OXIDATIVE METABOLISM OF MONONUCLEAR PHAGOCYTES

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INTRODUCTION

The process of phagocytosis in polymorphonuclear leukocytes and in macrophages is associated with dramatic changes of oxidative metabolism. These changes include an increase in oxygen consumption, in $O^{\cdot}_2$ and H_2O_2 generation, and in glucose catabolism through the hexose monophosphate (HMP) pathway (Sbarra & Karnovsky 1959; Iyer et al. 1961; Rossi & Zatti 1964; Paul & Sbarra 1968; Gee et al. 1970; Rossi et al. 1972; Klebanoff & Hamon 1972; Babior et al. 1973; Curnutte & Babior 1974; Rossi et al. 1975; Karnovsky et al. 1975; Root et al. 1975). A similar stimulation of oxidative metabolism is also induced in the absence of phagocytosis when the phagocytic cells are exposed to a variety of membrane perturbing agents (Strauss & Stetson 1960; Graham et al. 1967; Zatti & Rossi 1967; Rossi et al. 1971; Patriarca et al. 1971a; Karnovsky 1972; Romeo et al. 1973; Repine et al. 1974; Kakinuma 1974; Goetzl & Austen 1974; Goldstein et al. 1975). These metabolic changes are usually referred to as 'the respiratory burst' of phagocytes.

It is widely accepted that the biological significance of the respiratory burst is that of providing a battery of highly reactive compounds that can be used for the killing of micro-organisms either in the phagocytic vacuole or in the extracellular environment. The triggering mechanism of the respiratory burst, as well as the nature and the localization of the enzyme responsible for the increase of O_2 consumption and $O^{\cdot}_2$ and H_2O_2 production, are still controversial (Karnovsky 1962; Rossi et al. 1972; Patriarca et al. 1977).

It is widely accepted that the first step in the O_2 consumption

consists in the activation of an oxidase that catalyzes the univalent reduction of O_2 with formation of $O^{\cdot}_2$. Evidence has been obtained in our and other laboratories that the physiological electron donor for this reaction is NADPH (Iyer et al. 1961; Rossi & Zatti 1964, 1968; Zatti & Rossi 1965; Patriarca et al. 1971b; Rossi et al. 1972; Hohn & Lehrer 1975; DeChatelet et al. 1975; Babior et al. 1975, 1976). The main fate of $O^{\cdot}_2$ is to dismutate to H_2O_2 ($2O^{\cdot}_2 + 2H^+ \rightarrow H_2O_2 + O_2$) either spontaneously, with a rate constant of $1x10^5\ M^{-1}sec^{-1}$ at pH 7.4, or by catalysis of superoxide dismutase (SOD), with a rate constant of $2x10^9\ M^{-1}sec^{-1}$ at pH 7.4 (McCord et al. 1977). Part of the $O^{\cdot}_2$ is released outside the cell or into the phagocytic vacuole, where it can also dismutate to H_2O_2. The H_2O_2 formed inside the cell can be degraded by two mechanisms, a catalatic one, in which $\frac{1}{2}O_2$ is given back for each molecule of H_2O_2 ($H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$), and a peroxidatic one, in which a reduced compound is oxidized and H_2O is formed ($H_2O_2 + RH_2 \rightarrow 2H_2O + R$). The main reactions for H_2O_2 degradation in phagocytic cells are catalyzed by NaN_3-sensitive catalase and peroxidase, and by NaN_3-insensitive glutathione peroxidase. Part of the H_2O_2 escapes degradation and is released outside the cells or into the phagocytic vacuole.

On the basis of the above reactions, the following conclusions can be drawn: 1) The O_2 consumption that is measurable during the respiratory burst does not correspond to the total amount of O_2 reduced to $O^{\cdot}_2$, since this compound does not accumulate. The stoichiometric relationships between the actual O_2 consumed and the $O^{\cdot}_2$ generated depend on the mechanisms of H_2O_2 degradation and on the amount of H_2O_2 accumulated. If all H_2O_2 is degraded by a catalatic mechanism, four $O^{\cdot}_2$ are generated as intermediates for one O_2 actually consumed. If, on the contrary, all the peroxide is degraded by a peroxidatic mechanism or is accumulated, two $O^{\cdot}_2$ are formed for one O_2 actually consumed. Thus, the actual stoichiometric relationship between the measurable oxygen consumed and the $O^{\cdot}_2$ generated as intermediate can vary between 1:4 and 1:2, the variance depending on the relative importance of the various reactions for H_2O_2 degradation. The variable factor in the ratio $O_2:O^{\cdot}_2$ is O_2, while the amount of $O^{\cdot}_2$ generated does not change. 2) The steady-state rate of O_2

consumption and $O^{\cdot}_2$ and H_2O_2 generation and recovery, depends on the activity level of the NADPH-oxidase and on the rate at which $O^{\cdot}_2$ and H_2O_2 are utilized in the cell or released into the surrounding medium. 3) By using cytochrome c, which is directly reduced by $O^{\cdot}_2$, and scopoletin or other compounds such as homovanillic acid, which are oxidized by H_2O_2 in the presence of horseradish peroxidase, only the amounts of $O^{\cdot}_2$ and of H_2O_2 released outside the cell can be measured. 4) The amounts of $O^{\cdot}_2$ and H_2O_2 that are released depend on the rate of their formation, the rate of their intracellular degradation, and, wherever the oxidase is located, the rate of their diffusion across the plasma membrane.

It is widely known that the stimulation of the oxidative metabolism in phagocytic cells, measured as O_2 consumption and HMP activity, is highly variable, depending on cell type, on the sources of phagocytic cells, on the animal species, on the type of stimulant used and, in macrophages, on the functional state (elicited, activated) (Myrvik 1972; Rossi et al. 1975; Karnovsky et al. 1975; Nathan & Root 1977; Johnston et al. 1978). However, the greatest variability, concerns both the absolute measurable values of $O^{\cdot}_2$ and H_2O_2 and, chiefly, the relationships between these values and the O_2 actually consumed. The extreme cases of this variability are represented on the one hand by polymorphonuclear leukocytes of mammalian species, and on the other by alveolar macrophages of rabbit and guinea pig. The enhanced O_2 consumption is always associated with a consistent production and release of $O^{\cdot}_2$ and H_2O_2 in polymorphs, whereas none or only traces of these intermediates appear to be produced in alveolar macrophages of many mammalian species (Paul et al. 1970; Gee et al. 1970; Klebanoff & Hamon 1975; DeChatelet et al. 1975; Drath & Karnovsky 1975; Biggar et al. 1976; Tsan 1977).

To explain this extreme variability, the following hypotheses can be advanced: 1) The association between O_2 consumption and $O^{\cdot}_2$ and H_2O_2 generation is not a general feature of all the phagocytic cells. 2) During the respiratory burst, only a portion of O_2 is reduced, with formation of these intermediates, and this amount varies in different cell types. 3) In all phago-

cytic cells the stimulation of O_2 consumption is associated with the generation of $O^{\cdot}_2$ and H_2O_2, but in some cells these intermediates are degraded as fast as they are formed.

We have reinvestigated these problems in rabbit alveolar macrophages. In this paper evidence is provided that in these cells: 1) The stimulation of O_2 consumption is associated with the generation of $O^{\cdot}_2$ and H_2O_2. 2) The activities of the enzymes responsible for the intracellular utilization of $O^{\cdot}_2$ and H_2O_2 are so high that the actual release of these intermediates is very low. 3) The state of activation induced by BCG is associated with a greater respiratory burst and with a change of the equilibrium between the rate of formation, the rate of degradation, and the rate of diffusion across the plasma membrane of $O^{\cdot}_2$ and H_2O_2, so that the intermediates are released into the extracellular medium in larger amounts, both as absolute value and as percentage of the amount generated.

The first part of this paper deals with a comparative study on the various events of the respiratory burst in BCG-activated alveolar macrophages (AM) and in polymorphonuclear leukocytes (PMNL) of peritoneal exudate. In the second part the differences between normal and BCG-activated AM are presented.

MATERIALS AND METHODS

Preparation of the cells

Alveolar macrophages (AM) were obtained by tracheobronchial lavages with physiological saline from normal rabbits and rabbits injected intravenously 15 days earlier with 10 mg BCG (kindly supplied by Istituto Vaccinogeno Antitubercolare, Milan, pended in 1 ml sterile saline containing 2% Tween 80.

Polymorphonuclear leukocytes (PMN) were isolated from peritoneal exudates induced by the injection of 100 ml 1% sodium caseinate solution, as described elsewhere (Rossi et al. 1978). After lysis of the contaminating erythrocytes by a brief hypotonic shock, the cells were centrifuged for 7 min at 200 g and resuspended in Krebs-Ringer phosphate (KRP) buffer (pH 7.4) containing 0.5 mM $CaCl_2$ and 5 mM glucose. Differential counts

were carried out on May-Grünwald-Giemsa stained smears. The cell preparations from normal animals contained 90-98% macrophages and those from BCG-infected animals 82-90%. These latter preparations showed more heterogeneity in cell size, cells with basophilic cytoplasm, and 2-4% giant multinucleated cells. The contaminating granulocytes amounted to 1-3% in both AM preparations. The peritoneal exudates contained 85-95% PMN.

Metabolic assays

Oxygen consumption was measured polarographically with a Clark type oxygen electrode as previously described (Romeo et al. 1973b).

Hydrogen peroxide was measured fluorimetrically by the conversion of the non fluorescent compound 3-methoxy-4-hydroxyphenilacetic acid (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. Two experimental procedures were employed: with the first, the accumulated H_2O_2 was measured in the supernatants of samples withdrawn from the electrode vessel where oxygen consumption was continuously recorded, 4 min after the addition of the stimulatory agent. Aliquots of such supernatants (10-100 µl) were added to a spectrophotofluorimetric cuvette containing 2.5 ml KRP, 0.02 mM HVA, and 20 µg HRP, and the increase in fluorescence was compared with appropriate standards of H_2O_2. This procedure was employed for the H_2O_2 determinations reported in the columns I and II of Table 1. In the second procedure, 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 in the supernatants of samples withdrawn from the electrode vessel, 4 min after the addition of the stimulatory agent. This procedure, which measures H_2O_2 as it is formed or released, was also employed in the other experiments. The kinetic pattern of H_2O_2 release during the respiratory burst was also monitored by recording the increase in fluorescence with a CGA model DC3000 spectrophotofluorimeter.

Superoxide anion was measured by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c in samples withdrawn from the electrode vessel 4 min after the addition of the stimulatory agent (Dri et al. 1978). The determinations were carried out in reaction mixtures from which HVA and HRP were omitted. This was done to prevent the re-oxidation of reduced cytochrome c by HRP in the presence of H_2O_2 released by the cells.

All values for O_2 consumption and for $O^{\cdot-}_2$ and H_2O_2 released from AM given in the Tables throughout the paper are given after subtraction of the values of contaminating PMN.

The production of $^{14}CO_2$ from 1-^{14}C-glucose was determined as described previously (Romeo et al. 1973b).

Enzyme assays were performed on total homogenate of cells, or 100,000 g pellet, or supernatant, as indicated in the Tables. Superoxide dismutase activity was assayed according to McCord et al. (1977), catalase activity according to Bellavite et al. (1977), glutathione peroxidase and reductase according to Gennaro et al. (1978), peroxidase activity according to Romeo et al. (1973c), and glucose-6-phosphate dehydrogenase according to Patriarca et al. (1973).

RESULTS AND DISCUSSION

O_2 consumption and $O^{\cdot-}_2$ and H_2O_2 release from PMN and BCG-activated AM

The results concerning O_2 consumption and $O^{\cdot-}_2$ and H_2O_2 release by rabbit PMN and BCG-activated AM during phagocytosis are presented in Table 1. The data in column I show that in PMN the respiratory burst is associated with a consistent release of $O^{\cdot-}_2$ and of H_2O_2, whereas the respiratory increment of AM is accompanied by the release of only a small amount of $O^{\cdot-}_2$. From the values for $O^{\cdot-}_2$ and H_2O_2 released by both AM and PMN it appears that the stoichiometric relationship between the amount of $O^{\cdot-}_2$ and that of H_2O_2 does not correspond to the expected stoichiometry of 2:1, as indicated by the reaction of dismutation. In fact, since the released $O^{\cdot-}_2$ dismutates to H_2O_2, the expected

value for H_2O_2 in the extracellular medium of PMN and AM should be at least about 57 and 2.2, respectively. This discrepancy can be explained by the procedures employed for the measurement of the two compounds. $O^{\cdot}_2$ was measured by adding cytochrome c at the beginning of the respiratory burst. Under these conditions the free radical is trapped as soon as it is released from the cell. H_2O_2, on the contrary, was measured at the end (4 min) of the recording of O_2 consumption, i.e., by withdrawing samples from a separate electrode chamber (containing no cytochrome c) and by adding HRP and HVA. Thus, with this system only the amount of the peroxide that escaped degradation was measured.

When the respiratory burst occurs in the presence of NaN_3 (column II), which inhibits catalase and peroxidase, the actual amount of O_2 consumed is associated with an almost stoichiometric accumulation of H_2O_2 in PMN, whereas only traces of the peroxide become measurable in AM. It is worthwhile to point out here that in this condition the stoichiometric relationship between O_2 and H_2O_2 in PMN indicates that about 70% of the peroxide derives from intracellular sources and about 30% from dismutation of the released $O^{\cdot}_2$. In AM in the same condition almost all of the H_2O_2 is degraded. When H_2O_2 is measured by adding HRP and HVA to the incubation medium to trap the peroxide as it is released or formed from $O^{\cdot}_2$, a definite amount of H_2O_2 becomes detectable in the AM suspending medium (column III).

Comparison of the amounts of O_2 actually consumed and of H_2O_2 measured makes it evident that the percentage of consumed O_2 recovered as H_2O_2 is very low in AM and very high in PMN. These results clearly indicate that the main mechanisms for H_2O_2 degradation involve NaN_3-sensitive reactions (catalase and peroxidase) in PMN and NaN_3-insensitive reactions (glutathione peroxidase) in AM.

This group of results permits us to draw the following conclusions: 1) The use of appropriate devices makes it possible to show that AM too are able to release H_2O_2 during the respiratory burst; and 2) in any case, the amount of the peroxide detectable outside the cells represents a very low percentage of the O_2 actually consumed.

Table 1. O_2 consumption and $O^{\cdot-}_2$ and H_2O_2 release by phagocytosing PMN and BCG-activated AM from rabbits

	I		II + NaN_3		III + NaN_3 + HRP + HVA	
	PMN (3)	AM (5)	PMN (5)	AM (6)	PMN (3)	AM (6)
O_2	135.2 ± 13.8	78.9 ± 7.6	195.9 ± 12.9	76.2 ± 14.0	203.6 ± 29.4	71.3 ± 12.8
	(31.5 ± 5.4)	(116.0 ± 13.3)	(21.3 ± 5.6)	(111.7 ± 18.5)	(18.8 ± 5.4)	(101.3 ± 12.9)
$O^{\cdot-}_2$	115.4 ± 39.6	4.5 ± 2.2	105.9 ± 20.7	6.0 ± 1.5	-	-
	(9.2 ± 8.1)	(0)	(9.2 ± 8.1)	(0)		
H_2O_2	10.1 ± 3.0	(0)	179.3 ± 17.2	0.2 ± 0.1	163.0 ± 25.2	7.1 ± 1.9
	(0)		(6.2 ± 2.7)	(0)	(7.8 ± 3.5)	(0)
Percentage O_2 recovered as H_2O_2	7.4	(0)	91.5	0.26	80.0	10.0

The differences between phagocytosing and resting cells are reported. The values are expressed as nmol/4 min/ $1.5x10^7$ cells ± SEM. Resting values are given in parentheses.
Assay medium: $1-2x10^7$ PMN or AM in 2 ml KRP containing 0.5 mM $CaCl_2$ and 5 mM glucose. Opsonized Bacillus mycoides were used as stimulatory agent (ratio cell to bacteria 1:100). 2 mM NaN_3.

Table 2. Enzyme activities in rabbit PMN and BCG-activated AM

	PMN (6)		AM (8)	
	U*/10^7 cells	Specific activity	U*/10^7 cells	Specific activity
o GSH-peroxidase	6.2 ± 1.9	36.5	753.0 ± 137.0	941.2
o GSSG-reductase	5.1 ± 0.7	30.0	52.7 ± 4.0	57.1
o Catalase	39.8 ± 3.0	284.3	262.0 ± 31.0	340.2
Δ Peroxidase	323.0 ± 125.0	978.8	20.4 ± 3.0	19.4
□ SOD	1.3 ± 0.3	2.5	9.4 ± 1.7	4.8

Values are means ± SEM. The number of experiments is given in parentheses. o = measured in 100,000 g supernatant, Δ = measured in 100,000 g pellet, □ = measured in total homogenate.

* nmol NADPH/min for GSH-peroxidase and GSSG-reductase, μmol H_2O_2/min for catalase, nmol tetraguaiacol/min for peroxidase. One unit of superoxide dismutase (SOD) is the amount of enzyme that causes a decrease of 0.0125 OD/min in the reduction of cytochrome c.

Activities of the enzymes responsible for $O^{\cdot}_2$ and H_2O_2 degradation in PMN and BCG-activated AM

The difference between the release of $O^{\cdot}_2$ and H_2O_2 in PMN and in AM prompted us to investigate the activities of the reactions involved in the intracellular degradation of these intermediate products of O_2 reduction. The results in Table 2 show that the activity of glutathione peroxidase, glutathione reductase, catalase, and superoxide dismutase is higher in AM than in PMN, whereas the peroxidase activity is higher in PMN. The pattern of the enzymatic activities indicates that, compared to PMN, AM have very active mechanisms for H_2O_2 degradation. Since catalase usually has a low affinity for H_2O_2 and the activity of peroxidase is very low, it is likely that the main mechanism of H_2O_2 utilization involves the activity of glutathione peroxidase coupled with that of glutathione reductase (glutathione cycle). This is in agreement with the data presented by others (Vogt et al. 1971).

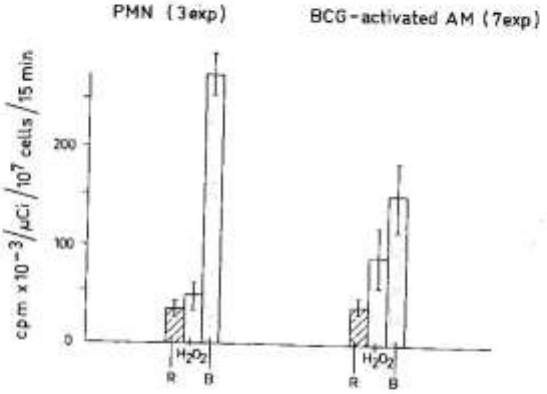

Fig. 1. Effect of bacteria or H_2O_2 addition on the $^{14}CO_2$ production from 1-^{14}C glucose by rabbit PMN and BCG-activated AM. Assay medium: $3x10^6$ cells in 1 ml KRP containing 0.5 mM $CaCl_2$, 0.2 mM Glucose and 0.2 μCi 1-^{14}C glucose. Opsonized Bacillus mycoides were used as stimulatory agent (ratio cell/bacteria, 1/100). Vertical bars indicate the SEM. R = resting cells. B = cells plus bacteria.

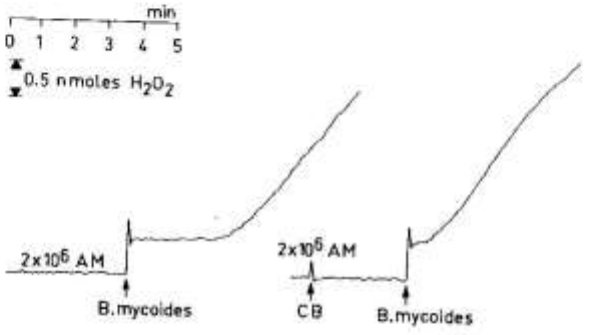

Fig. 2. Spectrophotofluorometric assay of H_2O_2 production by BCG-activated AM during phagocytosis in the absence and in the presence of cytochalasin B. Assay medium: 2.5 ml KRP containing 5 mM glucose, 0.5 mM $CaCl_2$, 0.02 mM HVA, 20 μg HRP, and 2 mM NaN_3. Ratio cell/bacteria 1/100. CB 5 μg/ml.

The utilization of H_2O_2 via the glutathione cycle is coupled with an increased activity of the HMP pathway. On this basis the efficiency of the glutathione cycle can be investigated by measuring the effect of externally added H_2O_2 on $^{14}CO_2$ production from 1-^{14}C-glucose, which in these cells indicates the rate of glucose oxidation via the HMP pathway. The results in Fig. 1 show that the HMP-stimulatory effect of exogenous H_2O_2 is much greater in AM than in PMN. Furthermore, in AM the intensity of the stimulation of the HMP pathway activity by 0.05 mM H_2O_2 closely approximates the maximal stimulation induced by phagocytosis.

Effect of cytochalasin B on the respiratory burst of BCG-induced AM

The results presented above raise the problem of whether the small percentage of H_2O_2 accumulated represents the total amount that is formed or the amount that escapes the intracellular degradation. To investigate this point, we treated the cells with cytochalasin B (CB), which increases the release of lysosomal enzymes and $O^{\cdot}_2$ by phagocytic cells during the respiratory burst (Zurier et al. 1973; Goldstein et al. 1975; Roos et al. 1976; Root & Metcalf 1977). Fig. 2 shows the spectrophotofluorometric recording traces of H_2O_2 production in NaN_3-treated AM during phagocytosis. It can be seen that CB markedly reduces the lag of fluorescence increase, which means that this drug increases the rate of the release of H_2O_2 or of its precursor $O^{\cdot}_2$ through the plasma membrane. It is worth pointing out that CB inhibits phagocytosis and reduces the intensity of the associated respiratory burst (Malawista et al. 1971; Romeo et al. 1977; Root & Metcalf 1977).

In an attempt to acquire more insight into the effect of CB, we carried out simultaneous measurements of O_2 consumption and $O^{\cdot}_2$ and H_2O_2 production by PMN and AM in the presence of CB. The data in Table 3 show the following. 1) By inhibiting the rate of phagocytosis, CB induces a decrease in the intensity of the stimulation of oxygen consumption in both PMN and AM. 2) CB enhances the release of $O^{\cdot}_2$. In fact, despite a decrease in the total amount of O_2 univalently reduced, as shown by the decrease

Table 3. O_2 consumption and $O^{\cdot-}_2$ and H_2O_2 release by phagocytosing rabbit PMN and BCG-activated AM in the absence and in the presence of cytochalasin B

	PMN (3)	AM (6)	CB PMN (3)	CB AM (4)
O_2	203.6 ± 29.4	71.3 ± 12.8	79.3 ± 8.7	38.1 ± 5.7
	(18.8 ± 5.4)	(101.3 ± 12.9)	(60.3 ± 18.4)	(116.1 ± 16.8)
$O^{\cdot-}_2$	105.9 ± 20.7	6.0 ± 1.5	114.9 ± 15.3	24.5 ± 9.1
	(9.2 ± 8.1)	(0)	(24.7 ± 0.4)	(6.2 ± 3.1)
H_2O_2	163.0 ± 25.2	7.1 ± 1.9	77.4 ± 28.8	9.6 ± 1.7
	(7.8 ± 3.5)	(0)	(29.4 ± 4.5)	(3.6 ± 2.6)
Percentage O_2 recovered as H_2O_2	80.0	10.0	97.6	25.0

The differences between phagocytosing and resting cells are reported. The values are expressed as nmol/4 min/1.5×10^7 cells ± SEM. Resting values are given in parentheses. For assay conditions, see Table 1. 5 µg/ml CB, 2 mM NaN_3.

in the measurable O_2 consumed, the amount of $O^{\cdot-}_2$ released is markedly increased in both PMN and AM. 3) CB also increases the amount of H_2O_2 measurable in the extracellular medium. In AM the percentage of O_2 actually consumed recovered as H_2O_2 rises from 10 to 25% in the presence of CB, whereas in PMN the amount of H_2O_2 recovered in the extracellular medium is virtually equal to all the O_2 consumed. 4) When CB is present, the stoichiometric relationship between $O^{\cdot-}_2$ and H_2O_2 in AM indicates that all H_2O_2 formed in the extracellular medium derives from dismutation of the $O^{\cdot-}_2$ released. In PMN, CB also changes the stoichiometric relationship between $O^{\cdot-}_2$ and H_2O_2 released. When the drug is absent, the amount of H_2O_2 deriving from the dismutation of the released $O^{\cdot-}_2$ is about 30%, while in the presence of CB is about 70%. This indicates that the effect of

CB on the release of these intermediates is not due to total or partial inhibition of vacuole formation, but to an increased rate of diffusion of $O^{\cdot}_2$ across the plasma membrane.

Apart from the mechanisms by which CB causes these effects, which are in agreement with the findings of others (Roos et al. 1976; Root & Metcalf 1977), the results of this set of experiments indicate that the type of the respiratory burst in AM is similar to that of granulocytes. In other words, the univalent reduction of oxygen followed by formation of H_2O_2 is operative and substantial in AM as well.

In an attempt to get a better insight into the effect of CB, which by inhibiting the phagocytic act reduces the respiratory increment, we employed a soluble stimulatory agent, the lectin concanavalin A (Con A).

Fig. 3 shows the spectrophotofluorometric recording traces of H_2O_2 production in NaN_3-treated AM during the stimulation of the metabolism by Con A. Con A alone induces a very slight increase in fluorescence, thus indicating a very small release of H_2O_2 (trace A). When the lectin is added to CB-treated AM, a very rapid and marked increase in fluorescence takes place, which indicates that under these conditions a substantial amount of peroxide is recovered outside the cells (trace B). A similar effect is obtained when CB is added after Con A (trace C).

The amount of H_2O_2 measured in CB-treated AM was unexpectedly high compared with that detected in the experiments presented above. This fact prompted further experiments in order to investigate the effect of CB on all of the events of the respiratory burst induced by Con A. Data obtained by the simultaneous measurement of oxygen consumption, $O^{\cdot}_2$ and H_2O_2 given in Table 4 are following: 1) Con A alone induces increased oxygen consumption in association with a consistent release of $O^{\cdot}_2$ and H_2O_2 in PMN, whereas in AM the amount of the intermediates is markedly lower. The relationship between the O_2 consumption and the release of the intermediates is similar to that observed when the stimulation is induced by phagocytosis (Table 1). 2) In both PMN and AM, CB markedly enhances the activation of oxidative metabolism triggered by Con A, measured as O_2 consumed and $O^{\cdot}_2$

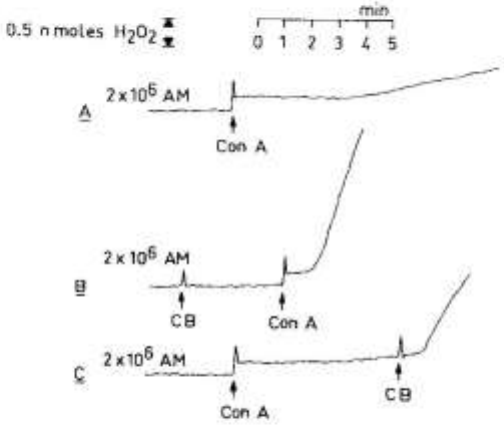

Fig. 3. Spectrophotofluorometric assay of H_2O_2 production by BCG-activated AM stimulated by concanavalin A in the absence and in the presence of cytochalasin B. For experimental conditions, see Fig. 2. Concanavalin A 100 µg/ml.

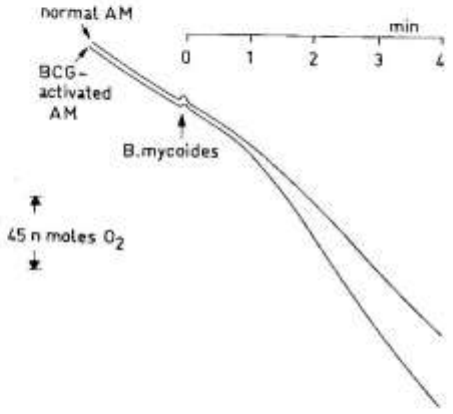

Fig. 4. Polarographic assay of O_2 consumption by normal and BCG-activated AM during phagocytosis. Assay medium: 2 ml KRP containing 1.5×10^7 cells, 5 mM glucose, and 0.5 mM $CaCl_2$. Ratio cell/bacteria 1/100 .

Table 4. O_2 consumption and $O^{\cdot}_2$ and H_2O_2 release by rabbit PMN and BCG-activated AM stimulated by concanavalin A in the absence and presence of cytochalasin B

	Con A		Con A + CB	
	PMN (3)	AM (4)	PMN (3)	AM (4)
O_2	52.6 ± 17.8	24.3 ± 5.7	106.3 ± 30.4	75.6 ± 6.9
	(18.8 ± 5.4)	(101.3 ± 12.9)	(60.3 ± 18.4)	(116.1 ± 16.8)
$O^{\cdot}_2$	30.3 ± 13.9	3.7 ± 1.8	98.1 ± 41.1	48.4 ± 9.8
	(9.2 ± 8.1)	(0)	(24.7 ± 0.4)	(6.2 ± 3.1)
H_2O_2	37.6 ± 21.4	2.1 ± 1.5	93.4 ± 42.7	33.8 ± 2.2
	(7.8 ± 3.5)	(0)	(29.4 ± 4.5)	(3.6 ± 2.6)
Percentage O_2 recovered as H_2O_2	71.5	8.6	87.8	44.7

The differences between concanavalin A-treated and resting cells are reported. The values are expressed as nmol/4 min/1.5×10^7 cells ± SEM. Resting values are given in parentheses. For assay conditions, see Table 1. 5 μg/ml CB, 100 μg/ml concanavalin A and 2 mM NaN_3.

and H_2O_2 formation and release. These results clearly suggest that the increased amount of $O^{\cdot}_2$ and H_2O_2 detected in the extracellular medium is due to a double effect of CB. The first of these effects is a potentiation of the stimulatory activity of Con A with increased generation of $O^{\cdot}_2$ and H_2O_2. The mechanism by which CB induces this stimulation is unknown. It should be pointed out here that CB does not modify the amount of ^3H-labelled lectin bound to the surface of the cell (data not shown). The second effect consists in an increased availability of both of the intermediate products of oxygen reduction in the extracellular medium. Under these experimental conditions the amount of H_2O_2 measured in the extracellular medium reaches a value close to 50% of the O_2 actually consumed. The mechanism under-

Table 5. Percentage of O_2 recovered as H_2O_2 in BCG-activated rabbit AM during the respiratory burst induced by phagocytosis and concanavalin A

	Phagocytosis	Con A
	6.5 ± 2.8 (4)	0.3[0-0.6] (4)
+ NaN_3	8.9 ± 2.0 (9)	5.8 ± 2.8 (6)
+ NaN_3 + CB	25.7 ± 7.7 (4)	44.7 ± 7.4 (6)

Table 6. Ingestion of bacteria (Bacillus mycoides) by normal and BCG-activated rabbit AM

	Normal	Normal + NaN_3	BCG-activated	BCG-activated + NaN_3
Percentage macrophages containing bacteria	97	94	96	96
Average number of bacteria/cell	10	11	10	11
Percentage cells containing 2-4 bacteria	8	17	12	10
Percentage cells containing 5-10 bacteria	55	30	52	42
Percentage cells containing > 10 bacteria	37	53	36	48

For experimental details see text.

lying this increased availability might be related to an increased rate of diffusion (permeability) of $O^{\cdot}_2$ and of H_2O_2 through the plasma membrane, or to their production at sites less accessible to enzymes active in their degradation, or to an inhibition of some of the mechanisms of their degradation. The latter possibility is unlikely, because, as we have seen, CB does not modify the activities of SOD, catalase, peroxidase, glutathione peroxidase and reductase, as measured in the appropriate cell fractions.

Although we need further investigations to clarify the mechanisms by which CB causes this double effect, the results obtained with this experimental model, that is, Con A plus CB, show that in AM too a respiratory burst can occur in which the values of the various parameters (oxygen consumed, $O^{\cdot}_2$ and H_2O_2 released) and their quantitative relationship lie in the range of the values obtained in the granulocytes of the rabbit and other mammalian species.

The results presented so far can be summarized as follows: 1) The respiratory burst in BCG-induced AM is associated with the production of $O^{\cdot}_2$ and H_2O_2. 2) In these cells the equilibrium between the rate of formation of these intermediates, the rate of their degradation, and the rate of their release is such that, under physiological conditions, only small amounts of $O^{\cdot}_2$ and H_2O_2 are released outside the cells. This equilibrium can be modified by appropriate experimental conditions, and the intermediates can be released at higher rate. Table 5 gives the percentage of O_2 actually consumed recovered as H_2O_2 during the respiratory burst induced by phagocytosis and by Con A under various conditions.

The respiratory burst in normal and in BCG-induced AM

It is known that the activated state of macrophages is associated with a greater stimulation of HMP during phagocytosis (Myrvik et al. 1972; Romeo et al. 1974; Karnovsky et al. 1975; Rossi et al. 1975). The polarographic traces of O_2 consumption during phagocytosis (Fig. 4) show that in BCG-activated AM the rate of oxygen uptake is also higher than in their normal

Table 7. O_2 consumption and $O^{\cdot-}_2$ and H_2O_2 release by rabbit normal and BCG-activated AM during phagocytosis in the absence and presence of NaN_3

		Resting		Phagocytosing - Resting	
			NaN_3		NaN_3
O_2	Normal	136.7 ± 12.6 (4)	73.1 ± 9.5 (8)	66.1 ± 12.6 (4)	52.6 ± 13.4 (7)
	BCG-activated	130.5 ± 10.0 (9)	89.8 ± 9.9 (9)	97.7 ± 14.1 (9)	80.6 ± 13.3 (9)
$O^{\cdot-}_2$	Normal	0 (5)	0 (5)	0.4[0-1.4] (4)	0.8[0-2.3] (6)
	BCG-activated	0 (4)	0 (4)	5.0 ± 1.4 (4)	6.0 ± 1.2 (7)
H_2O_2	Normal	0 (5)	0 (5)	0 (4)	0.3[0-0.6] (4)
	BCG-activated	0 (4)	0 (4)	6.5 ± 1.2 (4)	7.2 ± 1.1 (9)

The values are expressed as nmol/4 min/$1.5x10^7$ cells ± SEM. Number of experiments in parentheses. For assay conditions, see Table 1.

counterparts. Since this difference could be due to a higher rate of and capacity for ingestion of bacteria, we measured the ingestion of bacteria by normal and BCG-activated AM. The measurements were carried out on samples of cells withdrawn from the chambers of the oxygen electrode during the recording of the respiration and stained according to May-Grünwald-Giemsa. The results presented in Table 6 clearly show that under these conditions the rate of phagocytosis is similar in normal and BCG-activated AM. Thus, the greater stimulation of O_2 consumption by BCG-induced AM reflects an enhanced metabolic perturbability of these cells during phagocytosis.

Table 7 shows comparative data on the O_2 consumption and the $O^{\cdot-}_2$ and H_2O_2 release by normal and BCG-induced AM. In the latter cells the amount of $O^{\cdot-}_2$ and H_2O_2 recovered in the extracellular medium during the respiratory burst is higher than in the former cells. This finding is confirmed by the kinetic analysis of H_2O_2 release from normal and BCG-activated AM during the respiratory burst induced by different stimulants and in the presence of CB (Fig. 5). The spectrophotofluorometric traces show that in normal NaN_3-treated AM. H_2O_2 is only measurable during the respiratory burst induced by bacteria or by Con A when CB is present. In BCG-activated AM, however, H_2O_2 is released under all of the conditions of stimulation, in both the absence and the presence of CB. It is worth mentioning that the amounts of $O^{\cdot-}_2$ and H_2O_2 released are higher in BCG-activated AM, both as absolute values and in relation to the amount of O_2 actually consumed. The data in Table 8 summarize this phenomenon with respect to the release of H_2O_2.

The results presented so far indicate that compared with normal AM, BCG-activated AM show a larger respiratory burst and a higher release of $O^{\cdot-}_2$ and H_2O_2 into the extracellular medium.

To understand the reason for the different release of $O^{\cdot-}_2$ and H_2O_2 in the extracellular environment, we compared the activities of the enzymes responsible for the intracellular degradation of the two intermediates in normal and BCG-activated AM. The data presented in Table 9 show that there is a marked difference between the enzyme-activity patterns of normal and BCG-activated AM. In the latter cells the activity of gluta-

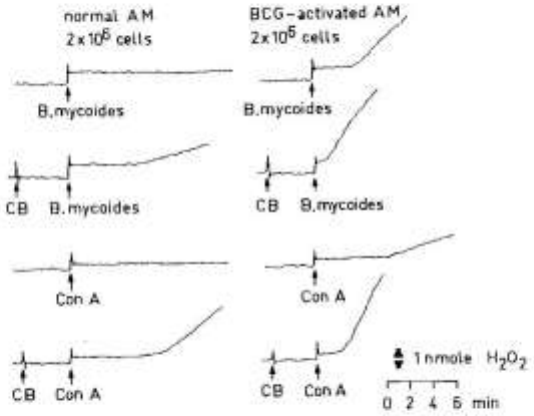

Fig. 5. Spectrophotofluorometric assay of H_2O_2 production by normal and BCG-activated AM stimulated by Bacteria and concanavalinA in the absence and in the presence of cytochalasin B. For experimental conditions, see Figs 2 and 3.

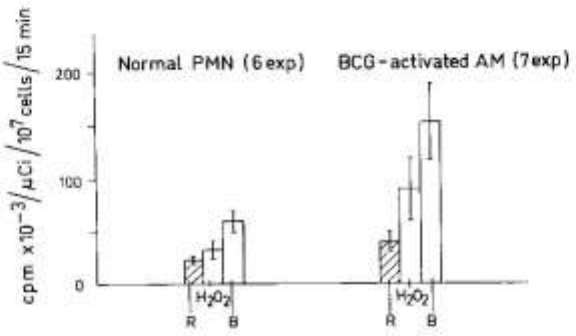

Fig. 6. Effect of bacteria or H_2O_2 addition on the $^{14}CO_2$ production from 1-^{14}C glucose by normal and BCG-activated AM. For experimental conditions, see Fig. 1. R = resing cells. B = cells plus bacteria.

Table 8. Percentage of O_2^- recovered as H_2O_2 in normal and BCG-activated rabbit AM during the respiratory burst induced by phagocytosis and concanavalin A

	Normal		BCG-activated	
	Phagocytosis	Con A	Phagocytosis	Con A
	0 (4)	0 (5)	6.5 ± 2.8 (4)	0.3[0-0.6] (4)
+ NaN_3	0.6[0-2] (4)	0 (5)	8.9 ± 2.0 (9)	5.8 ± 2.8 (6)
+ NaN_3 + CB	4.5 ± 2.5 (4)	26.2 ± 8.9 (4)	25.8 ± 7.7 (4)	44.7 ± 7.4 (6)

Table 9. Enzyme activities in rabbit normal and BCG-activated AM

	Normal (5)		BCG-activated (8)		
	U*/10^7 cells	Specific activity	U*/10^7 cells	Specific activity	
o GSH-peroxidase	254.2 ± 36.0	306.2	753.0 ± 137.0	941.2	p <0.01
o GSSG-reductase	38.4 ± 4.8	46.2	52.7 ± 4.0	57.1	p <0.025
o Catalase	313.5 ± 12.0	368.8	262.0 ± 31.0	340.2	NS
Δ Peroxidase	62.8 ± 19.2	69.8	20.4 ± 3.0	19.4	p <0.05
□ SOD	16.3 ± 3.1	9.0	9.4 ± 1.7	4.8	p <0.05
o G6P-DH	197.3 ± 34.5	234.8	459.0 ± 73.2	533.7	p <0.01

The mean ± SEM is reported. The number of experiments is given in parentheses. o = measured in 100,000 g supernatant, Δ = measured in 100,000 g pellet, □ = measured in total homogenate. The significance of the differences was calculated according to Student's t-test.

* nmol NADPH/min for GSH-peroxidase, GSSG-reductase, and G6P-DH, μmol H_2O_2/min for catalase, nmol tetraguaiacol/min for peroxidase. One unit of superoxide dismutase (SOD) is the amount of enzyme that causes a decrease of 0.0125 OD/min in the reduction of cytochrome c.

Table 10. Extra respiration and $O^{\cdot}_2$ and H_2O_2 release in NaN_3-treated BCG-activated AM during the respiratory burst induced by phagocytosis and concanavalin A in the absence and presence of CB

	Phagocytosing		Con A-treated	
	(6)	CB (4)	(4)	CB (4)
Extra O_2 consumption	71.3 ± 12.8	38.1 ± 5.7	24.3 ± 5.7	75.6 ± 6.9
$O^{\cdot}_2$	6.0 ± 1.5	24.5 ± 9.1	3.7 ± 1.8	48.4 ± 9.8
H_2O_2	7.1 ± 1.9	9.6 ± 1.7	2.1 ± 1.5	33.8 ± 2.2
H_2O_2 derived from dismutation of $O^{\cdot}_2$ released (nmoles)	3.0	12.25	1.85	24.2

The values are given as nmol/4 min/1.5×10^7 cells. For assay conditions, see Tables 1 and 4.

thione reductase and, particularly, of glutathione peroxidase, are higher and those of peroxidase and superoxide dismutase are lower than in normal AM. This pattern indicates that BCG-induced cells are endowed with a greater capacity to degrade H_2O_2, mostly by the glutathione cycle, and have a lower capacity to catalyze the enzymatic dismutation of $O^{\cdot}_2$. Furthermore, we do not know whether the observed enzymatic activities reflect an actual difference in the total and in the relative capacity of the cells to utilize $O^{\cdot}_2$ and H_2O_2. However, the higher stimulation of HMP pathway activity by exogenous H_2O_2 in BCG-induced AM (Fig. 6) indicates that, at least under conditions of overloading, the glutathione cycle is more efficient in the activated cells than in the normal cells.

The observation that BCG-activated AM release more H_2O_2 during the respiratory burst, in spite of the greater efficiency of the glutathione cycle, deserves some comment. At first sight it would appear that in these cells the activity of glutathione

peroxidase (300% increase; Table 9) should counteract the higher generation of the peroxide due to the greater respiratory burst (50% increase; Table 7). Some hypotheses can be advanced to explain this discrepancy: 1) It is likely that in BCG-activated AM the rate of dismutation of $O^{\cdot}_2$ catalyzed by SOD is inadequate with respect to the enhanced generation of the radical. This hypothesis is supported by the finding that the release of $O^{\cdot}_2$ is also higher in these cells during the respiratory burst. Furthermore, if the intermediate that escapes the degradation is mostly $O^{\cdot}_2$, a consistent amount of H_2O_2 found in the extracellular environment should derive from the spontaneous dismutation of the released $O^{\cdot}_2$. This seems to be the case (Table 10). 2) A second explanation, which does not rule out the first, is that the glutathione cycle is actually unable to cope with the higher rate of H_2O_2 generation during the respiratory burst. In fact, despite a high activity of glutathione peroxidase, the actual efficiency of the glutathione cycle is controlled by the activity of the key enzyme glutathione reductase, whose activity is only slightly increased in BCG-activated AM. Furthermore, the increased activity of the NADPH oxidase during the respiratory burst may compete for NADPH with glutathione reductase, and this represents another limiting factor for the activity of the key enzyme and hence for the efficiency of the glutathione cycle. 3) Finally, it could not be excluded that a modification of the plasma membrane takes place in BCG-activated AM, resulting in a higher rate of diffusion of $O^{\cdot}_2$ and of H_2O_2. This hypothesis could be consistent with the finding that in the activated cells the greater release of $O^{\cdot}_2$ and of H_2O_2 occurs independently of their maximal rate of generation as, for example, when the intensity of the respiratory burst induced by bacteria is decreased by CB.

Apart from these explanations, which at present are conjectural and require further investigation, the data presented show that the state of activation induced by BCG in alveolar macrophages is associated with: 1) an increased stimulation of the oxidative metabolism; 2) a modification of the equilibrium between the rate of formation, the rate of degradation, and the rate of diffusion across the plasma membrane of $O^{\cdot}_2$ and of H_2O_2,

such that these intermediates are released into the extracellular environment in higher amount both as absolute value and as compared to the amount of oxygen actually consumed. These modifications indicate that, although the release of $O^{\cdot-}_2$ and H_2O_2 is extremely low compared with that occurring in other phagocytic cells such as PMN, the efficiency of the bactericidal and cytocidal mechanisms linked to the respiratory burst is greater in the activated macrophages than in normal AM.

SUMMARY

The respiratory burst of rabbit AM is associated with the formation of superoxide anion and hydrogen peroxide. In normal cells these intermediates are not released, owing to the high efficiency of the intracellular mechanisms for their transformation and degradation. When appropriate devices are used in vitro a small amount of both superoxide anion and H_2O_2 will be released into the extracellular environment.

The state of activation of AM induced by BCG is associated with an enhanced metabolic perturbability. In activated AM the greater respiratory burst is also accompanied by a modification of the equilibrium between the rate of formation and the rate of degradation of the intermediate products of oxygen reduction, and as a result, during phagocytosis part of the intermediates is released into the extracellular environment in higher amounts, both as absolute values and as percentage of the amount generated. Thus, the process of activation of AM increases the efficiency of the bactericidal mechanisms linked to the respiratory burst.

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