ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1992, p. 1091–1096
0066-4804/92/051091-06$02.00/0

Vol. 36, No. 5

Parasiticidal Effect of Clindamycin on *Toxoplasma gondii* Grown in Cultured Cells and Selection of a Drug-Resistant Mutant

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Received 19 November 1991/Accepted 3 March 1992

Clindamycin, which has been reported to have no significant in vitro activity against *Toxoplasma gondii*, actually markedly inhibits the growth of this parasite in infected human fibroblasts. When measured 3 days after treatment, the concentration required to reduce parasite growth by 50% is about 1 ng/ml. Some observers failed to note this inhibition because of its markedly delayed onset. At 6 ng/ml, clindamycin is parasiticidal, and the rate and extent of parasite killing increase with higher drug concentrations. With the aid of chemical mutagenesis, we isolated a parasite mutant that is approximately 100-fold more resistant to clindamycin than is the wild type. Lincomycin inhibits *T. gondii* at a higher 50% inhibitory concentration, about 100 ng/ml. The clindamycin-resistant mutant is partially cross-resistant to lincomycin.

While Derouin et al. (3) observed that clindamycin concentrations as low as 0.5 ng/ml inhibited the growth of *Toxoplasma gondii*, at least three other laboratories have reported that the drug has no in vitro activity (5, 6, 8). These latter observations are in marked contrast both to the well-established efficacy of clindamycin phosphate against toxoplasmosis in infected mice (1, 6) and to the preliminary evidence for the successful use of clindamycin phosphate to treat toxoplasmic encephalitis in AIDS patients (2). We show here that clindamycin is actually a potent parasiticidal agent in cultured cells infected with *T. gondii*, with a 50% inhibitory concentration (IC_{50}) that is among the lowest reported for any antitoxoplasma drug. This activity previously escaped detection by several laboratories because the drug has little effect on parasite multiplication until many cell divisions have occurred.

MATERIALS AND METHODS

The host cells for all experiments were confluent cultures of human fibroblasts that were maintained as previously described (13), except that the medium for growth contained 10% calf serum and that for infection contained 1% fetal bovine serum. The parasites were our cloned line (13) of the RH strain or mutants derived from this clone. The parasites were maintained by serial subculturing in human fibroblasts. Infected cells were disrupted by forced extrusion through a 25-gauge needle to release intracellular parasites. The newly released parasites were assayed by a plaque procedure (13). In all final experiments, this procedure was carried out with 25-cm^2 flasks and the plaques were counted without staining. For preliminary plaque assays with multiwell trays, the monolayer was first fixed with 0.3 N trichloroacetic acid and then stained with Coomassie blue. Parasites were cloned by the detection of a single plaque 6 days after infection of 0.15-cm^2 wells with suitably diluted suspensions of extracellular *T. gondii*. Exponentially growing intracellular parasites were mutagenized with ethylnitrosourea as previously described (12). The growth of *T. gondii* was measured in 2-cm^2 wells by pulse-labeling with 1.0 μCi of [^{3}H]uracil per ml for 4 h. Incorporation into acid-precipitable material was measured as previously described (12). This incorporation is highly specific for the parasite because the host cell lacks the required salvage enzyme, uracil phosphoribosyltransferase (11). Labeling with radioactive uracil has been shown to be a reliable linear function of the number of parasites present (9). When the growth of the wild-type parasite was to be measured 1 day after infection, the inoculum was 10^5 parasites per well. For measurement of growth 2, 3, and 5 days after infection, 1.5×10^4, 5×10^2, and 1.5×10^2 parasites, respectively, were used per well. To measure the growth of the clindamycin-resistant mutant by using [^{3}H]uracil incorporation 3 days after infection, we used a larger inoculum, 1.5×10^3 parasites per well, because the mutant grew more slowly than the wild type. Parasites were washed free of the drug by centrifugation at $750 \times g$ for 10 min.

[5,6-^{3}H]uracil (20 Ci/mmol) was purchased from Moravek Biochemicals. All other materials were purchased from Sigma Chemical Co. Unless otherwise specified, clindamycin was used in the hydrochloride form.

RESULTS

Phase-contrast microscopic observations of human fibroblasts infected with *T. gondii* in the presence of various concentrations of clindamycin up to 100 μg/ml revealed no significant inhibition of parasite multiplication 1 day later. This lack of effect during the first 30 h of treatment was quantitated by use of the incorporation of [^{3}H]uracil, a parasite-specific precursor (11). As shown in Fig. 1, only slight inhibition of parasite growth was seen at 25 or 100 μg of the drug per ml. Microscopic observations made during the second day of infection in the presence of clindamycin concentrations up to 25 μg/ml revealed that the initially infected cells had released their intracellular parasites and that these progeny parasites had infected and begun to multiply in adjacent cells. However, at some time during the progression of these secondary infections, further growth of intracellular *T. gondii* slowed and then ceased. Thus, the amount of inhibition observed appeared to be a function of the time of observation. This conclusion was documented by measuring parasite growth at various intervals after treatment with clindamycin. Growth at the end of 1, 2, 3, and 5 days of incubation was measured by use of the incorporation of [^{3}H]uracil during a 4-h pulse. As shown in Fig. 2, the apparent antitoxoplasma effect of clindamycin was markedly

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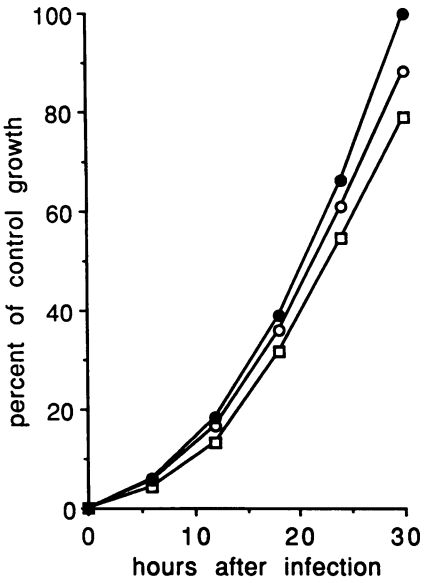

FIG. 1. Effect of various concentrations of clindamycin on the incorporation of [^{3}H]uracil into human fibroblast cultures infected with *T. gondii*. Confluent monolayers in 2-cm^2 wells were infected with about 0.4 parasite per cell and incubated at 37°C in the presence of clindamycin at 0 (●), 25 (○), and 100 (□) μg/ml. Quadruplicate wells at each drug concentration were labeled with [^{3}H]uracil (1.0 μCi/ml) for successive 6-h intervals starting 1 h after infection. The incorporations at successive intervals were summed to provide a kinetic picture of parasite growth. Total incorporation by cultures without the drug was used as the 100% value.

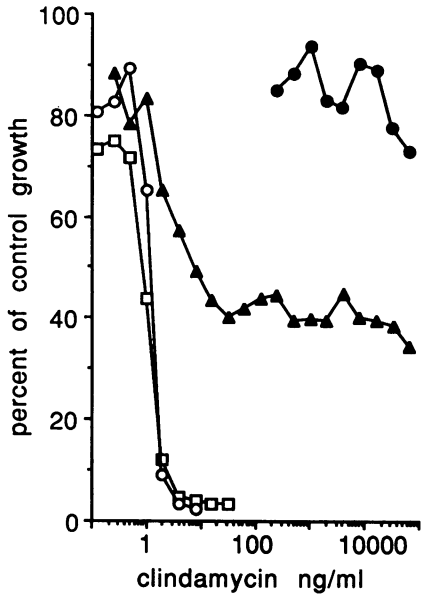

FIG. 2. Effects of various concentrations of clindamycin on the growth of *T. gondii* measured 1 (●), 2 (▲), 3 (□), and 5 (○) days after treatment. The drug was added at the time of infection. Each point is the average of two independent experiments each based on [^{3}H]uracil incorporation by quadruplicate wells. The 100% values were determined by the labeling of infected cultures incubated without the drug.

dependent on the duration of treatment. The drug had little effect at concentrations as high as 64 μg/ml during the first day. After 2 days of treatment, a concentration of clindamycin as low as 2 ng/ml resulted in some inhibition of parasite growth. However, even drug concentrations as high as 64 μg/ml did not reduce [^{3}H]uracil incorporation to near zero. Instead, clindamycin concentrations over a broad range resulted in a plateau that was roughly 40% the control value. The significance of this plateau will be considered in the Discussion. At the end of 3 days, the IC_{50} was about 1 ng/ml and inhibition was nearly complete. When growth in the presence of clindamycin was measured by use of the incorporation of [^{3}H]uracil 5 days after treatment, the inhibition curve was identical to that seen after 3 days, again with an IC_{50} of about 1 ng/ml. Consistent with these observations, clindamycin at 1 ng/ml reduced the plaque size, measured 7 days after treatment, to roughly half the control size, while 2 ng/ml completely blocked the formation of plaques. Clindamycin phosphate was also active against *T. gondii*, with an IC_{50} of about 1 ng/ml 5 days after treatment (data not shown).

We considered the possibility that the antitoxoplasma effect of clindamycin was mediated by toxicity for the host cell. Uninfected cultures were treated with medium that contained 10 μg of clindamycin per ml, 10,000 times the IC_{50}. One day later, the drug was removed by 10 washes over a 3-h period and then the cultures were infected in fresh medium. The parasites grew as well in the treated and washed cultures as they did in the untreated and washed control cultures (data not shown). Thus, we found no evidence of an irreversible toxic effect on the host cell. Consistent with this conclusion, 10 μg of clindamycin per ml had no cytotoxic effect on the host cell that could be detected by phase-contrast microscopy.

We used three different plaque assay protocols to determine whether clindamycin was parasiticidal or parasitostatic. In our first test for the parasiticidal effect of clindamycin, we incubated freshly prepared extracellular *T. gondii*, at 10^7 parasites per ml, in medium that contained 1% fetal bovine serum and 20 μg of clindamycin per ml or no drug. After 4 h, the drug was removed by centrifugation and by the dilutions required to carry out a plaque assay. In triplicate experiments, the drug-free control showed 61% ± 6% survival of the parasites, compared with the initial titer. In the clindamycin-treated samples, 58% ± 6% of the parasites survived. The difference between the drug-treated and control samples was not significant. Longer treatments were not studied because of the complication of spontaneous loss of viability in the untreated controls. The lack of effect of clindamycin on extracellular parasites at a concentration 20,000 times the IC_{50} could reflect the slow onset of parasite inhibition noted above. Therefore, we studied the parasiticidal effect of clindamycin on intracellular parasites, because such experiments could be carried out over much longer periods of time.

In our second examination of the parasiticidal activity of clindamycin, we infected monolayer cultures in 25-cm^2 flasks with small numbers of extracellular *T. gondii* in the presence of 0, 6, and 18 ng of clindamycin per ml. One and two days later, the drug was removed from these cultures by rinsing them four times with Hanks' (4) balanced salt solution. These cultures were incubated with fresh drug-free medium for 7 additional days, and the macroscopic plaques were counted. The adequacy of drug removal by the rinsing procedure was demonstrated by the inclusion of mock-infected cultures treated with 18 ng of clindamycin per ml.

TABLE 1. Effect of clindamycin treatment for 24 and 48 h on the ability of microscopic foci of *T. gondii* produced during the treatment to go on to make macroscopic plaques 7 days after removal of the drug

Clindamycin concn (ng/ml)	Expt	24-h treatment		48-h treatment	
		No. of parasites/focus[a]	% Foci able to make plaques after removal of drug[b]	No. of parasites/focus[a]	% Foci able to make plaques after removal of drug[b]
0	1	19	100	330	100
	2	11	100	190	100
	Avg	15	100	260	100
6	1	20	131	136	15
	2	12	100	113	12
	Avg	16	116	125	14
18	1	17	77	100	4
	2	12	77	110	4
	Avg	14	77	105	4

[a] Parasites were counted microscopically after disruption of replicate cultures. The number of foci was measured by a plaque assay of the initial inoculum.
[b] For clindamycin at 0 ng/ml, the percentage was assumed a priori to be 100.

These cultures were rinsed in parallel with the infected cultures. Freshly prepared extracellular parasites added to the rinsed, mock-infected cultures produced plaques of the expected number and size (data not shown). The principle of this experiment is that each infectious parasite in the inoculum will produce a heavily infected cell at the end of 24 h and a small cluster of infected cells at the end of 48 h. We call both the single infected cells and the small clusters of infected cells foci of infection because they give rise to macroscopic plaques in drug-free medium. If a single viable intracellular parasite remains at a focus of infection after 24 or 48 h of clindamycin treatment, the further multiplication of this parasite should produce a macroscopic plaque during the subsequent 7 days of incubation in drug-free medium. The clindamycin-induced reduction in the number of foci that contained at least one viable parasite is shown in Table 1. The initial number of foci was measured by a plaque assay of the inoculum done in the absence of drug. Nearly all of the foci contained at least one viable parasite at the end of 24 h of treatment with clindamycin, as evidenced by the fact that they went on to produce plaques after the drug was removed. However, at the end of 48 h of treatment with 6 and 18 ng of clindamycin per ml, only 14 and 4%, respectively, of the foci were able to progress to macroscopic plaques after the clindamycin was removed.

We noted that *T. gondii* initially multiplied extensively in the presence of clindamycin concentrations that ultimately were highly inhibitory. We documented the extent of multiplication in the above-described experiment by releasing intracellular parasites from replicate cultures treated with 0, 6, or 18 ng of the drug per ml and counting them with the aid of a hemacytometer. Table 1 shows the average number of parasites per focus. As expected, extensive multiplication in the drug-free controls yielded an average of 260 parasites per focus 48 h after infection. This value is consistent with the doubling time of about 6 h that we regularly observe with our cloned RH strain. Remarkably, the cultures treated with 18 ng of clindamycin per ml for 48 h yielded an average of 105 parasites per focus. However, only 4% of these foci were able to make a macroscopic plaque when the clindamycin was removed.

In the second assay for parasiticidal activity described above, it could be argued that the proximity of dead intracellular parasites somehow prevented the still-viable parasites in the microfoci from going on to make plaques when the clindamycin was removed. To examine this possibility, we infected replicate cultures with extracellular *T. gondii* in the presence of various concentrations of clindamycin. At 1 and 2 days after infection, the intracellular *T. gondii* organisms were released from infected cells, pooled with parasites present in the extracellular medium, and washed free of the drug by repeated centrifugation. The washed parasites were diluted for a plaque assay. The adequacy of this washing was demonstrated by including mock-infected cultures treated with the highest concentration of clindamycin. These cultures were processed in parallel with the infected ones, and the mock-infected material was added to fresh cultures as in a plaque assay. Freshly prepared extracellular parasites added to these cultures produced plaques of the expected number and size (data not shown), proving that the clindamycin had been removed. The results of this experiment revealed a substantial parasiticidal effect at all concentrations tested (Fig. 3). The number of viable *T. gondii* remained constant during the first day of drug treatment but decreased markedly during the second day. The interpretation of these killing curves will be considered in the Discussion.

Our observation that clindamycin had a delayed lethal effect on *T. gondii* allowed us to select a resistant mutant from a mutagenized population of parasites. Two cultures, each containing about 4×10^7 actively growing intracellular parasites, were treated for 4 h at 37°C with ethylnitrosourea at 140 and 180 μg/ml. Plaque assays of viable parasites in these two cultures and in an untreated control culture revealed 31 and 10% survivors, respectively. The parasites in the mutagenized cultures were subcultured daily for 3 days to allow phenotypic expression of any induced mutations. The two cultures of mutagenized parasites were then pooled, and 2×10^7 parasites were allowed to infect approximately 10^7 cells in medium that contained 25 ng of clindamycin per ml. The parasites were subcultured daily in the presence of the same concentration of drug until actively growing parasites emerged 6 days later. These parasites were cloned in the presence of 25 ng of clindamycin per ml, and two clones were frozen in liquid N_2. All subsequent experiments were done with one of these clones, named Cln^R-2 (RH) in accordance with the recently proposed uniform genetic nomenclature for *T. gondii* (14). We routinely maintained this clone in medium that contained 10 ng of clindamycin per ml. However, the mutation that con-

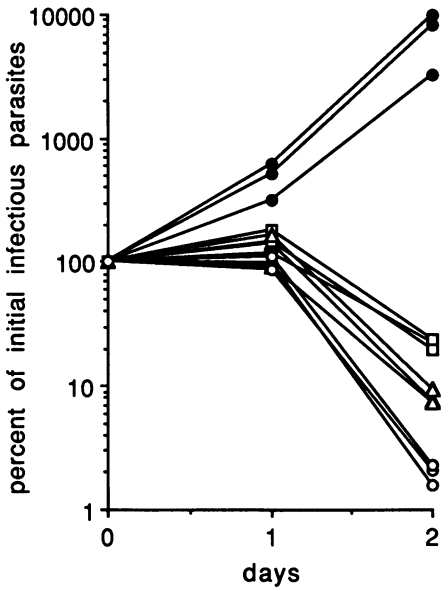

FIG. 3. Parasiticidal effect of clindamycin at 0 (●), 6 (□), 18 (△), and 54 (○) ng/ml on intracellular *T. gondii*. Replicate cultures in 25-cm^2 flasks were infected with 5×10^4 parasites. After 1 or 2 days, infected cultures were disrupted and the extracellular parasites were washed free of the drug by centrifugation. Viable parasites were measured by a plaque assay. The zero-time 100% value was determined by a plaque assay of the initial inoculum.

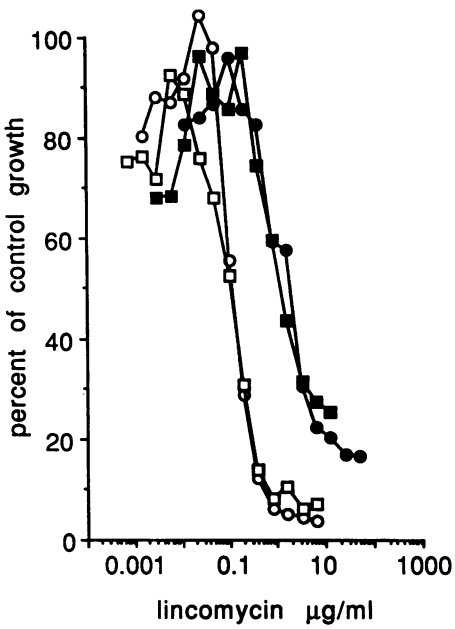

FIG. 5. Inhibition of the growth of the wild type (open symbols) and Cln^R-2 (closed symbols) by lincomycin in independent duplicate experiments. Growth was measured by use of the incorporation of [^{3}H]uracil during a 4-h pulse 3 days after infection. Each point is the average of results from quadruplicate wells. Incorporation by infected cultures without the drug was used as the 100% value.

ferred drug resistance appeared to be stable, since growth in the absence of the drug for 4 weeks had no effect on the degree of resistance (data not shown). The extent to which Cln^R-2 was resistant is shown in Fig. 4, which depicts [^{3}H]uracil incorporation 3 days after drug treatment. The mutant was approximately 100-fold more resistant to clindamycin than was the wild type.

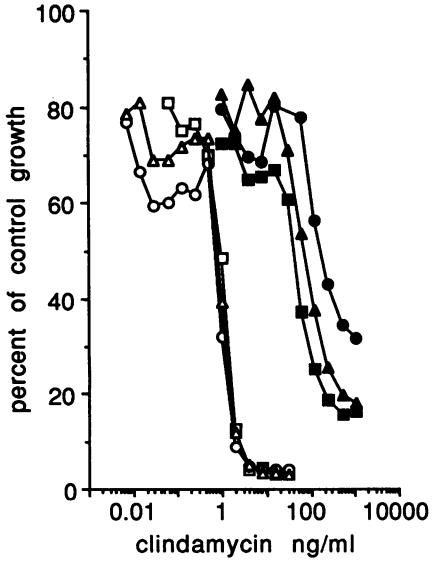

FIG. 4. Inhibition of the growth of the wild type (open symbols) and Cln^R-2 (closed symbols) by clindamycin in independent triplicate experiments. Growth was measured by use of the incorporation of [^{3}H]uracil during a 4-h pulse 3 days after infection. Each point is the average of results from quadruplicate wells. Incorporation by infected cultures without the drug was used as the 100% value.

Clindamycin is a chlorine-containing analog of lincomycin. Thus, it was of interest to examine the in vitro antitoxoplasma activity of lincomycin. As shown in Fig. 5, lincomycin was 100-fold less active than clindamycin, although in terms of absolute concentrations, its IC_{50} of 100 ng/ml was reasonably low. Mutant Cln^R-2 was partially cross-resistant to lincomycin; the IC_{50} for this mutant was 10-fold higher than that for the wild type.

DISCUSSION

In accordance with the previous observations of Derouin et al. (3), our results show the potent in vitro activity of clindamycin against *T. gondii*. The IC_{50} for this parasite, about 1 ng/ml, is in the range of the lowest MICs reported for various bacteria (reviewed by Steigbigel [15]). Other antitoxoplasma drugs with in vitro IC_{50}s in the low-nanogram range include monensin, with an IC_{50} of 0.5 ng/ml (10), and lasalocid (10) and arprinocid-*N*-oxide (12), both with an IC_{50} of approximately 20 ng/ml. The action of clindamycin is markedly delayed. A concentration 64,000-fold higher than the IC_{50} had no substantial effect on parasite multiplication during the first day of infection (Fig. 1 and 2). This delayed onset of action is probably the reason why other investigators reported no in vitro activity of clindamycin against *T. gondii* at 25 μg/ml (8) or 100 μg/ml (5). The supposed lack of in vitro antitoxoplasma activity of clindamycin phosphate led to the suggestion that this form of the drug, which is effective in vivo (1), might not be hydrolyzed to clindamycin in vitro (6). This hypothesis is now invalid, since we observed that both clindamycin and clindamycin phosphate had equal in vitro antitoxoplasma activities. Some component of our infected tissue cultures must provide the enzy-

matic activity that cleaves off the phosphate or, less likely, clindamycin phosphate itself has antitoxoplasma activity.

When parasite growth was measured by use of [^{3}H]uracil incorporation 2 days after treatment, a peculiar inhibition curve was observed (Fig. 2). Quite low concentrations of clindamycin resulted in a partial inhibition (40% that in the control) that remained constant over at least a 2,000-fold range of drug concentrations. The likely explanation for this response is that all inhibitory concentrations of clindamycin allow substantial parasite growth during the first 2 days of treatment and that the resulting progeny parasites are capable of [^{3}H]uracil incorporation. Table 1 shows that parasite growth was 40% that in the control after 2 days of incubation with 18 ng of clindamycin per ml. However, as argued below, many of these parasites were dead, as determined by plaque assays. After 3 days of drug treatment, most of the parasites became incapable of [^{3}H]uracil incorporation.

Both of our assays for the long-term effect of clindamycin on intracellular *T. gondii* revealed parasiticidal activity. However, each of these assays underestimated both the magnitude and the rapidity of this activity, because neither took into account the extensive multiplication of *T. gondii* in the presence of the drug that is documented in Table 1. In the experiment in which cultures were treated with 18 ng of clindamycin per ml for 48 h, the foci of infection, which began with a single parasite, contained an average of 105 parasites. When the clindamycin was removed, 96% of these foci did not contain even one infectious *T. gondii* organism. Thus, the 4% survival of plaque-forming foci greatly underestimated the parasiticidal activity of the drug, because all but a few of the roughly 105 parasites within those foci able to go on to form a plaque were probably dead when the drug was removed. When infected cultures treated with 18 ng of clindamycin per ml for 24 h were disrupted and the number of viable parasites was measured in a plaque assay, there was no decrease in the number of infectious parasites present compared with the zero-time number (Fig. 3). However, Table 1 shows that the parasites multiplied about 15-fold during the first day in the presence of this concentration of the drug. Most of these progeny parasites were thus already dead, as determined by a plaque assay 1 day after treatment with clindamycin. Thus, the parasiticidal effect of the drug measured on a per-parasite basis is already substantial after 24 h.

The mechanism of the antibacterial action of clindamycin is well established (reviewed by Steigbigel [15]). The drug binds to the larger (50S) subunit of the bacterial ribosome, blocks the transpeptidation reaction, and causes a rapid breakdown of polysomes. Thus, bacterial protein synthesis is blocked. The drug does not bind to the corresponding (60S) mammalian ribosomal subunit, accounting for the specificity of its action. The antitoxoplasma activity of clindamycin is likely to be based on a similar mechanism. The parasite offers two potential targets for clindamycin binding, the larger subunits of the cytoplasmic ribosomes and of the mitochondrial ribosomes. The inhibition of cytoplasmic protein synthesis by *T. gondii* seems a less likely alternative, since the cytoplasmic ribosomes of the parasite are presumably of the eucaryotic type. Moreover, the markedly delayed onset of the inhibition of parasite growth would not be expected if cytoplasmic protein synthesis were inhibited. The possibility that the cytoplasmic protein synthesis of *T. gondii* is sensitive to clindamycin is open to direct experimental testing.

Mitochondrial protein synthesis seems a more likely target of clindamycin action against *T. gondii*. Since mitochondria are thought to have a procaryotic origin, their ribosomes, in some eucaryotic species, may have retained sensitivity to antibiotics that affect bacterial ribosomes. Such a mitochondrial mechanism has been suggested for the antimalarial activity of tetracyclines (7). The inhibition of mitochondrial protein synthesis could result in markedly delayed antiparasitic activity if the intracellular parasites were not absolutely dependent on oxidative phosphorylation for energy but could also use glycolysis. In this model, the mitochondria of intracellular parasites are assumed to be essential only for the biosynthesis of some essential metabolite, but a sufficient reserve of this metabolite exists to allow substantial multiplication of the parasite before the lack of this metabolite becomes lethal. Alternatively, exceptionally slow penetration of clindamycin into the mitochondria of the intracellular parasites could explain the delayed action of the drug.

The availability of a *T. gondii* mutant resistant to clindamycin could aid in the study of the antiparasitic mechanism. Three modes of bacterial resistance to clindamycin have been described (reviewed by Steigbigel [15]). An altered protein of the 50S ribosomal subunit can confer resistance simultaneously to erythromycin and to clindamycin. Alternatively, an alteration in the methylation pattern of the 23S rRNA associated with the 50S ribosomal subunit can result in resistance to clindamycin. Finally, the drug can be inactivated by adenylation through the action of a nucleotidyltransferase. Since the two latter mechanisms are plasmid mediated, they are probably less likely to be relevant for *T. gondii*. Each of these potential mechanisms is open to experimental testing. A difference between the wild type and Cln^R-2 in a mitochondrial ribosomal protein or in the methylation of mitochondrial rRNA would provide strong circumstantial evidence as to the target of clindamycin in *T. gondii*.

ACKNOWLEDGMENT

This research was supported by grant AI-25187 from the National Institutes of Health.

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