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 O₂ (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 O₂. Diminished persistence of the radical in the presence of O₂ in a metronidazole-resistant strain strongly suggests a deficiency of O₂ 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 O₂.

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 mm²). 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 (~ 3 x 10⁸ 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 O₂ 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 x 10⁸ 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 O₂ electrode chamber (Radiometer) and placed in the cavity (type TE₁₀₂) 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 O₂ 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):
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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, 10⁵; scan time, 16 min; time constant, 1 s.

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 O₂ and switching back to a gas phase of N₂ 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, 9.17 GHz; gain 5 × 10⁴. Cell concentration, 3 × 10⁸ 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 O₂ 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 N₂, 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 O₂ (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 O₂ 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 O₂ in the mobile gas phase (Fig. 4). Comparison of the O₂ 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 O₂ tension. Thus the ESR signal intensity was halved in strain 30001 under an atmosphere which contained about 3 kPa O₂, whereas only about 2 kPa O₂ was necessary to halve that with
strain 85. Also whereas in the former strain radical signals were still detectable at O₂ partial pressures above 8 kPa, the resistant strain 85 showed no detectable free radicals at above 6 kPa O₂.

Chromium oxalate is a very efficient spin-broadening agent, upon collision 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 10⁹ organisms ml⁻¹) in order to detect free radicals, which are never released into the
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REFERENCES


