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Pneumocystis carinii: Oxygen Uptake, Antioxidant Enzymes, and Susceptibility to Oxygen-Mediated Damage

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The ability of *Pneumocystis carinii* obtained by alveolar lavage of rats with glucocorticoid-induced pneumocystosis to utilize molecular oxygen, the concentrations of selected antioxidant enzymes, and the susceptibility of *P. carinii* to in vitro killing by oxygen radical-generating systems have been evaluated. As expected of an organism which has been found to convert radiolabeled glucose to CO_2, the parasite utilizes molecular oxygen. No evidence for pathways of oxygen utilization other than the cytochrome pathway was found; cyanide virtually abolished oxygen consumption. Although readily detectable levels of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase were present in the *P. carinii* preparations, only superoxide dismutase was present at levels that suggested that the activity was indeed a property of the parasite. Almost certainly, *P. carinii* does not possess effective concentrations of catalase. In addition, it was found that *P. carinii* is susceptible to the lethal actions of hydrogen peroxide and superoxide, but the parasite seems to be resistant to the effects of a hydroxyl radical-generating system.

As a surface parasite of the alveolar type I cells (8, 23), *Pneumocystis carinii* is continually exposed to molecular oxygen as well as to its more reactive derivatives generated in metabolic pathways using molecular oxygen. Although it is reasonable to assume that a parasite so situated would be able both to use molecular oxygen and to catalyze detoxification of reactive oxygen moieties, there is no direct evidence that *P. carinii* possesses any of these capacities. Determining the susceptibility of *P. carinii* to the lethal effects of oxygen radicals is also relevant to understanding the susceptibility of the parasite to secretory products of inflammatory cells (7, 12). The studies reported here show that, although *P. carinii* is capable of utilizing oxygen by cyanide-sensitive pathways, the organism possesses low levels, if any, of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase. The parasites are susceptible to micromolar concentrations of hydrogen peroxide and to superoxide generated by cell-free systems, but they are unaffected by a hydroxyl ion-generating system.

MATERIALS AND METHODS

P. carinii was obtained from the lungs of Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, Wis.) that became overtly ill after prolonged (weeks to months) maintenance on low-protein chow and prednisolone-tetracycline–containing drinking water (15). The animals were killed by air embolism through a tail vein, and the heart and lungs were removed en bloc. After perfusion of the pulmonary circulation with 0.05 M phosphate-buffered saline (pH 7.4) containing 0.6 mM EDTA, the alveolar spaces were lavaged with phosphate-buffered saline–EDTA. Contaminating host inflammatory cells were removed by adherence to glass in medium 199 containing 10% heat-inactivated newborn calf serum, penicillin, and gentamicin (TCM) in an atmosphere of air and 5% CO_2, and the clumps of *P. carinii* cells and nonadherent host cells were disrupted by repeated passage through 27-gauge needles. The process was monitored by phase-contrast microscopic examination. Only preparations in which over 98% of the material was *P. carinii* were used for these studies; in most cases, *P. carinii* cells outnumbered host cells by at least 200 to 1. The final preparations, containing 1 $\times 10^8$ to 5×10^8 *P. carinii* cells per ml, contained <500 bacteria per ml (usually gram-negative rods) when cultured on brain heart infusion agar a level of contamination which does not contribute measurable activity in any of the assays (16). No attempts were made to grow mycoplasma, which I have not succeeded in isolating from the lavage fluids from similar chronically tetracycline-treated rats (16). *P. carinii* was stored on ice in TCM and used within 24 h of harvest. Because it is rare that more than a single rat is ready for harvest of *P. carinii* on any given day, organisms were derived from a single animal for each day's experiments.

Oxygen consumption. For determination of oxygen consumption, *P. carinii* suspensions were incubated in Earle salts solution (ESS) without glucose, with 0.1% albumin and antibiotics, in a Clark-type polarographic oxygen detector (YSI model 53 oxygen monitor) with a chart recorder. After it was determined that rates of oxygen consumption in ESS without glucose, ESS with glucose, and medium 199 containing 10% serum did not differ, subsequent measurements were conducted in TCM, since the organisms seem to maintain their integrity and ability to metabolize glucose in TCM longer than they do in defined salt solutions (16).

Antioxidant enzymes. Detection and quantitation of antioxidant enzyme systems in 0.1% Triton X-100-lysed *P. carinii* preparations were carried out using published methodology. Superoxide dismutase was quantitated by the method of McCord and Fridovich (11) by spectrophotometrically measuring the inhibition of reduction of cytochrome *c* (type III; Sigma Chemical Co., St. Louis, Mo.) by xanthine oxidase-xanthine-generated superoxide at 550 nm with bovine blood superoxide dismutase (Sigma type I) as the standard. Only total levels of the enzyme were determined. Glutathione peroxidase was determined by the method of Paglia and Valentine (13) by spectrophotometrically measuring the reduction of 0.28 mM NADPH by glutathione at 340 nm in the presence of 36 mM azide and glutathione reductase (Sigma). Catalase activity was determined by measuring the liberation of O_2 from a 25 mM solution of H_2O_2 in phosphate-buffered saline, using a polarographic electrode as previously described by Mandell (10), with bovine liver catalase (Sigma) as a standard. The concentration of H_2O_2 in stock solutions

TABLE 1. Oxygen consumption by *P. carinii* in vitro[a]

Medium	No. of expts.	O consumption (μl/h per mg of protein)[b]
ESS without glucose	3	1.4 ± 0.3
ESS + glucose (7 mM)	3	1.0 ± 0.1
ESS + glutamate	2	1.2, 1.5
ESS + pyruvate	2	1.2, 1.4
ESS + D-(+)-lactate	1	1.1
ESS + D-(−)-lactate	1	1.1
TCM	5	1.3 ± 0.4
TCM + KCN	3	0.1

[a] Electrode O consumption subtracted.
[b] For calculations of microliters of O, an O content of 5 μl of O per ml of ESS at 100% saturation by air at 37°C is assumed.

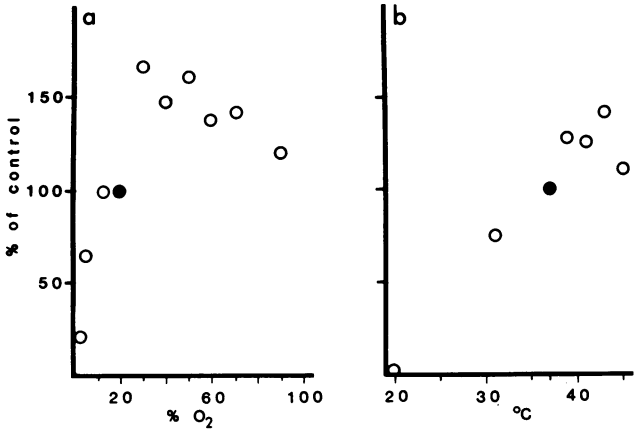

FIG. 1. Influence on consumption of oxygen by *P. carinii* of altered oxygen concentration in the medium at 37°C (a) or altered temperature at ambient oxygen tension (b). Values represent means of closely agreeing (±10%) results from two separate experiments. Oxygen consumption (microliters per min per milligram) under various conditions (○) is expressed as the percentage of consumption at ambient oxygen tension and 37°C (●).

was determined by measuring the absorbance of H_2O_2 solutions in deionized water, using $E = 43.6\ M^{-1}\ cm^{-1}$. For all assays, rat erythrocytes and rat alveolar macrophages were also analyzed both as references for which there are published data (6) concerning activity of these enzymes and to estimate the possible contribution of residual host cells to the measured activity of *P. carinii*-enriched preparations. Data for oxygen consumption and enzyme levels were normalized for protein content estimated by the method of Lowry et al. (9), using egg-white lysozyme as the standard.

Susceptibility to oxygen radical killing. Susceptibility of *P. carinii* to the lethal action of hydrogen peroxide in the presence and absence of iodide (5 mM) and lactoperoxidase (5 mU/ml; Sigma and Calbiochem, Lajolla, Calif.) was tested in a medium composed of 0.05 M citrate phosphate-buffered (pH 5.2, 6.2, or 7.2) saline with 7 mM glucose and 0.1% bovine serum albumin. Additional experiments in which hydrogen peroxide was generated in TCM by the addition of glucose oxidase (0.5 μg/ml) were conducted. In the same buffer systems, as well as in TCM, the effects of superoxide anions generated by the action of xanthine oxidase (0.04 U/ml) on xanthine (5×10^{-5} M) and of hydroxyl ions generated by the action of xanthine oxidase on acetaldehyde (20 mM) were also evaluated. For these assays, the effects of the addition of exogenous catalase (500 to 1,000 U/ml) and superoxide dismutase (250 U/ml) on *P. carinii* survival were also determined. To eliminate nonspecific protein effects in the inhibition studies, boiled enzymes were also tested. All assays were carried out in capped tubes and incubated at 37°C for 2 h on a tilt-table aliquot mixer. *P. carinii* controls were similarly incubated in TCM. Exposure to the oxidants was terminated by the addition of a 15-fold excess of cold phosphate-buffered saline and two rinses with centrifugation ($500 \times g$). Viability of *P. carinii* was then determined by measurement of the conversion of [*U*-^{14}C]glucose to $^{14}CO_2$ after 2 h of incubation in ESS without unlabeled glucose at 37°C as previously described (16). Apparent survival was calculated from the ratio of $^{14}CO_2$ production in test and control samples.

RESULTS

The initial experiments were carried out to further characterize the basic metabolic capabilities of *P. carinii* (16). Direct measurements of oxygen consumption with a polarographic electrode (Table 1 and Fig. 1) indicate that *P. carinii* utilizes molecular oxygen almost entirely by cyanide-sensitive pathways. Oxygen consumption was unaffected by the addition of glucose, lactate, pyruvate, or glutamate. Extraction of oxygen from the medium was relatively uniform over a range of oxygen concentrations (from ca. 10 to 50% oxygen), with measurable consumption occurring at oxygen concentrations of as low as $pO_2 = 8$ Torr. The organisms tolerated brief (15-min) exposures to oxygen concentrations of as high as 60% but were killed, as determined by a lack of further oxygen consumption or subsequent [^{14}C]glucose metabolism in 20% oxygen, by concentrations in excess of 70% for 5 to 10 min. Also, metabolic activity, as determined by oxygen consumption, as most active in the temperature range expected in the mammalian host and was unmeasurable at ambient environmental temperatures (Fig. 1b). Similarly, oxygen consumption (in microliters per minute per milligram of protein) was roughly proportional to the quantity of oxygen dissolved in the medium in the concentration range of 5 to 30% oxygen, but consumption declined gradually at higher concentrations (Fig. 1a).

The measured concentrations of the antioxidant enzymes catalase, superoxide dismutase, and glutathione peroxidase are summarized in Table 2, along with values for rat macrophages and erythrocytes. Superoxide dismutase levels in the *P. carinii*-enriched preparations were ca. 10% of the levels

TABLE 2. Activity of antioxidant enzyme systems

Cells	Enzyme activity		
	Catalase (U/mg of protein)	Glutathione peroxidase (μmol of NADPH per mg of protein)	Superoxide dismutase (U/mg of protein)
P. carinii	0.4 ± 0.1	0.06 ± 0.002	1.4 ± 0.4
Rat			
Macrophages[a]	18 (45)[b]	4.6 (77)	8.0 (6)
Erythrocytes[c]	600 (1,500)	7.3 (122)	21.4 (15)

[a] Glass-adherent alveolar macrophages from normal rats ($n = 2$).
[b] Ratio of activity of rat cell preparation to that of *P. carinii*-enriched preparation is indicated in parentheses.
[c] Washed erythrocytes from normal rats ($n = 2$).

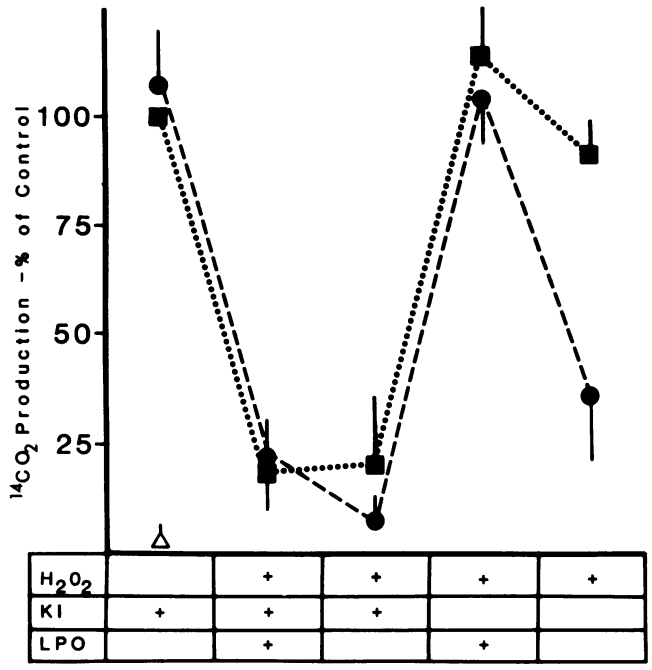

FIG. 2. Viability of *P. carinii* as determined by [*U*-^{14}C]glucose metabolism after 2 h of exposure in vitro to combinations of H_2O_2 (10 μM), iodide (KI), and lactoperoxidase (LPO) indicated along the abscissa. Results are shown for experiments conducted at pH 5.2 (△), 6.2 (●), and 7.2 (■). Means and standard errors of results from at least three experiments are plotted.

found in rat cells, whereas the measured activity of the other two enzymes was so low that trace contamination of the preparations with rat cells would have been sufficient to account for the measured levels. In the case of catalase, as few as one erythrocyte per 1,500 *P. carinii* cells could have produced the activity detected, a contamination level which present techniques do not exclude. Thus, it is tentatively concluded that *P. carinii* cells possess intrinsic superoxide dismutase activity but may not possess either catalase or glutathione peroxidase.

The susceptibility of *P. carinii* to in vitro killing by hydrogen peroxide, superoxide anions, and hydroxyl radicals is depicted in Fig. 2 and 3. In Fig. 2, the susceptibility of *P. carinii* to H_2O_2 (10 μM) at pH 6.2 and 7.2 is shown; increasing the concentration of H_2O_2 to 100 μM caused no additional killing, and 1 μM H_2O_2 was ineffective. Also evident in Fig. 2 is the susceptibility of *P. carinii* to low pHs. The optimum pH for measuring peroxide-peroxidase-mediated killing of microorganisms is generally considered to be ca. 5 (7), but the assay could not be carried out at the pH because of the lack of survival of *P. carinii* at pH 5.2 in either β-alanine or citrate phosphate-buffered saline. The lethal effect of the peroxide required iodide (5 mM) for maximal expression, but it was not enhanced by the addition of lactoperoxidase from two sources (Sigma and Calbiochem). The lactoperoxidase samples from both suppliers were reactive in an iodine fixation assay for peroxidase and were virtually devoid of catalase activity. Both preparations promoted killing of *Staphylococcus aureus* 502A by H_2O_2 in iodide-containing media at pH 5.2 (data not shown). Although lactoperoxidase usually promotes killing of microbes, in some situations the addition of the enzyme has conferred protection (1). The addition of catalase (≥200 U/ml) to the reaction mixtures completely prevented H_2O_2-mediated killing in these assays. H_2O_2 generated by the addition of glucose oxidase (0.5 μg/ml) to the medium was also lethal to *P. carinii* (11 ± 6% survival) and was less efficiently prevented by catalase (55 ± 8% survival with 2,000 U of catalase per ml; 14 ± 4% survival with 200 U of catalase per ml).

Superoxide generated by the action of xanthine oxidase on xanthine (17) was also lethal to *P. carinii* (Fig. 3). The addition of catalase was not protective, but the addition of superoxide dismutase (250 U/ml) or superoxide dismutase plus catalase (200 U/ml) was protective. There was no statistically significant improvement in survival noted after the addition of catalase to superoxide dismutase. Also apparent in Fig. 3 is the lack of effect of hydroxyl radicals generated by the action of xanthine oxidase on acetaldehyde (17). Other experiments in which the concentrations of both agents were either increased or decreased by 3- and 10-fold increments were conducted; again, no effects were noted (data not shown).

DISCUSSION

These data further extend the basic understanding of some of the functional capacities of *P. carinii*, especially those related to the life of the organism in the alveolar spaces and to the possible mechanisms of host defenses against the parasite. As would be expected of an organism which is adapted to life at the alveolar surface, *P. carinii* seems to actively consume molecular oxygen by pathways which appear to involve the cyanide-sensitive, cytochrome-mediated systems usually found in aerobic organisms. Somewhat

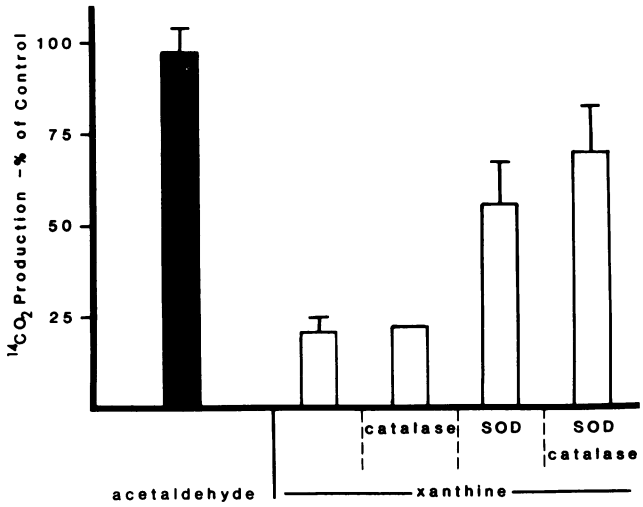

FIG. 3. Susceptibility of *P. carinii* to in vitro killing by xanthine oxidase containing oxygen radical-generating systems. Components present, in addition to xanthine oxidase, are indicated along the base line. Hydroxyl radicals generated by the action of xanthine oxidase on acetaldehyde did not kill *P. carinii* (solid bar). Superoxide generated by the action of xanthine oxidase on xanthine was lethal to the parasite, an effect that was diminished by inclusion of superoxide dismutase (SOD), with or without catalase, but not by catalase alone (open bars). Viability was determined as in Fig. 2, and results are plotted as the mean of results from two experiments or means and standard errors of results from three or more experiments.

surprising, however, and of potential significance in considering the role of the host in providing a suitable environment for growth of *P. carinii* is the observation that *P. carinii* preparations appear to possess limited quantities of classic antioxidant enzymes. Most aerotolerant and aerobic bacteria, as well as more complex eucaryotic organisms, contain and secrete a variety of antioxidant enzymes. When the enzymes are present, catalase and superoxide dismutase are found intracellularly and are secreted into the environment, whereas glutathione peroxidase activity is restricted to the cytoplasm. The data may best be interpreted as indicating that *P. carinii* cells probably possess superoxide dismutase, may not possess glutathione peroxidase, and almost certainly lack catalase. Until a method of purifying *P. carinii* from lavage fluids which allows the recovery of large numbers of *P. carinii* cells with a contamination from host cells of less than 0.01% is devised or in vitro growth of large numbers of organisms is feasible, it is unlikely that these interpretations will be substantially altered.

Available clinical and experimental data suggest that humoral and cellular immunity (20, 22, 24) as well as polymorphonuclear leukocytes (2, 14, 15) may participate in host defenses against *P. carinii* infection. Although the majority of patients with pneumocystosis currently being diagnosed have the acquired immunodeficiency syndrome and presumably suffer from a defect predominantly altering T-cell function (5), it is important to recall that both clinical and experimental data suggest roles for immunoglobulins (20, 21, 24) and neutrophils (2, 14, 15) in defense against the parasite. Although polymorphonuclear leukocyte function is often not considered in evaluations of host responses to *P. carinii*, patients with chronic granulomatous disease complicated by pneumocystosis have been reported (14), and in rats, gram-negative bacterial pneumonias which induce a polymorphonuclear leukocytic exudate protect against the subsequent development of steroid-induced pneumocystosis (15). The data presented here suggest that several mechanisms known to be active in phagocytic cells are capable of killing *P. carinii* in vitro. We previously noted that normal macrophages rapidly digested *P. carinii* in vitro (19). It is possible that the susceptibility of the organism to death at low pHs, as is expected to occur within phagolysosomes (7), could be involved in this rapid death within nonimmune macrophages. In addition, phagocytosing polymorphonuclear leukocytes (7) as well as activated macrophages (12) are known to secrete, into phagocytic vacuoles as well as into the surrounding medium, a variety of reactive oxygen moieties. The susceptibility of *P. carinii* to death within a short time after exposure to either superoxide or hydrogen peroxide at pH levels which may reasonably be expected to occur intracellularly and extracellularly in the alveolar spaces suggests that these mechanisms may be involved in host defenses against the organism. Acute pyogenic inflammation could promote the release of quantities of superoxide and peroxide that would be sufficient to kill *P. carinii* even in the absence of significant phagocytosis of the parasite by the inflammatory cells. Similarly, macrophages activated either specifically to *P. carinii* antigens or reacting to stimulation with another antigen which induces cellular immunity could also, by either direct phagocytosis or by secretion of reactive compounds, kill intraalveolar *P. carinii* cells. Whether the action of lymphokines on cells other than macrophages (3, 18), which may also be capable of generating superoxide and peroxides (4), could also contribute to the death of the parasites or to an inhibition of growth or excystment is an unanswered question.

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