Strain of *Trichomonas vaginalis* Resistant to Metronidazole and Other 5-Nitroimidazoles

JOSEF G. MEINGASSNER* AND JOSEFINE THURNER†

Sandoz Forschungsinstitut, A-1235 Vienna,1 and II. Universitäts-Hautklinik, A-1090 Vienna,2 Austria

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A strain of *Trichomonas vaginalis* (IR-78), recently isolated from a patient afflicted with recurrent symptomatic trichomoniasis, showed resistance to metronidazole, tinidazole, and nimorazole in vitro as well as in vivo. In a serial dilution test using cysteine monohydrochloride-peptone-liver infusion-maltose medium, *T. vaginalis* IR-78 was only resistant under aerobic conditions. Under anaerobic conditions it was as susceptible as the normal reference strain. The minimal lethal concentrations of metronidazole, tinidazole, and nimorazole for IR-78 were 100, 50, and 50 μg/ml aerobically and 0.4, 0.4, and 0.2 μg/ml anaerobically, respectively. The efficacy of metronidazole, tinidazole, and nimorazole was assessed in vivo by oral administration to mice simultaneously infected with IR-78 both subcutaneously and intraperitoneally. The CD₅₀ (dose needed to cure 50% of infections) of each compound was significantly higher for the subcutaneous than for the intraperitoneal infection. In contrast, there was little difference in CD₅₀ for these infections in mice inoculated with a susceptible trichomonas strain. The CD₅₀'s for all three compounds against intraperitoneal and subcutaneous infections with IR-78 were 2 to >70 times higher than for susceptible strain E. Both forms of infection with IR-78 could always be cured with therapeutically acceptable doses of tinidazole and nimorazole; subcutaneous infections could not be cured with tolerated doses of metronidazole.

Although there are clinical reports (1, 2, 4, 6, 8, 9, 13, 21) that document the refractoriness of infections with *Trichomonas vaginalis* to treatment with metronidazole, susceptibility tests have failed to demonstrate conclusively that the parasites isolated from such cases after treatment were resistant to this drug (12, 15). Thus, resistance of *T. vaginalis* has not been generally accepted as the factor responsible for failure of metronidazole therapy (22), since reinfection, irregular medication, poor absorption of the drug, and its inactivation by the vaginal flora have not been excluded (11, 15, 20).

A strain of *T. vaginalis*, unequivocally resistant to metronidazole, was recently isolated from a female patient who had not responded to two courses of treatment with this agent. The current report is concerned with the isolation of this strain and its in vitro and in vivo susceptibility to metronidazole and other 5-nitroimidazole derivatives.

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MATERIALS AND METHODS

Strains of *T. vaginalis*. The IR-78 strain of *T. vaginalis* on which this study is focused was derived from a patient, aged 42, with an acute vulvovaginitis yielding a discharge containing 10^7 trichomonads per ml. Two successive treatment courses, each at a dose of 500 mg of metronidazole twice daily for 5 days, did not relieve symptoms or eradicate the *T. vaginalis*. A third course of 1.0 g twice daily for 10 days effected symptomatic relief, but trichomonads were still present in the vaginal discharge 3 days after the last drug dose. A fourth treatment course undertaken 5 weeks later led to clinical and parasitological cure. This course included 1.0 g of metronidazole twice daily for 10 days combined with 500-mg metronidazole vaginal pessaries once daily and an additional oral dose of 1 mg of estriol (Ovestin, Organon) once daily for the same period.

The IR-78 strain was isolated from the vagina after the third treatment. A sample of the discharge, obtained by swab, was rinsed into cysteine monohydrochloride-peptone-liver infusion-maltose (CPLM) medium, as modified by de Carneri et al. (7), and incubated at 37°C. To eliminate bacterial contaminants, the first three passages were made in medium containing 1,000 IU of penicillin G and 1,000 μg of streptomycin sulfate per ml. Five additional passages through medium containing 20 μg of tetracycline per ml were needed to inhibit *Mycoplasma* spp. associated with the *T. vaginalis*. CPLM medium without antibiotics was used thereafter. Analysis of this material for both anaerobic and aerobic bacterial contamination was negative. After an additional transfer, axenic cultures were frozen in liquid nitrogen. For comparative tests, passages were made four times weekly for 15 weeks.
Overnight cultures were used for susceptibility tests. In vitro tests were performed with serial passages 10 and 68; in vivo tests were performed after passage 15. 

Experiments with the IR-78 strain were carried out in parallel with normal susceptible strains. Strain A was used in in vitro tests; strain E was used in tests with animals. These strains, cultured by four passages in CPLM medium weekly, were used because their susceptibility to metronidazole in vitro and in vivo was documented previously in comparison to other strains (16).

Antimicrobial agents. Stock solutions of metronidazole, tinidazole, and nimosazole were prepared in 10% dimethyl sulfoxide–0.2% Tween 80 solution. Serial doubling dilutions were prepared in either microdilution plates with microdiluters or in tubes by using pipettes. The dilutions to obtain final concentrations ranging from 0.05 to 100 μg/ml were made with CPLM medium.

In vitro susceptibility measurements. Three different assays were used in testing susceptibility of the IR-78 strain and reference strain A, since previous experiments showed that assay conditions may play a role in the in vitro detection of nitroimidazol-re-resistant protozoan parasites (18). Assays were performed either in stoppered tubes (tube test) or in multiwell plates incubated at 37°C in a normal atmosphere or under anaerobic conditions (tray tests). For the tray tests, 50 μl of CPLM medium containing 1,000 IU of penicillin G and 1,000 μg of streptomycin sulfate per ml was pipetted into each well of a Microtiter plate (Flow no. M24 ART). Next, a 50-μl stock solution (0.8 mg/ml) of the test compound was pipetted into the first well of a row. Twofold serial dilutions were then made from rows 1 to 12 with a 50-μl diluter (Flow no. M341). Next, 0.15 ml of a diluted culture giving a final concentration of ~50,000 cells per ml was added. The plates were covered with lids (Flow no. M42 AR) and incubated at 37°C for 48 h aerobically in a humid chamber or in an anaerobic jar (GaelPak anaerobic system; Baltimore Biological Laboratory, GaelPak no. 70304, disposable gas generator envelopes). The tube test was carried out in test tubes (16 by 160 mm) in a similar manner, except that total volumes of 7.5 ml were used. The final concentrations of test compounds and the number of cells per milliliter were identical in both tray and tube tests. The tubes were sealed with rubber stoppers and incubated for 48 h at 37°C.

Two evaluations with three replications for each serial dilution were performed. Controls without treatment and controls with solvent only were included in each evaluation.

The evaluation was based on determining the minimal lethal concentration. The wells of the plates and the tubes were examined with an inverted microscope at ×40 magnification for evidence of motile trichomonads. The highest dilution in which no motile organisms were seen after the incubation period was recorded as the minimal lethal concentration. These findings were confirmed for the plate tests by reexamination on the following day after the drug-containing medium was changed to a medium without drug.

In vivo susceptibility measurements. Five hundred and four female NMRI mice, 10 to 12 g in weight, were used in these studies. Each subject was inoculated in three areas, with 4 × 10^6 trichomonads in each shaved flank and 1.2 × 10^6 cells intra perito neally. Treatment via the oral route was started 2 h after the infection and was repeated at 18 and 24 h after infection. Experiments were terminated 6 days after inoculation. The absence of lesions in the region surrounding the site of subcutaneous inoculations and of motile trichomonads in 48-h cultures (CACH medium; 19) of washings of the abdominal cavity were used as measurements of the activity of the test compounds.

The drugs were dissolved in 10% dimethyl sulfoxide–0.2% Tween 80 solution. They were given in volumes of 0.1 ml/10 g of body weight.

To calculate the CD₅₀ (dose needed to cure 50% of infections) and CD₃₀, two experiments, each using 12 animals for each experimental drug dose, were performed. Between six and eight different doses were used for each drug. For infections with the resistant strain IR-78, concentrations between 3 × 50 and 3 × 800 mg/kg were used, whereas infections produced by susceptible strain E were treated using 3 × 5 to 3 × 35 mg/kg. Control experiments using the dimethyl sulfoxide-Tween 80 solvent alone showed no effect. Similarly, the rate of spontaneous cure was zero.

RESULTS

In vitro studies. Table 1 shows that there was a striking difference in the activity of metronidazole against strains IR-78 and A. In the aerobic tray test strain IR-78 proved to be highly resistant. Minimal lethal concentrations differed 32-fold. In contrast, there was no difference in the susceptibilities of these two strains when tested similarly under anaerobic conditions. Only slight differences were observed in the tube test. IR-78 exhibited a decreased susceptibility to two other antitrichomonad nitroimidazoles, i.e., tinidazole and nimosazole. Minimal lethal concentrations obtained at reexamination 24 h after the change of medium did not differ significantly from the direct reading in the plate tests; therefore, the data are not shown in the table. The stability of the strain was demonstrated by the fact that passages 10 and 68 of IR-78 showed the same susceptibilities.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Strain</th>
<th>Tray tests</th>
<th>Tube test</th>
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<td></td>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
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<tr>
<td>Metronidazole</td>
<td>IR-78</td>
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<td>A</td>
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<tr>
<td>Tinidazole</td>
<td>IR-78</td>
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<td></td>
<td>A</td>
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<td>0.8</td>
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<tr>
<td>Nimosazole</td>
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<td>50</td>
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ance of IR-78 was not decreased by passing four times weekly for 15 weeks in medium without drug.

In vivo studies. Table 2 clearly shows that IR-78 was highly resistant to metronidazole as compared with strain E when tested in mice bearing both intraperitoneal and subcutaneous infections. The degree of resistance was markedly dependent on the route of infection. Thus, to achieve a CD₅₀, a dose of more than 3 × 800 mg/kg had to be used for the subcutaneous infection as compared to 3 × 122 mg/kg for the subcutaneous infection. This difference was specific to the resistant strain and was not seen with strain E. The metronidazole analogs tinidazole and nimorazole exhibited similar characteristics, as might be expected from their reported similarity (3, 5, 17).

DISCUSSION

T. vaginalis IR-78 was isolated from a patient who did not respond to normal therapy with metronidazole. The strain exhibited metronidazole resistance in vitro only under aerobic conditions, thus confirming our earlier suggestion that in vitro assay conditions can play a decisive role in identifying resistant strains of T. vaginalis (18). Since microaerophilic conditions exist in the tube test used, it appears to be an undesirable technique in studying this problem. The plate test, which can be carried out in either an aerobic or anaerobic environment, seems to be the method of choice for studies with this strain.

The reported similarity in the mechanisms of action of metronidazole, tinidazole, and nimorazole (14) has been confirmed in that IR-78 showed a markedly decreased susceptibility to all three compounds.

Unfortunately, isolates of T. vaginalis made before or after the first unsuccessful metronidazole treatment were not available for susceptibility testing. Therefore, it is not known whether this strain became resistant in this patient during repeated treatments or whether the patient was infected with a resistant strain.

The development of refractive resistance of T. vaginalis to metronidazole in this case and other patients would receive support from a recent report showing that a strain of Bacteroides fragilis developed resistance to this compound during long-term therapy (10).

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LITERATURE CITED

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