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Inborn Errors of Immunity and Phagocytosis

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Molecular bases of the metabolic excitability of phagocytes
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A number of oxidative reactions, lethal to many bacteria, fungi, certain viruses and mycoplasmas, are activated by phagocytosis in polymorphonuclear leukocytes (PMNL) and macrophages.

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. 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-
trode. 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 x 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). Scopoletin (7-hydroxy-6-methoxycoumarin) 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. 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.
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$_2$O$_2$ 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](image)

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
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.

**STOICHIOMETRIC RELATIONSHIP BETWEEN CONSUMPTION OF O₂ AND GENERATION OF O₂⁻ AND H₂O₂**

The steady-state rate of consumption of O₂ and generation of O₂⁻ and H₂O₂ 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 O₂ consumption by reducing the number of phagocytic events and the rate of surface internalization.

Secondly, it depends on the activity levels of the primary O₂ reductase(s) and on the rates at which O₂⁻ and H₂O₂ are utilized in the cells and in the surrounding medium. In the assumption that the reduction of O₂ essentially proceeds via a one-electron pathway, the steady-state rate of oxygen consumption by activated leukocyte results from the rates of the following reactions:

1. \( \text{O}_2 + \text{RH} \rightarrow \text{O}_2^- + \text{H}^+ + \text{R}^- \)
2. \( \text{O}_2^- + \text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \)
3. \( \text{H}_2\text{O}_2 \rightarrow \frac{1}{2}\text{O}_2 + \text{H}_2\text{O} \)

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 O₂-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. Finally, the rate of reaction 3 is controlled by catalase, an enzyme which in phagocytes is either soluble or particulate.

In the presence of ferricytochrome c, the extracellularly released O₂, instead of undergoing dismutation (reaction 2), is oxidized to molecular oxygen:

4. \( \text{O}_2^- + \text{c}yt\ c (\text{Fe}^{3+}) \rightarrow \text{O}_2 + \text{c}yt\ c (\text{Fe}^{2+}) \)
This duplicates the amount of \( \text{O}_5 \) converted back to \( \text{O}_2 \) (see reaction 2). Thus, addition of ferricytochrome \( \text{c} \) to \( \text{O}_2 \)-releasing phagocytes is expected to diminish the overall rate of \( \text{O}_2 \) consumption, this effect being neutralized by exogenous SOD.

Treatment of phagocytosing leukocytes with poisons of haem-enzymes, such as sodium azide (\( \text{NaN}_3 \)), causes the inhibition of catalytic breakdown of \( \text{H}_2\text{O}_2 \) (reaction 3), thereby somewhat increasing the overall rate of \( \text{O}_2 \) consumption. Hydrogen peroxide may be utilized in other reactions, such as that catalysed by \( \text{NaN}_3 \)-sensitive peroxidase(s):

\[
5. \quad \text{H}_2\text{O}_2 + \text{AH}_2 \rightarrow 2\text{H}_2\text{O} + A
\]

and that catalysed by \( \text{NaN}_3 \)-insensitive glutathione peroxidase:

\[
6. \quad \text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

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 \( \text{NaN}_3 \) 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 \( \text{O}_2 \) consumption and recovery of generated \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \), 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 \( \text{NaN}_3 \) increases the overall \( \text{O}_2 \) consumption, which is on the contrary decreased by cytochrome \( \text{c} \). By virtue of the \( \text{NaN}_3 \)-insensitivity of the \( \text{O}_2 \)-generating enzyme(s)\(^{17-31} \), the recovery of \( \text{O}_2 \) is virtually unaffected by this inhibitor. Conversely, the inhibition of catalase and peroxidase by \( \text{NaN}_3 \) causes a great increase in the accumulation of \( \text{H}_2\text{O}_2 \).

**TABLE 16.2** \( \text{O}_2 \) consumption, extracellular \( \text{O}_2 \) recovery and \( \text{H}_2\text{O}_2 \) accumulation in phagocytosing PMNL

<table>
<thead>
<tr>
<th></th>
<th>(-\text{NaN}_3)</th>
<th>(+\text{NaN}_3)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(-) + \text{cyt} \text{c} ‡</td>
<td>(-) + \text{cyt} \text{c} ‡</td>
</tr>
<tr>
<td>( \text{O}_2 ) Consumption†</td>
<td>( 72.7 \pm 10.6 )</td>
<td>( 56.3 \pm 8.8 )</td>
</tr>
<tr>
<td>( \text{O}_2 ) Recovery†</td>
<td>( &lt; 2 )</td>
<td>( 65.5 \pm 15.5 )</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 ) Accumulation†</td>
<td>( &lt; 2 )</td>
<td>( &lt; 2 )</td>
</tr>
</tbody>
</table>

* Data taken from Dri et al.\(^{14}\)
† nmol/2 min/2 × 10⁷ cells (increments with respect to resting cells)
‡ The effects of cytochrome \( \text{c} \) 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 \( \times 10^7 \) cells. The reduction of this amount of O$_2$ should ultimately lead to the generation of 94.5 nmol of H$_2$O$_2$ (reactions 1 + 1' + 2), of which about 90% are recovered. This suggests that not more than 10% of the produced H$_2$O$_2$, 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 \( \times 10^7 \) cells. On account of reaction 3, this means that 43.6 nmol of H$_2$O$_2$/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$_2$O$_2$ actually produced during this time, thereby suggesting that the residual 44% of H$_2$O$_2$ 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\textsuperscript{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$_2$O$_2$.

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. \[2O_2^- + 2H^+ \rightarrow \frac{1}{2}O_2 + H_2O\]
which shows that for 2O₂ reduced to 2O₂⁻ only 1/4O₂ is actually consumed. Thus, the ratio between the decrease of O₂ consumption caused by cytochrome c and the extracellularly recovered O₂ 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 H₂O₂ because of the presence of NaN₃, 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 O₂ reduced in reactions 1 + 1' is recovered extracellularly as O₂⁻ and that most of the H₂O₂ produced, either by dismutation of O₂⁻ or as primary product of O₂ reduction, is utilized in NaN₃-sensitive peroxidatic and catalatic reactions.

SUBSTRATE AND LOCALIZATION OF THE OXIDASE (O₂⁻-GENERATING ENZYME)

The coupling of enhanced O₂ 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 Quastel²⁶, Rossi and his co-workers have described a particulate NADPH oxidase, whose activity is several-fold increased in phagocytosing cells²³,²⁵,³⁷,³⁸. 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 H₂O₂³⁷. Subsequently, Patriarca et al.²⁵,³⁸ demonstrated that NADPH oxidation by the granule fraction is maximal at pH 5.5 and is activated by Mn²⁺. 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 interiorization²⁹.

At pH 5.5 the oxidation of NADPH is inhibited by SOD³⁸, thereby suggesting that the oxidation of the nucleotide is O₂⁻-dependent. This finding is consistent with the observation later reported by Babiour et al.¹⁷,⁴⁰ that the granule fraction of PMNL exhibits an NADPH-dependent O₂⁻-generating enzyme activity (pH 5.5), which is increased more than 30 times by phagocytosis.

Both NADPH oxidase (pH 5.5, Mn²⁺)⁴¹,⁴² and NADPH-dependent O₂⁻-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, vesicles derived from the plasma membrane and other subcellular organelles. Takanaka and O'Brien and Roos 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, 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.

This postulated localization of the NADPH oxidase appears, however, to be in contrast with some experimental observations. In fact, Rossi et al. 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. and Segal and Peters 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, Babiör, 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. 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 that transfers hydrogen from NADPH to newly formed NADH. The activity of this enzyme, however, is too low to account for an adequate supply of NADPH to the HMP cycle. Alternatively, a glutathione-metabolizing pathway has been proposed, whose net effect is to reduce a portion of H2O2 generated in the metabolic burst. The contribution of this pathway to the generation of NADPH, however, does not appear to be very large, at least in the early stage of the metabolic activation. In fact, Weening et al. 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 O2 generated by the reduction of O2 by NADH at the plasma membrane oxidizes NADPH via a non-enzymatic chain reaction in the cytosol. This possibility, however, seems rather unlikely, because O2 is presumably very rapidly converted to O2 and H2O2 by cytoplasmic SOD. Furthermore, the O2-dependent oxidation of NADPH is markedly inhibited by NADH at concentrations close to the physiological ones.

In conclusion, there are arguments for both a granular NADPH-dependent and a plasma membrane NAD(P)H-dependent O2 generation, but the problem of the localization and substrate specificity of the O2 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 O2 and H2O2 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 O2 and H2O2. Both a failure in the activation of an NADPH-dependent O2 reductase and the absence of an NADH-dependent O2 reductase 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 H2O2 has been proposed to cause O2 consumption to stop, which was observed after a normal initial stimulation in PMNL of patients with severe glutathione reductase activity. Furthermore, the decreased or absent respiratory burst in glucose-6-phosphate dehydrogenase-deficient PMNs 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 O2
reduction (O$_2$-generation) and of glutathione reduction (H$_2$O$_2$ 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 catalytic breakdown of H$_2$O$_2$ or by an increased reduction of O$_2$, thereby preventing the conversion of H$_2$O$_2$ 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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