Antioxidant defences in the microaerophilic protozoan *Trichomonas vaginalis*: comparison of metronidazole-resistant and sensitive strains

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The sensitivity of the microaerophilic protozoan *Trichomonas vaginalis* to oxygen and products of its reduction, and the antioxidant defences employed by this organism, were investigated. Studies revealed that this amitochondrial flagellate is sensitive to oxygen tensions above those experienced *in situ* in the vagina (i.e. > 60 μ M) and that metronidazole-resistant strains (CDC 85 and IR78) were more sensitive to elevated oxygen levels than a metronidazole-sensitive isolate (1910). In the presence of radical scavengers, inactivation of organisms at 60 μ M oxygen was significantly lessened. Investigation of the antioxidant enzymes present in this organism revealed that activities of peroxide-reducing enzymes (e.g. catalase and general peroxidase) were not detectable, but that a cyanide-insensitive, azide-sensitive superoxide dismutase was present in cell extracts. Measurement of thiol-cycling enzymes indicated that NADPH could drive the reduction of oxidized glutathione (thiol reductase); however, the corresponding peroxidase activity was not detected. Analysis of thiols in whole cells of *T. vaginalis* indicated that glutathione was absent, but high levels of other thiols, propanethiol, methanethiol and H₂S, were present. No significant differences were detected in thiol levels or antioxidant enzyme activities on comparison of metronidazole-sensitive and resistant strains. These results indicate that the sensitivity of *T. vaginalis* to oxygen above physiological levels is due to the lack of adequate peroxide-reducing enzymes and radical-scavenging mechanisms.

Keywords: *Trichomonas vaginalis*, oxygen free radicals, superoxide dismutase, thiol-cycling enzymes, glutathione

INTRODUCTION

The microaerophilic protozoan parasite *Trichomonas vaginalis* possesses many characteristics of anaerobic organisms, including sensitivity to oxygen. *In vivo*, *T. vaginalis* is exposed to an environment of fluctuating nutrient levels and oxygen concentrations; measurements at the vaginal epithelium indicate that this is a micro-aerobic environment (Wagner & Levin, 1978; Rashad *et al.*, 1992). The fermentative metabolism of this organism is sensitive to oxygen fluctuations in this range (Paget &

Lloyd, 1990; Ellis *et al.*, 1992). However, the mechanisms of cellular protection against potentially cytotoxic oxygen metabolites have hardly been studied in this organism, although drugs generating oxygen radicals are used for chemotherapy of trichomoniasis (Moreno & Docampo, 1985; Docampo, 1990). This has important implications for *T. vaginalis*, where metronidazole-resistant isolates have defective oxygen-scavenging mechanisms (Lloyd & Pedersen, 1985; Yarlett *et al.*, 1986).

Oxidant stress is associated with the generation of reactive oxygen-derived species which are ultimately responsible for damage to a wide variety of cellular components (enzymes, DNA, membrane lipids, etc.). All living cells are prone to the toxic effects of oxygen and its reduction

Abbreviations: DABCO, 1,4-diazabicyclo[2.2.2]octane; PFOR, pyruvate:ferredoxin oxidoreductase; SOD, superoxide dismutase; POD, peroxidase.

products. Adequate defence against oxidative stress usually requires the presence of a number of protective mechanisms, including the detoxifying enzymes catalase, superoxide dismutase (SOD), general peroxidase (POD) and the glutathione-cycling enzymes (Halliwell & Gutteridge, 1989). Factors predisposing to oxygen sensitivity include inadequate oxygen-detoxifying enzymes, the presence of oxygen-sensitive enzymes central to the metabolism of the cell, lack of cellular reducing power and a high oxygen consumption rate (Morris, 1979).

T. vaginalis avidly consumes oxygen at low levels (Lloyd *et al.*, 1989) and oxygen consumption systems are present in the cytosol (Thong & Coombs, 1987; Linstead & Bradley, 1988), and in the hydrogenosomes (Čerkasov *et al.*, 1978; Müller & Lindmark, 1978; Lloyd *et al.*, 1983). The compartmentation of the oxygen labile-enzymes pyruvate:ferredoxin oxidoreductase (PFOR) and hydrogenase within the hydrogenosome may provide some protection from oxygen and its reduction products. Defence mechanisms operating in *T. vaginalis* against oxygen and products of its reduction (e.g. hydrogen peroxide, superoxide, singlet oxygen, hydroxyl radical) have not been studied in detail, although there has been a report of the presence of SOD (Müller, 1990).

In this study we identified antioxidant enzymes in metronidazole-resistant and sensitive strains of *T. vaginalis*, and the sensitivity of this organism to oxygen. We propose that cytosolic and hydrogenosomal oxidases and SOD form major defences; however, sensitivity of this human pathogen to oxygen levels above those experienced *in situ* may be due to deficiencies in peroxide-reducing enzymes and the generation of toxic radicals which are quenched in the presence of exogenous radical scavengers.

METHODS

Organisms. *T. vaginalis* strains 1910, NYH-286 (ATCC 50148), C1-NIH (ATCC 30001), RU-393 (ATCC 50142), IR78 (ATCC 50138) and CDC 85 (ATCC 50143) were cultured in TYM medium (pH 6.2), without agar, supplemented with 10% (v/v) heat-inactivated horse serum (Diamond, 1957). Reducing agents (cysteine and ascorbate) were omitted from the growth medium for 1910, IR78 and CDC 85 cultures which were used in oxygen susceptibility and enzyme studies. The *in vitro* MIC for metronidazole required by the strains determined from previous studies indicated that 1910, C1-NIH and NYH 286 were metronidazole-sensitive (Yarlett *et al.*, 1986; Ellis *et al.*, 1992) and that IR78, CDC 85 and RU 393 were metronidazole-resistant (Müller *et al.*, 1980; Yarlett *et al.*, 1986). Stock cultures (10 ml) were maintained at 37 °C and subcultured daily. When required, 500 ml cultures were grown to late exponential phase, harvested by centrifugation (500 g for 5 min) and washed twice in Doran's buffer (KCl, 16 mM; CaCl₂, 6 mM; NaCl, 75 mM; NaH₂PO₄·3H₂O, 30 mM; pH 7.0) (Doran, 1969). This was prepared using distilled water rendered oxygen-free by boiling and cooling under nitrogen. Cell-free extracts were obtained by resuspending the final washed pellet in breakage buffer (225 mM sucrose, 20 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 20 mM Tris/HCl, 10 mM potassium phosphate; pH 7.0) and disruption of the organisms by three cycles of freezing in liquid nitrogen and thawing. Homogenates were kept in sealed tubes under

nitrogen at 4 °C and assayed immediately. Anaerobic precautions were maintained throughout. Organisms were counted using a haemocytometer.

Oxygen electrode. A Clark-type oxygen electrode open for gas exchange (Lloyd *et al.*, 1989; Ellis *et al.*, 1989) was used to measure the oxygen affinity of strain 1910 and the survival of three strains of *T. vaginalis* (1910, CDC 85 and IR78) in the presence of oxygen. The incubation volume was 5.5 ml and the cell suspension was stirred at 790 r.p.m. Washed suspensions of *T. vaginalis* ($4-5 \times 10^6$ ml⁻¹) were maintained at defined dissolved oxygen levels (within the range 0–220 µM) for 4 h at 37 °C in the presence or absence of D-glucose (30 mM). Samples (50 µl) were removed every 20 min and the motility observed microscopically as a measure of cell viability. The effects of scavengers of oxygen metabolites on the survival of *T. vaginalis* were also determined by maintaining organisms at 60 µM oxygen and supplementing the incubation buffer with one of the following: mannitol (50 mM), histidine (10 mM), DABCO (1 mM), sodium benzoate (10 mM) or catalase (2000 units ml⁻¹). All experiments were repeated at least twice.

Enzyme assays. Spectrophotometric assays were performed at 37 °C. SOD activity was measured by the method of McCord & Fridovich (1969) using bovine SOD (Sigma) as standard. To characterize the type of metal centre present in this enzyme, the extracts were incubated for 1 h at 37 °C with one of the following: KCN (1 mM), NaN₃ (5 mM) or H₂O₂ (0.5 mM) and then assayed (Tannich *et al.*, 1991). Non-specific peroxidase activity was assayed by the method of George (1953) and catalase as described by Cohen *et al.* (1970). The presence of the broad-spectrum thiol-cycling enzyme (XSSX) reductase and peroxidase were determined as described by Bergmeyer *et al.* (1974) and Lawrence & Burk (1976). One unit of enzyme activity corresponds to 1.0 µmol product formed or substrate catabolized min⁻¹. Protein was estimated by the method of Bradford (1976).

Analysis of reduced thiols. Triplicate samples of 10⁶ cells were pelleted in 2 ml microfuge tubes (10000 g, for 2 min). The supernatant was carefully removed and the cell pellet was immediately resuspended in 50 µl 40 mM HEPPS/NaOH, 2 mM EDTA, pH 8.0. Thiols were derivatized by the addition of 2 mM monobromobimane (Thiolite, Calbiochem) in absolute ethanol (50 µl) and heated at 70 °C for 30 min. After cooling, the samples were deproteinized with 4 M methanesulphonic acid (pH 1.5 with LiOH). After standing for 15 min at 4 °C, derivatized protein was removed by centrifugation (10000 g, 2 min) and the supernatant removed for analysis by HPLC. Thiols were separated by reverse-phase HPLC on a Beckman/Altex C-18 column (4.5 × 250 mm; particle size 5 µm) (Beckman Instruments) using a Perkin-Elmer Series 410 (North America Instrument Division) fitted with a Rheodyne loop (10 µl) injector. The HPLC separation method was as described by Fairlamb *et al.* (1987) and consisted of solvents A [0.25% (w/v) D-camphor sulphonate (Calbiochem), pH 2.64] and B [0.25% (w/v) D-camphor sulphonate containing 25% *n*-propanol (Calbiochem)]. Thiols were eluted using a flow rate of 1 ml min⁻¹ starting with an isocratic step at 10% B for 20 min followed by a linear gradient to 50% B for 40 min and then an isocratic step at 50% B for 10 min. Starting conditions were regenerated in 5 min and maintained for a further 15 min prior to the next injection. Fluorescent compounds were detected using a Perkin-Elmer LS-1 fluorescence detector fitted with a 1 µl flow cell, using an extinction wavelength of 375 nm and an emission wavelength of 480 nm. Monobromobimane standards of glutathione, propanethiol, methanethiol (Aldrich) and H₂S were prepared as described for the samples.

RESULTS

Enzyme activities in cell-free extracts

Homogenates of *T. vaginalis* strains 1910, IR78 and CDC 85 were assayed for antioxidant enzymes. Neither catalase nor non-specific peroxidase activities were detectable [limit of detection about 5 mU (mg protein)⁻¹]. SOD activity was present in all strains (Table 1). This enzyme was inhibited by H₂O₂ and NaN₃, but not by KCN, indicating that the activity was probably due to the presence of FeSOD. The extent of inhibition by azide varied between strains. Measurement of thiol-cycling enzymes indicated that NADPH-driven reduction of glutathione occurred. The corresponding peroxidase activity was not detectable.

Survival of *T. vaginalis* in the presence of oxygen

Washed whole-cell suspensions of *T. vaginalis* 1910, IR78 and CDC 85 were maintained without the addition of D-

Table 1. Superoxide dismutase and thiol-reductase activities in cell-free extracts of *T. vaginalis*

Strain	Thiol reductase [mU (mg protein) ⁻¹]	Superoxide dismutase* [U (mg protein) ⁻¹]	Inhibition of SOD (%) by		
			KCN	NaN ₃	H ₂ O ₂
1910	66 ± 15	2.7 ± 0.6	0	10	70
IR78	45 ± 9	3.4 ± 0.4	0	30	70
CDC 85	10 ± 4	6.3 ± 1.1	0	48	74

* SOD enzyme activity is defined as the amount of enzyme required to inhibit the reduction of cytochrome *c* by 50%.

Results presented are means ± SD from three separate experiments.

Fig. 1. Survival of washed suspensions of metronidazole-sensitive (1910) and resistant (CDC 85 and IR78) strains of *T. vaginalis* ($4-5 \times 10^6$ ml⁻¹) in the presence of dissolved oxygen. Results presented are means from at least two separate incubations. ○, Strain 1910; ▲, strain IR78; ■, strain CDC 85; t_{1/2} the time taken for 50% of the organisms to become non-viable.

Table 2. Effects of D-glucose and exogenous oxygen metabolite scavengers on the survival of metronidazole-resistant and sensitive strains of *T. vaginalis* at 60 μM oxygen

Incubations contained $4-5 \times 10^6$ organisms ml⁻¹. Results presented are mean values from at least duplicate incubations.

Addition	t _{1/2} (min)*		
	1910	IR78	CDC 85
None (control)	92	68	61
D-Glucose	227	149	340
Catalase	97	59	107
DABCO	82	78	107
Histidine	120	92	93
Benzoate	154	151	129
Mannitol	240	109	162

* Time taken for 50% organisms to become non-viable.

Table 3. Thiols in *T. vaginalis* isolates

Values are expressed as nmol (10⁶ organisms)⁻¹ ± SD from triplicate determinations. Glutathione was undetectable, limits of detection 0.1 nmol (10⁶ organisms)⁻¹. Metronidazole-resistant isolates are RU 393, IR78 and CDC 85.

Strain	Propanethiol	Methanethiol	H ₂ S
C1-NIH	3.60 ± 0.98	0.91 ± 0.12	3.40 ± 1.4
NYH 286	3.10 ± 0.87	1.20 ± 0.22	4.10 ± 0.9
IR78	2.70 ± 0.80	0.65 ± 0.12	0.60 ± 0.07
CDC 85	2.19 ± 0.96	0.75 ± 0.42	0.50 ± 0.05
RU 393	3.64 ± 1.15	0.75 ± 0.05	1.20 ± 0.35

glucose at defined oxygen concentrations. Inactivation rates were measured at half times for loss of viability. All organisms tested retained full motility for at least 2 h at < 15 μM oxygen (Fig. 1), whereas higher dissolved oxygen levels led to diminished viability. Average values for the time taken for 50% of the organisms to be non-viable (t_{1/2}) revealed that the metronidazole-resistant isolates (IR78 and CDC 85) were inactivated at lower oxygen levels than the sensitive strain 1910. Addition of D-glucose (30 mM) sustained the viabilities of all three strains at 60 μM (Table 2). Addition of oxygen-metabolite scavengers also increased the duration of cell viability under these conditions (Table 2). The most pronounced effects were observed with benzoate or mannitol.

Thiol analysis of *T. vaginalis* isolates

Glutathione was not consistently detectable in whole-cell extracts of *T. vaginalis* by HPLC (Table 3). A minor signal occasionally appeared at the same retention time as glutathione in some samples (Fig. 2), but this was not consistent and probably originated from the growth medium. Significant amounts of other thiols,

Fig. 2. HPLC analysis of *T. vaginalis* thiols. (a) *T. vaginalis* C-1 NIH (10^6 organisms); (b) *T. vaginalis* IR78 (10^6 organisms); (c) standards: 1, propanethiol (0.35 nmol) retention time 31.6 min; 2, glutathione (0.3 nmol) retention time 39.4 min; 3, methanethiol (0.35 nmol) retention time 34.9 min; 4, H_2S (0.35 nmol) retention time 54.31 min. Thiols were separated as described in Methods.

propanethiol, methanethiol and H_2S , were detected in these organisms. The relative amounts of thiols did not appear significantly different in metronidazole-resistant and sensitive strains (Table 3).

DISCUSSION

T. vaginalis copes well with oxidative stress at the low levels which are reflective of the vagina. However, at levels exceeding these (i.e. $> 60 \mu M$) this organism

becomes rapidly non-viable, indicating that the parasite is well adapted to those levels encountered *in situ*, but less able to cope above physiological oxygen tensions. The mechanisms leading to cell inactivation during oxidative stress are complex, and in this study we attempted to identify protective mechanisms employed by this parasite and propose shortcomings that may ultimately lead to cellular damage and death.

T. vaginalis possesses protective enzymes such as SOD and NADH oxidases (Linstead & Bradley, 1988; Ellis *et al.*, 1992), but lacks the major peroxide-reducing enzymes catalase and general peroxidase. The SOD activity detected was an order of magnitude lower than that in the cattle parasite *Trichostrongylus axei* (Lindmark & Müller, 1974; Kitchener *et al.*, 1984). Inhibition of SOD activity by H_2O_2 , and to a lesser extent azide, suggests the presence of FeSOD in *T. vaginalis*, which is characteristic of other parasitic protozoa such as *Entamoeba histolytica* (Tannich *et al.*, 1991), the trypanosomatids (Meshnick & Eaton, 1983; Trang *et al.*, 1983) and *T. foetus* (Lindmark & Müller, 1974; Kitchener *et al.*, 1984). This enzyme has been identified as a potential chemotherapeutic target in these parasites, as FeSOD enzymes are absent in mammalian cells (Meshnick & Eaton, 1983).

The presence of glutathione reductase activity suggested that some thiol-cycling detoxification mechanisms may operate in *T. vaginalis*, but glutathione peroxidase activity was absent. *T. vaginalis*, however, does not contain detectable glutathione; instead high levels of other thiols, propanethiol, methanethiol and H_2S are present. Thus, the thiol-peroxidase activity of *T. vaginalis* may be specific for a particular thiol and unable to operate with glutathione as substrate. Typical aerobic cells contain high levels of low molecular mass thiols, usually glutathione, which form a key detoxification mechanism and also protect enzymes bearing $-SH$ groups which are considered obvious targets for oxygen attack. Other amitochondrial eukaryotes such as *Giardia duodenalis* and *E. histolytica* also lack detectable glutathione (Brown *et al.*, 1993; Fahey *et al.*, 1984), and thiols other than glutathione have been proposed to function in detoxification reactions in *G. duodenalis*, where cysteine is the major low molecular mass thiol (Brown *et al.*, 1993), and in trypanosomatids, where the glutathione-conjugate trypanothione functions in a unique detoxification reaction (Smith *et al.*, 1991). Thus, *T. vaginalis* may utilize low molecular mass thiols other than glutathione in the detoxification of oxygen reduction products.

Externally added scavengers of oxygen metabolites prolonged the survival of *T. vaginalis* in the presence of $60 \mu M$ oxygen. Most effective were the radical scavengers benzoate and mannitol (Halliwell & Gutteridge, 1989), suggesting that hydroxyl radical is formed. In the presence of intracellular oxygen, toxic metabolites can be formed from growth medium components (Morris, 1979). In the case of *T. vaginalis*, high levels of iron are present in the culture medium. The catalytic role of transition metals, particularly iron and copper, has been implicated in the catalysis of free radical reactions (Halliwell & Gutteridge,

1989). Traces of redox-active, non-protein-bound and soluble iron or copper catalyse the transformation of the moderately inactive superoxide radical anion ($O_2^{\cdot-}$) to the highly active hydroxyl radical ($OH^{\cdot}$) via the Haber-Weiss reaction. Under physiological conditions these metal ions are complexed to low and high molecular mass cellular components and thus serve as catalytic centres for the repeated formation of free radicals. Similarly, the presence of high levels of thiols in *T. vaginalis* may have detrimental effects at elevated oxygen levels. High levels of thiols, most notably thioglycollate, markedly enhanced the bactericidal effect of oxygen on moderately anaerobic bacteria (Griffiths & Shoesmith, 1977). Thus at elevated oxygen tensions, these oxygen-scavenging molecules may generate toxic products.

The presence of high levels of exogenous D-glucose markedly enhances the oxygen tolerance of *T. vaginalis*. Indeed, many anaerobes have improved tolerance to oxygen under 'reducing' conditions (Morris, 1979). It is proposed that damage from oxygen metabolites is minimized when oxygen is utilized as a terminal electron acceptor and internal redox couples are kept fully reduced, thereby minimizing potentially harmful effects on other aspects of metabolism. The hydrogenosomal terminal oxidase of *T. vaginalis* has a high affinity for oxygen (Lloyd *et al.*, 1982), which is somewhat surprising as it is non-cytochrome and a high affinity for oxygen is not characteristic of flavoprotein oxidases (Lloyd & Edwards, 1978; Lloyd & Coombs, 1989). Metronidazole-resistant strains of *T. vaginalis* were inactivated more rapidly than the sensitive strain at oxygen levels characteristic of those encountered *in situ* in the vagina. The affinity of the hydrogenosomal oxygen consumption system of these strains for oxygen was fourfold lower than that for metronidazole-sensitive isolates (Yarlett *et al.*, 1986). Thus for any given extracellular oxygen concentration within the range 0–20 μ M, the corresponding intracellular oxygen concentration will be higher in the metronidazole-resistant strains than the sensitive strains, as the former have defective oxygen-scavenging systems. This is the most likely explanation for their diminished oxygen tolerance. It also accounts for the directly demonstrated enhanced metronidazole nitro radical anion quenching demonstrated *in vivo* by oxygen in the resistant strains (Lloyd & Pedersen, 1985). Antioxidant enzymes such as SOD were present in these isolates at levels similar to that of the sensitive strain, as were the cytosolic oxidase activities previously reported (Ellis *et al.*, 1992). This suggests that the oxygen protection mechanisms in metronidazole-resistant *T. vaginalis* are not as efficient as those operating in the sensitive strain, as resistant isolates are more susceptible to oxidative damage at physiological levels, and indicates that the hydrogenosomal oxidase plays a major role in the protection of *T. vaginalis* from the toxic effects of oxygen at those levels encountered in the vagina.

In summary, the survival of *T. vaginalis* in the presence of oxygen is dependent on the delicate balance of oxidant stress and defence mechanisms. Clearly, very low levels of oxygen (< 0.25 μ M) stimulate growth of this pathogen

(Paget & Lloyd, 1990), but the ability to deal with oxidative stress deteriorates at elevated oxygen levels. As with all cells, the aetiology of oxygen toxicity is multifactoral. Protective enzymes such as NAD(P)H oxidases and SOD, the rapid consumption of oxygen by the hydrogenosomal oxidase, and scavenging by cellular thiols most probably keep intracellular toxic oxygen radicals at low levels *in situ* in the vagina. At elevated oxygen (> 60 μ M) however, the generation of harmful oxygen metabolites probably exceeds the rate of detoxification and thus results in irreversible cellular damage and cell death.

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