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In Vitro Effects of Four Macrolides (Roxithromycin, Spiramycin, Azithromycin [CP-62,993], and A-56268) on *Toxoplasma gondii*

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The effect of four macrolides against intracellular *Toxoplasma gondii* was determined in three different in vitro systems. Unactivated murine peritoneal macrophages were infected with the virulent RH strain of *T. gondii*. The activity of the macrolides was first measured with [^{3}H]uracil, which is incorporated by the parasite but not the host cell. The 50% inhibitory concentrations (IC_{50}s) and 95% confidence limits were calculated at 54 (38 to 73), 140 (98 to 201), 147 (101 to 214), and 246 (187 to 325) μM for roxithromycin, azithromycin (CP-62,993), A-56268, and spiramycin, respectively. Inhibition of *Toxoplasma* growth was confirmed by microscopic examination of the infected macrophages after treatment with roxithromycin. Compared with untreated controls, roxithromycin concentrations near the IC_{50}s decreased the number of infected cells, the number of tachyzoites per vacuole, and the number of cells containing rosettes (i.e., clusters of more than eight tachyzoites). After treatment with the four macrolides, tachyzoites were released from the macrophages and subcultured in HeLa cells, which are nonprofessional phagocytes, to assess the viability of the remaining parasites. This showed that the macrolides at concentrations corresponding to four times their 90% inhibitory concentrations (IC_{90}s) had no significant killing effect. At 8 times the IC_{90}, roxithromycin showed an incomplete killing effect, similar to that of the combination of pyrimethamine (0.41 μM)–sulfadiazine (99.42 μM). All macrolides tested showed inhibitory effects against intracellular *T. gondii*, but amounts of azithromycin and A-56268 corresponding to the IC_{90} appeared to be toxic against the host macrophages, which might have had nonspecific activity against *Toxoplasma* metabolism.

Toxoplasmosis, a very common parasitic disease, poses a great hazard to immunocompromised hosts and, when the disease is contracted during pregnancy, to the fetus in utero. In patients with the acquired immune deficiency syndrome *Toxoplasma* encephalitis is a major cause of morbidity and mortality (16, 21, 39).

Currently, the most effective treatment for both congenital and acquired toxoplasmosis is the combination of pyrimethamine-sulfadiazine (or trisulfapyrimidines) (10, 13, 37). However, treatment fails to eliminate the encysted form of the parasite (35). In patients with the acquired immune deficiency syndrome with *Toxoplasma* encephalitis who were treated with this combination, early treatment response has been followed by a high rate of toxicity, which required the cessation of therapy. A relapse often followed, with mortality rates approaching 70 to 92% (16, 21, 39). Also, because pyrimethamine is potentially teratogenic, its use during the first trimester of pregnancy is not recommended (29).

Some macrolide antibiotics have shown some activity against toxoplasmosis. Spiramycin has proven efficiency against murine toxoplasmosis (6, 14) and has been used in Europe for many years in an attempt to prevent transmission of the infection from pregnant women to their fetuses without evidence of harmful effects on fetal development (8, 9).

Recently, two newer macrolides have shown promising properties. One is roxithromycin, which has an antibacterial potency comparable to that of erythromycin (2, 18) and which appears to be effective in treating mice with acute toxoplasmosis (5, 6) and murine *Toxoplasma* encephalitis (17). Another is azithromycin (CP-62,993), a new 9-methyl derivative of erythromycin which has also been found to have antitoxoplasmic activity in similar murine models (F. G. Araujo, D. R. Guptill, and J. S. Remington, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 234, 1987). However, no studies aimed at comparing these drugs with regard to their antitoxoplasmic activities have been undertaken so far. Moreover, no data are available on the eventual killing effect of macrolides against *Toxoplasma gondii*. Hence, the inhibitory and killing effects of four macrolides against intracellular tachyzoites of *T. gondii* were investigated. Besides roxithromycin and azithromycin, we included A-56268, a new 6-*O*-methyl derivative of erythromycin (12), and as a standard, the combination of pyrimethamine-sulfadiazine.

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MATERIALS AND METHODS

Antimicrobial agents. All drugs were supplied in powder form by their respective manufacturers. Roxithromycin (molecular weight, 837.04; batch no. 5S 0484) was provided by Roussel-UCLAF, Paris, France; spiramycin (formed with spiramycin I [molecular weight, 843], spiramycin II [molecular weight, 885], and spiramycin III [molecular weight, 899]; batch no. 5643251) was provided by Rhône-Poulenc Pharma-Suisse S.A., Geneva, Switzerland; azithromycin (molecular weight, 749; batch no. 14462-120-1F) was provided by Pfizer Limited, Sandwich, England; pyrimethamine (molecular weight, 248.71; batch no. 712807), sulfadoxine (molecular weight, 310.34), and sulfadiazine (molecular weight, 250.28; batch no. 454871) were provided by F. Hoffmann-La Roche & Cie. S.A., Basel, Switzerland; A-56268 (molecular weight, 747.97; batch no. 84-420-AL) was

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provided by Abbott Laboratories, North Chicago, Ill. Roxithromycin, spiramycin, azithromycin, A-56268, and pyrimethamine were prepared extemporaneously in 95% ethanol and diluted in 0.1 M phosphate buffer and then in culture medium. The final pH was adjusted at 7.2. Sulfadiazine and sulfadoxine were prepared by dissolving them with a minimal amount of 0.1 M NaOH, adding phosphate buffer, and then further diluting the solution with culture medium at a concentration of 500 μg/ml.

Cells and media. (i) Macrophages. Unactivated peritoneal macrophages were obtained from normal female Swiss-Webster mice (weight, 25 ± 1 g; Madörin AG, Füllinsdorf, Switzerland) by washing their peritoneal cavities with 2 ml of Hanks balanced salt solution (HBSS; GIBCO AG, Basel, Switzerland) which contained 5 units of heparin per ml, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The cell suspensions obtained from 10 to 12 mice were pooled and then collected in siliconized tubes and centrifuged in Ficoll-Paque (Pharmacia, Uppsala, Sweden) at 400 × *g* at 4°C for 15 min. The cells were then suspended at a concentration of 4×10^6 cells per ml in medium 199 (M199; Fakola AG, Basel, Switzerland) containing 10% heat-inactivated (60 min, 56°C) fetal calf serum (FCS; Fakola), penicillin (100 U/ml), and streptomycin (100 μg/ml). Penicillin and streptomycin were used for preventing the risk of contamination of the cultures when the macrophages from the peritoneal cavities of mice were harvested. These antibiotics were removed by the washings before challenge with *T. gondii*.

The phagocytic ability of marophages was tested by their phagocytosis of heat-killed *Candida albicans* (30 min, 100°C) and by use of the acridine orange fluorochrome stain (38). Cell viability was also tested by the trypan blue exclusion test.

A total of 500 μl of the suspension containing 2×10^6 cells was placed into each well of 24-well plates (2 cm^2; Nunc, Roskilde, Denmark) and four-chamber slides (2 cm^2; Lak-Tek; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) and then incubated at 37°C (in a 5% CO_2–95% air atmosphere) for 1 to 4 h to allow macrophages to adhere. To remove nonadherent cells, monolayers were washed gently two times with prewarmed HBSS. Monolayers were then reincubated with M199–10% FCS without penicillin or streptomycin. The percentage of mononuclear phagocytes on the monolayers was assessed by nonspecific esterase staining (kit no. 90-A1; Sigma Chemical Co., St. Louis, Mo.) and myeloperoxidase staining (kit no. 391-A; Sigma).

(ii) HeLa cells. HeLa cells, an epithelium-like cell line (ATCC CCL2), were maintained under continuous passage and were used for viability tests. Cells were suspended in minimum essential medium containing 10% FCS. Forty-eight hours before they were used, 5×10^5 cells were seeded into each chamber of four-chamber slides (Lab-Tek).

***Toxoplasma* serology.** The dye test was performed as described by Sabin and Feldman (36) by using a micromodification (11) to ascertain that the biological products used in this study did not contain antibodies against *T. gondii*.

***T. gondii* strain, infectious challenge, and antimicrobial treatment of macrophage monolayers.** The virulent RH strain of *T. gondii*, which was maintained by continuous subinoculation in mice, was used. Tachyzoites were obtained by harvesting the peritoneal exudate of Swiss-Webster female mice with HBSS (4°C) containing 5 U of heparin per ml on day 2 of infection. Parasites were released by forcefully passing them through a 27-gauge needle with a 10-ml plastic syringe (7) and were filtered free of host cells through 3.0-μm-pore-size polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.) (26). Parasites were collected in siliconized tubes, centrifuged at 500 × *g* for 10 min (4°C), suspended in M199–3% FCS, counted in a hemacytometer, and adjusted to 4×10^6 tachyzoites per ml.

A 500-μl amount of M199–3% FCS that contained 2×10^6 tachyzoites was placed on the adherent peritoneal exudate cell cultures of each well or slide. After 1 h of incubation, cultures were washed with HBSS, and 950 μl of M199–10% FCS was placed into each well or slide culture. Dilutions of antimicrobial agents were then added under a volume of 50 μl and maintained for 24 or 40 h. According to a microbiological assay, no significant decay of the antibiotic activity was observed in the medium during the experiments.

Incubations. Unless indicated otherwise, incubations were performed at 37°C in a humid atmosphere of 5% CO_2–95% air. The pH of the medium was adjusted to 7.2

Controls for toxicity. Adherent macrophage monolayer cells were incubated for 40 h with 1, 2, and 4 times the 90% inhibitory concentration (IC_{90}) of roxithromycin and 1 and 2 times the IC_{90}s of the other macrolides. The supernatant was discarded, and the monolayers were washed twice with calcium- and magnesium-free phosphate-buffered saline (pH 7.2) and incubated for 3 min at 37°C with 200 μl of 0.05% trypsin (GIBCO) in calcium- and magnesium-free phosphate-buffered saline. A 1,300-μl amount of M199–10% FCS was added to each well to stop the action of the trypsin. The cells were removed from the wells by two gentle pipettings and centrifuged at 200 × *g* for 5 min. This procedure allowed the consistent removal of most of the adherent macrophages, as determined by microscopic examination of the wells thereafter. The supernatant was discarded, and the cells were suspended in calcium- and magnesium-free phosphate-buffered saline. A 50-μl amount of the cell suspension was incubated with the same amount of trypan blue (0.2% in normal saline), and those macrophages that excluded trypan blue after a 3- to 5-min incubation time at room temperature were considered viable. The viability was calculated by the following formula: percentage of viable cells = (number of viable cells/total number of cells) × 100.

Assessment of antitoxoplasmic activity. (i) [^{3}H]uracil incorporation. The intracellular growth of *T. gondii* in monolayer cultures was determined by measuring the incorporation of [^{3}H]uracil into acid-precipitable material by use of a filtration procedure (24). The uracil is incorporated by *T. gondii* and not by monolayer cells due to the presence, in significant amounts, of the uracil phosphoribosyltransferase (EC 2.4.2.9) in the parasite (26, 31).

Twenty hours after the addition of antimicrobial agents, treated and untreated infected monolayers were pulsed with 10 μCi of [5,6-^{3}H]uracil (specific activity, 50 Ci/mmol; Amersham International, Buckinghamshire, England) and reincubated for 20 h. Monolayers were dissolved in 1% sodium dodecyl sulfate containing 100 μg of unlabeled uracil (Sigma Chemie, Deisenhofen, Federal Republic of Germany) per ml, stored at 4°C for 1 h, and then precipitated with ice-cold 0.3 N trichloroacetic acid. The precipitates were collected on glass filters (diameter, 25 mm; type GF/C; Whatman Ltd., Maidstone, England). The material retained by the filters was washed with cold 0.3 N trichloroacetic acid, rinsed with 95% ethanol, dried, and counted in a liquid scintillation spectrophotometer.

(ii) Light microscopy studies. Twenty four hours after the addition of the antimicrobial agents, monolayers growing in slides (Lab-Tek) were fixed with 0.4% 9-aminoacridine (Sigma) in 50% (vol/vol) ethanol–water, Giemsa stained, and

then mounted. The percentage of infected cells, the number of *T. gondii* tachyzoites per vacuole, and the number of infected cells containing a rosette were determined by microscopic examination (×1,000) of 200 infected cells. A rosette was defined as eight or more *Toxoplasma* tachyzoites within a single vacuole.

(iii) Viability test. The viability of the possible intracellular parasites remaining after a treatment of the monolayers with the antimicrobial agents was assessed by testing their ability to invade HeLa cells. Monolayers growing in chamber slides (Lab-Tek) were treated for 24 h with macrolides in twofold increasing concentrations beginning with 1× the IC_{90}. At this point, monolayers were gently washed two times with prewarmed HBSS. The parasites were released from the macrophages by adding 500 μl of a solution of 5 mM Tris–75 mM saccharose in distilled water (pH 8.0) for 15 min. This provoked lysis of the macrophages by osmotic shock. By contrast, according to trypan blue and acridine orange staining (28), the tachyzoites did not seem to be affected by this treatment. Then, 500 μl of fourfold concentrated HBSS was added to each chamber to restore the molarity. The remaining parasites were centrifugated at 500 × *g* for 10 min and washed with HBSS. Parasites were suspended in minimum essential medium–3% FCS and used to challenge HeLa cells. After 1 h of contact, HeLa cell monolayers were washed with HBSS, fixed with 0.4% 9-aminoacridine, Giemsa stained, mounted, and assessed microscopically for the number of infected cells.

Statistics. The differences between test and control groups were analyzed by using Student's *t* test. All experiments were performed a minimum of 3 times. The 50% inhibitory concentrations (IC_{50}s), the 95% confidence limits, and the IC_{90}s were calculated by probit analysis by the method of Litchfield and Wilcoxon (22).

RESULTS

[^{3}H]uracil incorporation. Different control experiments were first performed (Fig. 1). Uninfected macrophages and free tachyzoites did not incorporate significant amounts of tritiated uracil after 40 h of incubation. By contrast, macrophages infected with *T. gondii* showed marked uptake which was not altered by the presence of drug excipient. Increased

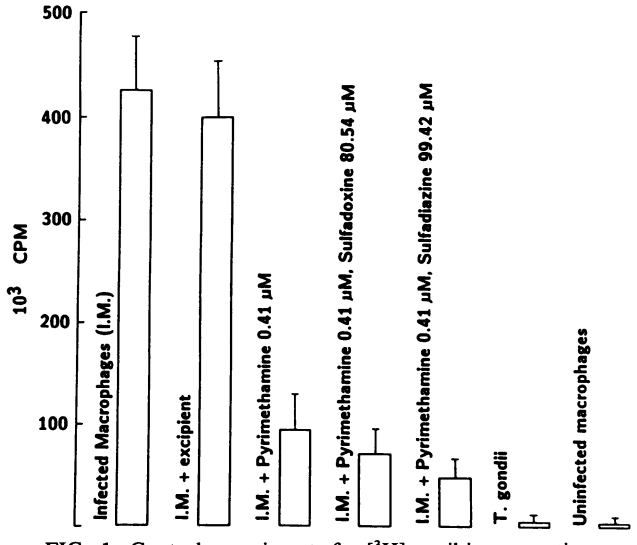

FIG. 1. Control experiments for [^{3}H]uracil incorporation.

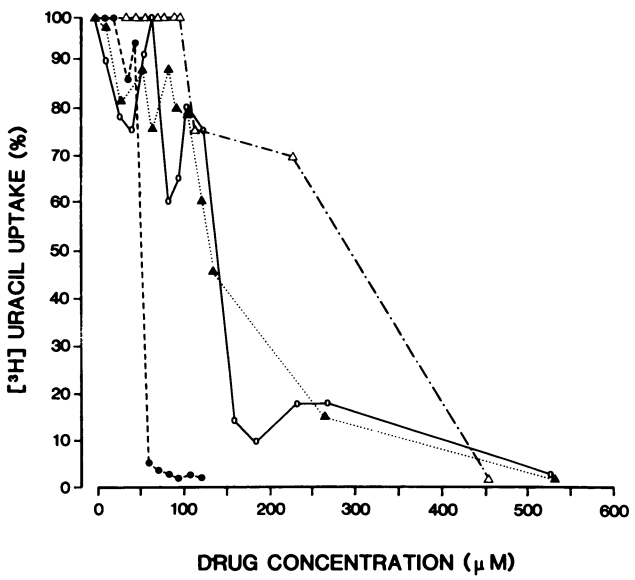

FIG. 2. Incorporation of [^{3}H]uracil by intracellular *T. gondii* after treatment with different concentrations of macrolides. Symbols: ●, roxithromycin; ○, A-56268; ▲, azithromycin; △, spiramycin.

inhibition of [^{3}H]uracil incorporation by infected macrophages was produced by pyrimethamine, pyrimethamine-sulfadoxine, and pyrimethamine-sulfadiazine ($P < 0.05$ compared with control). The four macrolides also inhibited [^{3}H]uracil incorporation (Fig. 2), allowing the calculation of the IC_{50}s and the IC_{90}s (Table 1).

Light microscopy studies. According to the results of the [^{3}H]uracil experiments, roxithromycin was the most active macrolide. Hence, this compound was selected for light microscopy studies to assess the inhibition of parasite multiplication. A significant reduction of the three criteria selected was observed, but numerous tachyzoites were still seen at a drug concentration corresponding to the IC_{90}s, as calculated above (Table 2). Compared with that of roxithromycin, the activity of pyrimethamine (0.41 μM)–sulfadiazine (99.42 μM) was similar with regard to the percentage of infected cells or the mean number of *T. gondii* per vacuole, but was superior with regard to the percentage of rosettes. Pyrimethamine alone had no effect compared with the controls at the concentration tested (0.41 μM).

Viability test. The four macrolides showed no killing effect when tested at concentrations corresponding to 1, 2, and 4 times their IC_{90}s, as calculated by the uracil incorporation study (Fig. 3). In the case of spiramycin, azithromycin, and A-56268, concentrations exceeding 1 mg/ml produced vacuolization of the macrophage cells. Therefore, only roxithromycin was tested at 8 and 16 times the IC_{90}, with which an incomplete killing effect was observed that was comparable to that of the combination of pyrimethamine-sulfadiazine.

TABLE 1. [^{3}H]uracil incorporation: IC_{50}s and IC_{90}s

Drug	IC_{50} (μM) (95% fiducial range)	IC_{90} (μM)
Roxithromycin	53.76 (38.48–72.84)	69.29
Azithromycin	140.18 (97.61–201.30)	333.77
A-56268	147.25 (101.40–213.81)	368.13
Spiramycin	246.32 (186.61–325.15)	338.98

TABLE 2. Results of light microscopy studies[a]

Drug (concn [μM])	% Infected cells at 24 h	Mean no. of *T. gondii*/ vacuole	% Infected cells at 24 h with rosettes
None (0)	23.5	8.9	62.5
Pyrimethamine (0.41)	16.4	6.2	40.6
Roxithromycin			
11.94	16.2	6.7	41.5
23.89	14.3[b]	6.8	44.5
35.84	10.6[b]	5.4[b]	35.5[b]
47.78	9.9[b]	4.8[b]	26.2[b]
59.73	12.4[b]	5.3[b]	21.0[b]
71.68	9.0	4.2[b]	15.5[b]
Pyrimethamine-sulfadiazine (0.41–99.42)	10.4[b]	3.7[b]	5.5[c]

[a] Data are the means of three replicate samples.
[b] $P < 0.05$, compared with control.
[c] $P < 0.01$, compared with control.

TABLE 3. Results of macrophage viability tests after 40 h of exposure to macrolides

Drug (concn [μM])	% Viable cells[a]
None (0)	95
Roxithromycin	
69.29	95
138.58	88
277.16	69
Azithromycin	
333.77	72
667.54	48
A-56268	
368.13	36
736.26	26
Spiramycin	
338.98	95
677.96	92

[a] Values are means of three determinations.

Toxicity test. Results obtained with the toxicity test are presented in Table 3. Roxithromycin and spiramycin did not significantly alter the viability of macrophages at concentrations corresponding to 1 and 2 times the IC_{90}. Roxithromycin at 4 times its IC_{90} and azithromycin and A-56268 at 1 and 2 times their IC_{90}s affected the viability of the cells.

DISCUSSION

Results of the uracil incorporation studies indicated that the macrolides are able to block the nucleotide synthesis of the intracellular parasites. Results of the light microscopy studies verified this effect, in that they showed that the multiplication rate of the parasite was hampered, at least with roxithromycin. Finally, results of the viability tests indicated that most of the tachyzoites continued to be able to invade HeLa cells, which are nonprofessional phagocytes. Hence, the macrolides tested exhibited essentially inhibitory properties against *T. gondii*. Only limited killing effects were observed at high concentrations of roxithromycin.

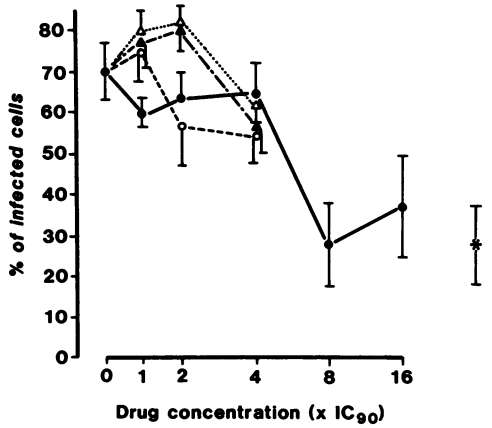

FIG. 3. Results of the viability test. Percent infected cells represents the percentage of HeLa cells infected after subinoculation by *T. gondii* pretreated with macrolides on macrophage monolayers. IC_{90}s were calculated after the [^{3}H]uracil incorporation studies. The isolated asterisk represents results obtained with pyrimethamine (0.41 μM)–sulfadiazine (99.42 μM). Symbols: ●, roxithromycin; ○, A-56268; ▲, azithromycin; △, spiramycin. Bars indicate the standard deviation.

In the case of roxithromycin, the light microscopy studies verified the uracil experiments in that they showed that the multiplication rate of the parasite was altered. By contrast Luft (23) has shown that roxithromycin, at dosages of up to 100 μg/ml, has no effect against the percentage of cells infected with *T. gondii* and the number of *T. gondii* per infected vacuole. Different incubation times with the antimicrobial agents (16 h versus 24 h in our study) and the pH of the media may account for this discrepancy. Macrolides are known to loose part of their in vitro potency at acidic pHs (15), and acidity is common with cultured macrophages (1). We checked carefully that the pH was maintained at 7.2 during all the experiments, while the actual pH was not mentioned by Luft (23).

However, the toxicity of macrolides on macrophages is an issue to be considered. At concentrations corresponding to the IC_{90}s, roxithromycin and spiramycin seemed to have no effect on the viability of macrophages, contrary to the results for azithromycin and A-56268; and this nonspecific effect of azithromycin and A-56268 might well have affected the toxoplasma metabolism.

The amount of drug that produced an observable effect in our system appeared to be significantly higher than the amount that was attainable in serum and the different tissues of humans given a conventional dose of that drug. Peak levels of roxithromycin in plasma have been found (32) to be 9.1 and 10.8 μg/ml after three single oral doses of 300 or 450 mg per healthy human, respectively; and the tissue/serum ratio has been shown to be equal or inferior to 1 in most cases (3). The concentration of spiramycin in tissue and plasma also appeared to be notably lower than the IC_{50} calculated in our study (19). However, both roxithromycin (5, 6, 17) and spiramycin (6, 14) have shown some activity in animal models, in which the corresponding drug (5, 34) concentrations in plasma were again lower than our IC_{50}s. This indicates that the correlation between this in vitro model and the clinical situation cannot be made on a simple weight basis.

Since *T. gondii* is a eucaryotic cell, the activity of macrolides against the parasite is somewhat surprising. In early studies (30) it was demonstrated that erythromycin inhibits bacterial protein synthesis by binding to the 50S ribosomal unit, which prevents elongation of the peptide chain. Spiramycin and carbomycin (and probably all macrolides) act similarly (27). Erythromycin does not bind to mammalian 80S ribosomes, and this accounts in part for its selective toxicity (25). Therefore, to explain the macrolide activity against *T. gondii* we provide the following two hypotheses. (i) Macrolides may interact with the parasite ribosome, as they do with bacteria. However, fractionation of RNA extracted from *T. gondii* by sedimentation revealed the presence of three major RNA components sedimenting at 24S, 19S, and 4S to 5S (33), a pattern different from that obtained with bacteria. Thus, to prove this hypothesis, a macrolide-binding site must be demonstrated in the ribosomal components of *T. gondii*. (ii) Macrolides may have a nonribosomal mode of action. Among the possibilities here, a stimulating effect of the macrolides on the microbicidal activity of the phagocytes cannot be ruled out. Investigations have shown, for instance, that roxithromycin, even at a concentration as low as 0.1 μg/ml, significantly enhances the phagocytosis and killing of *Staphylococcus aureus* by neutrophils (20).

Whatever their mode of action, macrolides need to penetrate into the macrophage to exert their activity against intracellular *T. gondii*. In a recent study (4), it has been shown that all macrolides are not similar in this respect. In macrophages of human and animal origin, roxithromycin accumulated more consistently and significantly than erythromycin, the former thus reaching considerably higher intracellular/extracellular concentration ratios. As a result, differences in the inhibitory effects among the four macrolides found in our study do not necessarily reflect corresponding differences in intrinsic activity. Indeed, different intracellular pharmacokinetics may also have to be considered.

In conclusion, macrolides may provide a safe and effective alternative to current therapy for the treatment of toxoplasmosis. Taking into account the fact that the macrolides exhibited an incomplete killing effect on *T. gondii*, synergistic combinations of these antibiotics with other compounds should be explored, as recently reported for roxithromycin and gamma interferon (17). Clinical studies are warranted to determine the relative efficacy and safety of these drugs in humans.

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