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EFFECT OF METRONIDAZOLE ON *ENTAMOEBA HISTOLYTICA IN VITRO*

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Hsin-Sheng Lo and Fong-Ling Yueh (1984) Effect of metronidazole On *Entamoeba in vitro*. *Bull. Inst. Zool., Academia Sinica* **23**(1): 39-45. Metronidazole is a nitro-imidazole derivative with marked activity against a number of parasitic protozoa and many anaerobic bacteria both *in vivo* and *in vitro*. This work was to investigate the effect of metronidazole on *Entameoba histolytica in vitro*.

The present results indicate that metronidazole was very effective in inhibiting the multiplication of *E. histolytica in vitro*. The minimum inhibitory dose was found to be 0.2 μg/ml which is 2 to 5 times lower than the previously reported value. Assays on the concentration of metabolic intermediates revealed that metronidazole was capable of reducing ATP concentration in amebae. The results may suggest that inhibition of ATP production is probably the mechanism of amebicidal action of metronidazole *in vitro*.

Metronidazole was originally described by Cosar and Jolou (1959) as a nitro-imidazole derivative with marked *in vitro* and *in vivo* activities against *Trichomonas vaginalis*. This compound exhibits similar activity against *Entamoeba histolytic* (Powell *et al.*, 1966, Tanowitz *et al.*, 1973), *Giardia lamblia* (Garrod and O'Grady, 1971), spirochaetes (Stephen *et al.*, 1966) as well as many anaerobic bacteria (Nastro and finegold, 1972, Prince *et al.*, 1969). Presently, metronidazole is regarded by most clinicians as the treatment of choice for urogenital trichomoniasis and has gained widespread acceptance for the treatment of intestinal and extra-intestinal amebiasis.

Despite the widespread use of metronidazole there have been few studies attempting to demonstrate the mode or site of antimicrobial action of this compound. Previous studies by Edwards and Mathison (1970) suggested that metronidazole inhibited directly, or indirectly, the hydrogenase step of the phosphoroclastic reaction in *T. vaginalis*. Such inhibitory reaction led to a decrease in hydrogen evolution in this parasite.

As to *E. histolytica*, Tanowitz *et al.* (1975) reported that metronidazole has a profound selective toxicity against this organism *in vitro*. But the precise mechanism of amebicidal action was not described. Reeves *et al.* (1974) suggested that assays on concentrations of metabolic intermediates in *E. histolytica* can be employed for investigating possible sites of action by anti-amebic drugs. If a drug inhibits the action of an enzyme the metabolite(s) ahead of this site should increase in concentration while those occurring later in the pathway should decrease. Such approach may be feasible in studying the site of action of metronidazole on *E. histolytica in vitro*. This report postulates the probable site of amebicidal action of metronidazole as suggested by the alteration of concentration of metabolic intermediates after administration of the drug.

MATERIALS AND METHODS

Organism

Axenically cultivated *E. histolytica*, strain HK-9, was employed in the study. It was maintained in this laboratory in a modified medium identical to the TYI-S-33 medium of Diamond *et al.* (1978) except that 1,000 units per ml penicillin G and 0.5% Panmede were added. Cells were grown in screw-capped culture tubes (16×125 mm) each containing 14 ml of the culture medium at 36°C. Routine subculture was made by inoculating 100,000 trophozoites into the subsequent culture tubes after 72 to 96 hr incubation at 36°C.

Counting of amebae

The number of amebae per culture was counted with a Fuchs-Rosenthal counting chamber.

Preparation of cell extracts

Large lots of *E. histolytica* were grown in 125 ml screw-capped culture flasks each containing 110 ml of the culture medium. The inoculum was approximately 600,000 amebae per flask. Ten to 20 million cells were harvested per flask after 72 to 90 hr incubation at 36°C. The cells were packed and washed by centrifugation in a balanced salt buffer, pH 7.0, containing 10 mM potassium phosphate; 20 mM KCl; 0.5 mM $MgCl_2$; 100 mM NaCl and 0.1 mM $Ca(NO_3)_2$. The packed cell volume was noted and then subjected to the treatment of cold 6% perchloric acid. Perchloric acid was stirred into the paste with a thin glass rod. Subsequent steps were carried out under cold or frozen conditions. The acid-killed cells were again ruptured by 20 passes in a motor-driven glass-pestle homogenizer. The cell suspension was then centrifuged for 30 min at 32,500×*g*. The supernatant fluid was withdrawn and neutralized with N KOH. After 30 min in an ice bath this solution was centrifuged as previously and the supernatant fluid or cell extract was removed from the precipitated salts. The volume of cell extracts was measured.

Assay of metabolic intermediates

The metabolic intermediates in the cell extracts of *E. histolytica* were assayed by enzymatic procedures employing spectrophotometric determination of the oxidation or reduction of pyridine nucleotides. The value of 6.22 was taken as the millimolar extinction coefficient for NADH or NADPH at the wavelength of 340 nm. A Gilford model 250 spectrophotometer with multiple-speed recorder was employed for the assays. In each assay, a control cuvette lacking amebal cell extract was employed and the assaying enzymes were added to it as well as to the experimental cuvette. The oxidation or reduction of pyridine nuclectides was monitored at 340 nm. When reaction was completed, the difference of optical density between the experimental and the control cuvettes was taken for the calculation of the amount of intermediate being determined.

In the following description of assay methods the amount of enzmymes applied was given in μg protein and the volume for each assay was 1 ml.

Glucose 6-phosphate, fructose 6-phosphate and adenosine triphosphate (ATP)

The amounts of glucose 6-phosphate, fructose 6-phosphate and adenosine triphosphate were assayed by adding successively 8 μg glucose 6-phosphate dehydrogenase, 6 μg glucose phosphate isomerase, and 20 μg hexokinase to cuvettes containing 50 mM Tris/HCl buffer, pH 8.0, 1 mM $MgCl_2$, 1 mM glucose, 0.5 mM NADP and sample.

Triose phosphate and fructose bisphosphate

Triose phosphate and fructose bisphosphate were assayed by adding successively 20 μg of a mixture of glycerol 3-phosphate dehydrogenase and triosephosphate isomerase, and 50 μg of aldolase to cuvettes containing 50 mM Tris/HCl buffer, pH 8.0, 1 mM $MgCl_2$, 0.2 mM NADH and sample.

Phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate

The assays for phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate were carried out by adding successively 10 μg of pyruvate kinase, 10 μg enolase, and 6 μg of phosphoglyceromutase plus 50 nmol of 2, 3-diphosphoglycerate as cofactor to cuvettes containing 50 mM imidazole/HCl buffer, pH 6.5, 2 mM $MgCl_2$, 2 mM NADH, 50 μg lactate dehydrogenase and sample.

Pyruvate and oxaloacetate

Pyruvate was determined by adding 25 μg lactate dehydrogenase to cuvettes containing 50 mM Tris/HCl buffer, pH 8.0, 0.2 mM NADH and sample. Oxaloacetate was assayed by adding 5 μg of malate dehydrogenase to similar cuvettes. Assays for pyruvate and oxaloacetate were made as soon as the cell extract was prepared.

L-Malate and ethanol

L-Malate was assayed by an adaptation of the method Ochoa (1955) employed to assay citrate condensing enzyme. Malate dehydrogenase, 10 μg, was added to cuvettes containing 50 mM Tris/HCl buffer, pH 8.0, 1 mM acetyl-CoA, 0.2 mM NAD, 20 μg of citrate synthase and sample. Ethanol was determined by adding 50 μg of alcohol dehydrogenase to cuvettes containing 50 mM Tris/HCl buffer, pH 8.0, 0.2 mM NAD and sample.

Materials used

All the enzymes, substrates and cofactors were purchased from the Sigma Chemical Company, St. Louis, Missouri, U. S. A. Panmede is an ox-liver extract and was purchased from the Paines and Byrne Limited, Greenford, England. Other reagents were of analytical grade.

RESULTS

Effect of metronidazole on lag-phase amebae

The effect of metronidazole on lag-phase *E. histolytica* was assessed by inoculating stationary-phase amebae into the culture tubes containing the drug. The inoculum was 4×10^4 amebae per tube. Cell counts were made 72 hr and 96 hr post-inoculation, respectively. The results on the effect of various concentrations of metronidazole on *E. histolytica* are given in Table I. It appears that metronidazole exhibited inhibitory effect on lag-phase amebae at a concentration as low as 0.2 μg per ml.

TABLE 1

Effect of various concentrations of metronidazole on lag-phase *Entamoeba histolytica*. Inoculum was 4×10^4 amebae. Numbers of amebae are the average of determinations made on triplicate cultures

Concentrations μg/ml	No. amebae/tube post-inoculation 72 hr	No. amebae/tube post-inoculation 96 hr
0	6×10^5	1.2×10^6
0.2	9×10^4	9×10^4
0.4	0*	0*
0.6	0*	0*
0.8	0*	0*
1.0	0*	0*
2.0	0*	0*
3.0	0*	0*

0* means no viable ameba was found during counting.

Effect of metronidazole on log-phase amoebae

The effect of metronidazole on log-phase *E. histolytica* was determined by adding the drug into the culture tubes containing exponentially growing amebae. Cell counts were made on each culture prior to the addition of metronidazole. After adding the drug, observations were made on the shape and mobility of amebae by means of a light microscope and cell counts were performed at varying time intervals. A number of morphological changes occurred soon after addition of metronidazole. The trophozoites became rounded and less motile as compared with those in the control culture tubes in which metronidazole was not added. Soon thereafter, the trophozoites started to detach from the glass and formed clumps in the culture medium. Those organisms that had rounded up and detached

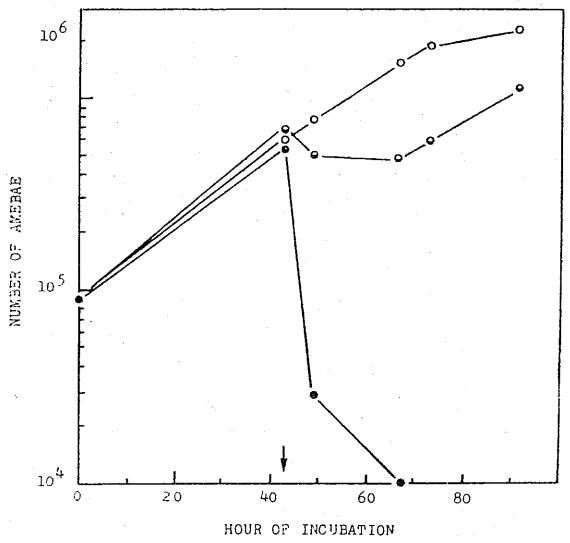

Fig. 1. Effect of metronidazole on the multiplicatiion of exponentially-growing *Entamoeba histolytica* (○—○ control, ◒—◒ 1 μg, ●—● 2 μg) The experimental points are the average of determinations made on triplicate cultures. Arrow indicates the time when metronidazole was added.

from the glass were considered to be dead. The effect of metronidazole on the multiplication of exponentially-growing amebae is shown in Fig. 1. In this experiment, only two concentrations of metronidazole, 1 μg/ml, and 2 μg/ml, were tested. Preliminary tests showed that metronidazole at lower concentrations only exhibited mild effect of inhibition. Metronidazole at 1 μg/ml completely inhibited the multiplication of *E. histolytica in vitro* while at 2 μg/ml killed most of the organisms immediately.

Effect of metronidazole on concentrations of metabolic intermediates in *E. histolytica*

In order to investigate the probable site of amebicidal action of metronidazole, assays on the concentrations of metabolic intermediates in cell extracts of *E. histolytica* were performed. Twelve to 14 culture flasks of amebae were used for each experiment. After 68 hr incubation at 36°C the culture flasks were divided into two groups: the control group in which metronidazole was not added

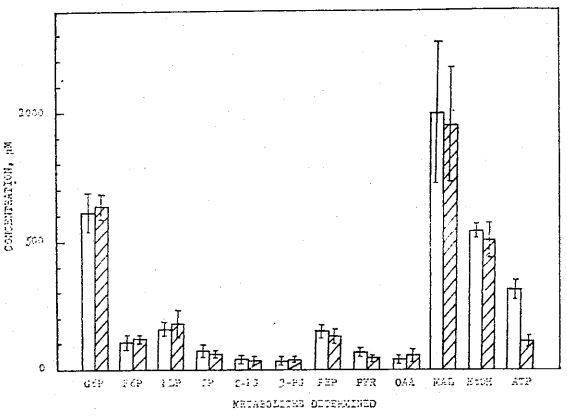

Fig. 2. Effect of metronidazole on the concentrations of metabolic intermediates in the axenic strain of *Entamoeba histolytica*. The open bars represent the mean of five experiments made on the cell extracts prepared from the control culture flasks. The bars with oblique lines represent the mean of five experiments made on the cell extracts prepared from the experimental culture flasks in which 1μg/ml of metronidazole was added. The vertical line at the center of each bar reflects the standard deviation of the mean for the determinations. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose bisphosphate; TP, triose phosphate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; MAL, L-malate; EtOH, ethanol; ATP, adenosine triphosphate.

and the experimental group in which metronidazole was added at a concentration of 1 μg/ml. After additional 10 hr incubation the cells were harvested and the cell extracts were prepared as described in Materials and Methods. The results on the concentrations of metabolic intermediates before and after addition of metronidazole are given in Fig. 2. These concentrations were recalculated from the analytical data by presuming that the intracellular fluid volume of *E. histolytica* is 0.8 ml per ml centrifugally packed cell volume (Reeves *et al.*, 1974).

It appears that metronidazole did not cause any significant changes in the concentrations of glycolytic intermediates in *E. histolytica* except that ATP concentration was markedly decreased. These results may imply that metronidazole does not exert its chemotherapeutic effect by the attack of glycolytic pathway, instead it inhibits the production of ATP in *E. histolytica*.

DISCUSSION

Although metronidazole has been available for a number of years for the successful therapy of several protozoal diseases, notably those caused by *E. histolytica* and *T. vaginalis*, relatively little is known regarding the precise mechanism of its action. As far as the trichomonads are concerned, several hypotheses have been proposed for the possible mode of action of metronidazole *in vitro*. The specificity of action of metronidazole depends upon the ability of the metronidazole-sensitive organisms to reduce the nitro group of the compound (Coombs, 1976, Müller and Lindmark, 1976). Müller *et al.* (1977) were of the opinion that there is an important link among organisms sensitive to the drug in which ferredoxin or similar proteins act as electron transport components in important metabolic pathways. There were evidence supported the view that the reduction of metronidazole was in connection with ferredoxin or comparable proteins (Coombs, 1976, Müller *et al.*, 1977). Edwards and Mathison (1970) postulated that metronidazole inhibited the clostridial type hydrogenase component of the phosphoroclastic reaction in *T. vaginalis*. The site of action would be the enzyme itself, or the electron transfer protein ferredoxin. Nevertheless, different view was raised by Coombs (1976) and O'Brien and Morris (1972) that the hydrogenase was not inhibited by metronidazole, whereas the inhibition of hydrogen evolution was rather due to the low redox potential of metronidazole which acted *in vitro* as a preferential alternative acceptor of electrons originated from pyruvate oxidation in the phosphoroclastic reaction. Further evidence showed that pyruvate: ferredoxin oxidoreductase of *Tritrichomonas foetus* (Lindmark and Müller, 1976) and pyruvate dehydrogenase of *Clostridium* spp. (Coombs, 1976) were also unaffected by metronidazole. In experiments involving [^{14}C]metronidazole in *T. vaginalis* and *T. foetus*, the radio-label was observed to be bound to proteins (Ings *et al.*, 1974, Müller *et al.*, 1976) and to DNA (Ings *et al.*, 1974). Further experiments with [^{14}C]-labelled adenine, thymidine and uridine revealed that metronidazole interfered with the nucleic acid synthesis (Ings *et al.*, 1974). The fine-structure study on the effects of metronidazole upon *T. vaginalis* suggested that protein synthesis and perhaps also other anabolic processes were affected by metronidazole (Nielson, 1976). It is somewhat discouraging that, despite the significant activity, so little is actually understood about the nature of the active inhibitory effect of metronidazole in trichomonads.

As to *E. histolytica*, only few attempts have been made to study the mode of amebicidal action of metronidazole despite its wide use in treatment of intestinal and extra-intestinal amebiasis. Tanowitz *et al.* (1975) showed that metronidazole has a selective toxicity for *E. histolytica* as opposed to Hela cells and *Trypanosoma cruzi*. Their *in vitro* data suggested that the ability of different cells to incorporate metronidazole was a possible explanation for the selective toxicity. These authors also demonstrated that the hydrogenase pathway was not present in axenic strain of *E. histolytica* and that metronidazole had no effect on phosphoroclastic reaction. It was later proven that pyruvate synthase and ferredoxin are present in this organism (Reeves *et al.*, 1977, Reeves *et al.*, 1980).

Reeves *et al.* (1974) reported the intracellular concentrations of metabolic intermediates in a monoxenic strain of *E. histolytica* and first suggested that assays on these concentrations can be used in investigating the site of action of metronidazole. The present study employed an axenic strain of *E.*

histolytica to study the effect of metronidazole on its growth and on the concentrations of its metabolic intermediates *in vitro*.

In view of the results, several findings are of significance. Firstly, metronidazole was very effective in inhibiting the growth of *E. histolytica in vitro*. The minimum inhibitory dose was found to be 0.2 μg/ml which is 2 to 5 times lower than the previously reported value (Tanowitz *et al.*, 1973). Secondly, the concentrations of metabolic intermediates in axenic strain of *E. histolytica* were different from that of a monoxenic strain. This would suggest probable involvement of bacterial associate in amebal metabolism. Thirdly, metronidazole did not cause any significant changes in the concentrations of metabolic intermediates in *E. histolytica in vitro* except that it significantly reduced the intracellular concentration of ATP. It can be thus postulated that metronidazole exerts its inhibitory effect on the production of ATP in amebae.

In aerobic organisms, the oxidative phosphorylation is the main process for the production of ATP which occurs primarily in mitochondria. Since *E. histolytica* is practically an anaerobe and lacks mitochondria, the oxidative phosphorylation is not operative (Miller *et al.*, 1961, Serrano *et al.*, 1977, Weinbach *et al.*, 1974). In amebae, there are two known steps which produce ATP are the phosphoenolpyruvate to pyruvate (Reeves, 1968) and the acetyl-CoA to acetate pathways (Reeves *et al.*, 1977). According to the results metronidazole did not alter the concentrations of amebal glycolytic intermediates, it is unlikely that glycolytic pathway is the site where metronidazole attacks. Whether there are any other unknown pathways which may be linked to the inhibition of ATP production by metronidazole needs further investigation.

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Metronidazole 於試管中對痢疾阿米巴之影響

羅 新 生　　岳 鳳 玲

Metronidazole 係 nitro-imidazole 之衍生物，對於幾種寄生原蟲及多種厭氧性細菌有顯著之活性。本研究之目的，在於探討 metronidazole 於試管中對痢疾阿米巴之影響。

本研究之結果指出，metronidazole 對痢疾阿米巴體外生長有很强之抑制作用，最低有效劑量爲 0.2 微克/毫升，較以往報告之數值低 2 至 5 倍。由代謝中間物濃度測定之結果發現，metronidazole 可降低 ATP 之濃度，因此推測抑制 ATP 之生成，可能是 metronidazole 對抗痢疾阿米巴之作用機轉。