

Inhibition of *Toxoplasma gondii* Protein Synthesis by Azithromycin

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Azithromycin was shown to specifically inhibit the protein synthesis of *Toxoplasma gondii* in experimental systems by using free tachyzoites and *T. gondii*-infected mouse macrophages. RNA synthesis of the parasite was not affected by azithromycin. Inhibition of protein synthesis was also proportional to the relative anti-*Toxoplasma* activity of three macrolides.

Azithromycin, a 15-membered macrolide derived from erythromycin (2, 9, 10), is a potent inhibitor of bacterial protein synthesis (13). The purpose of this study was to determine whether azithromycin interferes with specific physiological processes in an eukaryotic microorganism such as *Toxoplasma gondii* as it does in procaryotes. Therefore, assays were developed to determine the effect of azithromycin and other macrolides on the protein synthesis of intracellular and free tachyzoites of *T. gondii*.

A mouse macrophage cell line (RAW 264.7, ATCC TIB 71) was used for the cultivation of *T. gondii* RH. Macrophages were maintained in Dulbecco's modified eagle medium (GIBCO/BRL Canada, Burlington, Ontario, Canada) containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 0.1 µg of fungizone per ml, at pH 7.2 (3). For the inhibition studies, antimicrobial agents were omitted from the culture medium.

The growth of *T. gondii* was measured by the incorporation of [5,6-³H]uracil into trichloroacetic acid (TCA)-precipitable material as previously described (3, 4, 12). There was continuous growth of the parasite and the incorporation did not reach a plateau, indicating that the growth of *T. gondii* was limited neither by the host cells nor by a depletion of the culture medium. The growth of the parasite was significantly reduced by about 50% after 24 h of incubation in the presence of 10 µg of azithromycin (Pfizer Inc., Central Research Division, Groton, Conn.) per ml. The reduction of incorporation of [5,6-³H]uracil was apparent only after more than 6 h of incubation (Fig. 1).

RNA and DNA syntheses were measured for both intracellular and free tachyzoites of *T. gondii* by using [5,6-³H]uracil and [6-³H]thymidine, respectively. Macrophages were seeded in 24-well tissue culture plates at a concentration of 10⁶ cells per well. A total of 10⁶ tachyzoites were added to each well and were allowed to penetrate the cells during 30 min at 37°C. For experiments performed on free tachyzoites, 10⁷ *T. gondii* cells were placed in each well and incubated for 30 min at 37°C prior to the addition of drug. Then, 10 µCi of [5,6-³H]uracil or [6-³H]thymidine and various concentrations of macrolides were added. At various times, cells were lysed with H₂O. Lysates were digested with DNase 1 or RNase A (Sigma Chemical Co., St. Louis, Mo.) for 1 h at

37°C. TCA precipitates were collected on glass fiber filters (Whatman GF/C). Radioactivity was determined by using a Beckman LS 6000TA liquid scintillation system.

T. gondii can salvage and convert uracil into thymidine (8). Therefore, by providing [5,6-³H]uracil to the parasite, it was possible that both RNA and DNA would be labeled. However, in the intracellular and free tachyzoites, [5,6-³H]uracil was almost exclusively found in the RNA, with 96.5% of radiolabel associated with the RNase-susceptible fraction after 4 h of incubation. The amount of [5,6-³H]uracil incorporated into DNA was negligible and represented only 3.5% of the total incorporation. Under the conditions used, incorporation of thymidine was not detectable over a 4-h incubation.

Protein synthesis was measured in *T. gondii*-infected macrophages as well as in free tachyzoites by using [³⁵S]methionine in an RPMI-1640 Select-Amine kit (GIBCO/BRL Canada) without methionine and cystine and containing 10% fetal bovine serum (5). Tachyzoites or infected macrophages were incubated in the culture medium for 1 h at 37°C. At time zero, 15 µCi of [³⁵S]methionine was added to each well, and the incorporation was stopped at various times by the addition of cold methionine (15 µg/ml) and cold cystine (50 µg/ml). TCA precipitates were collected and radioactivity was determined as described above.

After 1 h of incubation in the presence of [³⁵S]methionine, infected macrophages showed an incorporation as high as 33,697 ± 4,098 cpm while uninfected cells did not incorporate any methionine. These results indicated that the protein synthesis measured was essentially due to the growth of *T. gondii*. *T. gondii* is an intracellular parasite which cannot be grown in axenic culture. Free tachyzoites, in culture flasks devoid of host cells, may remain alive for a limited time which extended beyond the limit imposed on our experimentation. Free tachyzoites incorporated detectable levels of [³⁵S]methionine at 1 h (42,242 ± 2,511 cpm).

The effect of azithromycin on protein and RNA syntheses was measured in infected macrophages and free tachyzoites of *T. gondii* and is presented in Fig. 2A and B, respectively. In this assay [³⁵S]methionine and [5,6-³H]uracil incorporations were measured during a 1-h exposure to various concentrations of azithromycin. In both the infected cells and the free tachyzoites, protein synthesis was gradually inhibited by increasing concentrations of azithromycin (Fig. 2A and B). Infected macrophages showed an activation of RNA synthesis at concentrations of azithromycin ranging

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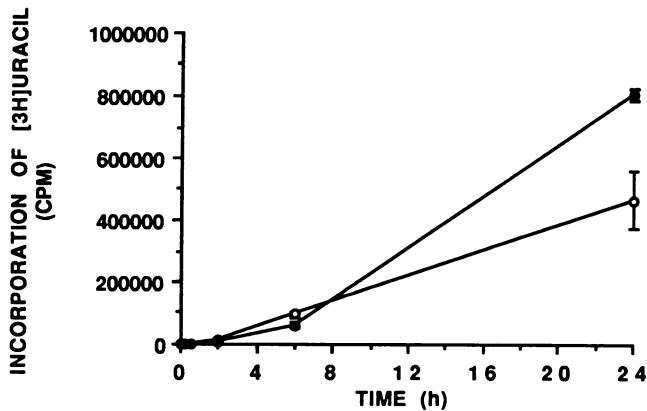


FIG. 1. Kinetics of the intracellular growth of *T. gondii* in cultured mouse macrophages (RAW 264.7) in the absence of drug (closed symbols) and in the presence of 10 µg of azithromycin per ml (open symbols). Growth was measured by the selective incorporation of [³H]juracil during a 24-h incubation period at 37°C. Each point represents the mean of two determinations ± standard error.

from 10 to 30 µg/ml. At concentrations of drug exceeding 30 µg/ml, RNA synthesis was significantly inhibited. In both free tachyzoites and infected macrophages, protein synthesis was inhibited by low concentrations of azithromycin

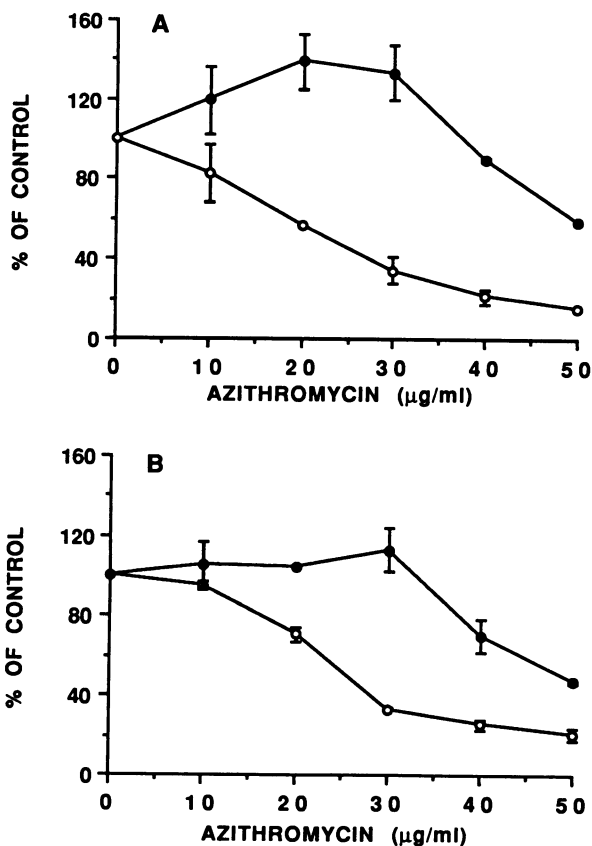


FIG. 2. Effect of increasing concentrations of azithromycin on the protein synthesis (open symbols) and RNA synthesis (closed symbols) of intracellular *T. gondii* (A) and free tachyzoites of *T. gondii* (B). The synthesis of macromolecules was measured during 1 h of incubation at 37°C. Each point represents the mean of four determinations ± standard error.

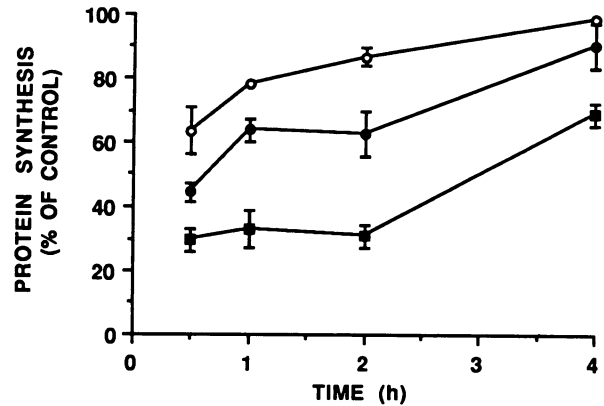


FIG. 3. Recovery of protein synthesis in free tachyzoites of *T. gondii* after a 1-h exposure to 10 (○), 30 (●), and 50 (■) µg of azithromycin per ml. Each point represents the mean of two determinations ± standard error.

while RNA synthesis was affected only at drug concentrations that had a profound effect on protein synthesis (>30 µg/ml).

Protein synthesis of free tachyzoites was also measured during 4 h in a drug-free environment, after a 1-h exposure to azithromycin (Fig. 3). The degree of inhibition of protein synthesis was proportional to the concentration of drug. After exposure to 10 and 30 µg of azithromycin per ml, protein synthesis gradually returns to the level of the control (98.62% ± 0.01% and 74.41% ± 0.03% of the untreated control after 4 h, respectively) (Fig. 3). However, a 1-h exposure to 50 µg of azithromycin per ml had a more lasting effect and protein synthesis remained at 30% of that of the untreated control for 2 h and only reached 68.85% ± 3.50% of that of the untreated control after 4 h of incubation in the absence of drug (Fig. 3).

The relative potency of inhibition of *T. gondii* protein synthesis for azithromycin, erythromycin, and dirithromycin is shown in Fig. 4. Erythromycin or dirithromycin had essentially no effect on the protein synthesis of free parasite

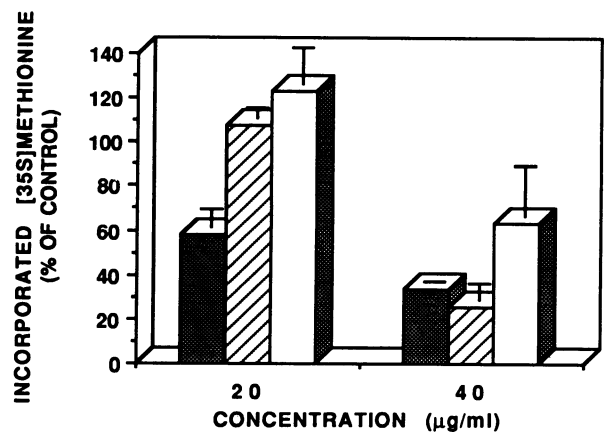


FIG. 4. Effect of azithromycin (solid bars), erythromycin (hatched bars) and dirithromycin (open bars) on the incorporation of [³⁵S]methionine by free tachyzoites of *T. gondii*. Cultures were incubated during 1 h at 37°C in the presence or absence of drugs at 20 or 40 µg/ml. Each bar represents the mean of two determinations ± standard error.

at a concentration of 20 $\mu\text{g/ml}$, whereas an equivalent concentration of azithromycin inhibited protein synthesis resulting in an incorporation of [^{35}S]methionine that was $57.54\% \pm 8.15\%$ of that of the control. When the drug concentration was raised to 40 $\mu\text{g/ml}$, azithromycin and erythromycin provoked an equivalent inhibition of protein synthesis and incorporation of [^{35}S]methionine was $33.20\% \pm 0.11\%$ and $25.60\% \pm 7.18\%$ of that of the control, respectively. The effect of dirithromycin was less pronounced than that of the two other macrolides, but it reduced incorporation of [^{35}S]methionine to $63.13\% \pm 22.71\%$ compared to that of the control. We have previously determined that the concentrations of azithromycin, erythromycin, and dirithromycin which inhibited 50% of the growth of *T. gondii* (IC_{50}) were 8.61 ± 2.51 , 14.38 ± 2.41 , and $>25 \mu\text{g/ml}$, respectively (4). The macrolides inhibited protein synthesis in free tachyzoites only at concentrations superior to the IC_{50} (Fig. 4).

It has been suggested that macrolide antibiotics may act indirectly during *T. gondii* infection through the stimulation of the immunological response of the host (11). However, azithromycin has demonstrated a very high activity against the parasite in vitro. This indicates that azithromycin may also act by direct interference with essential physiological processes of the parasite. Inhibition of protein synthesis by azithromycin was observed in both *T. gondii*-infected macrophages and free tachyzoites. Azithromycin is known to accumulate inside various types of cells, including macrophages, polymorphonuclear cells, and fibroblasts, at very high levels (6, 7). We have recently shown that azithromycin concentrated inside *T. gondii*-infected mouse macrophages and reached levels as high as 2 mg/ml. Concentrations of azithromycin inside infected cells are well above those needed to provoke the inhibition of the protein synthesis of the parasite (1).

Interference with RNA synthesis may be a consequence emerging from the inhibition of protein synthesis (Fig. 2). However, we must keep in mind that interference with both protein and RNA synthesis may ultimately play a role in the killing of the parasite. In tissue cultures, eradication of *T. gondii* could be achieved only by using high concentrations of azithromycin for a long period (4). Our results indicate that such treatment would be inhibitory to both physiological processes.

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