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Metronidazole Radical Anion Generation *in vivo* in *Trichomonas vaginalis*: Oxygen Quenching is Enhanced in a Drug-resistant Strain

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The nitro radical-anion of metronidazole has been detected *in vivo* in the sexually transmitted human parasite, *Trichomonas vaginalis*, under anaerobic conditions by electron spin resonance spectrometry. Exposure of organisms to oxygen decreased the intensity of the radical signal in both metronidazole-sensitive ATCC strain 30001 and in the metronidazole-resistant strain 85. The sensitive strain still gave radical signals at partial pressures of oxygen (> 6 kPa) sufficient to remove all detectable radicals from the resistant strain. This evidence suggests that the resistant strain has defective oxygen scavenging system(s).

INTRODUCTION

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] and other 5-nitroimidazoles with low mid-point redox potentials are often used as antimicrobial agents against bacteria and protozoa (Breccia *et al.*, 1982). Their highly effective action on the micro-organisms, combined with lack of toxicity to mammalian cells, depends on specific drug reduction to an active product, only possible in the anaerobic organisms possessing electron donors of sufficiently powerful reducing ability (Müller & Lindmark, 1976). Although the chemistry of metronidazole reduction is well understood, proceeding by way of the nitro radical anion, and the nitroso and hydroxylamine derivatives (Mason & Holtzman, 1975; Mason, 1979), whether all these intermediates occur *in vivo* has not been established, neither has the process of electron transfer from radical anion to O_2 (Wardman & Clarke, 1976) been shown to occur *in vivo*. The mechanism of cytotoxicity is also obscure, but hypothetical schemes involving binding of highly reactive intermediates to macromolecules have been proposed (Edwards *et al.*, 1973; Ings *et al.*, 1974; Edwards, 1979).

A number of reports of the isolation of metronidazole-resistant strains of the sexually transmitted human parasite *Trichomonas vaginalis* have emphasized the need to investigate the exact mode of action of metronidazole (Meingassner & Thurner, 1979; Müller *et al.*, 1980); *in vitro* resistance is observed only in aerobic assays (Milne *et al.*, 1978). Studies of drug uptake (Müller & Lindmark, 1976) and the enzymology of drug activation (Čerkasovová *et al.*, 1980; Müller & Gorrell, 1984) have failed to uncover differences between susceptible and resistant strains; although in another study, decreased metabolic activation or lowered NADH oxidase have been noted (Clackson & Coombs,1982).

Recently, electron spin resonance spectroscopy (ESR) was used to demonstrate *in vivo* generation of free radicals from nitroimidazoles in the cattle parasite, *Tritrichomonas foetus* (Moreno *et al.*, 1983). In this paper we measure directly the steady state intracellular level of the radical anion of metronidazole generated metabolically in *T. vaginalis* and demonstrate radical quenching by O_2. Diminished persistence of the radical in the presence of O_2 in a metronidazole-resistant strain strongly suggests a deficiency of O_2 scavenging mechanisms as the metabolic lesion underlying drug resistance in this organism.

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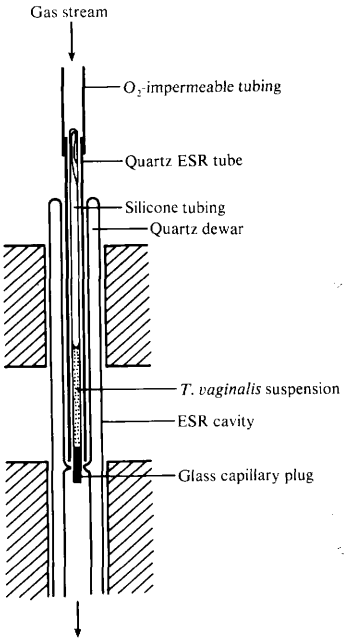


Fig. 1. System for obtaining ESR measurements in whole cell suspensions exposed to known partial pressures of O_2.

METHODS

Growth and harvesting of the organisms. Trichomonas vaginalis ATCC 30001 and metronidazole-resistant strain 85 (originally isolated by Dr J. Lossick, Center for Disease Control, 181S Washington Boulevard, Columbus, Ohio, USA) were grown axenically at 37 °C for 24 h in tryptone/yeast extract/maltose medium, adjusted to pH 6·4 before autoclaving and supplemented with 10% (v/v) heat-inactivated horse serum (Diamond, 1957; Müller *et al.*, 1980). Counting was in a modified Fuchs–Rosenthal haemocytometer (depth 0·2 mm, 1/16 mm^2). Organisms were harvested by centrifugation at 1000 *g* for 3 min at room temperature in a bench centrifuge, washed twice in 100 mM-potassium phosphate buffer at pH 7·5 and the sloppy pellets ($\sim 3 \times 10^8$ cells ml^{-1}) were loaded directly into silicone tubes after adding reductant (40 mM-glucose) and metronidazole (40 mM).

ESR measurements. Fig. 1 shows the experimental system used for exposing organisms to known O_2 partial pressures within the cavity of the ESR spectrometer at room temperature.

After addition of reductant (40 mM-glucose) and 40 mM-metronidazole (Sigma) the packed cell suspension (3×10^8 cells ml^{-1}) was immediately drawn into a piece of thin-walled silicone tubing (i.d. 1·47 mm, o.d. 1·96 mm; Silastic 602-235, Dow Corning Co., Midland, Mich., USA). After plugging with a small piece of plasticine-filled glass capillary, the tubing was slipped into an open-ended ESR quartz tube and held in position by folding back about 1 cm at the top. The ESR tube was then connected by oxygen-impermeable tubing to a gas mixer via an O_2 electrode chamber (Radiometer) and placed in the cavity (type TE_{102}) of a Varian E-104A spectrometer equipped with a variable temperature insert quartz dewar. After step changes in the gas phase the signal intensity adjusted to a new level in less than 5 min; this period was routinely allowed between switching of gas stream and scanning the spectrum.

Rat liver microsomes were prepared as described by Jørgensen & Johansen (1983), except that 3-methylcholanthrene (Sigma) was used for enzyme induction. Chromium oxalate, tri(oxalato)-chromium (III), was synthesized as the potassium salt according to Bailar & Jones (1935).

RESULTS

The metronidazole free radical, with hyperfine splitting characteristic of the nitro radical anion, was produced on reduction of the drug by electrons from NADPH catalysed by rat liver microsomal membrane preparation (Fig. 2). The radical was not detected while O_2 remained in the incubation mixture; under aerobic conditions it is reoxidized in a one-electron reaction which generates superoxide radical anions (Perez-Reyes *et al.*, 1980):

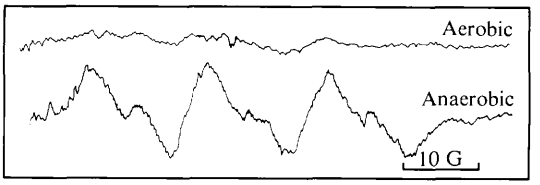


Fig. 2. ESR spectrum of the metronidazole radical anion formed in incubations containing microsomal protein (5 mg ml^{-1}) and a NADPH-generating system (Jørgensen & Johansen, 1983) before and after establishment of anaerobiosis. The metronidazole concentration was 30 mM. Incubation was in the aqueous sample cell (Varian E-248) with operating conditions as follows: field set, 3400 G (0·34 T); scan rate, 100 G; modulation amplitude, 4 G; microwave power, 20 mW; microwave frequency, 9·52 GHz; gain, 10^5; scan time, 16 min; time constant, 1 s.

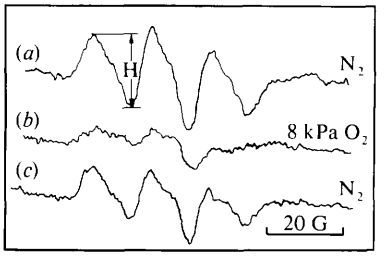


Fig. 3

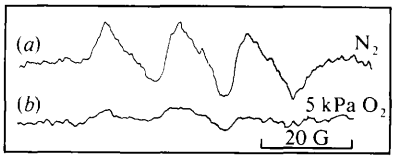
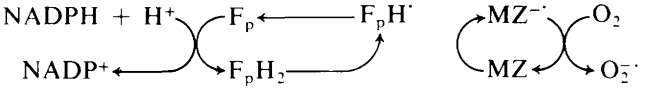


Fig. 4

Fig. 3. ESR spectra of metronidazole radical anions in *T. vaginalis* strain 30001. (*a*), (*b*) and (*c*) were obtained as successive scans after attainment of anaerobiosis, exposure for 5 min to 8 kPa O_2 and switching back to a gas phase of N_2 respectively. ESR height (H) was measured at field positions indicated (chosen to give maximum change in anaerobic–aerobic transition). Instrument settings and conditions were as in Fig. 2, except that field set was 3265 G; scan rate, 200 G; modulation amplitude, 8 G; microwave frequency, 9·117 GHz; gain 5×10^4. Cell concentration, 3×10^8 ml^{-1}.

Fig. 4. ESR spectrum of metronidazole radical anions in *T. vaginalis* strain 85; trace (*a*) was obtained anaerobically, then trace (*b*) was produced 5 min after switching to 5 kPa O_2 in the gas phase. Instrument settings and conditions were as in Fig. 3, except that microwave power was 10 mW.



The exact mechanism is not known.

When a packed non-proliferating suspension of *T. vaginalis* (metronidazole-sensitive strain 30001) was incubated with the drug in the presence of glucose in a silicone rubber tube in a stream of N_2, equilibration to anaerobiosis occurred within about 10 min (before commencement of the second scan) and was indicated by the attainment of maximal radical signal intensity (Fig. 3). The secondary hyperfine splitting could not be resolved due to the high modulation amplitude needed to optimize the signal to noise ratio. No signal was observed in the absence of metronidazole. Changing the gas phase to 8 kPa O_2 (40% air) gave almost complete disappearance of signal (H, measured as indicated). Switching back to anaerobic conditions never gave complete regeneration of original signal intensity. Stepwise increases in O_2 partial pressures resulted in progressive diminution of the free radical signal. In an identical experiment with strain 85, anaerobic conditions again allowed the formation of the metronidazole radical, and as for strain 30001, decreased signal intensity accompanied increasing O_2 in the mobile gas phase (Fig. 4). Comparison of the O_2 dependencies of the signal due to the free radical of the drug in several experiments with the sensitive and resistant strains (Fig. 5), indicates that the sensitive organisms were characterized by a greater persistence of metronidazole radicals at any given O_2 tension. Thus the ESR signal intensity was halved in strain 30001 under an atmosphere which contained about 3 kPa O_2, whereas only about 2 kPa O_2 was necessary to halve that with

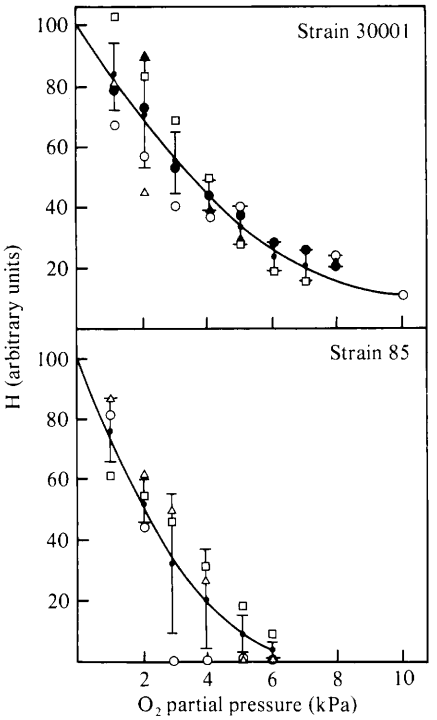


Fig. 5

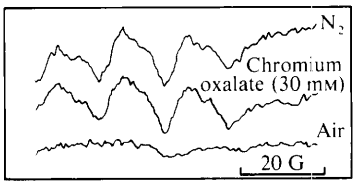


Fig. 6

Fig. 5. O_2 dependence of ESR signal height in metronidazole-sensitive and resistant strains of *T. vaginalis*. Different symbols refer to different batches of organisms. Means (•) together with 2 × SD indicated.

Fig. 6. Effect of 30 mM-tri(oxalato)-chromium (III) on the ESR spectrum of metronidazole in *T. vaginalis* strain 30001. Conditions and instrument settings as in Fig. 3.

strain 85. Also whereas in the former strain radical signals were still detectable at O_2 partial pressures above 8 kPa, the resistant strain 85 showed no detectable free radicals at above 6 kPa O_2.

Chromium oxalate is a very efficient spin-broadening agent, upon collison encounter it can broaden the narrow ESR signal of a free radical to invisibility. However, chromium oxalate cannot pass biological membranes (Berg & Nesbitt, 1979) and thus will not affect intracellular radicals. Addition of the reagent to a whole cell suspension of *T. vaginalis* failed to lower the intensity of the metronidazole radical signal (Fig. 6); this showed that the radical does not leave the organism but is present as an intracellular pool. Control experiments with metronidazole radicals generated using the microsomal system confirmed spin broadening by chromium oxalate where collisional interaction is found.

DISCUSSION

Although the metronidazole free radical has previously been detected by its magnetic resonance absorption in the cattle parasite *Tritrichomonas foetus* (Moreno *et al.*, 1983); this technique has not been applied to studies of drug reduction by the protozoon responsible for human trichomoniasis. The present work shows that a radical signal with resonances identical in magnetic field width and line shape with those produced by drug reduction in the presence of NADPH and a mammalian microsome membrane preparation is obtained in *T. vaginalis* suspensions under anaerobic conditions. Extremely high cell densities are required (of the order of 10^8 organisms ml^{-1}) in order to detect free radicals, which are never released into the

suspension medium, possibly due to the negative charge of the radical anion. These intracellular radical pools are quenched by O_2; restoration of anaerobic conditions after O_2 exposure never gives full recovery of steady state radical production.

Despite intensive efforts, the biochemical basis of metronidazole resistance observed clinically, and in certain strains isolated in pure culture, is still not understood (Müller & Gorrell, 1984). Drug uptake rates are similar in sensitive and resistant strains, and as it appears that no specific transport system is involved, it is difficult to envisage a resistance mechanism based on modified drug permeability. Metronidazole activation is dependent upon reduction by electrons donated by pyruvate via the hydrogenosomal enzyme pyruvate–ferredoxin oxidoreductase and mediated by the [2 Fe–2S] iron sulphur centre of ferredoxin (Marczak *et al.*, 1983; Yarlett *et al.*, 1984). This electron transport chain (Ohnishi *et al.*, 1980) is present and active at undiminished rates in cell free extracts prepared from a variety of different metronidazole-resistant strains (Müller & Gorrell, 1984).

One clue to the possible mechanism of metronidazole resistance comes from important observations (Milne *et al.*, 1978; Meingassner *et al.*, 1978; Beaulieu *et al.*, 1981) that all clinically resistant strains, hitherto isolated, manifest resistance *in vitro* only when examined in aerobic assays. Thus the minimal inhibitory concentrations of metronidazole (and other active 5-nitroimidazoles) for the sensitive and resistant strains are identical under anaerobic conditions. The present report shows that the metronidazole free radical, commonly assumed to be the toxic product of drug reduction, is produced *in vivo*, but is more persistent in organisms exposed to low partial pressures of O_2 in the case of the sensitive strain 30001 than in one resistant variant strain 85. Alternative mechanisms can be suggested for this persistence: (*a*) a greater rate of production of radicals in the sensitive strain, or (*b*) a smaller rate of radical disappearance in that strain. The previous biochemical studies with extracts referred to above make (*a*) unlikely. The data presented here show that a major factor in the rate of radical disappearance is the O_2 partial pressure of the gas phase to which the parasites are exposed. Furthermore, radicals disappear from the resistant strain at O_2 levels below those at which they persist in the sensitive strain. This suggests that the respiratory system(s) responsible for scavenging intracellular O_2 in *T. vaginalis* (the cytosolic NADH and NADPH oxidases and/or the hydrogenosomal O_2 uptake system; Lloyd *et al.*, 1983) may be less effective in the metronidazole-resistant strain so that at equivalent extracellular O_2 levels, intracellular O_2 remains at a higher concentration in the resistant strain, and permits more effective futile cycling between metronidazole and its cytotoxic electron reduction product. Although *in situ* measurements of vaginal O_2 concentrations have not been reported, it seems likely that strict anaerobiosis would rarely be encountered (perhaps only in very highly infected patients), and it is remarkable that the proposed lowered capacity for O_2 consumption in the resistant strain does not diminish its success as a parasite.

Further work is necessary to confirm the hypothesis of the diminished O_2 scavenging capabilities of the respiratory systems of strain 85 and to identify the basis for metronidazole resistance in other strains of *T. vaginalis*.

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