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# In Vitro Assays Elucidate Peculiar Kinetics of Clindamycin Action against *Toxoplasma gondii*

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**In order to characterize the delayed effect of clindamycin and macrolide antibiotics against *Toxoplasma gondii* tachyzoites (E. R. Pfefferkorn and S. E. Borotz, Antimicrob. Agents Chemother. 38:31–37, 1994), we have carefully examined the replication of parasites as a function of time after drug addition. Intracellular tachyzoites treated with up to 20 μM clindamycin (>1,000 times the 50% inhibitory concentration) exhibit doubling times indistinguishable from those of controls (~7 h). Drug-treated parasites emerge from infected cells and establish parasitophorous vacuoles inside new host cells as efficiently as untreated controls, but replication within the second vacuole is dramatically slowed. Growth inhibition in the second vacuole does not require continued presence of drug, but it is dependent solely on the concentration and duration of drug treatment in the first (previous) vacuole. The susceptibility of intracellular parasites to nanomolar concentrations of clindamycin contrasts with that of extracellular tachyzoites, which are completely resistant to treatment, even through several cycles of subsequent intracellular replication. This peculiar phenotype, in which drug effects are observed only in the second infectious cycle, also characterizes azithromycin and chloramphenicol treatment, but not treatment with cycloheximide, tetracycline, or anisomycin. These findings provide new insights into the mode of clindamycin and macrolide action against *T. gondii*, although the relevant target for their action remains unknown.**

The protozoan parasite *Toxoplasma gondii* is a ubiquitous human pathogen long recognized as a source of congenital neurological abnormalities (19). In recent years, this parasite has also acquired considerable notoriety as an opportunistic infection associated with AIDS (16). The ability of *T. gondii* parasites to persist as latent cysts in the tissues of infected patients mandates chronic treatment for infected AIDS patients, to guard against recrudescence. Unfortunately, the traditional therapeutic regimen of pyrimethamine plus sulfonamides (18) is not always suitable for prolonged treatment, because of the emergence of sulfa hypersensitivity and other adverse side effects (12, 14, 27).

Clindamycin (a lincosamide) and several macrolide antibiotics have proven effective for the treatment of AIDS-toxoplasmosis, usually in combination with pyrimethamine (6, 13, 15). These compounds are known to block protein synthesis in bacteria by interacting with the peptidyl transferase domain of 23S rRNA (5), but their target in *T. gondii* and related parasites remains unclear (1). Early difficulties in establishing a functional in vitro system to study clindamycin and macrolide action against *T. gondii* (4, 9, 17) can now be explained by the long lag period between drug administration and effect (20, 21). Nanomolar drug concentrations block parasite replication, but only 2 to 3 days after treatment—a remarkable delay, considering that the tachyzoite undergoes ~8 generations in this time.

In order to more precisely examine the peculiar kinetics of clindamycin and macrolide action against *T. gondii*, we have monitored parasite replication in the presence of drug at the level of individual parasitophorous vacuoles. As detailed below, under defined conditions of infection and growth in primary human foreskin fibroblasts, single *T. gondii* tachyzoites establish intracellular vacuoles in which they divide, synchronously, every ~7 h until their clonal progeny ultimately rupture the host cell (at ~48 to 56 h). The fate of emerging parasites can be monitored further (i.e., beyond the first infectious cycle) by controlled infection of a fresh host cell monolayer. Even very high concentrations of clindamycin have no effect on (i) intracellular parasite survival in the initial parasitophorous vacuole, (ii) lysis of the original host cell, (iii) extracellular survival, or (iv) invasion into the subsequent host cell. Replication rates within the second host cell are inhibited immediately upon entry, however, suggesting that the key event in clindamycin action may be establishment of the new parasitophorous vacuole.

## MATERIALS AND METHODS

Strain RH *T. gondii* tachyzoites (a clonal isolate originally obtained from Elmer Pfefferkorn, Dartmouth College) were maintained by serial passage in confluent monolayers of primary human foreskin fibroblasts (HFF cells; also obtained from E. Pfefferkorn) as previously described (22). HFF cell stocks were grown in Dulbecco's modified Eagle's medium containing 10% iron-supplemented newborn bovine serum, which was replaced with Eagle's minimum essential medium containing 1% dialyzed fetal bovine serum (Gibco, Grand Island, N.Y.) prior to parasite inoculation. Parasites were harvested shortly after complete lysis of the host cell monolayer and purified by filtration through 3-μm-pore-size polycarbonate filters (Nucleopore, Cambridge, Mass.). In some experiments, parasites were forcibly released by scraping of the host cell monolayer with a rubber policeman and passage through a 26-gauge needle, followed by filtration as above. Clindamycin hydrochloride, spiramycin, chloramphenicol, tetracycline, pyrimethamine, anisomycin, and cycloheximide were obtained from Sigma (St. Louis, Mo.); azithromycin was obtained from Pfizer Inc. (Groton, Conn.). Stock solutions (10 mM) of all drugs were prepared in 70% ethanol, and aliquots were stored at −80°C.

To assay the replication of intracellular parasites, confluent cultures of HFF cells in 25-$cm^2$ T-flasks were inoculated with $10^6$ freshly lysed-out filter-purified parasites. After incubation at 37°C for 8 h the supernatant medium was aspirated and replaced, to remove parasites that had not yet attached. Drugs were added at various times thereafter as indicated below. When drug removal was required, the infected monolayers were rinsed twice and fresh drug-free medium was added. Bioassays of supernatant medium from uninfected host cells incubated with clindamycin and rinsed as described above demonstrates that this procedure results in a >1,000-fold dilution of drug.

Parasite replication was monitored by counting the number of tachyzoites per

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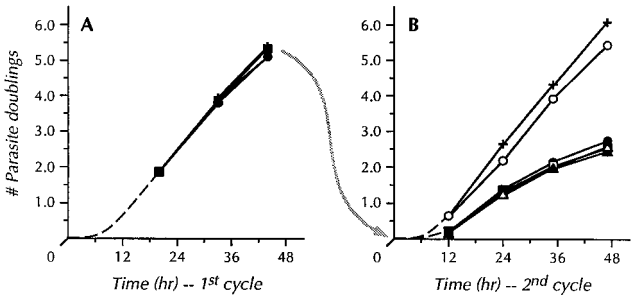


FIG. 1. Clindamycin treatment inhibits parasite replication only in the second infectious cycle. Intracellular parasites were treated with 0 nM, 100 nM, 1 μM, or 10 μM clindamycin for 2 h (8 to 10 h postinfection) or throughout the first cycle of infection. Following parasite-mediated lysis of the infected HFF cell monolayer (~56 h postinfection), purified extracellular tachyzoites were inoculated into fresh cultures (arrow) and monitored for a further 48 h without drug. The spectrum of vacuole sizes (number of tachyzoites per vacuole) was recorded every 12 h throughout this period, and numbers of parasite doublings were determined as the $\log_2$(parasite number). Data represent the averages for at least 50 randomly selected vacuoles; see Materials and Methods for further details. (A) First infectious cycle. (B) Second infectious cycle. +, untreated control parasites; ● and ○, 100 nM clindamycin treatment; ■ and □, 1 μM treatment; ▲ and △, 10 μM treatment. Filled symbols indicate continuous treatment throughout the first infectious cycle; open symbols (shown for panel B only) indicate 2-h treatment at the beginning of the first cycle. Samples for panel A were statistically indistinguishable by the two-tailed *t* test ($P > 0.05$) (29). For panel B, parasites treated with 100 nM drug for 2 h reproducibly replicated more slowly than controls ($P < 0.05$); all other samples replicated much more slowly than controls ($P \ll 0.01$) but were indistinguishable from each other.

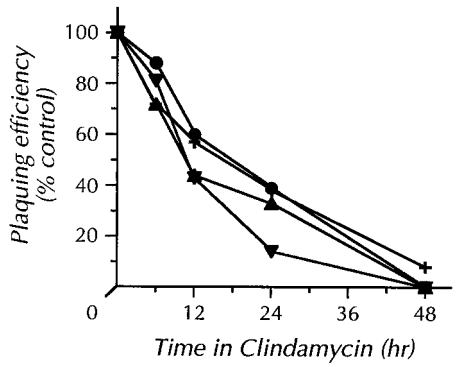


FIG. 2. Extracellular tachyzoites are unaffected by clindamycin treatment. Freshly harvested tachyzoites were incubated in culture medium containing various concentrations of clindamycin and assayed for plaquing efficiency (without drug) at various times. See Materials and Methods for further details. Although the viability of extracellular tachyzoites decreases rapidly, clindamycin treatment has no effect on this process. +, untreated controls; ▼, 5 nM; ●, 50 nM; ▲, 500 nM.

parasitophorous vacuole at 12-h intervals. To ensure random counts, fields to be scored were selected without microscopic examination, and all vacuoles within these fields were counted. Multiple fields were examined at high magnification (460-μm-diameter field) until at least 50 vacuoles were observed (typically 5 to 10 fields per sample). Because all *T. gondii* parasites within a single vacuole replicate synchronously, the number of divisions since infection can be determined as the $\log_2$(parasite number). Averages and standard deviations were calculated for each sample at each time point, and the statistical significance of between-sample differences was determined by a two-tailed *t* test (29). Replication rates (doubling times) were calculated during periods of stable logarithmic replication (usually 12 to 36 h postinfection). In some experiments, replication within individual vacuoles was also monitored by time-lapse video microscopy.

When parasites emerged from their host cells (~56 h postinfection in control cultures), tachyzoites were isolated, filtered, and inoculated into new flasks. Replication rates in the next cycle of infection were then assayed as described above. Parasite infectivity was assessed by counting the densities of parasitophorous vacuoles in randomly selected fields.

To examine the effect of drug treatment on extracellular *Toxoplasma* organisms, freshly lysed-out parasites were harvested and filter purified, and $10^7$ tachyzoites were inoculated into each of a series of tubes containing various concentrations of clindamycin (up to 10 μM) in a total volume of 1 ml of minimal essential medium plus 1% dialyzed fetal bovine serum. After incubation for various times at 37°C in a 5% $CO_2$ atmosphere, the parasite suspensions were gently mixed, and 100-μl aliquots (~$10^6$ tachyzoites) were inoculated into T25 flasks containing confluent HFF cells in 10 ml of medium. To provide identical conditions during infection, all flasks were adjusted to a final concentration of 100 nM clindamycin and incubated for 4 h. (Treatment for 4 h in 100 nM drug has no detectable effect on parasite replication, as shown below). After parasite invasion, cultures were rinsed twice in drug-free medium, and replication rates in the first and second vacuoles were monitored as described above. Plaque assays of drug-treated parasites, and one- and two-dimensional microtiter assays for the analysis of drug interactions, were carried out as previously described (22).

## RESULTS

**Clindamycin treatment has no effect on extracellular parasites or parasite replication in the first parasitophorous vacuole.** As shown in Fig. 1A, after an initial lag period necessary for parasite attachment and invasion, intracellular *T. gondii* tachyzoites exhibit exponential growth for at least 30 h in HFF cells. Until lysis of the host cell, the clonal progeny of a single infectious parasite are retained within the specialized parasitophorous vacuole. Host cells usually lyse during the sixth or seventh round of tachyzoite replication (from 64 to 128 or from 128 to 256 parasites), which typically occurs at ~48 to 56 h postinfection. Clindamycin concentrations up to at least 20 μM (>1,000 times the 50% inhibitory concentration for this drug; see below and reference 20) completely fail to inhibit parasite replication within the first parasitophorous vacuole (first cycle).

Treatment of extracellular tachyzoites also failed to inhibit parasite replication, as shown in Fig. 2. Although the plaquing efficiency of extracellular tachyzoites declines with a half-life of ~16 h, the rate of decline was not enhanced by treatment with up to 500 nM clindamycin for up to 24 h. Replication rates within individual parasitophorous vacuoles derived from treated and untreated samples were indistinguishable (data not shown). Because formation of visible plaques requires ~7 days of continuous parasite replication, it appears that extracellular parasites are completely refractory to clindamycin treatment.

**Inhibition of parasite replication following clindamycin treatment of intracellular parasites is seen only in the second parasitophorous vacuole.** In order to monitor parasite replication beyond a single infectious cycle, tachyzoites emerging from the first cycle of infection under clindamycin treatment were filter purified and reinoculated into a fresh cell culture (arrow in Fig. 1), as described in Materials and Methods. Clindamycin treatment had no effect whatsoever on parasite invasion: equal numbers of parasitophorous vacuoles were established by untreated controls and parasites which had been exposed to 100 nM clindamycin in the first infectious cycle (Fig. 3).

Parasite replication was drastically slowed, however, immediately upon entry into the second host cell, as shown by the solid symbols in Fig. 1B. Whereas control parasites divided with a log-phase replication time of ~6.6 h, parasites treated with clindamycin throughout the first infectious cycle exhibited replication times of >13 h in this experiment (the extent to which replication is suppressed in the second parasitophorous vacuole is somewhat variable between experiments; see below). Clindamycin concentrations as low as 10 nM (but not 1 nM; data not shown) are sufficient to inhibit parasite replication in the second vacuole. Interestingly, continuous drug treatment is not necessary for this effect: the presence of clindamycin during the second infectious cycle does not affect parasite replication (see below). In fact, treatment with high concentrations of clindamycin for as little as 2 h within the first parasitopho-

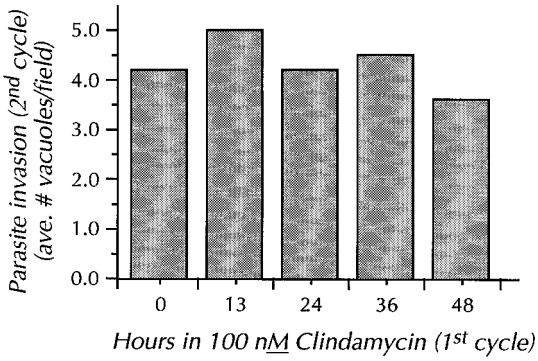


FIG. 3. Clindamycin has no effect on parasite invasion. *T. gondii*-infected host cells were treated with clindamycin for various periods and incubated until lysis. Emerging tachyzoites were purified and inoculated into fresh host cell cultures. After 24 h, the density of parasitophorous vacuoles (of any size) was determined in randomly selected fields. ave., average.

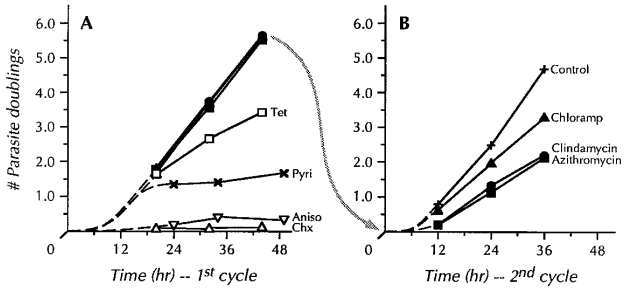


FIG. 4. A delayed effect is also observed with azithromycin and chloramphenicol, but not with other protein synthesis inhibitors or parasiticidal agents. Replication of intracellular tachyzoites in control cultures (+) and cultures containing 1 μg of azithromycin per ml (■), 20 μM chloramphenicol (Chloramp) (▲), 100 nM clindamycin (●), 1 μM cycloheximide (Chx) (△), 100 nM anisomycin (Aniso) (▽), 100 μM tetracycline (Tet) (□), or 1 μM pyrimethamine (Pyri) (x) was monitored. Azithromycin-, chloramphenicol-, and clindamycin-treated cultures were unaffected by drug in the first infectious cycle (A). The replication of tachyzoites produced by these cultures was inhibited only upon entry into a second host cell (in the absence of further treatment) (B). Data presented in this figure were compiled from several experiments, which accounts for the different time points for different drugs.

rous vacuole inhibited replication in the second vacuole as effectively as did continuous treatment (open versus closed squares in Fig. 1B). Note, however, that with 100 nM drug, continuous treatment in the first cycle was necessary to produce full inhibition in the second cycle (closed versus open circles). (See Discussion for further consideration of these results).

The reduced replication observed for clindamycin-treated parasites (in the second infectious cycle) is a general phenomenon observed in all parasitophorous vacuoles. This is indicated by the tight distribution of vacuole sizes in Table 1 (low standard deviations) and confirmed by video microscopy (data not shown). For example, at 36 h after inoculation in the second cycle of infection, 82% of the parasitophorous vacuoles in control cultures contained ≥16 parasites, while 88% of the parasites treated with 100 nM clindamycin (in the previous infectious cycle) produced vacuoles containing 4 or 8 parasites. In the drug-treated samples, all parasites continued to divide within the parasitophorous vacuole, but at a slower rate than controls. Even as early as 12 h after entry into the second host cell, differences between control and treated samples were highly significant ($P \ll 0.01$), as determined by the two-tailed *t* test. Most of these slowly growing parasites are never released from their host cells, and those which do emerge fail to replicate at all in the subsequent host cell (see below).

Figure 4 demonstrates that the delayed pharmacological effect observed for clindamycin is not typical of parasiticidal agents in general, or of protein synthesis inhibitors in particular. Anisomycin and cycloheximide both take immediate effect within the first parasitophorous vacuole. Pyrimethamine (an antifolate) blocks after the first parasite division. Tetracycline is weakly effective at high concentrations, and it gradually slows parasite replication within the first vacuole. In contrast, both chloramphenicol and macrolide antibiotics such as azithromycin and spiramycin (data not shown) exhibit a delayed effect on parasite replication similar to that observed for clindamycin, in which activity is observed only upon entry into the second parasitophorous vacuole (Fig. 4B).

As shown in Fig. 5, isobolograms show an additive (but not synergistic) effect of clindamycin and macrolide antibiotics (compare the inner solid curve with the dashed and dotted

TABLE 1. Size distribution of parasitophorous vacuoles in clindamycin-treated and control samples[a]

| Sample | Time (h) after entry into second vacuole | No. of vacuoles with the following no. of parasite doublings (no. of parasites/vacuole): | | | | | | | Avg no. of doublings per vacuole (SD)[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 (1) | 1 (2) | 2 (4) | 3 (8) | 4 (16) | 5 (32) | ≥6 (64)[c] | |
| Control | 12 | 18 | 32 | 0 | 0 | 0 | 0 | 0 | 0.64 (0.48) |
| Treated | 12 | 39 | 11 | 0 | 0 | 0 | 0 | 0 | 0.22 (0.41) |
| Control | 24 | 0 | 2 | 16 | 30 | 2 | 0 | 0 | 2.64 (0.62) |
| Treated | 24 | 2 | 28 | 19 | 1 | 0 | 0 | 0 | 1.38 (0.60) |
| Control | 36 | 0 | 0 | 0 | 9 | 18 | 21 | 2 | 4.32 (0.81) |
| Treated | 36 | 0 | 6 | 31 | 13 | 0 | 0 | 0 | 2.14 (0.60) |
| Control | 48 | 0 | 0 | 0 | 0 | 4 | 14 | 32 | 6.08 (1.06) |
| Treated | 48 | 0 | 0 | 14 | 35 | 1 | 0 | 0 | 2.74 (0.48) |

[a] Infected cultures were grown without drug or in the continuous presence of 100 nM clindamycin until lysis of the culture. Released parasites were inoculated into fresh HFF cultures without added drug, and the number of parasites per vacuole was scored for 50 randomly selected vacuoles at the times indicated. Parasites treated with clindamycin (in the previous vacuole) continued to replicate, but at a reduced rate. Nonzero values are underlined to facilitate comparison between samples. Averages are plotted in Fig. 1B (+, control; ●, treated); control and treated samples are significantly different ($P \ll 0.01$) at all time points, as determined by the two-tailed *t* test (29).

[b] SD, standard deviation.

[c] Includes host cells which lysed because of the parasite burden.

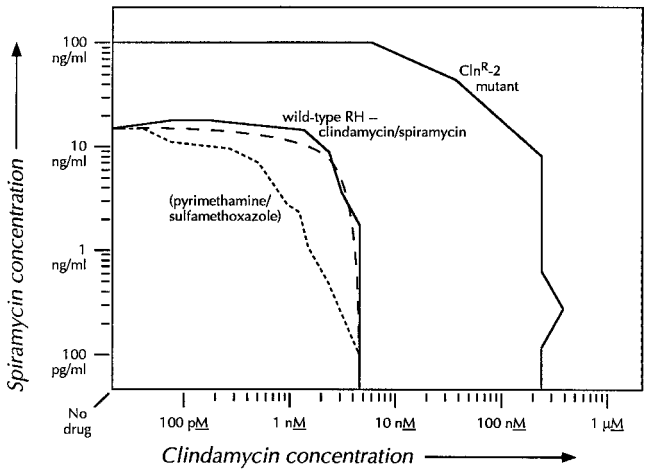


FIG. 5. Two-dimensional analysis of clindamycin-spiramycin interactions demonstrates an additive effect. Confluent HFF cell monolayers grown in microtiter wells were inoculated with RH-strain *T. gondii* tachyzoites in the presence of the indicated concentrations of clindamycin and spiramycin (note the log-log scale). Isobolograms reflect 50% inhibitory concentration measurements determined by optical scanning of stained plates (22). Wild-type parasites (inner solid line) were inhibited by ~5 nM clindamycin or ~15 μg of spiramycin per ml. Mixtures of these drugs show an additive effect (compare the inner solid line with the theoretical curve [dashed line]). In contrast, pyrimethamine plus sulfamethoxazole exhibits a fivefold synergistic effect (dotted line; normalized to the endpoints for comparison—actual 50% inhibitory concentrations were ~150 nM for pyrimethamine and ~1.5 μM for sulfamethoxazole). The Cln$^R$-2 mutant is highly resistant to both clindamycin and spiramycin (outer solid line).

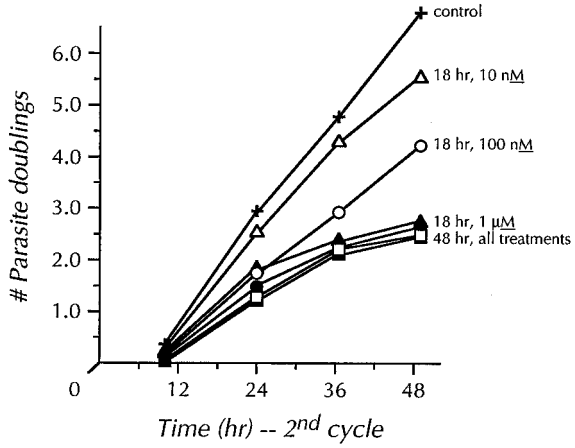


FIG. 6. Effects of clindamycin concentration on parasite replication in the subsequent parasitophorous vacuole. Following infection, intracellular parasites were treated for 18 or 48 h with various concentrations of clindamycin. After host cell lysis (intracellular replication and timing of host cell lysis were unaffected by drug treatment), freshly harvested parasites were inoculated into a second host cell culture in the absence of drug. +, untreated control; ▲ and △, 10 nM clindamycin; ● and ○, 100 nM clindamycin; ■ and □, 1 μM clindamycin. Filled symbols indicate continuous treatment throughout the first infectious cycle; open symbols indicate 18-h treatment. Samples treated for 18 h with different drug concentrations (in the previous infectious cycle) replicated at rates which were statistically distinct from each other and from that for the control sample ($P \ll 0.01$ by the two-tailed $t$ test); samples treated for 48 h with any drug concentration were statistically indistinguishable from each other and from the 18-h, 1 μM sample ($P > 0.05$).

curves), suggesting a common target. The clindamycin-resistant mutant Cln$^R$-2 (kindly provided by Elmer Pfefferkorn) (20) exhibits cross-resistance to spiramycin (outer solid line).

**Severity of the second cycle effect depends on concentration and duration of exposure to clindamycin in the first infectious cycle.** As noted above, the inhibition of parasite replication by clindamycin is dependent only on the presence of drug during the previous infectious cycle. Moreover, although nanomolar drug concentrations must be maintained throughout the previous infectious cycle to exert maximal effect, brief treatment with high concentrations of clindamycin is sufficient to inhibit replication (Fig. 1B). No concentration or duration of clindamycin treatment completely blocked parasite replication—continuous exposure to 10 μM clindamycin had precisely the same effect as treatment for 2 h or treatment with 100 nM clindamycin for 48 h (Fig. 1B). Reducing the concentration or the duration of treatment can produce an intermediate phenotype, however. As illustrated in Fig. 6, 18-h treatment with various concentrations of clindamycin slowed parasite doubling times (in the subsequent vacuole) from 6.5 h for untreated controls to 8.3 h (10 nM clindamycin), 10.1 h (100 nM clindamycin), or >20 h (1 μM clindamycin)—a rate indistinguishable from that observed for continuous treatment (with any drug concentration). Similarly, varying the duration of exposure to 100 nM clindamycin in the first vacuole produced replication times of ~7 to >30 h in the second vacuole, as shown in Fig. 7 and Table 2.

Primary data from the 48-h time point of this experiment are presented in Table 3, illustrating the low level of variation in the number of parasites observed per vacuole. It is clear from these data that intermediate replication rates are not the result of averaging together a mixture of vacuoles with short and long division times. Rather, each individual vacuole within the sample exhibits the replication rate characteristic of the duration and concentration of clindamycin exposure in the previous infectious cycle.

**Initiation of the second replication cycle—as opposed to time elapsed since clindamycin treatment—is the key factor**

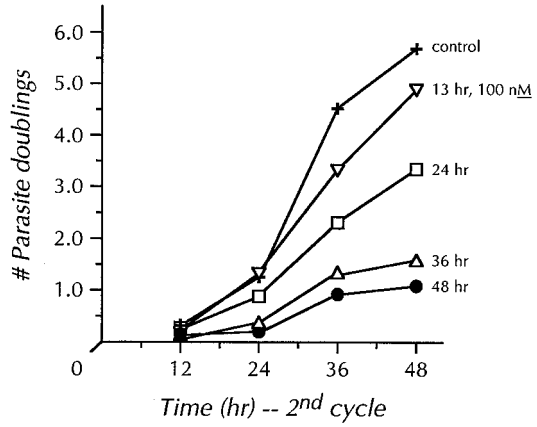


FIG. 7. Effects of the duration of clindamycin exposure on parasite replication in the subsequent parasitophorous vacuole. Clindamycin (100 nM) was added to cultures at various times postinfection. After lysis of the initial cultures (intracellular replication and timing of host cell lysis were unaffected by drug treatment), fresh flasks were inoculated with parasites in the presence or absence of drug. Data shown derive from experiments in which clindamycin was omitted from the second infectious cycle, but see Table 2 for calculated log-phase doubling times for all treatments. ●, drug added 8 h postinfection in the previous infectious cycle (resulting in 48-h exposure prior to host cell lysis); △, drug added 20 h postinfection in the previous infectious cycle (36-h exposure); □, drug added 32 h postinfection in the previous infectious cycle (24-h exposure); ▽, drug added 31 h postinfection in the previous infectious cycle (13-h exposure); +, untreated control. Statistical analysis (by the two-tailed $t$ test) indicates that control samples are distinct from the 13-h-treated parasites at $P < 0.02$; the 36-h and 48-h treatments are also distinct at $P < 0.02$, and all other pair-wise comparisons are distinct at $P \ll 0.01$.

TABLE 2. Replication rates of parasites exposed to 100 nM clindamycin at various times[a]

| Timing of treatment in the first infectious cycle (h postinfection) | Duration of treatment in the first infectious cycle (h) | Presence of clindamycin in the second infectious cycle | Doubling time in the second infectious cycle (h) |
|---|---|---|---|
| Expt 1 (Fig. 7) | | | |
| None | 0 | − | 6.7 |
| None | 0 | + | 7.0 |
| 43–56 | 13 | − | 7.5 |
| 43–56 | 13 | + | 8.2 |
| 32–56 | 24 | − | 12 |
| 32–56 | 24 | + | 13 |
| 20–56 | 36 | − | 24 |
| 20–56 | 36 | + | 20 |
| 8–56 | 48 | − | 33 |
| 8–56 | 48 | + | 35 |
| Expt 2 (Fig. 8) | | | |
| None | 0 | − | 6.8 |
| 9–27 | 18 | − | 11 |
| 18–36 | 18 | − | 12 |
| 27–49 | 22 | − | 10 |
| 9–49 | 40 | − | 18 |

[a] Parasites were exposed to 100 nM clindamycin in the first cycle of infection during the times indicated. Host cell lysis (at 56 and 49 h postinfection in experiments 1 and 2, respectively) was unaffected by drug treatment. After inoculation into fresh HFF cells in the presence or absence of clindamycin, the size distribution of parasitophorous vacuoles (numbers of parasites per vacuole) was monitored as described in Materials and Methods and doubling times were calculated over periods of log-phase replication. Experiment 1 demonstrates that doubling time in the second vacuole (the second infectious cycle) is dependent solely on the length of exposure to clindamycin in the previous parasitophorous vacuole and that continued drug treatment in the second vacuole is irrelevant. From experiment 2 it is clear that the relative timing of drug treatment within the first infectious cycle is unimportant.

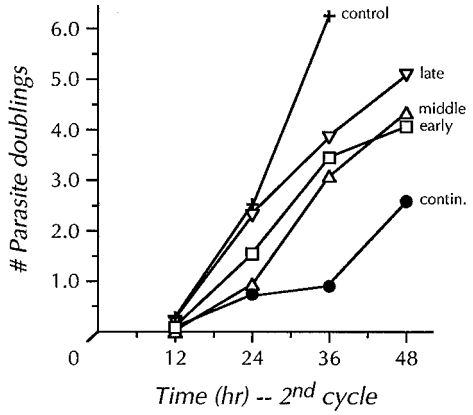


FIG. 8. Timing of clindamycin treatment within the first parasitophorous vacuole is irrelevant. Infected cell cultures were treated with 100 nM clindamycin at the beginning, middle, or end of the infectious cycle. Upon lysis of the host cells (parasite replication rates in the first cycle and the timing of emergence from the host cell were unaffected by drug treatment), replication in fresh cell monolayers without drug was monitored. +, untreated controls; □, 100 nM clindamycin treatment applied during the first half of the previous infectious cycle (9 to 27 h postinfection); △, treatment applied for 18 h in the middle of the previous infection (18 to 36 h postinfection); ▽, clindamycin treatment for 22 h at the end of the previous cycle (27 to 49 h postinfection); ●, continuous treatment (contin.) during the previous cycle. Regardless of the timing of drug administration (in the previous infectious cycle), parasite replication fell between the two extremes of the untreated and continuously treated samples (see the text for further discussion).

**governing parasite replication.** As shown in Fig. 7, replication rates of all treated parasites were reduced throughout growth in the second parasitophorous vacuole, regardless of the time at which drug treatment was initiated in the previous infectious cycle. Interpretation of these data is complicated, however, by the different lengths of drug exposure for the various samples tested. To more clearly assess whether the onset of the clindamycin effect is due to establishment of the second replication cycle per se, as opposed to the time elapsed since treatment, drug treatments of similar durations were applied at different times within the first infectious cycle, and replication in the subsequent vacuole was examined. As shown in Fig. 8 and tabulated in Table 2, replication in the second parasitophorous vacuole was slowed to similar extents in all treated samples, regardless of the timing of clindamycin exposure in the previous vacuole. Throughout the second infectious cycle, doubling times (slopes) for the early-, middle-, and late-treated samples were statistically indistinguishable from each other, but they were intermediate between those for the rapidly dividing control samples and the more slowly replicating continuously treated sample (the sole exception—an aberrantly high replication rate observed in the continuously treated sample between 36 and 48 h—was not observed at later time points). These experiments demonstrate that the extent to which replication is inhibited depends solely on the duration of previous

TABLE 3. Size distribution of parasitophorous vacuoles in the second infectious cycle, after treatment with clindamycin for various times in the first infectious cycle[a]

| Duration of treatment, first vacuole (h) | Time after entry into second vacuole (h) | No. of vacuoles with the following no. of parasite doublings (no. of parasites/vacuole): | | | | | | | Avg no. of doublings per vacuole (SD)[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 (1) | 1 (2) | 2 (4) | 3 (8) | 4 (16) | 5 (32) | ≥6 (64)[c] | |
| Control | 12 | 34 | 16 | 0 | 0 | 0 | 0 | 0 | 0.32 (0.47) |
| Control | 48 | 0 | 0 | 2 | 4 | 10 | 11 | 23 | 5.66 (1.89) |
| 13 | 48 | 0 | 0 | 2 | 3 | 17 | 16 | 12 | 4.88 (1.35) |
| 24 | 48 | 4 | 0 | 6 | 21 | 15 | 0 | 4 | 3.10 (1.20) |
| 36 | 48 | 12 | 9 | 17 | 12 | 0 | 0 | 0 | 1.58 (1.10) |
| 48 | 48 | 18 | 15 | 12 | 5 | 0 | 0 | 0 | 1.08 (1.00) |

[a] Parasites treated with 100 nM clindamycin in the first cycle of infection continued to replicate in the second cycle (compare their data with those for controls at 12 h after entry), but at rates dependent on the length of drug exposure (in the previous cycle). Vacuoles within each sample replicated at a characteristic rate, as indicated by the distinctive spectrum of vacuole sizes and the low standard deviations. To facilitate comparison between samples, categories encompassing the majority of the observed vacuoles are underlined. Averages provide the data for Fig. 7 (48-h time points). As described in the legend to Fig. 7, all sample pairs are significantly different ($P < 0.02$), as determined by the two-tailed $t$ test (29).

[b] SD, standard deviation.

[c] Includes host cells which lysed because of the parasite burden.

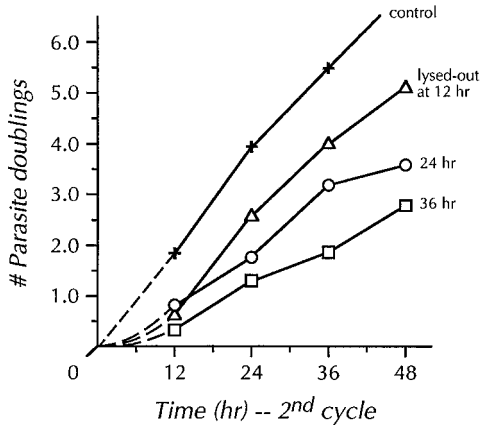


FIG. 9. Parasites forcibly released from cells replicate (in the second vacuole) at rates expected from the length of prior drug exposure. Infected host cells were scraped with a rubber policeman after replication in the presence of clindamycin for 12 (△), 24 (○), or 36 (□) h. Parasites were forcibly released and purified as described in Materials and Methods and reinoculated into fresh host cell cultures. Replication rates declined in the second infectious cycle, to an extent determined by the length of prior drug exposure ($P \ll 0.01$ by the two-tailed *t* test, but see the text for further discussion). For comparison, the expected replication rate for untreated parasites from a separate (but parallel) experiment is provided (+), and this rate extrapolated beyond the last measurable datum point (untreated parasites lysed out of their host cells prior to the 48-h time point in this experiment).

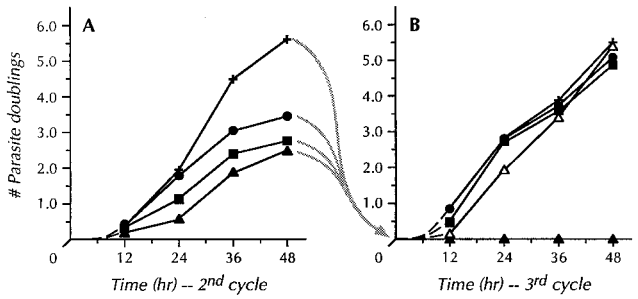


FIG. 10. In the third cycle after clindamycin treatment, parasites either recover or die. Intracellular parasites were treated with 100 nM clindamycin for 0 (+), 13 (●), 19 (■), or 25 (▲) h in the first cycle. Emerging parasites were reinoculated into a second host cell monolayer (A) and incubated without drug. When control parasites lysed their host cells (at ~60 h), treated parasites were forcibly released by syringe passage, filter purified, and inoculated into a third flask of cells (B). Parasitophorous vacuole sizes were scored at 12-h intervals throughout this period. Parasites which were exposed to clindamycin for 13 or 19 h (in the last cycle of infection) reverted to normal replication in the third infectious cycle, while the majority of the parasites originally treated for 25 h died in the third cycle. A small subpopulation of the 25-h treatment sample (△) replicated normally.

exposure and not on the relative timing of that exposure within the previous vacuole.

An alternative approach to examine the importance of establishing the second parasitophorous vacuole was also devised (Fig. 9). Parallel cultures were infected with *T. gondii* tachyzoites, treated with 100 nM clindamycin, forcibly released from the host cell at various times (by syringe passage), and inoculated into fresh HFF cell cultures. Parasites treated with drug for 24 h and forcibly released replicated in the subsequent infectious cycle more slowly than parasites treated for 12 h but more rapidly than those treated for 36 h. This pattern was not necessarily observed in every sample during each time segment—for example, parasites treated for 12 h (in the previous infectious cycle) appear to have divided more rapidly than the others in the segment of the second cycle from 12 to 24 h. However, within-sample differences in slope (obtained by comparing the individual segments of each curve) were not statistically significant, while between-sample variation in replication rate was clearly distinct ($P \ll 0.01$). In summary, replication rates are reduced within the second vacuole, to an extent determined by prior exposure to clindamycin, despite the fact that cultures exposed to the drug for the same length of time but left within the first vacuole continue to replicate normally (Fig. 1A).

**The fate of clindamycin-treated parasites beyond the second infectious cycle.** After ~5 to 7 days within the second parasitophorous vacuole, slowly replicating parasites eventually cease to divide, and they atrophy without ever lysing the second host cell (data not shown). Parasites exposed to low clindamycin concentrations for short times, however, do occasionally replicate sufficiently to lyse out of the second vacuole. We were therefore interested in monitoring their survival beyond the second parasitophorous vacuole. Because the timing of parasite emergence from the second parasitophorous vacuole is delayed to a variable extent (depending on the length of drug exposure in the first vacuole), a third infectious cycle was initiated synchronously by forcibly releasing parasites from the second vacuole at 60 h postinfection. As shown in Fig. 10, clindamycin treatment had the expected effect in the second cycle, reducing doubling times from 6.4 h (untreated controls) to 9, 11, and 13 h for parasites treated with 100 nM drug for 13, 19, and 25 h, respectively ($P < 0.01$) by two-tailed *t* test for all pairwise comparisons except 19 versus 25 h; differences between these two samples were statistically significant at the 24- and 36-h time points, however). In the third cycle, parasites treated for 13 or 19 h (two infectious cycles previously) recovered to grow as rapidly as untreated controls ($P > 0.5$), while most parasites treated for 25 h (or longer) established a third parasitophorous vacuole but failed to replicate at all. Very few parasites in the 25-h treatment sample (~10% of the total) were observed to replicate normally (open triangles in Fig. 10B).

## DISCUSSION

The mechanism of clindamycin and macrolide action against *T. gondii* has been the subject of considerable speculation in recent years. We have confirmed the delayed death phenotype observed when these drugs were used against *T. gondii* in vitro (20, 21) and examined the kinetics of this effect in detail, by directly measuring parasite replication as a function of the drug concentration and the duration and timing of drug exposure. Our studies indicate that even concentrations of clindamycin far in excess of the 50% inhibitory concentration have no immediate effect on parasite multiplication. Only once parasites emerge from the first host cell and establish a new parasitophorous vacuole (in a new host cell) is replication inhibited. For example, parasites treated with 100 nM clindamycin throughout the entire first infectious cycle replicated with doubling times of ~7 h, indistinguishable from those of controls (Fig. 1A). In the second vacuole, however, the replication of parasites was slowed to ≥18 h (Fig. 1B and Table 2). In contrast to one previous report (3), extracellular tachyzoites were insensitive to clindamycin in our study.

The extent to which replication is inhibited in the second parasitophorous vacuole is dependent on both the drug concentration and the duration of drug exposure during the previous cycle of infection. For example, treatment of intracellular parasites with 100 nM clindamycin for only 18 h (in the first

vacuole) produced doubling times of ~10 to 12 h in the second vacuole, intermediate between the doubling times of untreated and continuously treated controls (Table 2). As shown in Table 3, the replication rates reported are not the results of averaging various ratios of normally replicating and dead parasites; each vacuole within a given sample exhibits a replication rate characteristic of the concentration of clindamycin and the duration of exposure during the previous cycle. Parasites which are slowed sufficiently in the second vacuole never emerge from the host cell and eventually die. Those treated less extensively, however, may emerge to infect a third cell, whereupon they either revert to the normal replication rate or die (Fig. 10B).

Thus far, we have not observed intermediate growth rates in the second parasitophorous vacuole following treatment with high concentrations of clindamycin ($\geq$1 $\mu$M). This may be due to insufficient resolution of the exposure times tested. Alternatively, although treatment of host cells with high concentrations of clindamycin for several days prior to infection has no effect on parasite replication (and bioassays of the supernatant medium from these cells show no parasiticidal activity; data not shown), it remains possible that drug accumulation in infected cells artificially prolongs the period of intracellular exposure beyond the time when drug is present in the medium. Experiments examining the localization of azithromycin in *T. gondii*-infected cells have shown that >95% of the drug concentrates in host cell lysosomes and that virtually none is found in the parasitophorous vacuolar space (24).

The delayed effect of clindamycin, macrolides, and chloramphenicol seems to be dependent on the establishment of the second parasitophorous vacuole rather than the time elapsed since drug treatment, as the timing of drug exposure within the first vacuole is irrelevant—replication is slowed immediately upon entry into the second host cell. This is, perhaps, best understood by considering two parallel cultures, both subjected to a period of clindamycin treatment followed by incubation without drug. Parasites were forcibly released from one of the two cultures and reinoculated into fresh host cells, whereupon these tachyzoites invaded normally but were inhibited in their replication to the extent determined by the concentration of clindamycin and the duration of treatment prior to lysis (Fig. 9). Meanwhile, their sisters in the parallel flask continued to replicate normally (in the primary vacuole), despite an identical history of drug treatment.

The parasitophorous vacuole appears to be a key player in mediating the effects of clindamycin treatment. Parasite replication rates observed immediately upon entry into the vacuole are maintained throughout intracellular replication. It is as if the synthesis and/or storage of some factor which is essential for the establishment of a new vacuole is inhibited by treatment with clindamycin, but this damage manifests itself only when a new vacuole is established. It is unlikely that this effect is mediated by drug carried over by the parasite from the previous infectious cycle: as noted above, experiments with radiolabelled azithromycin indicate that very little drug enters the parasite, and most of that which does enter is sequestered in acidified lysosome-like organelles (24). It is intriguing to speculate on the relationship between parasite replication and recent studies of vacuolar structure and biochemistry (2, 23–26).

The delayed pharmacological effect observed for clindamycin is unusual among parasiticidal agents. Pyrimethamine (an antifolate) and the protein synthesis inhibitors cycloheximide, anisomycin, and tetracycline (as well as many other agents examined in our laboratory over the years) all inhibit parasite replication within the first parasitophorous vacuole (Fig. 4A). The distinctive delayed effect on parasite replication is seen only with clindamycin, macrolide antibiotics such as azithromycin and spiramycin, and chloramphenicol, suggesting a common target. Lincosamides, macrolides, and chloramphenicol are all known to act as protein synthesis inhibitors in bacteria, and all interact with the peptidyl transferase domain of 23S rRNA (5). It therefore seems likely that these structurally unrelated inhibitors of transpeptidation, all of which exhibit similar kinetics of action against *Toxoplasma* tachyzoites, act by inhibiting protein synthesis in *T. gondii* as well. Supporting this view, parasites resistant to clindamycin also show cross-reactivity to spiramycin (20) (Fig. 5).

Careful studies have failed to reveal any effect of macrolide or lincosamide antibiotics on cytoplasmic protein synthesis in *T. gondii*, however (1). Although these drugs are known to affect organellar ribosomes in various systems (11), it has not been possible to demonstrate any effect of clindamycin or azithromycin on mitochondrial protein synthesis (20). It is intriguing, however, that apicomplexan parasites possess a novel extrachromosomal element containing prokaryotic-type ribosomal genes (1, 8, 28).

Resistance to lincosamides, macrolides, and chloramphenicol in bacteria and chloroplasts is commonly associated with mutations affecting the peptidyl transferase loop of 23S rRNA (5, 7, 10, 11). Although sequence analysis of *T. gondii* ribosomal genes suggests that both cytoplasmic and mitochondrial ribosomes are resistant to clindamycin and macrolides, the putative ribosomal genes from the 35-kb element are likely to be sensitive to these drugs (1). Preliminary analysis of *T. gondii* mutants resistant to clindamycin or azithromycin (20) has failed to define sequence mutations associated with ribosomal genes of the 35-kb element (7a), but it will be interesting to examine protein synthesis from these mysterious ribosomes more directly.

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