ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1978, p. 1-3

Vol. 13, No. 1
Printed in U.S.A.

Assay Conditions and the Demonstration of Nitroimidazole Resistance in *Tritrichomonas foetus*

JOSEF G. MEINGASSNER,[1]* HUBERT MIETH,[1] RUDOLF CZOK,[1] DONALD G. LINDMARK,[2] AND MIKLÓS MÜLLER[2]

Sandoz Forschungsinstitut, A-1235 Vienna, Austria,[1] and The Rockefeller University, New York, New York 10021[2]

Received for publication 22 August 1977

Tritrichomonas foetus KV_1, a nitroimidazole-susceptible strain, and KV_1/M100, its nitroimidazole-resistant daughter strain, differed markedly in their in vivo susceptibility to metronidazole. In vitro susceptibility testing in multiwell plates and tubes with different trichomonad media containing no, or low concentrations of, ascorbate demonstrated that the resistant strain behaves like the susceptible one, if tested under anaerobic conditions (deep cultures in tubes or multiwell plates in anaerobic jars), but shows resistance if tested in the presence of air (multiwell plates exposed to air). In media containing high concentrations of ascorbate, no resistance was observed even in air. The results suggest that the two strains differ in the regulation of internal redox systems and underscore the role testing methods may play in the in vitro detection of nitroimidazole-resistant protozoan parasites.

The possible existence of nitroimidazole resistance in trichomonads has received much attention. A major approach to the study of this problem is provided by attempts to develop resistant strains experimentally with in vivo or in vitro drug pressure. Recently we developed, with in vivo exposure to metronidazole, a *Tritrichomonas foetus* strain that exhibits a high level of resistance to several 5-nitroimidazoles, as demonstrated in mice and in vitro tests (12). Similar observations were made also in a *T. foetus* strain in which resistance was induced in hamsters (1). In contrast, Benazet and Guillaume (2) and de Carneri et al. (4) observed in vivo resistance only and no significant in vitro resistance in *Trichomonas vaginalis* strains in which metronidazole or nimorazole resistance was induced in vivo. The latter authors thus questioned the value of the in vitro test in establishing the metronidazole susceptibility of fresh clinical isolates. The reasons for the discrepancies could lie in differences in the assay methods used. The effects of the medium and its components, as well as pO_2, were reported to influence the in vitro efficacy of metronidazole (3, 13, 16).

In this paper we demonstrate that in our resistant *T. foetus* strain the in vitro demonstration of resistance strongly depends on the assay method used. Preliminary results were reported previously (J. G. Meingassner and D. G. Lindmark, Abstr. Fifth Int. Cong. Protozool., New York, 1977, abstract no. 392).

MATERIALS AND METHODS

Organisms. Two axenic strains of the cattle parasite *T. foetus* were used. The metronidazole-susceptible strain KV_1 was obtained from J. Kulda (Prague, Czechoslovakia) in 1971 and was kept constantly in culture. The resistant strain, KV_1/M100, was developed by J.G.M. (12). The history of this strain is as follows. After 40 passages in vivo under increasing drug pressure, it was isolated from the host. The thus established culture was transferred in vitro 37 times without drug, frozen, and kept in liquid N_2 and reestablished in culture. This culture underwent 124 transfers when the experiments reported here were performed. Stock cultures of both strains were grown at 37°C in screw-cap tubes containing liquid thioglycolate medium (Baltimore Biological Laboratory [BBL], Cockeysville, Md.) supplemented with 10% horse serum. Transfers were made on Mondays, Wednesdays, and Fridays.

Susceptibility tests. The susceptibility of the organisms was determined by the assessment of growth and viability of cells exposed in liquid medium to different concentrations of metronidazole. Assays were performed in either multiwell plates (tray test) or test tubes (tube test).

For the tray tests, 24-h-old stock cultures were diluted with the selected medium to a concentration of about 50,000 cells/ml. To each well of sterile multiwell plates (Linbro Chemicals Co., Inc., New Haven, Conn.; model 96CV-TC) 1.4 ml of the cell suspension and 0.1 ml of drug solution were added. The plates were covered with Scotch 3M membrane and incubated at 37°C in a normal atmosphere ("aerobic" tray test) or in anaerobic jars (GasPak anaerobic system with GasPak no. 70304 disposable

gas generator envelopes; BBL) for 48 h ("anaerobic" tray test).

Tube tests differed from the tray test only in using test tubes (16 by 160 mm) with rubber stoppers instead of plates. The amounts of medium and drug solution were increased five times. The tubes were incubated at 37°C for 48 h.

Tests were always made in triplicate for each drug concentration. Each assay included controls both with no additions and with solvent only added.

Stock solutions (1.5 mg/ml) of metronidazole (pure compound; obtained from Bayer AG, Leverkusen, or G. D. Searle, San Juan) were prepared in 10% methyl sulfoxide in water containing 0.2% Tween 80. Serial dilutions were prepared with water in 12 steps to obtain a concentration range of from 100 to 0.05 μg/ml.

The 11 different media used are listed in Table 1. The commercial media were prepared according to manufacturers' instructions. Preparation of the others was done as described in the references given in Table 1. The pH of all media was adjusted to 7.2.

Evaluation of the results. The covered plates or closed tubes were examined with an inverted microscope (magnification, × 160). Concentration of cells was judged subjectively and scored from + (single mobile trichomonads per well or tube) to +++ (cell number corresponding to normal controls). The minimum inhibitory concentration is defined as the concentration giving growth between + and ++, and the minimum lethal concentration is defined as that in which no mobile organisms are seen.

RESULTS

The results are summarized in Table 1. It can be seen that the aerobic tray test is the only type of assay system in which a decreased susceptibility of the in vivo metronidazole-resistant strain could be detected with nonmodified media. The only exception was the complete CACH (15) medium, in which no difference was seen. One major difference between this and the other media was in the ascorbate content. Therefore, the effect of ascorbic acid was tested. Addition of ascorbic acid to TTYS (8) and fluid thioglycolate media led to increased susceptibility of the resistant strain, whereas the omission of ascorbic acid permitted demonstration of the resistance of the KV_1/M100 strain also in the CACH medium.

The tube test and anaerobic tray test did not reveal any differences in the metronidazole susceptibility of the two strains.

DISCUSSION

The present work demonstrates that the test methods used will determine whether one can demonstrate in vitro the resistance of an in vivo nitroimidazole-resistant *T. foetus* strain. One of the major conclusions is that in the absence, or at low concentrations (≲0.02%), of

TABLE 1. *Effect of metronidazole on susceptible and resistant T. foetus*

Medium	Reference	Aerobic tray test[a]				Tube test			
		KV_1		KV_1/M100		KV_1		KV_1/M100	
		MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
CM 161 (Oxoid)		0.8	1.6	50	100	0.2	0.8	0.2	0.8
CPLM	17	0.8	1.6	25	50	0.1	0.4	0.4	0.8
STS	17	0.8	1.6	50	100	0.2	0.4	0.2	0.4
TYM	7	0.4 (1.0)	1.6 (1.0)	25 (1.5)	50 (1.5)	0.8	0.8	1.6	1.6
Feinberg and Whittington	17	0.8	1.6	25	50	0.4	0.4	0.4	0.4
FTG[b] (BBL)		0.8 (0.5)	1.6 (1.5)	50 (0.5)	100 (1.0)	0.4	0.8	0.4	0.8
FTG with 0.1% ascorbid acid		0.8	1.6	1.6	25	0.4	0.8	0.4	0.8
CACH (complete)	15	0.4	0.8	0.4	0.8	1.6	3.1	0.4	0.8
CACH without ascorbic acid		0.8	3.1	12.5	50	–[c]	–	–	–
TTYS	8	0.8	1.6	25	100	0.8	1.6	0.8	1.6
TTYS with 0.1% ascorbic acid		0.2	0.4	0.4	1.6	–	–	–	–

[a] Number in parentheses gives result for the anaerobic tray test. MIC, minimum inhibitory concentration; MLC, minimum lethal concentration.

[b] FTG, Fluid thioglycolate.

[c] –, Not tested.

ascorbate, in vitro resistance appears only if oxygen is present in the medium, as is in the aerobic tray test. If oxygen is absent, as in the depth of the medium in the tubes and in the anaerobic tray test, no resistance can be observed. The presence of 0.1% ascorbate in the medium in the aerobic tray test mimics the effect of anaerobiosis. Since the media are complex and undefined, it is not clear yet whether this effect is due to ascorbic acid alone or an interaction with other components of the media is also necessary.

It is well documented that the antitrichomonad activity of metronidazole as well as of other 2- and 5-nitroimidazoles depends on the intracellular reduction of these compounds (6, 9–11, 14), and thus the biological efficacy of these compounds depends on the intracellular availability of "reducing power," possibly the availability of reduced low-redox-potential electron transport proteins similar to ferredoxin (14). Since the presence of oxygen is necessary for the in vitro expression of resistance and since this aerobic expression can be suppressed by ascorbate, a strong reducing agent, it is tempting to assume that one of the differences between the resistant and susceptible strains is in their ability to regulate their intracellular redox conditions. This assumption is being probed in our laboratories.

Since the mode of action of metronidazole seems to be similar in *T. foetus* and *T. vaginalis* (5, 10, 11, 14), it is possible that these findings also apply to the latter species. If so, they could go far in explaining why the in vivo resistance of some *T. vaginalis* strains was not observed in in vitro assays. At the same time they suggest that some reevaluation of published data on the metronidazole susceptibility of *T. vaginalis* isolates might be necessary and that the development of an in vitro assay more closely reflecting in vivo conditions could be of practical significance.

ACKNOWLEDGMENTS

J. G. Meingassner, H. Mieth, and R. Czok acknowledge the help of J. Kulda (Prague, Czechoslovakia) in providing the original KV_1 strain, R. Gönnert in supplying metronidazole, and the conscientious assistance of K. Stockinger. D. G. Lindmark and M. Müller acknowledge the courtesy of E. Roman (G. D. Searle, San Juan, P.R.) in providing metronidazole as well as the competent technical help of M. Sarachik.

Research at The Rockefeller University is supported by Public Health Service grant AI 11942 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Actor, P., D. S. Ziv, and J. F. Pagano.** 1969. Resistance to metronidazole by *Trichomonas foetus* in hamsters infected intravaginally. Science 164:439–440.
2. **Benazet, F., and L. Guillaume.** 1971. Induction of in-vivo resistance of *Trichomonas vaginalis* to nitrimidazine. Lancet ii:982–983.
3. **de Carneri, I.** 1966. Variation of the sensitivity of a strain of *Trichomonas vaginalis* to metronidazole after culturing in the presence or absence of the drug, p. 366–367. *In* A. Corradetti (ed.), Proceedings of the First International Congress of Parasitology (Rome, 21–26 September, 1964), vol. 1. Pergamon Press, New York.
4. **de Carneri, I., G. Achilli, G. Monti, and F. Trane.** 1969. Induction of in-vivo resistance of *Trichomonas vaginalis* to metronidazole. Lancet ii:1308–1309.
5. **de Carneri, I., A. Bianchi, S. Castellino, G. Meinardi, and F. Trane.** 1976. New data on the mechanism and speed of action of nitroimidazoles on prokaryota and eukaryota with and without mitochondria. Arzneim. Forsch. 26:765–769.
6. **Coombs, G. H.** 1976. Studies on the activity of nitroimidazoles, p. 545–552. *In* H. Van den Bossche (ed.), Biochemistry of parasites and host-parasite relationships. North-Holland, Amsterdam.
7. **Diamond, L. S.** 1957. The establishment of various trichomonads of animals and man in axenic cultures. J. Parasitol. 43:488–490.
8. **Diamond, L. S.** 1968. Improved method for the monoxenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amebae with trypanosomatids. J. Parasitol. 54:715–719.
9. **Edwards, D. I., M. Dye, and H. Carne.** 1973. The selective toxicity of antimicrobial nitroheterocyclic drugs. J. Gen. Microbiol. 76:135–145.
10. **Ings, R. M. J., J. A. McFadzean, and W. E. Ormerod.** 1974. The mode of action of metronidazole in *Trichomonas vaginalis* and other microorganisms. Biochem. Pharmacol. 23:1421–1429.
11. **Lindmark, D. G., and M. Müller.** 1976. Antitrichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles. Antimicrob. Agents Chemother. 10:476–482.
12. **Meingassner, J. G., and H. Mieth.** 1976. Cross resistance of trichomonads to 5-nitroimidazole-derivatives. Experientia 32:183–184.
13. **Müller, M., and D. G. Lindmark.** 1976. Uptake of metronidazole and its effect on viability in trichomonads and *Entamoeba invadens* under anaerobic and aerobic conditions. Antimicrob. Agents Chemother. 9:696–700.
14. **Müller, M., D. G. Lindmark, and J. McLaughlin.** 1976. Mode of action of nitroimidazoles on trichomonads, p. 537–544. *In* H. Van den Bossche (ed.), Biochemistry of parasites and host-parasite relationships. North-Holland, Amsterdam.
15. **Müller, W. A., and C. Gottschalk.** 1970. Standardisierung der parasitologischen Nachweisverfahren für Trichomonadeninfektionen. Angew. Parasitol. 11:170–176.
16. **Probst, R. T., J. F. Summersett, and R. K. Heming.** 1968. Interference of metronidazole activity against trichomonads by culture medium ingredients. J. Protozool. 15(Suppl.):20.
17. **Taylor, A. E. R., and J. R. Baker.** 1968. The cultivation of parasites in-vitro. Blackwell Scientific Publications, Oxford.