

Metronidazole resistance of *Trichomonas vaginalis* as a cause of treatment failure in trichomoniasis

A case report

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SUMMARY Six isolates of a strain (MRP-MT) of *Trichomonas vaginalis* obtained from a woman before and after unsuccessful treatment with metronidazole had an appreciably lower susceptibility to metronidazole both in vitro in the aerobic tube assay and in vivo in the mouse assay than did control strains from patients cured with standard doses of the drug.

Our results support recent evidence that metronidazole-resistant strains of *T vaginalis* do cause treatment failure. Resistance of these strains could be detected in vitro under only aerobic but not anaerobic conditions. The prevalence of metronidazole-resistant strains of *T vaginalis* should be kept under surveillance in order to estimate their clinical importance.

The patient harbouring the resistant strain MRP-MT was finally cured with increased doses of ornidazole.

Introduction

Resistance of *Trichomonas vaginalis* to 5-nitroimidazole drugs has been a matter of controversy.¹ Although drug resistance of trichomonads has not been shown convincingly in vitro there have been clinical reports of treatment failures. The possibility of the existence of metronidazole-resistant strains of *T vaginalis* has been met with considerable scepticism.²⁻⁴ Persistent trichomonal infections which could not be ascribed to reinfection were attributed to altered pharmacokinetics in patients⁵ or to a lowering of the drug concentration in the vagina by concomitant bacteria.^{4 6 7} Recently, however, a decreased susceptibility to metronidazole has been proved unequivocally in several strains of *T vaginalis* isolated from patients who were refractory to treatment.⁸⁻¹⁰

Meingassner *et al*¹¹ showed the importance of assay conditions in the in-vitro detection of resistance of trichomonads to 5-nitroimidazoles. Using a laboratory-developed drug-resistant strain of the

cattle parasite *Tritrichomonas foetus*¹² these workers found that the resistance of this strain could be ascertained in vitro only under aerobic conditions. Aerobic assay systems have been developed to allow reliable determinations of trichomonad susceptibility to 5-nitroimidazoles in vitro.^{8 10}

We report metronidazole resistance in a strain of *T vaginalis* isolated from a female patient who did not respond to several courses of treatment with 5-nitroimidazoles.¹³

Patient and methods

CASE REPORT

The patient was a 41-year-old woman who gave a four-year history of recurrent vulvo-vaginitis caused by *T vaginalis*. During this period she had been treated unsuccessfully seven times with metronidazole (Entizole, Polfa) and once with tinidazole (Fasigyn, Pfizer). In five of the treatment courses metronidazole (250 mg) was taken orally twice daily supplemented with 500 mg inserted vaginally once daily for 10 days. In one course the daily dose of metronidazole was increased to 1 g, and in another five 250-mg oral tablets (a total of 1250 mg metronidazole) were taken daily for three days.

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Tinidazole was given orally in 150 mg tablets twice daily for seven days.

When she was examined in our department the patient had an acute vulvo-vaginitis with an inflammatory discharge containing many trichomonads (isolate MRP-MT-1). She was given two successive courses of treatment with oral metronidazole (Flagyl, Specia) in a dosage of 500 mg twice daily for five days. Similar treatment was given to her husband. After completion of the treatment the symptoms disappeared and no trichomonads were found in a wet mount preparation. *T vaginalis* was cultured, however, from vaginal material (isolate MRP-MT-3). Within a month the symptoms had returned and numerous trichomonads were detected in the vaginal exudate.

To determine whether the treatment failure was due to poor absorption of metronidazole the patient was admitted to hospital and given metronidazole in a single oral dose of 2 g. The serum concentrations of the drug were determined two, four, and 24 hours after treatment. Satisfactory concentrations of metronidazole were present in the serum (table I). The treatment was completed by giving the patient metronidazole in a dosage of 1g twice daily for seven days. Despite symptomatic relief and the reappearance in the vagina of *Lactobacillus vaginalis* trichomonads were cultured from vaginal material (MRP-MT-8). The patient was eventually cured with ornidazole (Tiberal, Roche) 1 g orally twice daily supplemented with 500 mg inserted vaginally once daily for six days. The symptoms disappeared completely and *T vaginalis* could not be cultured from vaginal material.

TABLE I Serum concentrations of metronidazole after administration of a single oral dose of 2 g (polarographic determinations at -0.7 V)

Hours after administration of drug	Serum metronidazole concentration ($\mu\text{g/ml}$)
4	33
8	25
24	12

ISOLATION AND CULTIVATION OF *T VAGINALIS*

The metronidazole-resistant strain under study, *T vaginalis* MRP-MT, was isolated from the vagina of the patient on several occasions before and after unsuccessful treatment. A total of eight isolates were obtained during the two-year study of the patient. Six of these isolates were assayed for susceptibility to metronidazole in vitro.

Eight strains of *T vaginalis* which were susceptible to metronidazole (TV5-27, TV7-37, TV10-02, TV14-85, TV17-48, TV67-77, TV87-64, and

TV99-51) were isolated from patients who had attended our clinic. All patients had been successfully treated with a single course of oral metronidazole.

Trichomonads were isolated from vaginal secretions collected with a cotton swab from the posterior fornix of the vagina. Cultures were established and maintained in Diamond's (trypticase, yeast extract, maltose) medium (TYM),¹⁴ pH 6.0 with trypticase (BBL) replaced by tryptone (Oxoid, L-42). The medium was supplemented with 10% heat-inactivated horse serum. Concomitant micro-organisms were eliminated by migration in the DeCarneri tube¹⁵ and with the aid of antibiotics. All isolates were treated three times with kanamycin sulphate (2500 $\mu\text{g/ml}$), three times with penicillin G (5000, 2000 and 1000 IU/ml) and once with streptomycin sulphate (1000 $\mu\text{g/ml}$). The absence of contaminating micro-organisms was confirmed by standard sterility tests including assays for *Mycoplasma* spp. Axenised cultures with the addition of 5% dimethylsulphoxide were stored in liquid nitrogen.

The two reference metronidazole-resistant strains, obtained by courtesy of Dr J G Meingassner, Sandoz Forschungsinstitut, Vienna, were *T vaginalis* IR-78 from Vienna, Austria,⁸ and *T vaginalis* "Fall River" from the USA.¹⁰ The former was a fresh subculture of the original stabilate,⁸ and the latter had been maintained for several months by serial transfers. Both strains had been grown axenically. After arrival at our laboratory the strains were transferred to TYM medium and cryopreserved.

Cultures used in the experiments were derived from frozen stabilates and grown in TYM medium without antibiotics. Except for the "Fall River" strain, none of the strains was maintained in active culture at 37°C for longer than three weeks.

SUSCEPTIBILITY ASSAYS

Susceptibility of trichomonads to metronidazole in vitro was estimated as a minimal lethal concentration (MLC) of the drug using an aerobic tube assay. Suspensions of trichomonads (1×10^5 cells/ml) in 1 ml of the aerobic medium (TYM without cysteine and ascorbic acid) were exposed for 48 hours to twofold serial dilutions of metronidazole, and the viability of the parasites was assayed by subsequent cultivation in a drug-free medium. The assay was performed in 100 \times 15 mm centrifuge tubes, each containing 0.9 ml of a suspension of parasites in the aerobic TYM and 0.1 ml of the metronidazole solution. The tubes were closed with metal caps allowing equilibration with air. After incubation at 37°C for 48 hours the cells were centrifuged (1600 \times g), washed in saline, and resuspended in 5 ml of standard TYM. The metal caps were replaced by

non-toxic rubber stoppers and the tubes incubated at 37°C for seven days. The MLC at 48 hours (MLC_{48h}) was defined as the lowest concentration of metronidazole at which no viable trichomonads could be detected by cultivation. The assays were run in duplicate and repeated at least twice; additional dilutions were included to refine endpoints.

The in-vitro susceptibility to metronidazole was assayed also under anaerobic conditions in 13 × 100 mm screw-cap tubes (Bellico). Each tube contained 4.4 ml of standard TYM (with reducing compounds included), 0.1 ml of metronidazole solution, and 0.5 ml of trichomonad suspension (to make a final concentration of 1 × 10⁵ cells/ml). After 48 hours exposure to metronidazole the cells were washed with saline and resuspended in a drug-free medium.

The in-vivo susceptibility to metronidazole was assayed by estimating the DC₅₀ (dose needed to cure 50% of infections) using the subcutaneous mouse assay described by Meingassner and Thurner.⁸ Mice were inoculated subcutaneously in each flank with trichomonads which were in the logarithmic phase of growth. Metronidazole was given orally at two, 18, and 24 hours after inoculation. The therapeutic effect was evaluated on day 6 by determining whether viable parasites were present or absent at the site of inoculation (by microscopy and culture). Male albino SPF mice (18-20 g) supplied by Velaz (Prague, Czechoslovakia) were used in preliminary experiments performed in Prague. Thirty mice were inoculated with *T vaginalis* isolate MRP-MT-3 and 30 received the susceptible control strain TV17-48. The inocula contained 1 × 10⁶ cells in 0.5 ml of TYM medium. Groups of six mice were given the same dose of metronidazole. Further experiments on the metronidazole-resistant isolate MRP-MT-3 were performed by J G Meingassner in Vienna. Female NMRI mice (weight 10-12 g), supplied by Gassner (Salzberg, FRG) were inoculated subcutaneously and intraperitoneally. A total of 55 mice (two experiments) were inoculated subcutaneously (inocula 4 × 10⁵ cells) and 118 mice (three experiments) intraperitoneally (1.2 × 10⁶ cells). The same dose of the drug was given to groups of seven or eight mice. Untreated controls were included in the experiments. Curative doses were determined by means of a probit analysis.

METRONIDAZOLE

Pure metronidazole produced by Specia or Bayer was used throughout the experiments. For in-vitro assays stock solutions were prepared in glass-distilled water (50 mg/10 ml), and the substance was dissolved by autoclaving. Serial dilutions were made in aerobic TYM. For mouse assays the drug was dissolved and

administered in a 10% aqueous solution of dimethylsulphoxide and 0.2% Tween 80 (v/v).

SERUM CONCENTRATIONS OF METRONIDAZOLE
Serum concentrations of metronidazole were determined polarographically by the method of Kane¹⁶ using an LP-60 polarograph (Laboratory Supplies, Prague) at -0.7 V. Before the measurement the sera were diluted 1/10 with potassium chloride (final KCl concentration 0.001 mol/l).

Results

The data summarised in table II showed notable differences between the in-vitro susceptibility to metronidazole of MRP-MT isolates and that of control *T vaginalis* strains from patients responding favourably to a standard treatment. In an aerobic tube assay the mean MLC_{48h} (81 µg/ml) for the MRP-MT isolates was about 29 times higher than that for the control strains (TV) (2.8 µg/ml). The MLCs for the MRP-MT isolates were within the range of those for the reference metronidazole-resistant strains from Austria (IR-78) and the USA ("Fall River"). The resistance in vitro was detectable only in the aerobic assay. Under anaerobic conditions all assayed strains were susceptible to metronidazole with a mean MLC_{48h} of 2.15 µg/ml.

When the efficacy of metronidazole against infection with the isolate MRP-MT-3 was further assayed in vivo in subcutaneously and intraperitoneally infected mice the susceptibility of this isolate to metronidazole was low (table III). Its relative drug resistance was more pronounced in mice which had been inoculated subcutaneously. The DC₅₀ value of the MRP-MT-3 isolate, calculated from the subcutaneous assays performed in Prague, was about 20 times higher than that of the drug-susceptible control TV 14-85. The highest dose given (3 × 800 mg/kg) was insufficient to obtain a 100% cure. In Meingassner's experiments none of the mice infected subcutaneously was cured with the highest dose assayed (3 × 400 mg/kg). Intraperitoneal infections with the MRP-MT-3 isolate were successfully cured with metronidazole. The doses necessary for eliminating the infection, however, were relatively high (3 × 400 mg/kg) and the DC₅₀ values about eight times higher than that for the Viennese control strain E (table III).

The MLC values for six isolates of the MRP-MT strain (table II) showed that throughout the two years of study the protozoon did not respond to repeated treatment by an increase in resistance.

TABLE II *In-vitro* susceptibility to metronidazole of *T vaginalis* MRP-MT isolates compared with reference metronidazole-resistant strains (IR-78 and Fall River) and eight drug-susceptible strains

<i>T vaginalis</i> strain	MLC ₄₈ * of metronidazole (µg/ml)			Anaerobic assay
	Aerobic assay			
	Mean	n†	Range	
Resistant strains:				
MRP-MT-1	110	6	80-150	2·0
MRP-MT-2	65	4	40-100	2·5
MRP-MT-3	85	4	80-100	2·5
MRP-MT-5	80	4	80	2·0
MRP-MT-7	60	2	40-80	ND
MRP-MT-8	87	6	80-100	2·5
IR-78	52	8	40-100	(4·0)‡
Fall River	80	3	80	(2·0)‡
Susceptible strains:				
TV 5-27	1·25	2	1·25	ND
TV 7-37	5·0	2	5·0	ND
TV 10-02	2·0	4	1·25 - 2·5	ND
TV 14-85	5·6	8	5·0 - 10·0	2·0
TV 17 - 48	2·5	2	2·5	ND
TV 67-77	2·2	4	1·25 - 2·5	1·0
TV 87 - 64	2·5	2	2·5	1·0
TV 99-51	1·25	2	1·25	ND

*Minimal lethal concentration of metronidazole after 48 h exposure of parasites to the drug

†Number of determinations

‡Data from reference¹⁰ obtained by anaerobic tray test

ND = not determined

TABLE III *In-vivo* susceptibility to metronidazole of the *T vaginalis* isolate MRP-MT-3 compared with the drug-susceptible strains TV 14-85 and E

Strain	Assayed by	Subcutaneous assay		Intraperitoneal assay	
		No of mice	DC ₅₀ (mg/kg)(×3)*	No of mice	DC ₅₀ (mg/kg)(×3)
MRP-MT-3	Kulda <i>et al</i> Meingassner	30	300	94	115·9
		55	>400		
TV 14-85	Kulda <i>et al</i>	30	15		
E†	Meingassner		11·2		14·7

*Dosage of metronidazole given orally at two, 18, and 24 h

†Data on this strain is given in reference⁸

Discussion

Our results showed a relative resistance to metronidazole of the *T vaginalis* strain MRP-MT isolated in Prague from a patient with a resistant vulvo-vaginitis. Neither reinfection nor failure to take the prescribed doses of the drug could be regarded as being responsible for the persistent infection, because one of the unsuccessful courses of treatment was given while she was in hospital. Serum concentrations of metronidazole after oral administration indicated satisfactory absorption of the drug from the gut. The resistance of the trichomonads isolated from the patient was shown in laboratory assays under bacteria-free conditions, thereby excluding any interference by bacteria in the trichomonocidal ability of the drug. The low susceptibility of the MRP-MT strain to

metronidazole was detected *in vitro* by an aerobic tube assay as well as *in vivo* by determining the CD₅₀ in mice infected subcutaneously and intraperitoneally. The reliability of the laboratory tests was controlled by assaying in parallel the MRP-MT isolates, the reference metronidazole-resistant strains, and drug-susceptible strains isolated from patients cured successfully with standard doses of metronidazole. These data indicated that infection with a metronidazole-resistant strain was the primary cause of the treatment failure in our patient.

The properties of the MRP-MT strain were similar to those described recently by other workers.^{8 10 17} All these strains caused treatment-resistant diseases in man; their resistance was detected *in vivo* in subcutaneously infected mice and also *in vitro* if assay conditions were aerobic. The importance of using an aerobic system for the *in-vitro* demon-

stration of resistance was confirmed by others.¹⁸ Forsgren and Forssman⁹ identified their metronidazole-resistant strain using a tube assay which appeared to be essentially aerobic. On the other hand, the relatively low minimum inhibitory concentrations (MICs) reported for strains of *T vaginalis* from treatment failures in Britain (MIC 20-25 µg/ml) might have been accounted for by the use of an assay system in which there is a lower availability of oxygen.^{19 20}

The need for aerobic conditions to show resistance in an anaerobic organism seems to be an unusual phenomenon. Persisting doubts on the real existence of metronidazole-resistant strains of *T vaginalis* are therefore understandable. Incomplete anaerobiosis was the cause of a false metronidazole "resistance" in anaerobic bacteria.²¹ Even in trichomonads the presence of air suppressed the uptake of metronidazole²² and increased MLC values of the drug.¹¹ Recently published comparative data⁸⁻¹⁰ have shown differences in aerobic MLC values between *T vaginalis* strains isolated from readily curable trichomoniasis and from a treatment-resistant infection. This evidence is supported by the results of our study.

Laboratory experiments indicated that the resistance of *T vaginalis* to metronidazole involves an aerobic tolerance to the drug. Implications of this phenomenon for the drug-parasite interaction in situ need to be clarified. The number of resistant trichomonads in the vagina is appreciably reduced after treatment with metronidazole. Probably very few potentially resistant trichomonads meet with conditions in the human vagina which favour their aerobic drug-tolerance. Data on oxygen tension and redox potential in different parts of the infected vagina would clarify the physiological background of infections with metronidazole-resistant trichomonads. Biochemical mechanisms of *T vaginalis* resistance to metronidazole are not yet known.^{23 24} There is some evidence, however, that this aerobic type of resistance is not connected with enzyme deficiencies eliminating the pathway of anaerobic pyruvate metabolism essential for activation of 5-nitroimidazoles. Such enzyme lesions have been observed in metronidazole-resistant mutants of *Trichomonas foetus*^{25 26} and *Bacteroides fragilis*²⁷ but not in the drug-tolerant *T vaginalis*.^{24 28 29}

Metronidazole resistance of the strain of *T vaginalis* MRP-MT which was reisolated repeatedly from the vagina of our patient did not increase as a result of three unsuccessful courses of treatment; each of the courses apparently exerted considerable selection pressure on the trichomonads. Similarly, increase in resistance was not observed in a strain of

T vaginalis isolated in Sweden after treatment with tinidazole.⁹ In both cases, however, the patients had been treated with nitroimidazoles before initial isolation of the parasite. It is uncertain whether the resistance was induced by inadequate treatment or was intrinsic to the infecting organism.

Recent evidence indicates that metronidazole-resistant strains of *T vaginalis* cannot be regarded as unique in their incidence. Isolation of resistant strains from patients have been reported in Austria,⁸ Sweden,⁹ Britain,^{19 20} and the USA.^{10 17 18} This calls for a systematic follow-up of treatment failures and extensive surveys of a random population to establish the prevalence of resistant strains. Until then it would be premature to draw conclusions on the clinical importance of metronidazole resistance in strains of *T vaginalis*. In view of the absence of clinically available drugs which could replace 5-nitroimidazoles, however, the resistance of *T vaginalis* to metronidazole should be followed with concern.

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