

Oxytetracycline inhibition of mitochondrial protein synthesis in bovine lymphocytes infected with *Theileria parva* or stimulated by mitogen

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SUMMARY

Oxytetracycline (OTC) significantly inhibited cytochrome c oxidase activity in bovine lymphocytes infected with *Theileria parva* and in uninfected mitogen-stimulated lymphocytes. The inhibitory effect was detected *in vitro* within 24 h of treatment with drug concentrations as low as 1 µg/ml. Following mitogen stimulation of lymphocytes, concentrations of 3 and 10 µg/ml OTC completely inhibited an increase in cytochrome c oxidase activity for 48–72 h. This inhibitory activity was considered to be due to a direct effect on lymphoblast mitochondrial protein synthesis. As a consequence, adenosine triphosphate activity was significantly reduced in lymphocytes stimulated either by infection with *T. parva* sporozoites or by mitogen and then treated with OTC. The results also indicated that parasite mitochondrial protein synthesis was inhibited by OTC. The activity of OTC reported in this study could explain the suppression of disease following 'infection and treatment' immunization against East Coast fever and the *in vitro* drug-inhibition of schizont development.

Key words: *Theileria parva*, East Coast fever, oxytetracycline, mitochondrial protein synthesis, bovine lymphocytes, immunization.

INTRODUCTION

For the control of East Coast fever (ECF), caused by infection with *Theileria parva*, curative drugs, namely parvaquone, halofuginone and buparvaquone (Schein & Voigt, 1979; McHardy, Hudson & Rae, 1980; Dolan, 1981; McHardy & Wekesa, 1985) are available, but immunization using a method of 'infection and treatment' is at present the only practical method of protecting cattle against the disease. The immunization is based on the initial observations of Neitz (1953, 1957) that tetracyclines suppressed the development of disease following the application of ticks infected with *T. parva* to cattle and these were immune to homologous challenge. The development of methods for cryopreservation of the parasite and of slow-release formulations of tetracyclines has improved the original method and it is currently achieved by inoculation of cattle with *T. parva* sporozoites followed immediately by treatment with a long-acting formulation of oxytetracycline, usually at 20 mg/kg (reviewed by Purnell (1977) and Radley (1981). Recently, the anti-theilerial drug buparvaquone has been found to be effective when used at 2.5 mg/kg in infection and treatment immunization (McHardy & Wekesa, 1985; Mutugi *et al.* 1988); however, this drug is not yet commercially available.

The mode of action of OTC during immunization has been unclear (Irvin & Morrison, 1987). However, recent *in vitro* studies have shown that, following infection of bovine lymphocytes with *T. parva* sporozoites, the drug inhibits the development of the

schizont stage (Spooner, 1990). The inhibitory activity of OTC was detected at concentrations of 2.5–5.0 µg/ml which were comparable with estimated concentrations achieved during immunization. The mechanism by which OTC inhibits schizont development was not determined; however, similar drug concentrations have been shown to inhibit bacterial protein synthesis (Gale & Folkes, 1953; Connamacher & Mandel, 1965; Day, 1966). Mitochondrial protein synthesis in mammalian cells was also inhibited by concentrations of tetracyclines below 20 µg/ml (De Vries & Kroon, 1970; Gijzel, Strating & Kroon, 1972; Van den Bogert & Kroon, 1981*a*). These lower concentrations did not affect products of cytoplasmic protein synthesis such as cytochrome c and total protein synthesis was also unaffected (Gijzel *et al.* 1972; Van den Bogert & Kroon, 1981*a*; Van den Bogert *et al.* 1983*a*). Higher concentrations of tetracyclines (above 50 µg/ml) were required to inhibit cytoplasmic protein synthesis (De Jonge, 1973; Gijzel & Kroon, 1978).

In this study the action of OTC on mitochondrial protein synthesis was studied using bovine lymphocytes stimulated by concanavalin A (con A), following infection with *T. parva* sporozoites, or as a line persistently infected with *T. parva* schizonts. Drug effects were assessed using the activity of cytochrome c oxidase as an indicator of mitochondrial protein synthesis (De Vries & Kroon, 1970; Van den Bogert *et al.* 1981). This respiratory chain enzyme is specific to, and synthesized in part in the mitochondrion (Weiss, Sebald & Bucher, 1971;

Schatz & Mason, 1974; Tzagoloff, Macino & Sebald, 1979). Since inhibition of this enzyme could lead to a reduction in energy available within the host cell the effects of OTC on adenosine triphosphate (ATP) levels were also examined.

MATERIALS AND METHODS

Cell lines and culture methods

The cells used were a cloned line, H12, of bovine lymphoblastoid cells infected with the Muguga stock of *T. parva*, an uninfected T cell clone (reference A2) grown in the presence of T cell growth factor, the same clone infected with *T. parva* (Muguga) and a B cell line similarly infected. These cell lines and culture methods have been described previously (Brown & Grab, 1985; Brown & Logan, 1986; Lalor *et al.* 1986; Spooner, 1990). Bovine peripheral blood mononuclear cells (PBM) stimulated with 5 µg/ml con A, PBM infected with *T. parva* sporozoites, and schizonts isolated from infected lymphocytes following release by aerolysin (Sugimoto *et al.* 1988) were also used.

Cells were cultured in Leibovitz's L15 medium (Flow Laboratories, Ayrshire, Scotland) containing 20% (v/v) foetal bovine serum (Flow Laboratories). Duplicate flasks (Falcon 3013, Becton Dickinson, Heidelberg, W. Germany) were set up for each test and for controls and contained 10 ml of growth medium with cell concentrations of 2×10^5 /ml for the infected and uninfected lymphoblasts, 10^6 /ml for PBM stimulated with con A, and 4×10^6 /ml for PBM following infection with sporozoites.

Sampling and preparation of cells

At each sampling, cell counts were performed and cell viability determined by dye exclusion following staining with 1% trypan blue. Appropriate volumes (in duplicate) of cell suspensions were removed from each flask so that each contained the following number of viable cells. (i) 1×10^5 cells for studies of *T. parva*-infected T and B cell lines and uninfected T lymphoblasts. (ii) 1×10^6 cells for cultures of PBM stimulated with con A. (iii) 1×10^7 cells at day 3 and 5×10^6 cells at day 5 from cultures of PBM infected with sporozoites.

Sampled cells were pelleted by centrifugation at 200 g for 10 min, washed with 10 ml of PBS and centrifuged again. The pellets were resuspended in 1 ml of PBS and centrifuged (200 g for 10 min) in 1.5 ml tubes (No. 3810, Eppendorf Gerateban, Hamburg, W. Germany). The supernatant fraction was removed and the pellets resuspended in 0.2 ml of PBS for cytochrome c oxidase assays. For ATP assays, the pellet was resuspended in 0.1 ml of 0.7 M perchloric acid for 30 s, centrifuged at 10000 g for 5 min and the supernatant fraction used for assay. All samples were stored at -80°C until assayed.

Cytochrome c oxidase assays

A modification of the methods of Cooperstein & Lazarow (1951) and Borst, Ruttenberg & Kroon (1967) was used for cytochrome c oxidase assays. This modified method has been found to be sensitive, accurate and reproducible (Spooner, 1987). Samples to be tested were thawed rapidly and mixed with an equal volume (200 µl) of cold 1% digitonin (Sigma, D5628) in 0.03 M PBS (pH 7.4). The mixture was held on crushed ice for 15 min and then centrifuged at 10000 g for 1 min. Supernatant fractions were removed, stored on crushed ice and assayed within 1 h.

Cytochrome c (Sigma, C3131) was dissolved in 0.03 M PBS to give a 1.7×10^{-5} M solution, and reduced with 1.2 M sodium hydrosulphite (Sigma, S 1256) at 100 µl to 30 ml cytochrome c solution. To remove excess hydrosulphite the mixture was stirred vigorously for 1 h at room temperature. Three ml of reduced cytochrome c were added to quartz cuvettes and 100 µl of enzyme preparation added. The reactants were mixed rapidly and the optical density read every 20 s at 550 nm for 2 min at 25 °C, in a spectrophotometer (Beckman Instrument Company, California, USA, model DU7 with kinetic attachment). All assays were done in triplicate on two separate occasions, with control and test samples from one experiment always prepared and assayed at the same time. The rates of enzyme activity of test samples were compared directly as a percentage of control rates, since the rate is proportional to the cytochrome c oxidase concentration. When calculation of units of enzyme activity was required (one unit is defined as that amount of enzyme which will oxidize 1.0 µl mole of reduced cytochrome c to 1.0 µ mole of oxidized cytochrome c/min at pH 7.0 and 37 °C), activity was compared with that of standard cytochrome c oxidase preparations (Sigma, C5771) assayed at the same time (Cooperstein & Lazarow, 1951).

Adenosine triphosphate assays

A method to assay ATP similar to that of Stanley & Williams (1969) was used, with several modifications, and has been found to be sensitive and consistent (Spooner, 1987). The assay measured luminescence which was proportional to the ATP concentration. Cell extracts were thawed rapidly, diluted 1:10 in distilled water and held on crushed ice. One ml of firefly lantern extract (FLE 50, Sigma) was thawed and clarified at 10000 g for 5 min. The supernatant fraction was added to 5 ml of cold assay buffer (glycyl glycine, 0.03125 M; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.036 M, pH 7.8) and stored protected from light on crushed ice. One ml of FLE 50 assay mixture was added to counting vials (No. 6008117, Hewlett Packard Nederland BV, Amstelveen, Holland) which were placed in a liquid scintillation counter (Packard

Model 3330) to equilibrate for 10 min at room temperature. Then 10 μ l of diluted cell extract were added to each vial, the mixture swirled gently for 5 s, and the light emission counted. Readings were taken with the coincidence circuit turned off, the discriminator set at 0–1000, and the gain set at 65%. Counts normally stabilized (less than 1% decay rate of light emission) after the third reading and the assay count was taken as the mean of the next three readings. Triplicate assays were performed on two separate occasions, and when control and treated samples were compared, assays of similar samples (i.e. same day preparations) were carried out in close succession. The final sample count was calculated as the mean of the three assays and mean counts from control and test samples were compared directly.

Protein estimations

Protein estimations were carried out on isolated schizonts to calculate ATP or cytochrome c oxidase activity per unit of protein. Assays used a commercially available system (Bio-Rad Protein assay, Bio-Rad Laboratories, Richmond, California, USA) and protein concentrations were determined from a standard curve using known concentrations of bovine serum albumin.

Statistical methods

Data were analysed using an analysis of variance and the $p \times q$ repeated measures design. In the event of significant differences between treatments, the Newman Keuls Multiple Range test was used to identify which groups were different (Winer, 1971).

RESULTS

Using serial dilutions of a standard commercial cytochrome c oxidase preparation, the rate of

oxidation of reduced cytochrome c was confirmed to be proportional to the enzyme concentration. Treatment with 1% digitonin was found to be more efficient in releasing this enzyme from *T. parva*-infected lymphocytes than either sonication or freeze-thawing. Additional sonication for between 30 s and 2 min following digitonin treatment did not release more enzyme.

The cytochrome c oxidase concentration in *T. parva*-infected cells was determined from the slope plotted using standard enzyme preparations. A mean figure (from 8 assays) of 5.5 units of enzyme activity/ 10^7 infected cells was found during the logarithmic phase of growth.

Cytochrome c oxidase activity was determined following treatment of various cell lines with several concentrations of OTC for 3 days (Table 1). Significant reductions in enzyme activity of between 14 and 62% were detected within 24 h, with cell lines infected with *T. parva* and uninfected but stimulated T cells. Inhibition was observed with 1 μ g/ml OTC but was most marked following treatment with 10–100 μ g/ml OTC for 3 days, when enzyme activity was reduced by 65–77%.

Assays were also conducted with 0.33 or 3.3 μ g/ml OTC added directly to the assay mixture. Mean values of 98.5 and 103.5% of the control enzyme activity were found, indicating no direct inhibitory effect of this drug on the assay system.

Table 2 summarizes the effects of OTC on ATP and cytochrome c oxidase activity. The *T. parva*-infected H12 cell line was treated with 1, 10 and 30 μ g/ml OTC for 4 days. In treated cells reductions in ATP activity of 12–31% were found on days 1 and 2 and were most marked using 30 μ g/ml OTC. On day 3, ATP activity increased in treated cells and by day 4 were significantly higher than control values. During this experiment the effect of adding OTC directly in the assay was studied. Concentrations of 1 or 10 μ g/ml in the assay mixture had no significant

Table 1. The effect of oxytetracycline on cytochrome c oxidase activity in various cell lines (Enzyme activity is expressed as a percentage of control activity, determined from two separate assays.)

Cell type	Parasite	OTC μ g/ml	Day of treatment			
			0(+3 h)	1	2	3
T cell	<i>T. parva</i> (Muguga) reference line H12	100	—	37.6	38.1	22.3
		10	—	41.2	38.1	26.7
		1	—	62.2	70.1	83.0
T cell (clone A2)	<i>T. parva</i> (Muguga)	10	97.4	47.6	29.2	28.9
		1	98.7	73.2	72.9	77.2
T cell (clone A2)	Uninfected	10	92.9	60.7	48.7	34.7
		1	102.8	85.7	76.1	61.2
B cell	<i>T. parva</i> (Muguga) reference R1+1	100	—	—	—	7.6
		10	—	—	—	22.8

—, Not tested.

Table 2. The effect of oxytetracycline on ATP and cytochrome c oxidase activity in a *Theileria parva*-infected cell line (H12)

(Results are expressed as percentages of control activity and are the mean of two separate assays.)

OTC ($\mu\text{g/ml}$)	Day of treatment							
	1		2		3		4	
	ATP	CYT.OX	ATP	CYT.OX	ATP	CYT.OX	ATP	CYT.OX
30	87.5	41.0	68.7	39.3	87.9	25.0	143.4	32.4
10	83.9	42.6	87.5	22.7	101.8	25.0	121.7	32.4
1	89.3	75.2	87.3	71.6	113.9	44.3	129.0	94.1

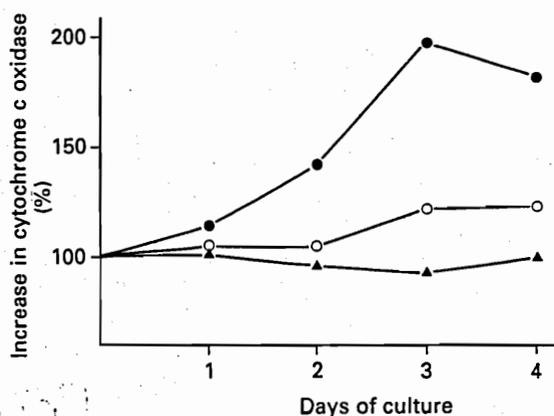


Fig. 1. The effect of oxytetracycline on cytochrome c oxidase activity in bovine lymphocytes stimulated with 5 $\mu\text{g/ml}$ con A. Drug concentrations in $\mu\text{g/ml}$ as follows: (○) 3; (▲) 10; (●) control. Enzyme level in unstimulated lymphocytes was considered to be 100%. Results are the mean of two similar experiments.

effect on the assay results. Reductions in cytochrome c oxidase activity due to OTC were also detected on days 1–3 in this experiment. In cells treated with 10 and 30 $\mu\text{g/ml}$ OTC, enzyme values showed a slight increase between days 3 and 4, but during this period control activity had decreased by 28%. Cells treated with 1 $\mu\text{g/ml}$ OTC showed a more marked rise in cytochrome c oxidase activity between days 3 and 4. In this experiment culture growth rates were also reduced by OTC and at day 4, viable cell counts after treatment with 1, 10 or 30 $\mu\text{g/ml}$ OTC were 74.5, 27.2 and 10.2% of control counts, respectively.

The effects of OTC on stimulation of bovine PBM by con A were also studied. Fig. 1 summarizes two experiments in which the effects of 3 and 10 $\mu\text{g/ml}$ OTC on cytochrome c oxidase activity were determined daily for 4 days. Enzyme activity was also determined in control stimulated PBM and in control unstimulated PBM. The activity of the enzyme in unstimulated cells was used as the baseline (100%) value above which increased cytochrome c oxidase activity due to stimulation was determined. OTC, at 10 $\mu\text{g/ml}$, completely inhibited any net increase in enzyme activity in response to con A

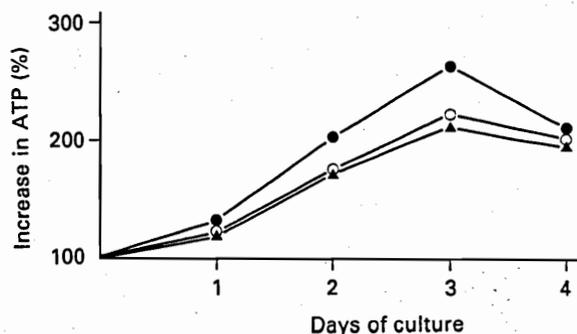


Fig. 2. The effect of oxytetracycline on ATP activity in bovine lymphocytes stimulated with 5 $\mu\text{g/ml}$ con A. The ATP level in unstimulated lymphocytes was considered to be 100%. Other details are as described in the legend to Fig. 1.

stimulation. A similar inhibitory effect was found with 3 $\mu\text{g/ml}$ OTC, followed by a limited increase in enzyme activity on days 3 and 4. The rate of increase of cytochrome c oxidase activity to day 3 was significantly ($P = < 0.01$) faster and the activity was significantly higher in control cells, then in cells treated with 3 or 10 $\mu\text{g/ml}$ OTC. Effects on ATP were also studied in these experiments and are summarized in Fig. 2. ATP synthesis was consistently reduced by 25–43% during the 3 day period after stimulation using OTC at 3 or 10 $\mu\text{g/ml}$. The effect on day 4 was less marked due to the more rapid decline in control ATP levels. Significant differences ($P = < 0.01$) in ATP activity were shown between control and treated cells on days 1–3.

Table 3 gives details of 2 experiments in which the action of OTC was studied on cytochrome c oxidase and ATP activity in bovine PBM following infection with *T. parva* sporozoites. While slight reductions in cytochrome c oxidase activity were found following treatment of infected cells with OTC for 3 days, no significant effects were detected on ATP levels. The cytochrome c oxidase and ATP activity in control infected PBM at day 3 indicated only a low level of stimulation when compared with uninfected PBM. When sampling was carried out on day 5 after infection, significant differences ($P = < 0.01$) in

Table 3. The effect of oxytetracycline on cytochrome c oxidase and ATP activity in bovine lymphocytes infected with *Theileria parva* sporozoites

(Enzyme activity is expressed as a percentage of the level in control infected PBM. ATP activity in treated cells is given as a percentage of the level in control infected PBM, after subtraction of the level in uninfected PBM, and shows the effect of OTC on ATP synthesis in response to infection of these cells. ATP in uninfected PBM is expressed as a percentage of the level in control infected PBM and indicates increased synthesis due to infection).

Treatment	Day sampled	CYT.OX	ATP
OTC 10 µg/ml	3	91.4	97.4
OTC 3 µg/ml		92.3	100.3
PBM (uninfected)		89.1	84.0
OTC 10 µg/ml	5	45.8	64.9
OTC 3 µg/ml		63.7	71.3
PBM (uninfected)		45.6	49.4

cytochrome c oxidase and ATP levels were found between control cells and cells treated with 3 or 10 µg/ml OTC. Enzyme and ATP activity in uninfected PBM were shown to be less than 50% of the control infected cells at this time.

Similar studies were carried out on schizonts isolated from cells treated with 10 µg/ml OTC for 3 days. No significant reduction in ATP levels was found in schizonts from treated cells when activity/mg of schizont protein was assessed. Cytochrome c oxidase activity in schizonts isolated from cells treated with OTC was reduced by 54.5% when compared with control schizont fractions and enzyme activity in treated host cell fractions (without schizonts) was reduced by 52.8% when compared with untreated host cell fractions.

DISCUSSION

Oxytetracycline was demonstrated to inhibit mitochondrial protein synthesis in uninfected bovine T cells, PBM stimulated either by mitogen or by infection with *T. parva* sporozoites, and in lymphoblastoid cells persistently infected with *T. parva* schizonts. Significant inhibition was detected within 24 h using 1–3 µg/ml OTC. A direct effect of the drug on cytochrome c oxidase activity in isolated schizonts was also detected, but may have resulted from contamination of the schizont preparation with host cell mitochondria.

The increased ATP synthesis detected in bovine PBM 1–3 days after mitogen stimulation and 5 days after sporozoite infection was significantly inhibited using 3 and 10 µg/ml OTC. ATP activity in an established *T. parva*-infected cell line was reduced for 2 days following treatment with OTC; however, on days 3–4, ATP activity in treated cells was higher

than in control cells. Interpretation of the results obtained with this cell line was difficult due to the pre-treatment levels of ATP which were apparently unaffected by OTC and also due to the cytostatic effect of this drug. The lower activity of ATP at day 4 in con A-stimulated control cultures probably resulted from growth inhibition due to the high cell densities in these cultures. There was a decline in the inhibitory effects caused by 1 µg/ml OTC on day 4. This concentration may be near the threshold of activity of the drug and the decline in effect may have resulted from the reported instability of OTC at neutral or alkaline pH (Van den Bogert & Kroon, 1981b).

The inhibition by OTC of the growth rate of *T. parva*-infected lymphocytes and uninfected but stimulated cells shown here, has also been reported in a more detailed study (Spooner, 1990). The drug was cytostatic and cell viability was unaffected by treatment with up to 40 µg/ml for 4 days. Growth rates recovered rapidly upon removal of OTC and within 5 days were similar to those of untreated cells.

Despite the apparently complete inhibition of mitochondrial protein synthesis by OTC in mitogen-stimulated PBM (i.e. no increase in cytochrome c oxidase level above the unstimulated PBM value), the amount of ATP increased significantly in these cells. ATP synthesis may have resulted from glycolysis which has been reported to be stimulated by con A (Ashman, 1984) and which is unaffected by 100–500 µg/ml OTC (De Jonge, 1973). ATP could also have been synthesized using reserves of mitochondrial enzymes. However, this may be unlikely since cytochrome c oxidase concentrations in *T. parva*-infected cells were found to be low and similar to those reported in Leydig tumour cells (Van den Bogert, Dontje & Kroon, 1983b). These concentrations are about 1% of those found in mammalian liver cells and as a consequence, lymphocytes, following infection with sporozoites or stimulation with con A, may be more sensitive to inhibitors of mitochondrial protein synthesis.

The integration of *T. parva* into the host cell following sporozoite infection is a critical phase of development and treatment at this stage with OTC *in vitro* inhibits the development of parasites to mature schizonts (Spooner, 1990). It is probable that this effect is due to inhibition by OTC of mitochondrial protein synthesis in the infected host cell or the resulting restriction of available energy. There may be other actions of OTC which could explain these results. Inhibition of cytoplasmic protein synthesis would decrease production of certain subunits of cytochrome c oxidase, leading to reduced enzyme concentration in mitochondria, but such inhibition has been reported at concentrations above 50–100 µg/ml OTC (De Jonge, 1973; Gijzel Kroon, 1978). Synthesis of ATP could be reduced by uncoupling of mitochondrial respiration, how-

ever, this effect has been reported with concentrations above 150 µg/ml OTC *in vitro* (De Jonge, 1973).

A direct effect of OTC on mitochondrial protein synthesis of the developing schizont seems possible from the results presented. Cytochrome c oxidase has been demonstrated between the inner and outer mitochondrial membranes of *Theileria annulata* sporozoites (Weber, 1980; Jura, Brown & Brocklesby, 1985) and presumably is present in *T. parva* sporozoite and schizont mitochondria. Drug inhibition of mitochondrial function in the parasite would also have deleterious effects on mitochondrial biogenesis and consequently on parasite development.

Support for a direct effect of OTC on *T. parva* schizonts comes from studies of the related parasite, *Plasmodium falciparum*. *In vitro* studies have shown that *P. falciparum* parasitaemia was reduced by treatment with tetracyclines and this was considered to result from drug-inhibition of parasite mitochondrial protein synthesis (Geary & Jensen, 1983; Blum *et al.* 1984; Divo, Geary & Jensen, 1985). More recent studies (Prapunwattana, O'Sullivan, & Yuthavong, 1988; Kiatfuengfoo *et al.* 1989) have confirmed these findings.

Concentrations of 1–3 µg/ml OTC found to inhibit mitochondrial protein synthesis *in vitro* are likely to be present in the serum of cattle for up to 48 h following a single dose of 20 mg/kg of a long-acting formulation (Terramycin LA, Pfizer) (Davey, Ferber & Kaye, 1985) which is used during immunization against ECF. Therefore, it appears probable that the effects of OTC, demonstrated in this study, occur during infection and treatment immunization. Suppression of development of the parasite at this stage of infection may allow the host immune response a greater opportunity to respond to the parasite thereby preventing disease. Whether the suppression of parasite development results from drug-inhibition of mitochondrial protein synthesis of the host cell or parasite or both, is unclear. A direct effect of OTC on this process in the parasite would have more immediate and probably more serious consequences on subsequent development in the host cell. These studies will be expanded when more highly purified schizont preparations are available for biochemical analysis.

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