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New Micromethod to Study the Effect of Antimicrobial Agents on *Toxoplasma gondii*: Comparison of Sulfadoxine and Sulfadiazine Individually and in Combination with Pyrimethamine and Study of Clindamycin, Metronidazole, and Cyclosporin A

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An in vitro method by which reagents, cells, and *Toxoplasma gondii* trophozoites are conserved (micromethod) was developed to quantitate the effect of antimicrobial agents on *T. gondii*. Sulfadoxine alone had no effect on *T. gondii* in vitro when evaluated with a macromethod, the new micromethod, or visual inspection of Giemsa-stained preparations. Sulfadoxine combined with pyrimethamine inhibited *T. gondii* more than did pyrimethamine alone, but the combination of sulfadoxine plus pyrimethamine was slightly less active than was the combination of sulfadiazine plus pyrimethamine. Neither clindamycin nor metronidazole, alone or in combination with sulfadiazine or pyrimethamine and sulfadiazine, had any effect on intracellular *T. gondii*. Brief exposure (10 min before and during challenge) to clindamycin had no effect on extracellular *T. gondii* when clindamycin was studied alone or with sulfadiazine or pyrimethamine plus sulfadiazine. Cyclosporin A inhibited *T. gondii* replication at concentrations of ca. ≥2 μg/ml.

In vitro assays have been used to evaluate the effects of antimicrobial agents on *Toxoplasma gondii* (2, 8, 9), but none of these methods have been designed to minimize the amounts of cells, *T. gondii* trophozoites, and reagents used. We developed such a system and then utilized this system to study the effects of a number of antimicrobial agents. Pyrimethamine and sulfadiazine were studied to determine whether the micromethod would demonstrate the inhibitory effects on *T. gondii* that have been described clinically and for other in vitro and in vivo assay methods. Clindamycin was studied because of its efficacy in the treatment of toxoplasmosis in mouse and rabbit models (1, 7, 10), and metronidazole was studied because it is effective against another protozoan, *Entamoeba histolytica*. Sulfadoxine was studied because it has a half-life that is substantially longer than those of sulfadiazine and the triple sulfonamides and it is available in combination with pyrimethamine, which has a half-life of days. Cyclosporin A was studied because of its antimalarial effect in vitro and in vivo (11) and the current development of compounds related to cyclosporin A that retain antimicrobial but not immunodepressive effects.

MATERIALS AND METHODS

Culture of peritoneal macrophages. (i) Preparation of cell suspension. Peritoneal exudate cells from Swiss Webster female mice (Laboratory Supply, Indianapolis Ind.) weighing 20 g each were cultured as previously described (4). Briefly, after harvesting, peritoneal exudate cells were suspended at a concentration of 4×10^6 cells per ml in medium 199 (GIBCO, Grand Island, N.Y.) that contained 10% heat-inactivated (60 min, 56°C) fetal calf serum (GIBCO), penicillin (100 U/ml), and streptomycin (100 μg/ml) (M199-FCS).

(ii) Macromethod. A 0.5-ml amount of the cell suspension was placed into each well of 24-well plates (2 cm^2; Linbro Scientific Inc., Hamden, Conn.) and four-chamber Lab-Tek slides (2 cm^2; Miles, Naperville, Ill.) which were then incubated at 37°C for 1 to 4 h to permit macrophages to adhere. Nonadherent cells were then removed, and cultures were washed with Hanks balanced salt solution (GIBCO).

(iii) Micromethod. A 0.1-ml amount of the cell suspension was placed into each well of 96-well plates (0.28 cm^2; Linbro Scientific Inc.). Cultures were incubated for 1 to 4 h at 37°C before challenge. Nonadherent cells were not removed from the cultures because eliminating this step simplified performance of the assay and did not alter the results.

Challenge of cultures with *T. gondii*. (i) Macromethod. Cultures were challenged with the RH strain of *T. gondii* as previously described (5, 6). Briefly, the RH strain of *T. gondii* was obtained by peritoneal lavage of mice infected intraperitoneally 2 days earlier. *T. gondii* trophozoites were filtered free of host cells, and 0.5 ml of M199-FCS that contained 2×10^6 trophozoites of the RH strain of *T. gondii* was placed on the adherent peritoneal exudate cell cultures in each well. After 1 h, cultures were washed with Hanks balanced salt solution, and 1 ml of M199-FCS was placed on the culture.

(ii) Micromethod. A 0.1-ml amount of M199-FCS that contained 4×10^5 trophozoites of the RH strain of *T. gondii* was added to each well containing peritoneal exudate cells in 0.1 ml of M199-FCS.

Antimicrobial agents. The following antimicrobial agents (obtained from the indicated manufacturers) were utilized: pyrimethamine (Burroughs Wellcome Co., Research Triangle Park, N.C.), sodium sulfadoxine (Roche, Basel, Switzerland), sodium sulfadiazine (City Chemical Co., New York, N.Y.), clindamycin phosphate (The Upjohn Co., Barceloneta, P.R.), metronidazole (G. D. Searle & Co., Chicago, Ill.), and cyclosporin A (Sandoz Pharmaceuticals, East Hanover, N.J.). All dilutions were made in medium 199, with the exception of pyrimethamine and cyclosporin A, which were initially dissolved in ethanol; pyrimethamine, at a concentration of 5 mg/ml; and cyclosporin A, at a concentration of 10 mg/ml. M199-FCS containing the amounts of ethanol necessary to dissolve the pyrimethamine and cyclosporin A was

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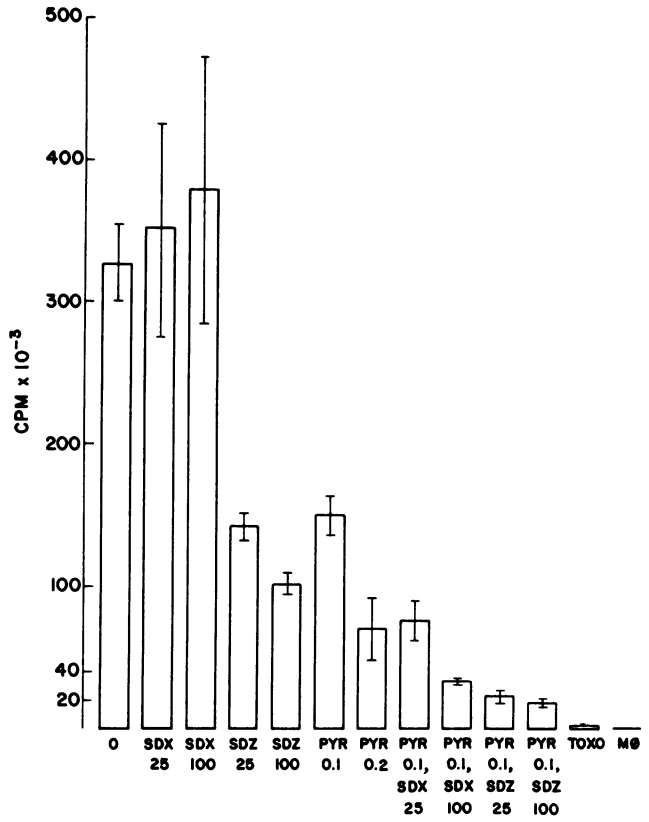

FIG. 1. Effect of antimicrobial agents on *T. gondii* evaluated by the macromethod. SDX, Sulfadoxine; SDZ, sulfadiazine; PYR, pyrimethamine; TOXO, *T. gondii* alone; Mϕ, macrophages alone. Concentrations of antimicrobial agents are shown in micrograms per milliliter; data are means ± standard deviation for four replicate wells.

shown to have no effect on *T. gondii* in separate experiments. Dilutions were freshly prepared for each experiment. In the macromethod, 1 h after the challenge was removed the antimicrobial agents were added in 50-μl aliquots to produce the following final concentrations (in micrograms per milliliter): pyrimethamine, 0.1 and 0.2; sodium sulfadoxine, 25 and 100; sodium sulfadiazine, 25 and 100; clindamycin, 5, 25, and 100; metronidazole, 5 and 50; and cyclosporin A, 0.1 to 10. Final concentrations of the antimicrobial agents were the same in the micromethod but were added in 25-μl amounts and the challenge was not removed. These antimicrobial agents remained in the cultures for the next 40 h. In other experiments carried out to evaluate effect of clindamycin on extracellular *T. gondii*, antimicrobial agents were incubated with *T. gondii* trophozoites for 10 min before challenge and then were present for the next 40 h.

Quantitation of effect of antimicrobial agents on *T. gondii*.
(i) Stained preparations. Monolayers were fixed with 0.4% aminoacridine in 50% ethanol and Giemsa stained. The percentage of infected cells, mean number of *T. gondii* trophozoites per vacuole, and mean number of cells per high-power field (×400) were determined as described previously (4–6).

(ii) Macromethod. At 20 h after the addition of antimicrobial agents to infected and uninfected wells, 10 μCi of [5,6-^{3}H]uracil (specific activity, 40 to 60 Ci/mmol; Amersham Corp., Arlington Hts., Ill.) was added and cultures were incubated for an additional 20 h. Incorporation of [^{3}H]uracil into acid-precipitable material was measured by a filtration procedure (6). Briefly, supernatants aspirated from each well were centrifuged at 850 × *g* for 10 min at 4°C. Monolayers and the sediment from the supernatants from each well were placed with 1% sodium dodecyl sulfate containing 100 μg of uracil (Sigma Chemical Co., St. Louis, Mo.) per ml for 1 h at room temperature. The disrupted supernatant from each well was then placed with cells from that well and stored for 1 h at 4°C. Trichloroacetic acid was added to the disrupted cells and supernatants to produce a final concentration of 5% trichloroacetic acid. The resulting precipitates were maintained at 4°C and collected on glass filters (25 mm, type A-E; Gelman Sciences, Inc., Ann Arbor, Mich.). Material retained by the filters was washed with cold 5% trichloroacetic acid, rinsed with 95% ethanol, dried, and counted with a liquid scintillation spectrophotometer (6).

(iii) Micromethod. At 20 h after the addition of antimicrobial agents, 25 μl of medium 199 containing 2.5 μCi of [5,6-^{3}H]uracil was added to each well, and cultures were then incubated for 20 h. Cells were dislodged by alternating vigorous washing and aspirating with isotonic saline and collected on glass filters (934-Ah; Whatman, Inc., Clifton, N.J.) with a multiple automated sample harvester (MASH II; M. A. Bioproducts, Walkerville, Md.). Filters were dried, and material retained by the filters was counted with a liquid scintillation spectrophotometer (6).

TABLE 1. Effect of sulfadoxine, sulfadiazine, and pyrimethamine on *T. gondii* evaluated in preparations examined by light microscopy

Antimicrobial agent (concn [μg/ml])[a]	% Infected cells at 24 h	% Infected cells at 24 h with ≥8 *T. gondii* trophozoites per vacuole	No. of cells per hpf at 24 h[b]
None (0)	30	100	80
SDX (25)	26	100	128
SDX (100)	34	100	80
SDZ (25)	12	100	87
SDZ (100)	24	100	139
PYR (0.1)	21	10	70
PYR (0.2)	18	10	156
PYR (0.1) + SDX (25)	17	12	200
PYR (0.1) + SDZ (25)	14	6	240

[a] SDX, sulfadoxine; SDZ, sulfadiazine; PYR, pyrimethamine.
[b] At 40 h, there was preservation of the monolayer by sulfadiazine (25 or 100 μg/ml) and pyrimethamine (0.1 and 0.2 μg/ml), as well as the combinations of pyrimethamine plus sulfadoxine or sulfadiazine. At 40 h, sulfadoxine (25 or 100 μg/ml) did not preserve the monolayer. hpf, High-power field.

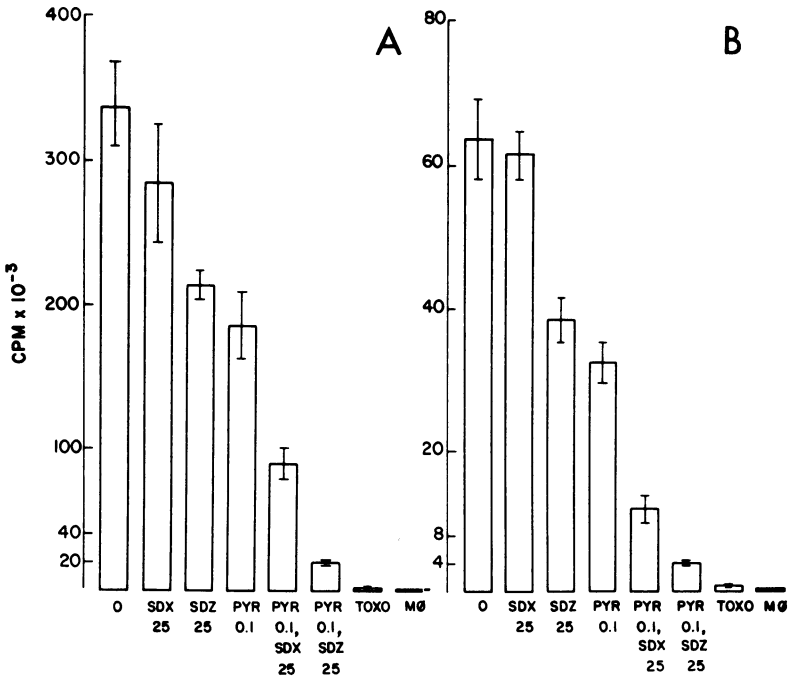

FIG. 2. Comparison of macromethod (A) and micromethod (B) in evaluating the effects of antimicrobial agents on *T. gondii*. SDX, Sulfadoxine; SDZ, sulfadiazine; PYR, pyrimethamine; TOXO, *T. gondii* alone; Mϕ, macrophages alone. Concentrations of antimicrobial agents are shown in micrograms per milliliter; data are means ± standard deviation for four replicate wells.

Statistics. Significance of differences was evaluated by the multiple-range test of Neumann Keuls (12). *P* values of ≤0.05 were considered to be significant.

RESULTS

Comparison of macromethod and micromethod. Initially, the macromethod was utilized to ascertain that results obtained with the macromethod accurately reflected the quantitation of the effect of antimicrobial agents by visual examination of stained preparations, as described by others (2). Data from experiments in which the macromethod was used to study antimicrobial agents (Fig. 1) reflected the same inhibition of *T. gondii* by pyrimethamine, sulfadiazine, and the combinations of sulfadiazine plus pyrimethamine and sulfadoxine plus pyrimethamine, as was seen on preparations examined with light microscopy (Table 1). Data obtained when effects of antimicrobial agents were evaluated by the macromethod and the micromethod simultaneously demonstrated the same effects of antimicrobial agents, (Fig. 2). Inhibition of *T. gondii* by antimicrobial agents was indicated by a reduction in the percentage of infected cells, reduction in the percentage of cells infected with ≥8 *T. gondii* trophozoites per vacuole, and lack of destruction of the cell cultures by *T. gondii* when antimicrobial agent-treated and control cultures were compared (Table 1). For example, when cultures treated with 0.1 μg of pyrimethamine plus 25 μg of sulfadiazine per ml were compared with cultures that contained no antimicrobial agent, the percentage of infected cells was reduced from 30 to 14%, the percentage of cells infected with ≥8 *T. gondii* trophozoites per vacuole was reduced from 100 to 6%, and there were more (240 versus 80) cells per high-power-(× 400) field in the cultures treated with pyrimethamine plus sulfadiazine. Inhibition of *T. gondii* by antimicrobial agents was indicated by a reduction in the incorporation of radiolabeled uracil into the nucleic acids of *T. gondii* trophozoites (Fig. 1 and 2). For example, when cultures treated with 0.1 μg of pyrimethamine and 25 μg of sulfadiazine per ml were compared with cultures that contained no antimicrobial agent, the incorpo-

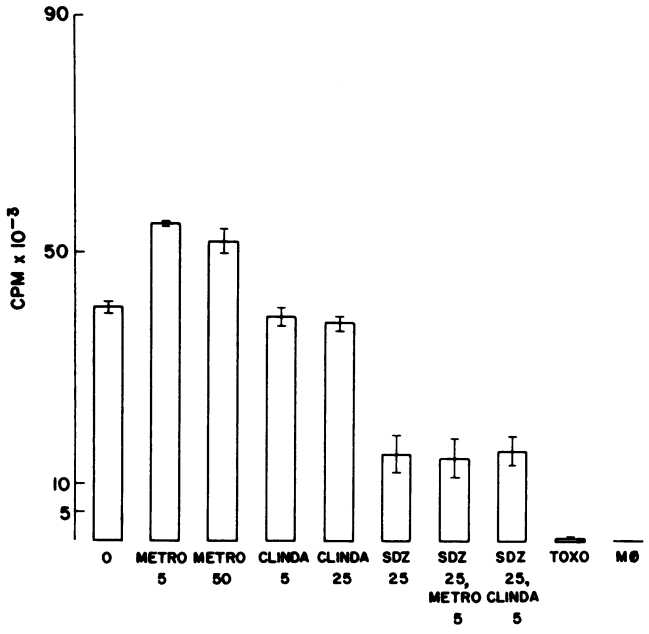

FIG. 3. Lack of effect of clindamycin and metronidazole on *T. gondii* evaluated with the micromethod. CLINDA, Clindamycin; METRO, metronidazole; SDZ, sulfadiazine; TOXO, *T. gondii* alone; Mϕ, macrophages alone. Concentrations of antimicrobial agents are shown in micrograms per milliliter; data are means ± standard deviation for four replicate wells.

TABLE 2. Effect of clindamycin alone and in combination with pyrimethamine and sulfadiazine on incorporation of uracil by *T. gondii* evaluated with the micromethod

Antimicrobial agent (concn [μg/ml])[a]	Uracil uptake (cpm) in the presence of drug[b]:	
	Before (10 min), during, and after challenge	1 h after challenge
None	34,792 ± 5,487	50,018 ± 10,702
CLINDA (25)	32,202 ± 6,799	41,313 ± 2,303
PYR (0.1) + SDZ (25)	3,412 ± 1,555	2,486 ± 171
CLINDA (25), PYR (0.1), + SDZ (25)	3,918 ± 588	2,381 ± 345
T gondii alone	870 ± 188	570 ± 108
Unchallenged macrophages	498 ± 115	426 ± 102

[a] CLINDA, Clindamycin; PYR, pyrimethamine; SDZ, sulfadiazine.
[b] Results are means ± standard deviation of four replicate wells.

ration of uracil into the nucleic acids of intracellular *T. gondii* was reduced from 3.25×10^5 to 2.5×10^4 cpm (Fig. 1) or from 6.5×10^4 to 5×10^3 cpm (Fig. 2). Uracil incorporated in extracellular *T. gondii* and cells other than those of *T. gondii* was minimal in all experiments.

Comparison of sulfadiazine and sulfadoxine. Sulfadoxine alone had no effect on *T. gondii* (Fig. 1 and 2; $P > 0.05$). Pyrimethamine combined with sulfadoxine inhibited *T. gondii* more than pyrimethamine alone (Fig. 1 and 2; $P < 0.05$), but the inhibitory effect of pyrimethamine plus sulfadiazine was greater than that of pyrimethamine plus sulfadoxine (Fig. 1 and 2; $P < 0.05$).

Lack of effect of metronidazole on intracellular *T. gondii*. Metronidazole (5 or 50 μg/ml) added after challenge had no effect on *T. gondii* (Fig. 3; $P > 0.05$). Metronidazole did not alter the antimicrobial effect of sulfadiazine (Fig. 3; $P > 0.05$).

Lack of effect of clindamycin on extracellular and intracellular *T. gondii*. Clindamycin had no effect on extracellular *T. gondii* at a concentration of 25 μg/ml (Table 2; $P > 0.05$) or 100 μg/ml (data not shown) or intracellular *T. gondii* at a concentration of 5 or 25 μg/ml (Fig. 3 and Table 2; $P > 0.05$). This antimicrobial agent did not change the antimicrobial effect of sulfadiazine alone or sulfadiazine plus pyrimethamine (Fig. 3 and Table 2; $P > 0.05$).

Inhibitory effect of cyclosporin A on *T. gondii*. Cyclosporin A concentrations of ≥2 μg/ml were inhibitory to *T. gondii* when evaluated visually (e.g., at 20 h after infection, the numbers of *T. gondii* trophozoites per vacuole were 6.2 [no cyclosporin A], 5.2 [2 μg of cyclosporin A per ml], and 2.1 [10 μg of cyclosporin A per ml] and by the micromethod (Tables 3 and 4). Effects on *T. gondii* were apparent after 20 h in culture.

TABLE 3. Effect of cyclosporin A on *T. gondii* within macrophages evaluated with the micromethod[a]

Cyclosporin A concn (μg/ml)	Uracil incorporation (cpm)[b]
0	88,065 ± 3,579
0.5	83,901 ± 3,263
1.5	79,692 ± 3,586
2.0	58,877 ± 9,293
5.0	55,118 ± 5,261
10.0	15,925 ± 1,286
0	3,960 ± 272[c]
0	626 ± 107[d]

[a] One representative experiment of six.
[b] Results are means ± standard deviation for four replicate wells.
[c] *T. gondii* alone.
[d] Unchallenged macrophages.

TABLE 4. Percentage of inhibition of *T. gondii* by cyclosporin A evaluated with the micromethod

Cyclosporin A concn (μg/ml)	% Inhibition[a]
0	0
0.5	22 ± 15
1.0	27 ± 20
1.5	27 ± 20
2.0	30 ± 16
3.0	27 ± 18
4.0	50 ± 16
5.0	58 ± 15
10.0	95 ± 6

[a] Results are the means of six experiments ± standard deviation. Percentage inhibition was calculated as 100 × [1 − (incorporation (counts per minute) of uracil by *T. gondii* in culture with antimicrobial agent/incorporation (counts per minute) of uracil by *T. gondii* in culture without antimicrobial agent)].

DISCUSSION

A micromethod was developed to quantitate the effect of antimicrobial agents on *T. gondii* within macrophages. This micromethod proved to be an effective way to evaluate the effect of antimicrobial agents on intracellular and extracellular (with brief exposure) *T. gondii* and conserved cells, *T. gondii* trophozoites, and reagents when compared with either of the other methods studied (macromethod, in which uptake of tritiated uracil into *T. gondii* nucleic acids is evaluated [2], or visual evaluation of Giemsa-stained preparations [8]). The results obtained when this micromethod was used to test pyrimethamine plus sulfadiazine correlated well with those obtained clinically by others and from the previously described in vivo methods and macromethods.

Sulfadoxine alone had no effect on *T. gondii* in vitro when evaluated with the macromethod, the new micromethod, or visual inspection of Giemsa-stained preparations, but sulfadoxine combined with pyrimethamine inhibited *T. gondii* considerably more than did pyrimethamine alone. The combination of sulfadoxine and pyrimethamine, however, was slightly less active than that of sulfadiazine and pyrimethamine. The significance of these data can be assessed only by comparison of such combinations in an appropriate model in vivo.

Clindamycin and metronidazole, alone or in combination with sulfadiazine or pyrimethamine and sulfadiazine, had no effect on intracellular *T. gondii*. Brief exposure of *T. gondii* to clindamycin alone had no effect on extracellular *T. gondii* and did not change the effect of the combination of sulfadiazine plus pyrimethamine or that of sulfadiazine as a single agent. This lack of effect of clindamycin on *T. gondii* is in contrast with in vivo results reported by others (1, 7, 10) and may be due to a need for more prolonged exposure of extracellular *T. gondii* to the antibiotic, the effect in vivo being due to a metabolite rather than to the parent compound, lack of penetration of clindamycin into mouse macrophages, which might be necessary for a sustained effect on *T. gondii* or an unrecognized artifact of this in vitro system. Clindamycin has been found to be actively transported into guinea pig alveolar macrophages (3), but whether clindamycin entered mouse peritoneal macrophages in our system was not determined.

Treatment with cyclosporin A resulted in some inhibition

of *T. gondii* replication within macrophages in vitro at approximately the maximum concentrations achieved in human serum. A near-maximal effect occurred only at concentrations higher than those achieved clinically. Although this observation is of interest and might influence the incidence and outcome of toxoplasmosis in organ transplant recipients treated wtih cyclosporin A, the immunodepression produced would detract from its possible usefulness as an antimicrobial agent. It will be of interest, however, to determine whether compounds currently being developed that are related to cyclosporin A but which are not immunodepressive retain the antimicrobial effects on *T. gondii*. Our study also demonstrates that the antimicrobial activity of cyclosporin A for *T. gondii* within macrophages would limit its usefulness for studying the roles of T lymphocytes and natural killer cells in protection in *T. gondii* infection. The mechanism of inhibition of *T. gondii* within macrophages by cyclosporin A remains to be determined.

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