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

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*Trichomonas foetus* KV, a nitroimidazole-susceptible strain, and KV/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 pO₂, 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, was obtained from J. Kulda (Prague, Czechoslovakia) in 1971 and was kept constantly in culture. The resistant strain, KV/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 N₂ and reestablished in culture. This culture underwent 124 transfers when the experiments 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...
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, 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**       | **Tube test**               |
|                             |                             | **KV**                      | **KV/M100**                 |
|                             |                             | **MIC**                     | **MLC**                     |
|                             |                             | **MIC**                     | **MLC**                     |
| CM 161 (Oxoid)              | 0.8                         | 1.6                         | 50                          | 100                         | 0.2                        | 0.8                        | 0.2                        | 0.8                        |
| CPLM                        | 0.8                         | 1.6                         | 25                          | 50                          | 0.2                        | 0.8                        | 0.2                        | 0.8                        |
| STS                         | 0.8                         | 1.6                         | 50                          | 100                         | 0.2                        | 0.4                        | 0.2                        | 0.4                        |
| TYM                         | 0.4                         | 1.6                         | 25                          | 50                          | 0.8                        | 0.8                        | 1.6                        | 1.6                        |
| Feinberg and Whitlington   | 0.8                         | 1.6                         | 25                          | 50                          | 0.4                        | 0.4                        | 0.4                        | 0.4                        |
| FTG (BBL)                   | 0.8                         | 1.6                         | 50                          | 100                         | 0.4                        | 0.8                        | 0.4                        | 0.8                        |
| FTG with 0.1% ascorbic acid | 0.8                         | 1.6                         | 1.6                         | 25                          | 0.4                        | 0.8                        | 0.4                        | 0.8                        |
| CACH (complete)             | 0.4                         | 3.1                         | 12.5                        | 50                          | 1.6                        | 3.1                        | 0.4                        | 0.8                        |
| TTYS                        | 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                         | 0                          | 0                          | 0                          | 0                          |

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

* FTG, Fluid thioglycolate.

* --, 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 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.

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LITERATURE CITED


