Chemiluminescence and superoxide production in *Acanthamoeba castellanii*: free radicals generated during oxidative stress

BERNARD DAVIES and STEVEN W. EDWARDS*

Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

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The amoeba *Acanthamoeba castellanii* generated both luminol- and lucigenin-enhanced chemiluminescence upon addition of the respiratory inhibitor sodium cyanide, but not upon the addition of sodium azide. Photon emission in the presence of lucigenin was three- to fourfold greater than that measured in the presence of luminol, but both forms of chemiluminescence were strictly dependent upon the presence of O_2 , indicating the requirement for oxidative reactions in these processes. Lucigenin-chemiluminescence measured during the phagocytosis of latex beads under identical conditions was, however, barely detectable above background levels. Cyanide similarly induced the formation of O_2^- , as indicated by the stimulation of superoxide-dismutase-inhibitable cytochrome *c* reduction, and the rate of production was similar to that previously observed during phagocytosis of latex particles or yeasts by these cells. Thus, in view of the similar rates of O_2^- production during cyanide exposure or phagocytosis, but disparate rates of lucigenin-chemiluminescence, these two treatments must activate different molecular processes leading to reactive oxidant production. The rates of cyanide-stimulated lucigenin-chemiluminescence were directly proportional to the O_2 tensions in the medium from 0 to 300 μM , indicating that the rates of free-radical-generating reactions were directly related to the oxygen tensions in the environment. The superoxide dismutase inhibitor diethyldithiocarbamate similarly stimulated lucigenin-chemiluminescence, with photon emission again being dependent upon O_2 tensions in the range 0–320 μM . A mechanism by which cells may limit O_2^- -generating reactions, and so reduce damaging free radical reactions, was observed when anaerobic suspensions were re-aerated. These data indicate that oxidative stress and phagocytosis provide two intracellular sources of free-radical generating reactions in these cells.

Introduction

Acanthamoeba castellanii is a phagocytic soil amoeba which possesses a complex array of terminal oxidases whose relative activities and contributions to whole-cell respiration show considerable variations during exponential growth. These oxidases include a mitochondrial (cyanide-sensitive) cytochrome oxidase, an alternative salicylhydroxamic-acid-sensitive (cyanide-insensitive) mitochondrial oxidase and a low-affinity cyanide-sensitive (azide-insensitive) component (Edwards & Lloyd, 1977, 1978; Lloyd *et al.*, 1979a). Additionally, these cells possess a cyanide-insensitive O_2^- -generating oxidase (resembling the NADPH oxidase of neutrophils and macrophages; Curnutte, 1988), which becomes activated during phagocytosis of heat-killed yeast and latex particles (Davies *et al.*, 1991). This latter oxidase is responsible for the lucigenin-dependent chemiluminescence which is produced during this process. A *b*-type cytochrome (Segal & Jones, 1978) becomes associated

with phagolysosomal membranes during engulfment of latex particles (Davies *et al.*, 1991) and hence the O_2^- -generating oxidases of *A. castellanii* and of 'professional phagocytes' may be structurally related.

The respiratory burst of 'professional phagocytes' may be monitored by the sensitive technique of chemiluminescence, which can be amplified using probes such as luminol (Allen & Loose, 1976) and lucigenin (Allen, 1981). The precise molecular processes that result in photon emission during oxidation of these probes are unknown, but the former measures peroxidase/ H_2O_2 -dependent reactions whereas the latter appears to be dependent upon O_2^- and/or H_2O_2 formation (Edwards, 1987). The O_2^- -generating oxidase of *A. castellanii*, unlike that of neutrophils and macrophages, produces little, if any, luminol-chemiluminescence during phagocytosis (because the cells are devoid of peroxidase), but lucigenin-chemiluminescence is readily detected (Davies *et al.*, 1991).

Previous work has shown that when cultures of *A.*

castellanii are exposed to the respiratory inhibitor sodium cyanide, they generate low levels of chemiluminescence (Lloyd *et al.*, 1979b). It was proposed that this O_2^- -sensitive photon emission was due to inhibition by cyanide of superoxide dismutase, the enzyme responsible for regulating the intracellular levels of O_2^- which is inevitably generated during oxidative metabolism (Halliwell & Gutteridge, 1985). Inhibition of superoxide dismutase by cyanide thus results in an increase in the steady-state level of O_2^- and this oxidant (or an oxidant derived from it, such as a product of lipid peroxidation) is responsible for photoemission.

The aim of this work was to identify the mechanisms by which O_2^- may be generated in these cells and to ascertain if cyanide-stimulated chemiluminescence was mediated by molecular processes which were similar to, or distinct from, those involved in the respiratory burst.

Methods

Growth and maintenance of the organism. *A. castellanii* was grown and maintained with shaking at 30 °C exactly as described previously (Edwards & Lloyd, 1977). 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.

Analytical methods. Superoxide anion (O_2^-) production by cell suspensions in PBS (phosphate-buffered saline: 0.9% NaCl; 10 mM-potassium phosphate buffer; pH 7.4) was measured by the method of Babior *et al.* (1973) using a Perkin-Elmer Lambda 5 spectrophotometer: suspensions (1 ml) contained 1×10^6 cells, 75 μ M-cytochrome *c*, and superoxide dismutase, as indicated. Chemiluminescence measurements were performed in 10 ml suspensions containing either 10 μ M-luminol or 50 μ M-lucigenin in a specially constructed 'open' system in which O_2 measurements could be made simultaneously with chemiluminescence measurements (Edwards *et al.*, 1987). In this system the O_2 tensions in the gas phase (and after equilibration, those in the liquid phase) were regulated using a digital gas mixer.

Results

Effect of cyanide and azide on chemiluminescence

Suspensions of *A. castellanii* were grown to the mid-exponential phase of growth ($1-2 \times 10^6$ cells ml^{-1}) and suspended in PBS. Ten millilitres of suspension were placed into the 'open' O_2 /chemiluminescence system (maintained at 30 °C) and lucigenin was added to 50 μ M. Under a gas phase of air (235 μ M- O_2 at 30 °C), the rate of unstimulated chemiluminescence was approximately 250 c.p.s. per 10^6 cells, but upon the addition of 1 mM-cyanide there was a rapid increase in this photon emission to 750 c.p.s. per 10^6 cells (Fig. 1, A). The rate of chemiluminescence rapidly declined over the next

Fig. 1. Lucigenin-dependent chemiluminescence. *A. castellanii* were grown to the mid-exponential phase (2×10^6 cells ml^{-1}), harvested and suspended in PBS at a concentration of 1.6×10^6 cells ml^{-1} . Ten millilitres of this suspension was placed in the 'open' O_2 electrode/chemiluminescence system (Edwards *et al.*, 1987), which was maintained at 30 °C, and lucigenin was added to 50 μ M (final concn). At the point indicated (↓) 1 mM-sodium cyanide was added. In (A) the gas phase comprised air (235 μ M- O_2); in (B) the gas phase comprised O_2 -free N_2 , and at the point indicated (↓) the gas phase was switched to air, using a digital gas mixer.

15 min, but could be restored if a further addition of cyanide was made (data not shown). This phenomenon is explained by the fact that the inhibitor is expelled from the liquid phase in such 'open' systems because of the continuous gas flow through the suspension (Lloyd *et al.*, 1979b). When suspensions were incubated under anaerobic conditions (by replacing air with O_2 -free N_2 in the gas phase, and monitoring the O_2 tension in the liquid) the endogenous rate of chemiluminescence was decreased to 150 c.p.s. per 10^6 cells and no increase in chemiluminescence was observed upon the addition of cyanide (Fig. 1, B). However, when the suspension was aerated by switching the gas phase to air, photon emission was detected. Additions of sodium azide (up to 5 mM) did not stimulate lucigenin-chemiluminescence (data not shown).

When 10 μ M-luminol replaced lucigenin in the incubations, a similar chemiluminescence emission was observed upon the addition of 1 mM-cyanide (Fig. 2, B), but the responses were lower than those observed for lucigenin (although it must be pointed out that the concentrations of luminol used in these experiments were lower than those of lucigenin). For example, unstimulated levels of photon emission were 200 c.p.s. per 10^6 cells, which increased by a further 125 c.p.s. per 10^6 cells upon the addition of cyanide. A similar O_2^- dependence of luminol-chemiluminescence was observed (Fig. 2, C) but again, sodium azide did not elicit a response (Fig. 2, A).

No differences in cyanide-stimulated lucigenin- or

Fig. 2. Luminol-dependent chemiluminescence. Experimental conditions were exactly as described in the legend to Fig. 1 except that the suspensions contained $10\ \mu\text{M}$ -luminol. In (A) $1\ \text{mM}$ -sodium azide was added and in (B) $1\ \text{mM}$ -sodium cyanide was added, at the points indicated ($\downarrow$): the gas phase comprised air. In (C) the gas phase comprised O_2 -free N_2 and at the point indicated ($\downarrow$) $1\ \text{mM}$ -sodium cyanide was added and then at ($\downarrow$) the gas phase was switched to air.

Fig. 3. Lucigenin-dependent chemiluminescence during phagocytosis. Experimental conditions were as described in the legend to Fig. 1, the gas phase comprising $235\ \mu\text{M}$ - O_2 . At the point indicated ($\downarrow$) latex beads (3×10^{10} , $1\ \mu\text{m}$ diameter in a total volume of $10\ \text{ml}$) were added and the chemiluminescence response measured.

luminol-chemiluminescence were observed when cells were isolated from cultures which were harvested at the early (cyanide-stimulated) or late (cyanide-sensitive) phases of exponential growth (Edwards & Lloyd, 1977), indicating no dependency on the activities of the alternative terminal oxidases.

Cyanide-stimulated and phagocytosis-induced chemiluminescence and O_2^- production

We have previously shown that phagocytosis of heat-killed yeast or latex beads by *A. castellanii* is accompanied by increased rates of lucigenin-dependent, but not luminol-dependent, chemiluminescence and O_2^- production (Davies *et al.*, 1991). Therefore, it was necessary to ascertain if cyanide-stimulated chemiluminescence was related to the molecular processes which are responsible for the respiratory burst and which

Fig. 4. Cyanide-stimulated O_2^- production. *A. castellanii* were grown to the mid-exponential phase of growth and suspended in PBS at a concentration of $1 \times 10^6\ \text{cells ml}^{-1}$. Cytochrome *c* was added to a final concentration of $75\ \mu\text{M}$ and superoxide dismutase ($20\ \mu\text{g ml}^{-1}$) was added to the reference cuvette. A_{550} was measured using a Perkin-Elmer Lambda 5 spectrophotometer. After following endogenous activity for 5 min, $1\ \text{mM}$ -cyanide was added to both sample and reference cuvettes and the increase in A_{550} measured. In (A) $20\ \mu\text{g ml}^{-1}$ (final concn) superoxide dismutase was added to the sample cuvette at the point indicated ($\downarrow$), whereas in (B) this was added to the suspension prior to the addition of cyanide.

become activated during phagocytosis. Under identical experimental conditions to those in Figs. 1 and 2, the lucigenin-chemiluminescence activated upon phagocytosis of latex beads by *A. castellanii* was just detectable above background levels (Fig. 3) using this apparatus. In order to measure rates of O_2^- production, cells were suspended in PBS containing cytochrome *c*, and O_2^- generation was measured as the rate of superoxide-dismutase-inhibitable reduction of this haemoprotein (Babior *et al.*, 1973). Endogenous rates of cytochrome *c* reduction were zero, indicating immeasurable rates of O_2^- production in untreated cells, but upon the addition of $1\ \text{mM}$ -cyanide, the rate of reduction increased rapidly, reaching a maximal rate of $2.5\ \text{nmol min}^{-1}\ \text{per } 10^6\ \text{cells}$ (Fig. 4, A). This cytochrome *c* reduction was abolished upon addition of superoxide dismutase (at $20\ \mu\text{g ml}^{-1}$), indicating its dependence upon the generation of O_2^- . When suspensions were incubated with superoxide dismutase prior to the addition of cyanide, the rate of cytochrome *c* reduction was greatly reduced (Fig. 4, B), confirming that the reduction of this haemoprotein was indeed due to the formation of O_2^- . We have previously shown that upon phagocytosis of latex beads by *A. castellanii*, O_2^- generation is also rapidly activated and maximal rates of production are 1.8 – $2.1\ \text{nmol min}^{-1}\ \text{per } 10^6\ \text{cells}$ (Davies *et al.*, 1991). Hence, in view of the comparable activated rates of O_2^- generation but disparate levels of lucigenin-chemiluminescence, we conclude that O_2^- generation via phagocytosis and upon cyanide addition arise from two separate molecular processes.

Fig. 5. O_2 -dependence of cyanide-stimulated chemiluminescence. Cells were incubated as described in the legend to Fig. 1 at 5×10^5 cells ml^{-1} . O_2 tensions in the suspensions were poised at pre-determined levels using a digital gas mixture and after a 5 min equilibration period, 1 mM-cyanide was added and the chemiluminescence response measured. 0% indicates the unstimulated background level and 100% represents 1000 c.p.s. per 10^6 cells.

O_2 dependence of cyanide-stimulated chemiluminescence

In order to determine the relative O_2 -dependence of cyanide-stimulated chemiluminescence in *A. castellanii*, cells were suspended in PBS (containing $50 \mu M$ -lucigenin) and the O_2 tension in the medium (monitored using an O_2 electrode) was poised at pre-determined levels throughout the range 0–235 μM . After equilibration at each O_2 tension for 5 min, 1 mM-cyanide was added to the suspension through an injection port so that the O_2 tension was not appreciably perturbed, and the magnitude of the chemiluminescence response was measured. Fig. 5 shows that the rate of cyanide-stimulated chemiluminescence was directly proportional to the O_2 tension in the medium. When suspensions were gassed with O_2/N_2 mixtures to attain O_2 tensions in the medium above 235 μM (the O_2 tension of air-saturated medium at $30^\circ C$), the rate of cyanide-stimulated chemiluminescence increased further. Thus, at 300 μM - O_2 , the activated response was approximately 20% greater than that observed at 235 μM - O_2 . These data indicate that under hyperoxic conditions, when it may be predicted that oxidant-scavenging systems such as superoxide dismutase activity may be saturated due to oxidative stress, rates of oxidant production detected by lucigenin-chemiluminescence are enhanced.

Effect of diethyldithiocarbamate on chemiluminescence

The above experiments confirm the idea that cyanide stimulates O_2^- production in *A. castellanii* via inhibition of superoxide dismutase (Lloyd *et al.*, 1979b). However, cyanide also inhibits a number of other enzymes involved in oxidative metabolism in these cells (such as

Fig. 6. Effect of diethyldithiocarbamate concentration on lucigenin-dependent chemiluminescence. Experimental conditions were as described in the legend to Fig. 1 except that the cell concentration was 5×10^5 cells ml^{-1} . Rates of endogenous lucigenin-dependent chemiluminescence were measured and then diethyldithiocarbamate was added at the final concentration indicated. The rate of chemiluminescence was monitored and the maximal rate obtained (by 30 min) was plotted as a function of the inhibitor concentration: 100% (the rate obtained at a concentration of 5 mM) represents 250 c.p.s. per 10^6 cells, whilst 0% represents the unstimulated background level.

cytochrome oxidase and catalase) and hence its effects on lucigenin-dependent chemiluminescence may not be solely due to inhibition of superoxide dismutase activity. Therefore, a more specific inhibitor of superoxide dismutase was sought, and to this end, the effects of diethyldithiocarbamate (Halliwell & Gutteridge, 1985) on this phenomenon were tested.

Suspensions of *A. castellanii* were incubated with various concentrations of diethyldithiocarbamate and subsequent rates of lucigenin-dependent chemiluminescence were measured. This inhibitor did indeed stimulate chemiluminescence, but its effects were different to those of cyanide in a number of ways. Firstly, the inhibitor induced a much more gradual increase in chemiluminescence compared with cyanide, being maximal by 30 min after addition: this may reflect a reduced ability of diethyldithiocarbamate to permeate the cells. Secondly, the maximal rate of chemiluminescence stimulated by diethyldithiocarbamate was only about one-third of that stimulated by cyanide. Thirdly, the diethyldithiocarbamate-stimulated chemiluminescence signal was more stable than that induced by cyanide, presumably because the former inhibitor was not expelled from the suspension during continuous aeration. The maximal enhancement of lucigenin-dependent chemiluminescence by diethyldithiocarbamate occurred at a concentration of 5 mM (Fig. 6) and this concentration of inhibitor was used in experiments to determine the O_2 -dependence of its action.

Suspensions of *A. castellanii* were incubated with $50 \mu M$ -lucigenin at an O_2 tension of 300 μM and diethyl-

Fig. 7. Effect of O₂ on diethyldithiocarbamate-stimulated lucigenin-chemiluminescence. Experimental conditions were as described in the legend to Fig. 1 except that the cell concentration was 5×10^5 cells ml⁻¹. The cells were incubated at an O₂ tension of 300 μM and at the point indicated (↓) diethyldithiocarbamate (DEDTC) was added to a final concentration of 5 mM. The chemiluminescence response was then measured and the gas phase was then switched to N₂, (↓) (a) shows the O₂ tension in the suspension whilst (b) shows the lucigenin-dependent chemiluminescence.

dithiocarbamate was added at a concentration of 5 mM (Fig. 7). The rate of chemiluminescence then increased slowly to reach a maximal value after about 30 min incubation. This rate was stable for up to a further 2 h. The suspension was then made anaerobic by switching the gas phase to N₂ and the rate of chemiluminescence declined as the O₂ tension in the suspension decreased, so that at zero O₂, the rate of chemiluminescence was equivalent to that of the unstimulated suspension. This approach was then modified to measure the O₂-dependence of diethyldithiocarbamate-stimulated lucigenin-dependent chemiluminescence. Suspensions of cells were incubated at an O₂ tension of 300 μM and diethyldithiocarbamate added to 5 mM. After 30 min incubation (when the rate of chemiluminescence had reached a steady value) the suspension was made anaerobic by switching the gas phase to N₂. As the O₂ tension decreased to 140 μM, there was only about a 15% reduction in the chemiluminescence emission, but below this tension, the rate of photon emission was more O₂ dependent (Fig. 8). At zero O₂, the rate of chemiluminescence was identical to that of unstimulated suspensions. However, when anaerobic suspensions were then re-aerated, the O₂ profile observed was quite different to that of aerobic suspensions becoming anaerobic. For example, at O₂ tensions up to 140 μM, the rate of chemiluminescence was always lower in anaerobic → aerobic suspensions than in aerobic → anaerobic suspensions. Above 140 μM-O₂, the anaerobic → aerobic

Fig. 8. O₂ dependence of diethyldithiocarbamate-stimulated lucigenin-chemiluminescence. Experimental conditions were as described in the legend to Fig. 1 except that the cell concentration was 5×10^5 cells ml⁻¹. Cells were incubated at 300 μM-O₂, (■) and diethyldithiocarbamate was added to a final concentration of 5 mM. After a stable chemiluminescence response was obtained (30 min) the gas phase was switched to N₂ and the rate of chemiluminescence was measured as a function of the O₂ tension. Anaerobic suspensions (●) were then re-aerated by switching the gas phase to 300 μM-O₂. Similar results were obtained in five other experiments (data not shown).

chemiluminescence was much more O₂ dependent and when tensions approached 300 μM, maximal rates of chemiluminescence were restored. Several identical cycles of chemiluminescence changes during aerobic → anaerobic → aerobic transitions could be monitored. These data indicate that anaerobic suspensions may be adapting to increasing O₂ tensions in the environment by reducing the activities of O₂⁻-generating pathways which may be potentially hazardous.

Discussion

The data presented here confirm and extend the idea that the respiratory inhibitor sodium cyanide induces chemiluminescence in the amoeba *A. castellanii* (Lloyd *et al.*, 1979b) and strongly indicate that this phenomenon is due, at least in part, to inhibition of superoxide dismutase which leads to the accumulation of O₂⁻: this radical itself, or species formed from it (such as lipid peroxides, carbonyl radicals, etc.), may be the oxidant species which are responsible for the photon emission detected by the probes used in this study (luminol or lucigenin). Because no differences in cyanide-stimulated chemiluminescence were detected in cultures isolated from either the early (cyanide-stimulated) or late (cyanide-sensitive) exponential phases of growth (Edwards & Lloyd, 1977), we conclude that the phenomenon of cyanide-stimulated photon emission is independent of the activities of the phosphorylating or alternative mitochondrial oxidases known to operate in these organisms. Mitochondrial oxidase involvement may also

be ruled out on the basis of the O_2 -dependence of the phenomenon (Lloyd *et al.*, 1983a).

Previous work has shown that like 'professional phagocytes' such as neutrophils and macrophages, *A. castellanii* can generate O_2^- during a cyanide-insensitive respiratory burst of O_2 uptake which is activated upon phagocytosis of latex beads or heat-killed yeast (Davies *et al.*, 1991). Furthermore, this process results in the generation of lucigenin-dependent, but not luminol-dependent, chemiluminescence, although the levels of photon emission (on a per cell basis) are some 200-fold lower than those observed in neutrophils. Luminol-chemiluminescence can be detected, however, when exogenous myeloperoxidase is added to phagocytosing suspensions of *A. castellanii* and this observation is explained by the fact that, unlike lucigenin, luminol requires the activity of an O_2^-/H_2O_2 -generating system and a peroxidase for maximal efficiency of photon emission. *A. castellanii* possesses no endogenous peroxidase activity and hence lucigenin-chemiluminescence is a more efficient probe for O_2^-/H_2O_2 than luminol in this experimental system.

Upon the addition of cyanide, but not azide, both lucigenin- and luminol-chemiluminescence were stimulated in suspensions of *A. castellanii* although the rate of photon emission in the presence of the former probe was about fivefold greater than that detected with the latter probe. Furthermore, cyanide, but not azide, stimulated superoxide-dismutase-inhibitable cytochrome *c* reduction and hence this assay provides direct evidence for the increased generation of O_2^- upon addition of this inhibitor. Similarly, phagocytosis of latex beads induces lucigenin-chemiluminescence, but this was barely detectable above background photon emission. In contrast, rates of cyanide-stimulated and phagocytosis-induced O_2^- production were comparable. Hence, we conclude that the molecular pathways which result in cyanide-stimulated and phagocytosis-induced O_2^- production, and lucigenin-chemiluminescence, are distinct from one another.

One likely process that may produce chemiluminescence upon addition of cyanide is that proposed by Lloyd *et al.* (1979b), namely inhibition of superoxide dismutase and subsequent accumulation of O_2^- . However, cyanide is a non-specific inhibitor of a number of haemoproteins involved in oxidative metabolism (such as cytochrome oxidase and catalase) and hence we sought a more specific inhibitor of superoxide dismutase. Therefore, we measured the effect of diethyldithiocarbamate on lucigenin-chemiluminescence and showed that this also could activate oxidant production. Its major difference to the action of cyanide was that it took longer to exert its effects (presumably because of permeability problems) but the chemiluminescence signal obtained was more

stable. This allowed us to determine the relative O_2^- affinities of diethyldithiocarbamate-stimulated O_2^- production and also to examine the effects of aerobic/anaerobic transitions on this phenomenon.

When the O_2 -dependency of cyanide-stimulated chemiluminescence was measured by poisoning the O_2 tensions in the suspensions prior to addition of inhibitor, there was a close correlation between the chemiluminescence response obtained and the O_2 tension between 0 and 300 μM . This observation fits the idea that rates of O_2^- production are directly proportional to the O_2 tension in the environment and that conditions of oxidative stress are associated with high levels of production of this oxidant: steady-state levels of O_2^- are thus normally regulated by superoxide dismutases which prevent the accumulation of hazardous levels of this species. Whilst O_2^- itself has only limited reactivity, other radical species invariably generated from this, such as $\cdot OH$, are extremely damaging. Similarly, diethyldithiocarbamate-stimulated chemiluminescence showed O_2 -dependence from 0 to 300 μM - O_2 , but in these experiments an unusual and striking adaptation to anaerobiosis was observed: when anaerobic suspensions were re-aerated, rates of lucigenin-chemiluminescence were much lower than those observed in corresponding aerobic cultures which had been made anaerobic. This may be interpreted as an attempt by the cells to shut down oxidative processes leading to O_2^- production in order to prevent the likelihood of tissue damage mediated either directly or indirectly by this oxidant. Adaptation of mitochondrial respiration to periods of anaerobiosis has previously been reported in this organism (Lloyd *et al.*, 1983b).

In conclusion, we have shown that *A. castellanii* possesses at least two distinct oxidative pathways that result in O_2^- formation and lucigenin-chemiluminescence. The first of these occurs via the respiratory-burst enzyme which is activated upon phagocytosis: this enzyme appears analogous to the NADPH oxidase of 'professional phagocytes' and may be instrumental in microbial killing. The second source of O_2^- arises from the inhibition of superoxide dismutase by cyanide or diethyldithiocarbamate. Further work is necessary to determine if perturbation of the activity of this enzyme is associated with features of oxidative stress, such as increased rates of lipid peroxidation.

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