assembly or disassembly of microtubules, and in prostaglandin biosynthesis. Experimental evidence obtained in various laboratories indicate that some of the processes previously listed can be influenced by the products of oxygen reduction during the respiratory burst (7,25,26,29,52).

It is obvious, however, that a demonstration of the involvement of these

compounds in some biological activities does not allow an association with the respiratory burst. In fact, these intermediates can be directly or indirectly generated in other enzymatic reactions not related with those responsible for the activation of respiration of the inflammatory cells.

ACKNOWLEDGMENT

BCG was kindly supplied by Istituto Vaccinogeno Antitubercolare, Milano. This work was supported by Grant No. 77. 0148.04 from the National Research Council of Italy (CNR). P. Bellavite is recipient of a fellowship from A. V. Rusconi Foundation, Varese, Italy.

NOTE ADDED IN PROOF

While this paper was in press two reports were published showing that the NADPH-oxidase would be located in the plasma membrane of granulocytes (Dewald, B. et al. (1979): *J. Clin. Invest.,* 63:21–29; Cohen, H. J. et al. (1978): *Fed. Proc.,* 37:(abs 40),1276.)

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