Superoxide generation during phagocytosis by Acanthamoeba castellanii: similarities to the respiratory burst of immune phagocytes

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Starved cultures of the soil amoeba Acanthamoeba castellanii suspended in phosphate-buffered saline exhibit stimulated O₂ uptake in response to phagocytosis of either heat-killed yeast or latex beads. This phagocytosis-dependent (cyanide-insensitive) oxidative activity was observed when cells were isolated from either cyanide-stimulated or -inhibited cultures, and was therefore independent of the relative activities of the alternative mitochondrial oxidases known to exist in this organism. The extra O₂ consumed during phagocytosis was stoichiometrically converted into O₂⁻ as detected by the rate of superoxide dismutase-inhibitable cytochrome c reduction. Phagolysosomal membranes isolated after uptake of latex beads were enriched in a b-type cytochrome which was spectrally similar to that present in immune phagocytes. Thus, the biochemical events during phagocytosis by either A. castellanii or immune phagocytes appear similar, suggesting that the 'respiratory burst' enzyme(s) responsible for O₂⁻ generation in these two cell types is structurally related.

Introduction

Acanthamoeba castellanii is a free-living soil amoeba which may phagocytose micro-organisms in order to satisfy its nutritional requirements. In the laboratory the organism may be successfully grown axenically in semi-defined liquid medium, but even when cultured under these conditions it still retains avid phagocytic activity when challenged. Thus, organisms grown in liquid media rapidly phagocytose bacteria, latex beads, erythrocytes and heat-killed yeast when these are presented to them.

Unlike immune phagocytes (such as polymorphonuclear neutrophils or mononuclear phagocytes), the mechanisms by which A. castellanii kill and digest their target organisms have not been extensively investigated. The immune phagocytes possess a broad spectrum of specialized killing processes, many of which have been partially characterized (Curnutte, 1988). Of these bacterial systems, the so-called O₂⁻-dependent pathways, i.e. those generating a series of reactive oxygen metabolites such as O₂⁻, H₂O₂ and HOCl during a respiratory burst of non-mitochondrial O₂ uptake (Babior, 1984), have received the greatest attention. Extensive experimentation has shown that these oxidants are essential for the killing of some (but not all) microbial pathogens because phagocytes from patients with genetic defects in the generation of these oxidants have an impaired ability to kill certain microbial pathogens both in vivo and in vitro (Holmes et al., 1967). The precise molecular processes necessary for the generation of these oxidants have not been fully defined, but the activation of a O₂⁻/H₂O₂-generating plasma-membrane-bound NADPH oxidase (Rossi, 1986) which contains a flavoprotein and unusual cytochrome b, is a key event.

Whilst the biological functions of phagocytosis in A. castellanii and immune phagocytes are quite different (being required for nutrition in the former and host protection in the latter), several lines of evidence have demonstrated a number of similarities in oxidative metabolism in the two cell types. For example, both exhibit cyanide-insensitive O₂ uptake and increased O₂ consumption during phagocytosis (Edwards & Lloyd, 1977; Edwards & Doulah, 1982; Brooks & Schneider, 1985). It was thus the aim of the present study to establish whether A. castellanii possesses an O₂⁻-generating 'respiratory burst' oxidase which becomes activated during phagocytosis.

Methods

Growth and maintenance of the organism. A. castellanii was grown and maintained with shaking at 30 °C exactly as described previously (Edwards et al., 1987). The growth medium comprised (% w/v): proteose peptone, 0.75; yeast extract, 0.75; glucose, 1.5. Organisms were counted (after a suitable dilution with 0.9% NaCl) using a Fuchs Rosenthal haemocytometer slide.
Isolation of phagolysosomes. This was performed by the method described by Korn (1974). A 1 l cell culture was grown to the mid-exponential phase of growth, harvested by centrifugation at 1000 g for 5 min and then cells were suspended in fresh growth medium at 2 × 10⁶ cells ml⁻¹. The suspension was then incubated at 30 °C with shaking for 30 min and latex beads (1-01 μm diameter) were added to give a latex concentration of 0·5 mg ml⁻¹. The culture was incubated for 45 min with gentle shaking (30 cycles min⁻¹) and harvested by centrifugation at 1000 g for 5 min. The cell pellet was washed three times with 20 mM-Tris/HCl, pH 7·0, and then suspended in a disruption buffer of 30% (w/v) sucrose, 10 mM-Tris/HCl, pH 7·0. After homogenization in a chilled Kontes hand homogenizer (usually 10 passages gave approximately 75% breakage, which was estimated microscopically), the extract was diluted in this buffer to give an equivalent cell density of 5 × 10⁷ cells ml⁻¹ and placed in a centrifuge tube. The extract was successively overlayed with 25%, 20%, and 10% (w/v) sucrose solutions (all containing 10 mM-Tris/HCl, pH 7·0) and then centrifuged at 131000 g for 1·5 h. After centrifugation, latex-filled phagolysosomes collected at the interface of the 20% and 10% sucrose solutions and were removed via a syringe after puncturing the wall of the tube. The phagolysosome preparation was diluted two-fold in 20 mM-Tris/HCl, pH 7·0, and sonicated by 3 × 10 s bursts at 0 °C. The released latex beads were collected by centrifugation at 5000 g for 15 min. The resulting supernatant containing phagolysosomal membranes was removed and centrifuged at 100000 g for 1 h and the pellet recovered.

Analytical methods. O₂ uptake was measured on 2 ml suspensions of cells in PBS (phosphate-buffered saline; 0·9% NaCl; 10 mM-potassium phosphate buffer, pH 7·4) at 0·7–1·8 × 10⁶ cells ml⁻¹ using a Clark-type O₂ electrode (Rank Bro.). Superoxide anion (O₂⁻) production was measured on cell suspensions in PBS (0·1–1·0 × 10⁶ cells ml⁻¹) by the method of Babior et al. (1973) using a Perkin-Elmer Lambda 5 spectrophotometer operating in the double-beam mode. Suspensions in the sample and reference cuvettes (1 ml) contained 75 μM-cytochrome c, latex particles and superoxide dismutase (20 μg ml⁻¹), as indicated. Rates of O₂ production were calculated from these absorption changes using the millimolar absorption coefficient of 18 (Ohnishi et al., 1967). Heat-killed yeast were prepared as described by Edwards & Doulah (1982) and difference spectra were recorded at room temperature using a Perkin-Elmer Lambda 5 spectrophotometer. Protein was measured by the Lowry method using bovine serum albumin as standard. Chemiluminescence was performed using an LKB 1251 luminometer in 1 ml suspensions containing either 10 μM-luminol or 25 μM-lucigenin (Edwards, 1987). Human myeloperoxidase was purified by the method of Pember et al. (1983) and its A₄₃₀/A₂₈₀ was 0·82.

Results

Stimulation of O₂ uptake during phagocytosis

During phagocytosis (or activation by other stimuli), neutrophils undergo a rapid phase of cyanide-insensitive O₂ uptake known as the respiratory burst. Unstimulated rates of O₂ uptake are low in these cells because of the absence of mitochondrial respiration (Klebanoff & Clark, 1978), and the respiratory burst reduces O₂ to O₂⁻ in a one-electron step, H₂O₂ being formed from the enzymic or spontaneous dismutation of O₂⁻ (Babior, 1984). A. castellanii possesses a number of terminal oxidases and previous work has shown that during exponential growth the apportionment of electrons via these alternative oxidases during respiration varies considerably (Edwards & Lloyd, 1977; Lloyd et al., 1979). Thus, during the early exponential phase of growth O₂ uptake is markedly cyanide-stimulated but it becomes increasingly cyanide-sensitive during the mid-to late-exponential phase.

Initial experiments on suspensions of A. castellanii in growth medium from either cyanide-stimulated or -sensitive cultures failed to show increased O₂ uptake during phagocytosis of either latex beads or heat-killed yeasts (data not shown). In contrast to neutrophils, mitochondrial respiration plays a major role in the overall oxidative metabolism of A. castellanii, and so organisms were grown in the presence of chloramphenicol, which substantially decreases mitochondrial respiration due to inhibition of cytochrome biosynthesis (S. W. Edwards, unpublished observations). Cultures were grown for 24 h at chloramphenicol concentrations of up to 2 mg ml⁻¹. Whilst cell growth was inhibited (e.g. 40% inhibition at 2 mg ml⁻¹), cells grown in the presence of this inhibitor and suspended in growth medium failed to show any appreciable stimulated O₂ uptake during phagocytosis.

Therefore, organisms were grown to the early- or mid-exponential phase of growth, harvested and suspended in PBS for various times and temperatures (0, 18 or 30 °C for periods of up to 20 h), in order to deplete the cells of oxidizable substrates and hence decrease mitochondrial respiration. Representative O₂ uptake rates of cells suspended in PBS are shown in Fig. 1 before and after the addition of phagocytic stimuli. When suspensions were washed with PBS and assayed within 4 h, the rate of
O₂ uptake upon the addition of heat-killed yeast was, in fact, reduced during phagocytosis (Fig. 1, A). However, when washed suspensions were incubated for 20 h at 4 °C before assay, the rate of unstimulated respiration was reduced by approximately 30% (Fig. 1, B, C), presumably due to exhaustion of endogenous mitochondrial substrates. In these starved suspensions the rate of O₂ uptake was clearly stimulated by over 40% upon the addition of either heat-killed yeast (Fig. 1, B) or latex beads (Fig. 1, C). This enhanced O₂ uptake during phagocytosis was observed when cells were isolated from either cyanide-stimulated or -sensitive cultures and was always unaffected by the addition of 1 mM-cyanide (data not shown).

Thus, starved A. castellanii clearly exhibit a respiratory burst of stimulated O₂ uptake during phagocytosis of either heat-killed yeasts or latex beads, but this activity is distinct from the phenomenon of cyanide-stimulated respiration.

**O₂-Generation during phagocytosis**

The primary product of O₂ reduction during phagocytosis of neutrophils is O₂⁻ (Babior et al., 1973) and the rate of O₂ uptake during the respiratory burst is dependent upon the concentration of particulate stimuli used, presumably reflecting the number of phagocytic vesicles formed per cell (Hallett et al., 1987). Whereas in unstimulated A. castellanii (starved in PBS) O₂⁻ generation was undetectable, upon the addition of latex beads the rate of production of this oxidant markedly increased (Fig. 2, A). This stimulated rate of cytochrome c reduction was inhibited by superoxide dismutase (Fig. 2, B), confirming that the assay indeed reflected O₂⁻ production. The rate of production was strictly dependent on the concentration of latex particles used to elicit the respiratory burst (Fig. 3): thus, when more phagocytic vesicles were produced, the rate of O₂⁻ generation correspondingly increased.

If all the extra O₂ uptake during phagocytosis was reduced to O₂⁻, then one would expect that the rate of stimulated O₂⁻ production to be equivalent to the extra O₂ consumed: the stimulated portion of O₂ uptake during phagocytosis was 1.8–2.3 nmol min⁻¹ per 10⁶ cells, whereas the maximal rate of O₂⁻ generation detected was 1.8–2.1 nmol min⁻¹ per 10⁶ cells, indicating that most, if not all of the extra O₂ consumed is converted to O₂⁻.

**Chemiluminescence**

When neutrophils phagocyte particles or are activated by other stimuli, reactive oxidant generation can be detected by luminol- or lucigenin-dependent chemiluminescence (Allen & Loose, 1976; Allen, 1981). In addition to the O₂⁻/H₂O₂ generating oxidase, neutrophils also possess the enzyme myeloperoxidase (Klebanoff & Clark, 1978), which is capable of producing HOCl and related compounds. Luminol-chemiluminescence

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Fig. 2. O₂ production during phagocytosis. Superoxide formation during phagocytosis was measured as the rate of superoxide-dismutase-inhibitable cytochrome c reduction, as described in Methods. Suspensions (10⁶ ml⁻¹, total volume 1 ml) contained 75 μM-cytochrome c at 30 °C. In (A) the reference cuvette and in (B) both the sample and reference cuvettes contained 20 μg superoxide dismutase ml⁻¹; phagocytosis was initiated by adding 10⁹ latex particles to each cuvette. Rates of cytochrome c reduction were then converted to rates of O₂⁻ using an eₘₐₜ of 18 (Ohnishi et al., 1967).

Fig. 3. Effect of latex bead concentration on O₂⁻ production. Experimental conditions were as described in the legend to Fig. 2 except that latex beads at the final concentration indicated were added to the cuvettes (the reference cuvette containing 20 μg superoxide dismutase ml⁻¹) to initiate phagocytosis. The maximal rate of O₂⁻ production measured at each bead concentration was then measured.
measures the activity of the myeloperoxidase-H₂O₂ system, whereas lucigenin-chemiluminescence measures myeloperoxidase-independent oxidants.

*A. castellanii* suspended in PBS exhibited low but detectable levels of lucigenin-chemiluminescence upon phagocytosis of latex beads (Fig. 4, A), indicating that during this process they generate O₂⁻ and/or H₂O₂. The maximal rate of lucigenin-dependent chemiluminescence detected was strictly dependent upon the cell concentration, but rates per cell (0.6 mV per 10⁶ cells), were considerably lower than those detected for neutrophils measured under identical conditions (100 mV per 10⁶ cells) (unpublished observations). In contrast, no luminol-dependent chemiluminescence was detected at any cell concentration used (Fig. 4, B). This assay requires the activity of the myeloperoxidase-H₂O₂ system (Davies & Edwards, 1989) and as *A. castellanii* does not possess peroxidase activity (S. W. Edwards, unpublished observations), this result was perhaps not too surprising. Therefore, exogenous purified human myeloperoxidase was added to *A. castellanii* suspensions containing luminal prior to the addition of latex beads: upon initiation of phagocytosis a burst of chemiluminescence was detected, which rapidly declined (Fig. 5). Addition of myeloperoxidase after this initial burst did not enhance chemiluminescence (data not shown).

**Absorption spectra of phagolysosomes**

From the above data it may be concluded that *A. castellanii* possesses an O₂⁻/H₂O₂-generating oxidase which appears analogous, at least functionally, to the plasma-membrane-bound NADPH oxidase of neutrophils. This NADPH oxidase contains an unusual cytochrome b which probably functions as the terminal oxidase, and this has an absorption maximum (in reduced minus oxidized difference spectra) at 426 nm (Segal & Jones, 1978). Phagolysosomes were prepared...
from *A. castellanii* and, after removal of latex particles, were analysed for cytochrome content. Fig. 6 shows that phagolysosomal membranes contain a haemoprotein with an absorption maximum at 426 nm, spectrally similar to the cytochrome *b* of the NADPH oxidase of neutrophils.

**Discussion**

The results presented here clearly show that *A. castellanii* possesses a respiratory burst-like oxidase system which is responsible for stimulated O$_2$ uptake during phagocytosis leading to O$_2$ formation. Thus phagocytosis in this organism has many similarities to the process more extensively studied in immune phagocytes, namely: (1) increased cyanide-insensitive O$_2$ uptake during phagocytosis; (2) stoichiometric conversion of this O$_2$, into O$_2^-$; (3) a direct relationship between the number of phagocytic vesicles produced per cell and the extent of oxidant production; (4) a b-type cytochrome present in phagolysosomal membranes. It may well be, and further experiments are clearly necessary to clarify this, that the enzyme complexes responsible for the respiratory burst in both *A. castellanii* and immune phagocytes are analogous and even more importantly, perhaps linked in evolutionary terms.

Stimulated O$_2$ uptake was not detected in freshly harvested cells suspended in either growth medium or PBS. Furthermore, attempts to reduce the contribution of mitochondrial respiration to the overall oxidative metabolism by growth in the presence of chloramphenicol did not facilitate detection of phagocytosis-dependent O$_2$ uptake. It was found to be necessary to starve the cells in PBS for 20 h at 4°C to reduce oxidizable mitochondrial substrates before respiratory burst activity could be measured. Under these experimental conditions no encystment of the cells was observed and the fact that they maintained high levels of O$_2$ uptake which was enhanced during phagocytosis is indicative that most, if not all, cells had retained their viability. It was expected that cells from cyanide-stimulated cultures would exhibit the greatest rates of phagocytosis-dependent O$_2$ uptake, but this was found not to be the case: starved cells from either cyanide-stimulated or -inhibited cultures exhibited approximately equal rates of stimulated O$_2$ uptake during phagocytosis. Thus, the capacity of the cells to mount a respiratory burst is independent of the apportionment of electrons via the alternative mitochondrial oxidases in this organism (Edwards & Lloyd, 1977; Lloyd *et al.*, 1979).

As *A. castellanii* can generate O$_2^-$ (and hence oxidants derived from this such as H$_2$O$_2$ and -OH, Halliwell & Gutteridge, 1985), it is possible that this oxidant is utilized (either directly or indirectly) to kill ingested bacteria during phagocytosis. In contrast to immune phagocytes, *A. castellanii* does not contain peroxidase activity and hence peroxidase-derived oxidants (e.g. HOCl) or peroxidase-mediated cytotoxicity cannot be employed. Addition of purified myeloperoxidase prior to the initiation resulted in luminol-chemiluminescence during particle uptake (Fig. 5). The rapid decline in chemiluminescence after the initiation of phagocytosis reflects intraphagolysosomal utilization of myeloperoxidase (rather than rates of H$_2$O$_2$ production), presumably because low levels of this enzyme are co-internalized during bead uptake: addition of myeloperoxidase when phagocytosis was proceeding did not result in photon emission as the extracellular enzyme could not gain access to intracellularly-generated H$_2$O$_2$ production. Established methods now exist for the purification of the NADPH oxidase complex (Curnutte, 1988) and the cDNA sequence of the neutrophil cytochrome *b* is available (Royer-Pokora *et al.*, 1986), so it should be possible to utilize immunological and gene cloning techniques to determine the structural similarities between the respiratory burst enzyme complexes in these two cell types.

**References**


