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Molecular bases of the metabolic excitability of phagocytes

D. Romeo, P. Dri, P. Bellavite and F. Rossi

A number of oxidative reactions, lethal to many bacteria, fungi, certain viruses and mycoplasmas, are activated by phagocytosis in polymorphonuclear leukocytes (PMNL) and macrophages[1-4].

The efficiency of these microbicidal systems depends on the continuous supply of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), the main products of the increased O_2 reduction in phagocytosing leukocytes[3, 5-14]. The mechanism of generation and utilization of these compounds has been the subject of extensive investigation in several laboratories. Suitable techniques have been set up to measure the rate and extent of O_2 consumption and of concomitant generation of O_2^-, H_2O_2 and $NADP^+$, especially in the early stage following cell exposure to phagocytosable particles.

METHODOLOGY

The most appropriate way of measuring the consumption of O_2 by phagocytes is that of following the rate of respiration of a cell suspension, before and after addition of particulate objects, by means of an oxygen elec-

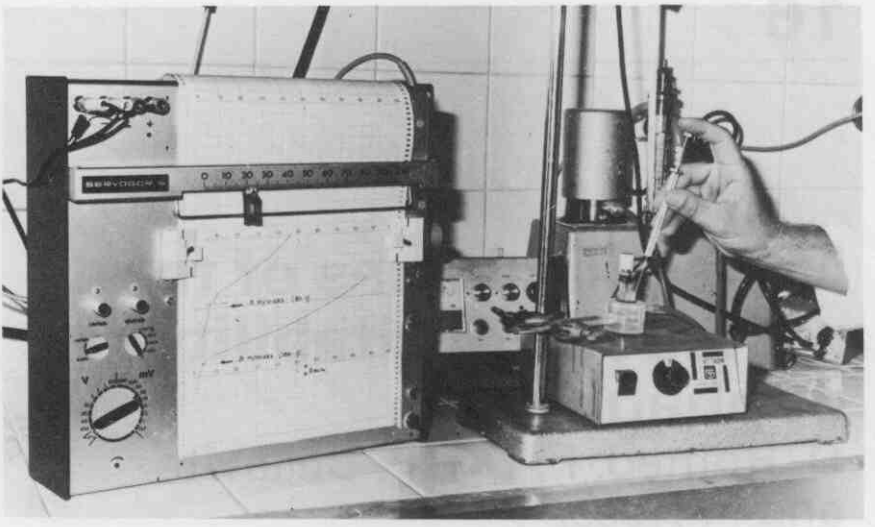


Figure 16.1 Polarographic assembly and recorded traces of oxygen consumption by PMNL

trode[15]. We currently use a Clark-type oxygen electrode attached to a thermostatically controlled (37 °C) plastic vessel. Each sample contains 2 ml of cell suspension ($1–2 \times 10^7$ cells) stirred magnetically and additions of activators of cell metabolism are made through a narrow puncture in the lid covering the vessel. Our polarographic set-up and a typical polarographic trace are shown in Figure 16.1.

The rate of hydrogen peroxide release from the cells can be measured fluorometrically by the decrease of scopoletin fluorescence in the presence of horseradish peroxidase (HRP)[12]. Scopoletin (7-hydroxy-6-methoxy-coumarin) emits a blue fluorescence when excited with light of 350 nm wavelength (emission 460 nm). In the presence of H_2O_2 it is oxidized by HRP yielding a loss of fluorescence which is directly proportional to the peroxide concentration in the medium. Hydrogen peroxide can also be determined colorimetrically with the ferrithiocyanate method[16]. Briefly, portions of a cell suspension are treated with trichloroacetic acid and, after removal of precipitated protein by centrifugation, reacted with ferrous ammonium sulphate and potassium thiocyanate. The absorption of the red thiocyanate complex formed in the presence of H_2O_2 is read at 480 nm. With the two methods, determinations of H_2O_2 on standard H_2O_2 solutions as well as on samples of phagocytes provide results which match very closely each other[14].

The assay of O_2^- is in general confined to the amount of this radical which is recovered outside the cell, where it reacts with exogenous ferricytochrome *c* in a stoichiometric relationship of 1:1[10]. The amount of O_2^--dependent reduction of cytochrome *c* is calculated from the difference of absorbance between the cytochrome *c* reduced in the absence of superoxide dismutase (SOD) and the cytochrome *c* reduced in the presence of SOD, by using an extinction coefficient[14] of 21.1 mM^{-1} cm^{-1}; O_2^- production can also be determined by measuring SOD-sensitive reduction of nitroblue tetrazolium (NBT) to formazan at 530 nm, by using an E_m M for formazan of 18.3 and a stoichiometric relationship between O_2^- formation and NBT reduction of two to one[4,17].

Dri *et al.*[14] have combined these techniques to obtain a simultaneous determination of O_2 consumption and recovery of O_2^- and H_2O_2 in the same cell suspension. Briefly, they measured the consumption of O_2 in the absence or in the presence of cytochrome *c* (to trap O_2^-) and of NaN_3 (to inhibit the peroxidatic and catalatic degradation of H_2O_2). At 2 min from the addition of phagocytosable particles to the leukocytes, portions of the cell suspension are quickly transferred from the vessel, where O_2 consumption is recorded, into an Eppendorf microtube and centrifuged (when measuring O_2^-, the microtubes contain SOD to prevent further cytochrome *c* reduction). The cell free supernatants are then used for the determination of H_2O_2 and of the extent of O_2^--dependent ferricytochrome *c* reduction.

Coupled to the enhanced O_2 reduction in phagocytosing leukocytes there is also an increased utilization of glucose in the oxidative route of the hexose monophosphate pathway (HMP)[18-23]. The yield of $^{14}CO_2$ from 1-[^{14}C]glucose can be evaluated either after a suitable incubation time or by continuous sampling from the O_2 electrode vessel[9]. In the former case, the leukocyte suspension is added to Erlenmeyer flasks, which are shaken at 37 °C in a Dubnoff incubator. After addition of labelled glucose and of suitable metabolic stimulants, the flasks are rapidly covered with a rubber cap. The reaction is terminated by injecting H_2SO_4 through the cap and $^{14}CO_2$, trapped in a centre well containing KOH, is quantitated by liquid scintillation spectrometry. Alternatively, labelled glucose can be added to the oxygen electrode vessel: in the course of the measurement of oxygen consumption, small portions of the cell suspension are withdrawn at suitable time intervals with a microsyringe, and rapidly injected into rubber-capped flasks containing H_2SO_4.

KINETICS OF STIMULATION OF THE OXIDATIVE METABOLISM OF PHAGOCYTES

The methods described above allow a continuous recording of the process of activation of oxidative metabolism in phagocytosing leukocytes. This has permitted us to observe that the onset of phagocytosis-associated stimulation of O_2 reduction to O_2^- and H_2O_2 and of HMP activity falls a few seconds after exposure of leukocytes to the phagocytosable objects. This is shown by the representative experiments of Figures 16.2 and 16.3.

The overall rate of the oxidative route of HMP is dependent on the cellular $NADP^+$ concentration[24]. Thus one would expect that the increased rate of glucose oxidation by phagocytosing PMNL is sustained by a sudden increase in the steady-state concentration of $NADP^+$. Rossi *et al.*[23] have indeed shown that 3 min after the exposure of leukocytes to bacteria there is a 3-fold increase in the $NADP^+$:NADPH ratio, whereas the steady-state concentrations of NAD^+ and NADH vary very slightly (Table 16.1).

Continuous monitoring of O_2 disappearance from the cell-suspending medium in the electrode vessel (Figures 16.1 and 16.2) indicates that the

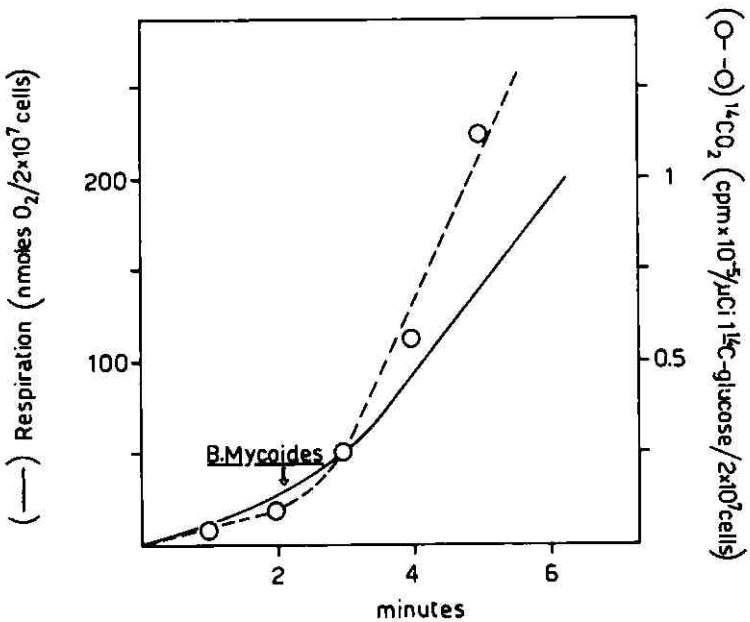


Figure 16.2 Simultaneous evaluation of the kinetics of stimulation of O_2 consumption and hexose monophosphate pathway activity in PMNL exposed to heat-killed opsonized bacteria

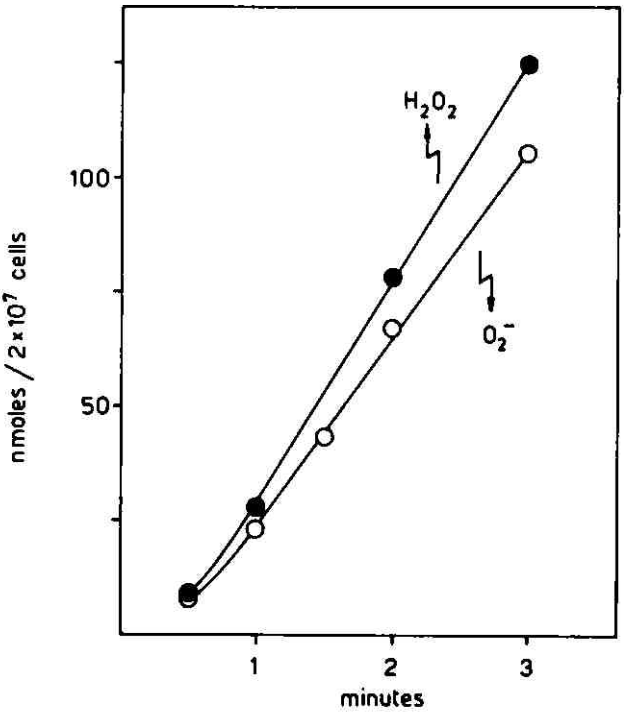


Figure 16.3 Recovery of O_2^- and H_2O_2 (+ NaN_3) generated by phagocytosing PMNs (heat-killed opsonized *B. mycoides* added at time zero)

TABLE 16.1 Nicotinamide adenine nucleotide concentrations in resting and phagocytosing (3 min) PMNL*.

	Resting cells		*Phagocytosing cells*	
	mM†	*Ratio*	*mM*†	*Ratio*
NADP⁺	0.031	0.11	0.065	0.31
NADPH	0.273		0.211	
NAD⁺	0.434	7.11	0.448	7.72
NADH	0.061		0.058	

* Data taken from Patriarca *et al.*[25]

† Based on a value of 0.35 μl cell water/million PMNL (Hawkins and Berlin[57])

rate of activated oxygen consumption is linear for at least a few minutes. Concomitantly, the sampling technique for determination of HMP activity, O_2^- and H_2O_2 (Figures 16.2 and 16.3) also confirms that the rate of activated metabolism is linear for a few minutes after exposure of leukocytes

to phagocytosable particles. This linearity of the rate of the metabolic events very likely reflects a linearity of the rate of the phagocytic process, which with increasing time involves an increasing number of cells and leads to increased number of surface membrane invaginations[26].

STOICHIOMETRIC RELATIONSHIP BETWEEN CONSUMPTION OF O_2 AND GENERATION OF O_2^- AND H_2O_2

The steady-state rate of consumption of O_2 and generation of O_2^- and H_2O_2 by a leukocyte population challenged with phagocytosable objects depends on a number of factors. First of all, as mentioned above, it depends on the rate at which an increasing number of cells become engaged in phagocytosis and on the rate at which the metabolism-activating endocytic events take place. For example, cytochalasin B causes a depression in O_2 consumption by reducing the number of phagocytic events and the rate of surface internalization[26].

Secondly, it depends on the activity levels of the primary O_2 reductase(s) and on the rates at which O_2^- and H_2O_2 are utilized in the cells and in the surrounding medium. In the assumption that the reduction of O_2 essentially proceeds via a one-electron pathway[13], the steady-state rate of oxygen consumption by activated leukocyte results from the rates of the following reactions:

$$1.\quad O_2 + RH \rightarrow O_2^- + H^+ + R^\cdot$$
$$1'.\quad O_2 + R^\cdot \rightarrow O_2^- + R$$
$$2.\quad 2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$
$$3.\quad H_2O_2 \rightarrow \tfrac{1}{2}O_2 + H_2O$$

Reactions 1 and 1′ have not yet been defined precisely and, as we will discuss below, the identity of RH, the cell localization and the nature of the oxidase (or O_2^--generating enzyme) have not yet been fully clarified. Reaction 2 may either proceed spontaneously or be catalysed by SOD, whose presence in the cytosol and in the granule fraction of PMNL has been detected by several investigators[4, 27-31]. Finally, the rate of reaction 3 is controlled by catalase, an enzyme which in phagocytes is either soluble or particulate[8, 23, 32, 33].

In the presence of ferricytochrome *c*, the extracellularly released O_2^-, instead of undergoing dismutation (reaction 2), is oxidized to molecular oxygen:

$$4.\quad O_2^- + \text{cyt } c\,(Fe^{3+}) \rightarrow O_2 + \text{cyt } c\,(Fe^{2+})$$

This duplicates the amount of O_2^- converted back to O_2 (see reaction 2). Thus, addition of ferricytochrome *c* to O_2^--releasing phagocytes is expected to diminish the overall rate of O_2 consumption, this effect being neutralized by exogenous SOD.

Treatment of phagocytosing leukocytes with poisons of haem-enzymes, such as sodium azide (NaN_3), causes the inhibition of catalatic breakdown of H_2O_2 (reaction 3), thereby somewhat increasing the overall rate of O_2 consumption. Hydrogen peroxide may be utilized in other reactions, such as that catalysed by NaN_3-sensitive peroxidase(s):

$$5.\quad H_2O_2 + AH_2 \rightarrow 2H_2O + A$$

and that catalysed by NaN_3-insensitive glutathione peroxidase[21]:

$$6.\quad H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

Thus, the higher the rate of reaction 3 (catalase) with respect to reactions 5 (peroxidase) and 6 (glutathione peroxidase) the larger will be the effect of NaN_3 on the rate of overall oxygen consumption.

With their sampling technique, carried out under appropriate conditions of assay linearity with respect to time and cell concentration, Dri *et al.*[14] have recently carried out a number of measurements of O_2 consumption and recovery of generated O_2^- and H_2O_2, which allow an experimental control of the stoichiometric relationships shown in the above reactions. Data referring to phagocytosing guinea-pig PMNs are summarized in Table 16.2. The table shows that, consistently with the expectations, the addition of NaN_3 increases the overall O_2 consumption, which is on the contrary decreased by cytochrome *c*. By virtue of the NaN_3-insensitivity of the O_2^--generating enzyme(s)[17, 31], the recovery of O_2^- is virtually unaffected by this inhibitor. Conversely, the inhibition of catalase and peroxidase by NaN_3 causes a great increase in the accumulation of H_2O_2.

TABLE 16.2 O_2 consumption, extracellular O_2^- recovery and H_2O_2 accumulation in phagocytosing PMNL*

	$-NaN_3$		$+NaN_3$	
	−	+ *cyt c*‡	−	+ *cyt c*‡
O_2 Consumption†	72.7 ± 10.6	56.3 ± 8.8	94.5 ± 15.8	64.0 ± 14.0
O_2^- Recovery†	−	65.5 ± 15.5	−	63.7 ± 14.2
H_2O_2 Accumulation†	< 2	< 2	84.8 ± 7.4	59.9 ± 11.1

* Data taken from Dri *et al.*[14]

† nmol/2 min/2 × 10^7 cells (increments with respect to resting cells)

‡ The effects of cytochrome *c* were neutralized by exogenous SOD

In the presence of NaN_3, the only back-production of O_2 should derive from decay of O_2^- (1 mol of O_2 generated per 2 mol of O_2 reduced in reactions 1 + 1′). Thus, the total amount of O_2 reduced should be twice that actually measured, i.e. by using the data of Table 16.2, an average of $94.5 \times 2 = 189$ nmol/2 min/2×10^7 cells. The reduction of this amount of O_2 should ultimately lead to the generation of 94.5 nmol of H_2O_2 (reactions 1 + 1′ + 2), of which about 90% are recovered. This suggests that not more than 10% of the produced H_2O_2, at least in the early phase of metabolic stimulation of PMNs, is disposed through NaN_3-insensitive pathways, including the glutathione-metabolizing pathway.

Table 16.2 also shows that the presence of NaN_3 causes an increment in O_2 consumption of 21.8 nmol/2 min/2×10^7 cells. On account of reaction 3, this means that 43.6 nmol of $H_2O_2/2 \times 10^7$ cells are degraded by catalase in the first 2 min following the metabolic stimulation. This value represents about 46% of the H_2O_2 actually produced during this time, thereby suggesting that the residual 44% of H_2O_2 is utilized in NaN_3-sensitive peroxidative reactions.

From Table 16.2 it also emerges that the addition of cytochrome *c* to phagocytosing cells does not fully suppress O_2 consumption, as would be expected if, under this condition, reactions 1 + 1′ and 4 prevailed over the other reactions. The possibility that this might be due to competition between O_2^- dismutation and oxidation by cytochrome *c* seems very unlikely. In fact, although at pH 7.4 the two reactions have comparable rate constants[34,35], the steady-state extracellular concentration of O_2^- should be several-fold lower than that of cytochrome *c*. A more likely explanation is that only a portion of O_2^- is accessible to cytochrome *c* extracellularly, the remaining part of it being subjected to dismutation either in the cytoplasm or in the phagocytic vacuoles. From the data of Table 16.2, which refer to measurements carried out in the early stage of the phagocytic stimulus, it appears that the amount of O_2^- oxidized by cytochrome *c* is about 34% of that produced according to the one-electron mechanism of reduction of O_2 (reactions 1 + 1′). An additional explanation is that, in contrast to our assumption, part of O_2 is reduced by a two-electron mechanism with direct generation of H_2O_2.

Some interesting considerations might finally be made on the stoichiometric relationship between the decrease of O_2 consumption caused by cytochrome *c* and O_2^- recovered extracellularly. By adding reactions 2 and 3, one ends up with the following overall reaction:

$$7. \quad 2O_2^- + 2H^+ \rightarrow {}^3/_2O_2 + H_2O$$

which shows that for $2O_2$ reduced to $2O_2^-$ only $\frac{1}{2}O_2$ is actually consumed. Thus, the ratio between the decrease of O_2 consumption caused by cytochrome *c* and the extracellularly recovered O_2^- should be 1:4. This calculated ratio is fully verified by the experimental data of Table 16.2. When catalase is prevented from acting on H_2O_2 because of the presence of NaN_3, this ratio should become 1:2 (see reaction 2). This is also fully consistent with the data of Table 16.2.

To summarize, our conclusions are that, at least in the early stage following the PMN exposure to phagocytosable particles, not more than one-third of the O_2 reduced in reactions 1 + 1′ is recovered extracellularly as O_2^-, and that most of the H_2O_2 produced, either by dismutation of O_2^- or as primary product of O_2 reduction, is utilized in NaN_3-sensitive peroxidatic and catalatic reactions.

SUBSTRATE AND LOCALIZATION OF THE OXIDASE (O_2^--GENERATING ENZYME)

The coupling of enhanced O_2 reduction to stimulation of HMP, sustained by an increased $NADP^+$: NADPH ratio, requires that an NADPH oxidative pathway is activated in phagocytosing leukocytes. Following the original discovery of an NADPH oxidase in PMNs by Iyer and Quastel[36], Rossi and his co-workers have described a particulate NADPH oxidase, whose activity is several-fold increased in phagocytosing cells[9, 23, 25, 37, 38]. This enzyme, which is recovered in the 20 000 *g* granule fraction of leukocytes, has been originally assayed both polarographically and spectrophotometrically at pH 7.2 and shown to lead to the generation of H_2O_2[37]. Subsequently, Patriarca *et al.*[25, 38] demonstrated that NADPH oxidation by the granule fraction is maximal at pH 5.5 and is activated by Mn^{2+}. The low pH optimum has been thought to support a role for the NADPH oxidase in the metabolic activation of PMNs, since the phagocytic vacuole, where the enzyme should exert its activity, is rapidly acidified after interiorization[39].

At pH 5.5 the oxidation of NADPH is inhibited by SOD[38], thereby suggesting that the oxidation of the nucleotide is O_2^--dependent. This finding is consistent with the observation later reported by Babior *et al.*[17, 40] that the granule fraction of PMNL exhibits an NADPH-dependent O_2^--generating enzyme activity (pH 5.5), which is increased more than 30 times by phagocytosis.

Both NADPH oxidase (pH 5.5, Mn^{2+})[41, 42] and NADPH-dependent O_2^- generating enzyme (pH 5.5)[40] have been reported to be insensitive to the phagocytic stimulus in PMNs of CGD patients, thereby suggesting that

the inability of these cells to carry out the respiratory burst may be due to lack of activation of O_2^--generating NADPH oxidation.

The localization of the NADPH-dependent O_2^--generating enzyme is still unclear. The granule fraction, to which it is associated, presumably contains, beside the two main populations of granules of PMNs[43], vesicles derived from the plasma membrane and other subcellular organelles. Takanaka and O'Brien[44] and Roos[45] have suggested that the enzyme is a component of the plasma membrane. A generation of O_2^- and H_2O_2 at the PMNs surface, which is favoured also by Baehner, Johnston, Root, Goldstein and their associates[4, 49, 50], would directly result in synthesis of these reactive compounds within the phagosome upon membrane internalization during particle ingestion. Furthermore, since the oxidative metabolism of phagocytes is excited not only by particulate objects but by a variety of surface-reactive stimuli, a plasma membrane localization of the oxidase would simplify the problem of understanding the mechanism of its activation[26].

This postulated localization of the NADPH oxidase appears, however, to be in contrast with some experimental observations. In fact, Rossi *et al.*[46] have shown that the specific activity of NADPH oxidase (pH 5.5, Mn^{2+}) in purified plasma membrane preparations of guinea-pig PMNs is lower than that of the nuclei + granules fraction. Furthermore, Patriarca *et al.*[47] and Segal and Peters[31] have demonstrated, by sucrose density zonal centrifugation, that in rabbit exudate and human blood PMNs, respectively, the enzyme is associated to the azurophilic granules.

The conclusions reached by Rossi, Babior, Lehrer, De Chatelet and their associates on the key role of NADPH oxidase in the metabolic stimulation of PMNL are not shared by other investigators. In particular, Karnovsky, Segal and their associates believe not only that the site of O_2 reductase activity is the plasma membrane, but also that the reduction of O_2 is accomplished by NADH[31, 48]. They base their conclusions on the observations that NADH stimulates the production of H_2O_2 within the phagosomes and on the plasma membrane of phagocytosing PMNL, as shown by cytochemical identification of H_2O_2 oxidation products, and that purified plasma membrane preparations of resting PMNL exhibit a SOD-inhibitable NBT reductase with high affinity for NADH. So far, however, no isolation of a plasma membrane fraction with stimulated NADH-dependent O_2^--generating activity has been described.

An NADH-dependent reduction of O_2 as a key event in the respiratory burst of phagocytes makes it necessary to postulate secondary pathways to account for the increased generation of $NADP^+$ from NADPH. One such secondary pathway involves the existence in PMNL of a nicotinamide

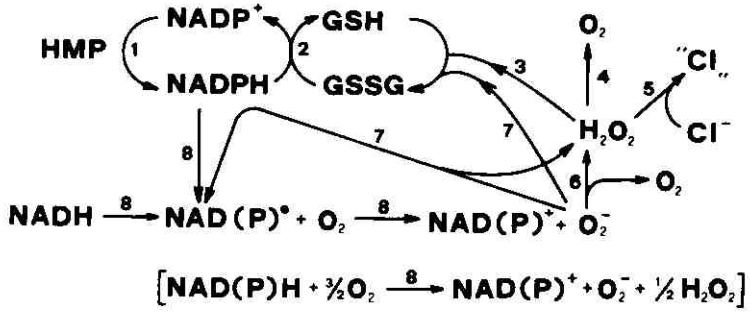
nucleotide transhydrogenase-catalysed reaction[51] that transfers hydrogen from NADPH to newly formed NAD^+. The activity of this enzyme, however, is too low[23,51,52] to account for an adequate supply of $NADP^+$ to the HMP[19]. Alternatively, a glutathione-metabolizing pathway has been proposed[21], whose net effect is to reduce a portion of H_2O_2 generated in the metabolic burst. The contribution of this pathway to the generation of $NADP^+$, however, does not appear to be very large, at least in the early stage of the metabolic activation. In fact, Weening *et al.*[53] have shown that in phagocytosing PMNs of a patient severely deficient in glutathione reductase activity the initial stimulation of HMP activity is only somewhat less than normal.

A third possibility would be that O_2^- generated by the reduction of O_2 by NADH at the plasma membrane oxidizes NADPH via a non-enzymatic chain reaction in the cytosol. This possibility, however, seems rather unlikely, because O_2^- is presumably very rapidly converted to O_2 and H_2O_2 by cytoplasmic SOD[4,27-30]. Furthermore, the O_2^--dependent oxidation of NADPH is markedly inhibited by NADH at concentrations close to the physiological ones[54].

In conclusion, there are arguments for both a granular NADPH-dependent and a plasma membrane NAD(P)H dependent O_2^- generation, but the problem of the localization and substrate specificity of the O_2 reductase in the intact cell has not yet been conclusively solved, nor has the possibility been ruled out that phagocytosis activates more than one oxidase.

CONCLUSIONS

The main reactions which are thought to lead to generation and utilization of O_2^- and H_2O_2 by phagocytosing leukocytes are illustrated in the scheme of Figure 16.4. The scheme represents a cycle that can be interrupted by a deficient activity of enzymes catalysing either the generation or the detoxification of O_2^- and H_2O_2. Both a failure in the activation of an NADPH-dependent O_2 reductase[41,42] and the absence of an NADH-dependent O_2 reductase[31] have been suggested to be the defect which interrupts the cycle in PMNL of CGD patients. On the other hand, a defective detoxification of excess H_2O_2 has been proposed to cause O_2 consumption to stop, which was observed after a normal initial stimulation in PMNL of patients with severe glutathione reductase activity[53]. Furthermore, the decreased or absent respiratory burst in glucose-6-phosphate dehydrogenase-deficient PMNs[55] is likely to be ascribed to a decreased rate of NADPH regeneration from $NADP^+$, which would cause a drop in the rate of NADPH-dependent O_2



1. Gluc-6-P DH & 6-P-Glucon DH
2. Glutathione Reductase
3. Glutathione Peroxidase
4. Catalase
5. Myeloperoxidase
6. Superoxide Dismutase
7. O_2^- - Dependent Non-Enzymatic Oxidations
8. NAD(P)H-Dependent O_2 Reductase

Figure 16.4 Scheme of metabolism of O_2^- and H_2O_2 in phagocytes (the reaction in square brackets refers to a hypothetical simultaneous monovalent and divalent reduction of O_2)

reduction (O_2^--generation) and of glutathione reduction (H_2O_2 detoxification).

Reagents and products of the reactions shown in the scheme of Figure 16.4 may interact in a rather complex way. The effects of inhibition or activation of any enzyme of the cycle can, therefore, be amplified by these interactions. For example, a drug added to phagocytosing PMNL can decrease O_2 consumption by simply causing an oxidative back-conversion of O_2^- to O_2 or by inhibiting the dehydrogenases of the HMP[56], without any interaction with the primary oxidase. On the contrary, an increase in O_2 consumption may be caused by inhibition of catalatic breakdown of H_2O_2 or by an increased reduction of O_2^-, thereby preventing the conversion of H_2O_2 and O_2^- to O_2, respectively. A stimulation of PMNL respiration caused by cell exposure to reduced nicotinamide nucleotides might be due to O_2^--dependent chain reactions[38] and not to supply of exogenous substrate to an oxidase[44]. In view of these considerations, conclusions concerning the role of enzymes of the cycle of Figure 16.4, based on measurements of a single biochemical parameter (O_2 consumption, for example) carried out with intact cells, are to be drawn with great caution.

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