Reprinted from

Inborn Errors of Immunity and Phagocytosis

Monograph based upon
Proceedings of the Fifteenth Symposium of
The Society for the Study of Inborn Errors of Metabolism

Edited by
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J. W. T. Seakins
and
R. A. Harkness

MTP PRESS LIMITED
International Medical Publishers
1979

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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[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-

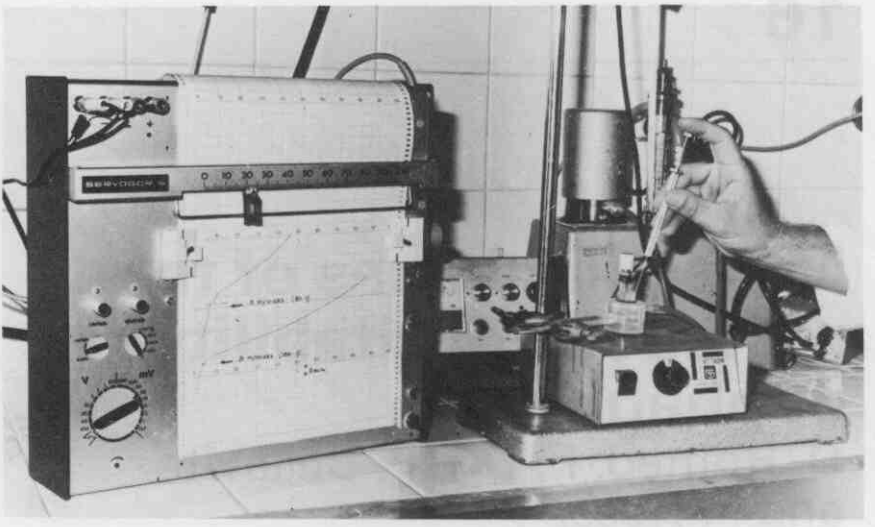

Figure 16.1 Polarographic assembly and recorded traces of oxygen consumption by PMNL

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

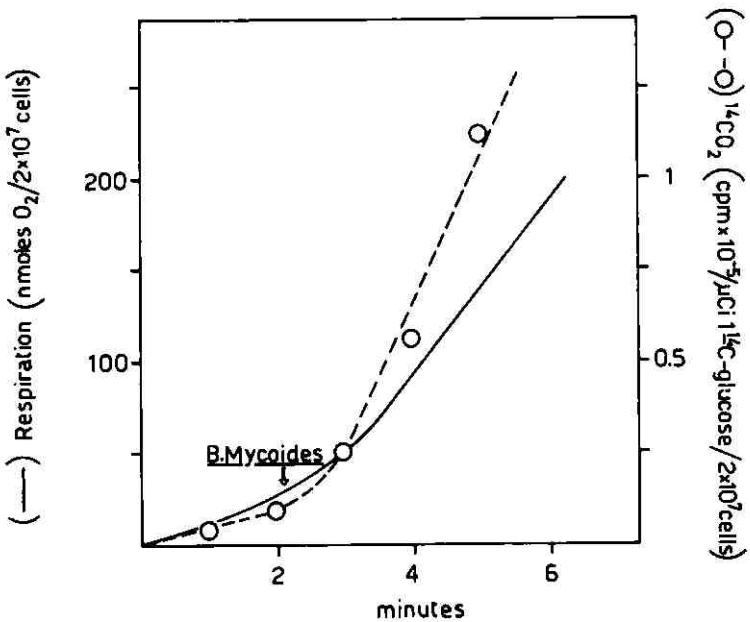

Figure 16.2 Simultaneous evaluation of the kinetics of stimulation of O_2 consumption and hexose monophosphate pathway activity in PMNL exposed to heat-killed opsonized bacteria

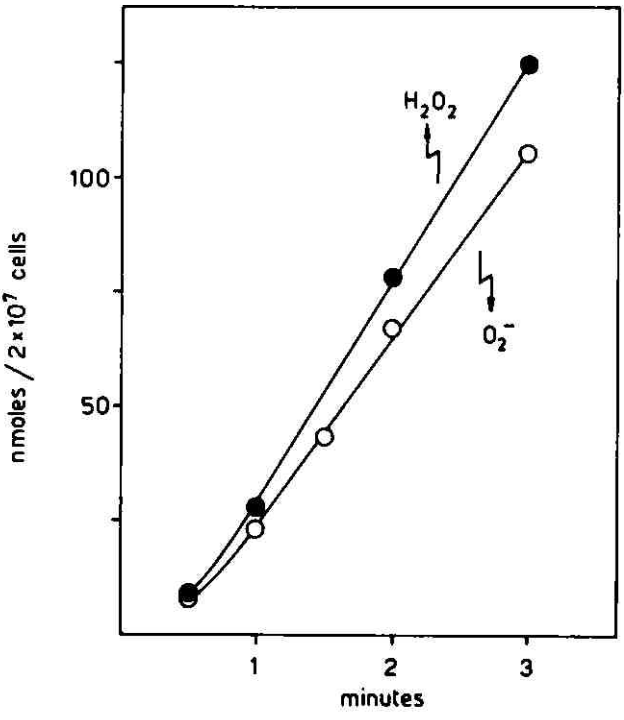

Figure 16.3 Recovery of O_2^- and H_2O_2 (+ NaN_3) generated by phagocytosing PMNs (heat-killed opsonized *B. mycoides* added at time zero)

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:

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