Comparison of Mutants of *Toxoplasma gondii* Selected for Resistance to Azithromycin, Spiramycin, or Clindamycin

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Azithromycin and spiramycin markedly inhibited the growth of *Toxoplasma gondii* in cultured human fibroblasts. However, 3 days of treatment were required to reveal their full antitoxoplasma activity. This delayed onset of inhibition was similar to that previously reported for clindamycin. Mutants of *T. gondii* resistant to azithromycin (Azi^R-1) and spiramycin (Spr^R-1) were isolated and compared with a previously described mutant resistant to clindamycin (Cln^R-2). Mutant Cln^R-2 was cross-resistant to all three antibiotics, while Azi^R-1 was cross-resistant only to spiramycin and Spr^R-1 was cross-resistant only to azithromycin. In short-term studies of protein synthesis by freshly prepared extracellular parasites, clindamycin and azithromycin were effective only at concentrations much greater than their 50% inhibitory concentrations in infected cultures and the resistant mutants did not differ from the wild type in antibiotic sensitivity. Thus, protein synthesis on cytoplasmic ribosomes of the parasite did not seem to be the target of these antibiotics. To determine whether mitochondrial protein synthesis in *T. gondii* was inhibited by clindamycin or azithromycin, wild-type parasites were grown in cultured cells in the presence of antibiotic concentrations well above the 50% inhibitory concentrations. Mitochondrial function, measured by oxygen uptake per purified extracellular parasite, did not decrease substantially, after the parasites had multiplied 11-fold in the presence of antibiotic. Thus, mitochondrial protein synthesis did not seem to be the target of clindamycin or azithromycin. An alternative target is protein synthesis in the putative apicomplexan organelle that has a 35-kb genome.

Toxoplasmic encephalitis is common among AIDS patients. The standard treatment of this opportunistic infection with pyrimethamine and sulfadiazine is generally effective. However, since many AIDS patients cannot tolerate prolonged administration of these drugs (11), alternative treatments are needed. Among the antibiotics known to be active against *Toxoplasma gondii* are the lincosamide clindamycin and the macrolides spiramycin and azithromycin. Clindamycin reduces mortality in infected mice (1, 12, 18) and, in combination with pyrimethamine, is as effective as pyrimethamine-sulfadiazine in the treatment of toxoplasmic encephalitis in AIDS patients (7, 14). Spiramycin is active against murine toxoplasmosis (2) and is thought to reduce the incidence and severity of congenital disease when used to treat women who acquired toxoplasmosis during pregnancy (17). Azithromycin reduces mortality in experimental murine toxoplasmosis (2). The mechanism of action of these antibiotics against *T. gondii* is unknown. An understanding of how they exert their antiparasitic activity might allow the design of more potent derivatives. One way to approach the mechanism of action is to characterize antibiotic-resistant mutants. We have isolated a mutant of *T. gondii* that is markedly resistant to clindamycin (21) and describe here mutants selected for resistance to azithromycin or spiramycin.

MATERIALS AND METHODS

All experiments were carried out with confluent monolayer cultures of human fibroblasts that were maintained as previously described (22). Cultures were infected in medium that contained 1% fetal bovine serum. The wild-type parasites were our cloned line (22) of the RH strain. The isolation of the clindamycin-resistant mutant Cln^R-2 has been re-

ported (21). The isolation of mutants resistant to azithromycin (Azi^R-1) and spiramycin (Spr^R-1) is described in this report. All parasites were maintained by subculture in human fibroblasts and were stored frozen in liquid N₂ (22). Freshly released extracellular parasites were prepared by disrupting infected cells by forced passage through a 25-gauge needle. The infectivity of extracellular parasites was measured by a previously described plaque assay (22), and the results were recorded in PFU. The parasites were cloned by microscopic identification of 0.16-cm² wells that contained a single plaque 6 days after infection with a suitably diluted suspension of extracellular parasites. Actively growing intracellular parasites were mutagenized by a 4-h treatment with 140 µg of ethylnitrosourea per ml, a lower concentration than the 180 µg/ml employed in previous studies (21). Recent experience and theoretical considerations agree that this lower mutagen concentration yields more mutants because it allows much greater survival (about 30%) than the higher concentration (about 7%). This factor outweighs the small increase in the fraction of survivors that have a particular mutation, which is roughly a linear function of mutagen concentration (24). The growth of *T. gondii* was measured by the incorporation of the parasite-specific precursor [³H]uracil (23) into infected monolayers in 2-cm² wells. Labeling with radioactive uracil has been shown to be a reliable linear function of the number of parasites present (19). Antibiotics to be tested for antitoxoplasma activity were added at the time of infection. When the growth of parasites was to be measured 1 day after infection, the inoculum was 10⁵ parasites. For measurement of growth 2, 3, and 4 days after infection 1.5 × 10⁴, 5 × 10², and 2 × 10² parasites, respectively, were used per well. The well cultures were labeled by the addition of 0.5 µCi of [³H]uracil, and acid-precipitable radioactivity was measured 4 h later as previously described (20).

Oxygen consumption was measured polarographically at

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FIG. 2. Inhibition of wild-type *T. gondii* (closed symbols) and mutant Azi^R-1 (open symbols) by azithromycin in triplicate experiments. Growth was measured by the incorporation of [³H]uracil during a 4-h pulse 3 days after infection. Each point is the average of results from triplicate wells. Incorporation of [³H]uracil by infected cultures without antibiotic was used as the 100% value.

In some bacteria, resistance to macrolides is inducible by subinhibitory concentrations (reviewed in reference 34). Since our mutants were selected and routinely maintained in the continuous presence of antibiotic, this mechanism could explain our results. To test for inducible resistance, wild-type parasites were grown for 7 days in the presence of a barely subinhibitory concentration of azithromycin (4 ng/ml). The antibiotic sensitivity of this pretreated parasite population was then compared with that of other wild-type parasites that had never been exposed to the antibiotic. No difference was seen when growth was measured 3 days after challenge with various higher concentrations of azithromycin (data not shown). Thus, we found no evidence of inducible resistance. Consistent with this conclusion, the IC₅₀s for azithromycin and spiramycin were unchanged after Azi^R-1 and Spr^R-1 were subcultured for 30 days in the absence of antibiotic.

The pattern of cross-resistance among these mutants and our previously described clindamycin-resistant mutant,

FIG. 3. Inhibition of wild-type *T. gondii* (closed symbols) and mutant Spr^R-1 (open symbols) by spiramycin in triplicate experiments. Growth was measured by the incorporation of [³H]uracil during a 4-h pulse 3 days after infection. Each point is the average of results from triplicate wells. Incorporation of [³H]uracil by infected cultures without antibiotic was used as the 100% value.

TABLE 1. Cross-resistance among mutants selected for resistance to clindamycin (Cln^R-2), azithromycin (Azi^R-1), or spiramycin (Spr^R-1)

Parasite	IC ₅₀ (ng/ml) ^a		
	Clindamycin	Azithromycin	Spiramycin
Wild type	0.9	10	12
Cln ^R -2	120	1,200	110
Azi ^R -1	1.3	400	700
Spr ^R -1	1.6	500	700

^a Measured 3 days after infection and antibiotic treatment. Each value is the average of two or three independent determinations, with growth at each antibiotic concentration measured in triplicate. The IC₅₀s for Azi^R-1 and Spr^R-1 are not identical to those shown in Fig. 2 and 3 because independent experiments were used to produce the comparative data for this table.

Cln^R-2, was examined by using data from a series of assays in which growth was measured 3 days after antibiotic treatment. Table 1 shows a pattern of partial cross-resistance among these three mutants. Mutant Cln^R-2 was substantially cross-resistant to both azithromycin and spiramycin. Although Spr^R-1 was cross-resistant to azithromycin and Azi^R-1 was cross-resistant to spiramycin, neither mutant was cross-resistant to clindamycin. Azi^R-1 and Spr^R-1 did not differ significantly in sensitivity to any of the antibiotics. Since these two mutants were isolated from the same pool of mutagenized parasites, they may well be identical. For this reason, we did not examine Spr^R-1 further and concentrated on a comparison of Azi^R-1 and Cln^R-2.

In bacteria, the action of macrolides and lincosamides is the result of inhibition of protein synthesis (reviewed in reference 9). If protein synthesis on cytoplasmic ribosomes were the target of these antibiotics in *T. gondii*, some difference might be expected between the mutants and the wild type in the antibiotic sensitivity of protein synthesis. Therefore, the incorporation of [³H]leucine was measured in freshly prepared extracellular parasites as a function of antibiotic concentration. These studies had to be short term because *T. gondii* does not multiply outside of its host cell and rapidly loses its biosynthetic capacity in the extracellular environment. Freshly prepared extracellular parasites were incubated for 1 h with various concentrations of antibiotic. [³H]leucine was then added, and incorporation into protein was measured 1 h later. Figure 4 shows that the antibiotics inhibited protein synthesis by extracellular wild-type parasites only at concentrations that were much greater than the IC₅₀s for growth in infected cultures measured 3 days after treatment (5,000-fold higher for azithromycin and more than 10⁵-fold higher for clindamycin at the IC₅₀s shown in Table 1). Moreover, the antibiotic sensitivities of protein synthesis by extracellular mutant and wild-type parasites could not reproduce the prolonged exposure needed to disclose the antibiotic sensitivity of actively growing intracellular parasites, they provide no evidence that protein synthesis on cytoplasmic ribosomes is the target of clindamycin or azithromycin.

Cytoplasmic ribosomes are not the only site of protein synthesis in *T. gondii*. The parasite has morphologically identifiable mitochondria (10). Since mitochondria synthesize a limited array of proteins on their own ribosomes (reviewed in reference 32), their protein synthesis is a potential target for the action of clindamycin and azithromycin. The long interval between the antibiotic treatment and the cessation of parasite growth allowed the design of an

FIG. 4. The effect of clindamycin and azithromycin on the incorporation of [^3H]leucine by extracellular wild-type and antibiotic-resistant *T. gondii*. Freshly prepared extracellular parasites were incubated at 37°C in medium with various concentrations of antibiotic for 1 h and then were labeled for an additional 1 h with [^3H]leucine. The data are the averages of three independent experiments, with duplicate determinations within each experiment. $\square$, wild type treated with clindamycin; $\circ$, Cln^R-2 treated with clindamycin; $\blacksquare$, wild type treated with azithromycin; $\bullet$, Azi^R-1 treated with azithromycin.

experiment to detect the inhibition of mitochondrial protein synthesis. We reasoned that if mitochondrial protein synthesis were immediately inhibited by clindamycin or azithromycin, mitochondrial activity on a per-parasite basis would decrease as the parasites multiplied in the presence of the antibiotic. Mutant and wild-type parasites were grown for 22 h in normal medium or in medium that contained clindamycin (18 ng/ml) or azithromycin (300 ng/ml), concentrations chosen to be at least 20 times the IC_{50} s. The extent of

parasite growth in the antibiotic-treated cultures was calculated by comparing the number of PFU in the initial inoculum and the number of progeny parasites released by lysing the infected cells 22 h later. The progeny parasites were enumerated microscopically because, at least in the case of clindamycin (21), some were not viable. In accord with the results shown in Fig. 2 for azithromycin and previous observations with clindamycin (21), the antibiotic-treated *T. gondii* multiplied extensively. Although we did not measure the growth of the control cultures precisely, the yield of parasites was similar to those of the antibiotic-treated cultures. In various experiments, the parasites multiplied 9- to 13-fold in the presence of antibiotic and thus had doubling times similar to those reported for growth in normal medium (21). The freshly released parasites were rapidly purified, and mitochondrial activity was measured by polarographic determination of oxygen uptake and calculated on a per-parasite basis. If the antibiotic treatment immediately blocked mitochondrial protein synthesis and allowed no further increase in mitochondrial activity, the 9- to 13-fold increase in parasite number should reduce the per-parasite oxygen uptake to 11 to 8% of the level seen of the control parasites. As shown in Table 2, this prediction was not fulfilled. The parasites grown in the presence of clindamycin had an oxygen uptake that averaged 72% of the control value, while those grown in the presence of azithromycin averaged 109% of the control. These data suggest that mitochondrial protein synthesis is not the primary target of clindamycin or azithromycin.

DISCUSSION

Azithromycin and spiramycin resemble clindamycin (21) in their delayed action against *T. gondii* in cultured cells. With all three antibiotics, little inhibition was seen until 3 days after treatment. This delay accounts for the roughly

TABLE 2. The effect of growth in the presence of clindamycin or azithromycin on oxygen uptake by *T. gondii*

Antibiotic and expt no.	Growth conditions ^a	Fold increase in parasites ^b	Oxygen uptake ^c	
			μmol/min/10 ⁹ parasites	% of control
Clindamycin				
1	Clindamycin	9.0	0.070	71
	Control	ND ^d	0.098	
2	Clindamycin	10.4	0.066	64
	Control	ND	0.104	
3	Clindamycin	13.8	0.007	81
	Control	ND	0.107	
Mean ± SD	Clindamycin	11.1 ± 2.5	0.074 ± 0.011	72 ± 9
	Control	ND	0.103 ± 0.005	
Azithromycin				
1	Azithromycin	12.9	0.123	94
	Control	ND	0.131	
2	Azithromycin	9.6	0.097	77
	Control	ND	0.126	
3	Azithromycin	11.3	0.179	156
	Control	ND	0.115	
Mean ± SD	Azithromycin	11.3 ± 1.7	0.133 ± 0.042	109 ± 44
	Control	ND	0.124 ± 0.008	

^a Clindamycin, 18 ng/ml; azithromycin, 300 ng/ml.

^b Calculated from the ratio of the yield of parasites 22 h after infection (measured by hemacytometer counts) to the number of infectious parasites in the inoculum (measured by plaque assay).

^c Measured in parasites purified from infected cells after 22 h of growth. All values are the means of triplicate determinations except for the first clindamycin experiment, which had duplicates.

^d ND, not done.

1,000-fold greater IC_{50} s previously reported for azithromycin (8 μ g/ml) and spiramycin (20 μ g/ml) in 2-day experiments (6). The observation that mutants selected for resistance to clindamycin, azithromycin, or spiramycin show a pattern of partial or complete cross-resistance (Table 1) suggests that these antibiotics share a common target in *T. gondii*. This conclusion is consistent with the markedly delayed onset of antiparasitic activity seen with all three antibiotics.

The antibacterial activity of the macrolides and lincosamides is known to result from their ability to inhibit protein synthesis by binding to the transpeptidation site of the larger ribosomal subunit (reviewed in reference 9). Thus, it is logical that these antibiotics should also affect *T. gondii* by inhibiting protein synthesis. We measured amino acid incorporation by extracellular parasites to test the hypothesis that protein synthesis on cytoplasmic ribosomes was the target of clindamycin and azithromycin. Freshly prepared extracellular parasites do not provide a perfect model for events that take place during exponential intracellular growth. Nonetheless, the incorporation of [3 H]leucine by extracellular parasites should occur through the same biochemical mechanisms used in intracellular growth and should reflect the antibiotic sensitivity of cytoplasmic ribosomes. We found that protein synthesis by extracellular parasites was inhibited only at antibiotic concentrations that were markedly greater than the IC_{50} s for intracellular growth measured 3 or more days after treatment. Similar inhibition has been reported for azithromycin-treated extracellular *T. gondii* (3). These observations might be significant for the antitoxoplasma effect of azithromycin and clindamycin if the uptake of antibiotics by the host cell or by intracellular parasites exposed the protein-synthetic machinery of *T. gondii* to elevated concentrations. However, concentration of these antibiotics from the extracellular medium by mammalian cells is not relevant, because the high intracellular concentrations of antibiotic are found in membrane-bound acidified intracellular compartments such as lysosomes (5, 16). The mechanism by which the antibiotics are concentrated is the familiar equilibrium distribution of a weak base between acidified and neutral compartments separated by a membrane that is permeable only to the un-ionized form of the weak base. Because the parasitophorous vacuole in which *T. gondii* grows is not acidified (28), the intracellular parasites are not exposed to the high antibiotic concentrations found only in acidified compartments of its host cell. *T. gondii*, itself, also concentrates azithromycin by a mechanism that is similar to that of mammalian cells. The elevated concentrations of antibiotic are found in an acidified compartment (13) and are not significant for protein synthesis taking place on cytoplasmic ribosomes of *T. gondii*. Thus, we do not consider the inhibition of protein synthesis in extracellular parasites by high antibiotic concentrations to be relevant to the antitoxoplasma activity of clindamycin or azithromycin. This conclusion is strengthened by the finding that antibiotic-resistant mutants did not differ from the wild type when protein synthesis by extracellular parasites was measured in the presence of clindamycin or azithromycin (Fig. 4). The observation that these antibiotics inhibit the growth of *T. gondii* only after a 2-day delay also argues against an effect on cytoplasmic protein synthesis in *T. gondii*. The parasites must synthesize protein at a nearly normal rate, as they multiply in the presence of high concentrations of antibiotics during the first 2 days of treatment (Fig. 1). We conclude that protein synthesis on the cytoplasmic ribosomes of *T. gondii* is unlikely to be the target of clindamycin or azithromycin.

Mitochondria are thought to have originated from bacteria

(26). Thus, protein synthesis in these organelles is a reasonable alternative target for antibiotics. We chose an indirect method to assess the effect of clindamycin and azithromycin on mitochondrial protein synthesis. When mitochondrial protein synthesis is inhibited, those proteins universally encoded in the mitochondrial genome, such as cytochrome *b* and certain components of cytochrome oxidase (32), can no longer be made. Since these proteins play essential roles in oxidative phosphorylation, mitochondrial function should not increase when parasites multiply in the presence of inhibitors of mitochondrial protein synthesis. We treated intracellular parasites with 20 times the IC_{50} of clindamycin or 30 times the IC_{50} of azithromycin. Consistent with previous experiments, the antibiotic-treated parasites grew as rapidly as the controls; yielding on average an 11-fold increase in 22 h. Significantly, mitochondrial function in the antibiotic-grown parasites, as measured by oxygen uptake on a per-parasite basis, was 72 to 109% of the control value (Table 2). Thus, total mitochondrial function must have increased roughly 8- to 11-fold in the presence of highly inhibitory concentrations of clindamycin and azithromycin. This increase argues against an immediate inhibition of mitochondrial protein synthesis. We realize that this conclusion is based on circumstantial evidence. Most mitochondrial proteins are encoded in the nuclear genome and are imported by the mitochondrion after they are synthesized (32). If all of the proteins specifically encoded in the mitochondrial genome of *T. gondii* were present in an 11-fold or greater excess, mitochondrial function on a per-parasite basis could remain constant while the parasites multiplied in the presence of antibiotic concentrations that inhibit further mitochondrial protein synthesis. Alternatively, the antibiotics could uncouple oxidative phosphorylation to just the proper extent to mask the effects of inhibiting mitochondrial protein synthesis. However, these alternative explanations seem less likely than the simple conclusion that mitochondrial protein synthesis is not the primary target of clindamycin or azithromycin in *T. gondii*.

Fortunately for the theory that clindamycin and azithromycin act by inhibiting protein synthesis somewhere in the parasite, *T. gondii* and other apicomplexans have a second extrachromosomal genome. Most of what is known about this 35-kb circular genome has come from studies of *Plasmodium falciparum* (reviewed in reference 35). Certain features of the *P. falciparum* 35-kb genome suggest that it may be related to the genomes of plastids. Like the mitochondrial genome, the 35-kb genome encodes rRNAs and thus probably has its own ribosomes. The 35-kb circular extrachromosomal genome of *T. gondii* has been less well studied than that of *P. falciparum*. However, this genome has been purified from *T. gondii* and identified by electron microscopy (4) and rRNA genes have been identified in it (25). If the 35-kb genome of *T. gondii* resides in an independent cytoplasmic organelle, that organelle probably carries out its own protein synthesis on ribosomes that contain the large and small RNAs encoded by its genome. Since plastids are thought to have their origin in cyanobacteria (26), their protein synthesis could be sensitive to antibiotics. Our current hypothesis is that clindamycin and azithromycin inhibit *T. gondii* by blocking protein synthesis in the putative organelle of apicomplexans that contains the 35-kb genome. In the context of this hypothesis, it may be significant that clindamycin has been reported to be useful in treating infection with two other apicomplexans, *P. falciparum* (8) and *Babesia microti* (37).

Resistance to macrolides and lincosamides is common in

bacteria. At least three mechanisms, all of which result in reduced binding of the antibiotic at the transpeptidation site of the large ribosomal subunit, are known. The first is inducible resistance of the MLS_B type that confers resistance to macrolides, lincosamides, and streptogramin B (reviewed in reference 34). Exposure of certain bacteria to subinhibitory concentrations of a macrolide induces the activity of an RNA methylase that dimethylates an adenine at the transpeptidation site of the larger rRNA. In *Escherichia coli*, this adenine is nucleotide 2058 of the 23S rRNA (30). We attempted to induce resistance to azithromycin by prolonged subculture in subinhibitory concentrations. Although we failed to demonstrate inducible resistance, an RNA methylation mechanism could explain the observed antibiotic resistance if the appropriate methylase were made constitutively in our mutants.

The second mechanism of resistance involves mutations that alter ribosomal proteins that play a role at the transpeptidation site. Two such proteins, L4 and L22, have been identified in *E. coli* mutants resistant to erythromycin (36). If resistance to clindamycin and azithromycin in *T. gondii* involves mutation of a nuclear gene encoding a ribosomal protein that is imported into the putative plastid-like organelle, it may be possible to locate and identify such a resistance gene by using recently described genetic mapping methods (27).

The third bacterial mechanism for resistance to lincosamides and macrolides is mutation of the large rRNA gene in the sequence that encodes the transpeptidation site. Several such mutations have been well characterized in *E. coli* strains that contain plasmids bearing rRNA genes. A mutation that changes nucleotide 2058 of the 23S RNA from adenine to uracil (29) produces an *E. coli* phenotype of resistance to macrolides and lincosamides that resembles that of Cln^R-2 . A mutation that changes nucleotide 2611 from a cytosine to a uracil results in an *E. coli* phenotype of resistance to macrolides but not lincosamides (33) that resembles that of Azi^R-1 . The suggestion that resistance to lincosamides and macrolides in *T. gondii* is encoded in the rRNA genes of the 35-kb genome has the advantage of being testable by comparing wild-type and mutant sequences near the transpeptidation site.

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