

In Vivo Assessment of Antimicrobial Agents against *Toxoplasma gondii* by Quantification of Parasites in the Blood, Lungs, and Brain of Infected Mice

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The in vivo effects of antimicrobial agents against *Toxoplasma gondii* were evaluated in mice that were infected intraperitoneally with 10^4 tachyzoites of the RH strain by determination of survival rates and study of the kinetics of growth of *T. gondii* in infected mice. At various intervals after infection, subcultures of serial dilutions of blood, lung, and brain homogenates were performed in fibroblast tissue cultures for determination of parasitic loads. Pyrimethamine (18.5 mg/kg per day), sulfadiazine (375 mg/kg per day), and clindamycin (300 mg/kg per day) were administered for 10 days from day 1 or day 4 after infection. Untreated control mice died within 9 days and showed early and predominant lung involvement. All mice treated with sulfadiazine administered from day 1 survived and were apparently healthy; parasitic loads decreased early after treatment, but a relapse was observed 5 days after the cessation of therapy. When pyrimethamine was administered from day 1, 7 of 11 mice died within 25 days; by determination of parasitic loads, the effect of pyrimethamine was only demonstrable from day 6, and a relapse was constantly observed after the cessation of therapy. When pyrimethamine and sulfadiazine were administered in combination, 100% of mice survived; when therapy was started at day 1, parasites remained undetectable; in mice treated from day 4, parasites were eradicated by day 8 but infection relapsed 8 days after the cessation of therapy. All mice treated with clindamycin from day 1 or day 4 died within 10 days, but parasitemia was always undetectable. These results indicate that study of the kinetics of parasitic loads in blood and organs may provide additional information on the effect of antimicrobial agents against *T. gondii* in regard to the evolution of the infection and may represent a reliable basis for the determination of therapeutic regimens in humans.

In vivo experimental studies of *Toxoplasma gondii* are usually performed in mice that are either acutely infected by intraperitoneal injection of tachyzoites or chronically infected by peroral ingestion or intraperitoneal injection of cysts. Another alternative, which better fits with the pathophysiology of toxoplasmosis in immunocompromised human patients, is to induce a reactivation in chronically infected mice by depleting the CD4⁺ lymphocyte subpopulation (19), administering anti-gamma interferon (18), or creating a model of intracerebral infection by local injection of tachyzoites (11, 12). Assessment of the efficacy of antimicrobial agents is based on the comparative study of mean survival times of untreated and treated mice, enumeration of brain cysts, and in some cases, histologic examination of organs and subinoculation to mice of dilutions of organ homogenates. The survival rate is an important parameter which can be determined easily but which is poorly informative regarding the mode of drug action. Since additional histological studies and mice subinoculations are time-consuming and may not be applied for extensive studies, we proposed the use of a tissue culture method for evaluating the kinetics of growth of *T. gondii* in infected mice. For the optimum reproducibility of infection, we chose to create acute infections in Swiss mice of the virulent RH strain and to monitor the kinetics of parasitic growth in blood and those organs which most commonly become infected in patients with acquired immunodeficiency syndrome, i.e., the brain and lung (15; C. Marche, M. Wolff, R. Mayorga, S. Matheron, D.

Salmon, and L. Matthiesen, 5th Int. Conf. AIDS, WBP23, p. 355, 1989).

This model was used to study the effect of pyrimethamine and sulfadiazine, either alone or in combination, and clindamycin by comparing their efficacies when administered 1 or 4 days after infection.

MATERIALS AND METHODS

Mice. Adult Swiss Webster female mice (age, 6 to 8 weeks; weight, 18 to 20g; Iffa Credo, Lyon, France) were used in all experiments.

Parasites. The virulent RH strain of *T. gondii* was maintained in mice by syringe passages of intraperitoneal fluid from infected mice at 3-day intervals. The peritoneal cavities of infected mice were washed with 5 ml of physiological saline, and then the tachyzoites that were collected were enumerated with a hemacytometer and adjusted to a concentration of 2×10^4 /ml. Mice were infected by intraperitoneal injection of 0.5 ml (i.e., 10^4 tachyzoites).

Determination of parasitic loads in infected mice. Parasitic loads were determined by subculturing of serial dilutions of blood, brain, and lung homogenates on fibroblast tissue cultures. Cultures of MRC5 fibroblasts (Bio-Mérieux, Lyon, France) were prepared as described previously (5) by using minimum essential medium (Eurobio, Paris, France) supplemented with glutamine (290 µg/ml), penicillin (50 IU/ml), streptomycin (50 µg/ml), and 10% heat-inactivated fetal bovine serum (Flow Laboratories, Paris, France). Twenty thousand cells in 200 µl of culture medium were seeded into each well of 96-well tissue culture plates and grown to

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confluence at 37°C in a moist 5% CO₂-95% air atmosphere. Culture plates were used within 10 days after preparation.

Infected mice were killed by cervical dislocation. Blood was collected immediately from the retroorbital sinus, and then lungs and brain were removed and washed with phosphate-buffered saline (PBS) solution-0.15 M NaCl (pH 7.2). Each organ was gently wiped, weighed, and then homogenized in a tissue grinder (Ultra Turrax; Janke and Kunkel Co., Staufen, Federal Republic of Germany) with 4 ml of PBS supplemented with penicillin and streptomycin. From each blood and organ suspension, serial fourfold dilutions were prepared in the culture medium, and then 40 µl of each dilution was inoculated into duplicate wells of the tissue culture plates. After 72 h of incubation at 37°C, plates were emptied, washed by immersion in PBS, fixed with cold methanol for 10 min, and air dried. Demonstration of *T. gondii* in the culture was performed by an indirect immunofluorescence assay as described previously (6). Briefly, 50 µl of a dilution of a rabbit anti-*T. gondii* antibody was added to each well and incubated for 30 min at 37°C. After two washings with PBS, 50 µl of a dilution of a fluorescein-labeled anti-rabbit immunoglobulin G conjugate (Diagnostics Pasteur, Marne, France) was added and incubated for an additional 30 min. After two washings, the plates were emptied and examined under an inverted fluorescence microscope at a magnification of ×100. The presence of parasitic foci was recorded in each well. The final titer was the last dilution for which the tissue culture contained at least one parasitic focus. The number of parasites per gram or milliliter (parasitic load) was calculated as follows: parasitic load = (reciprocal titer in tissue culture/volume [ml] or weight [mg]) × 1,000.

Kinetics of infection in untreated mice. Thirty mice were inoculated with 10⁴ parasites at day 0 and were allocated to two groups. One group of 10 mice (group A) was used for determination of survival rates. Another group of 20 mice (group B) was used for assessment of parasitic loads. At 2-day intervals from day 0 until the time of death, five mice of group B were sacrificed and examined for parasitic loads in blood, lungs, and brain, as described above.

Assessment of the effect of antimicrobial agents. Pyrimethamine (Sigma Chemical Co., St. Louis, Mo.) and sulfadiazine (Laboratoires Doms, Paris France) were obtained in powder form. According to Kovacs et al. (14), the dosage regimens were 18.5 mg/kg per day for pyrimethamine and 375 mg/kg per day for sulfadiazine, which were administered either alone or in combination. The drugs were given once a day by tube feeding. Clindamycin in liquid phosphate salt form (Laboratoire Upjohn, Paris, France) was administered at 300 mg/kg per day, as described by Araujo and Remington (1), by subcutaneous injections twice daily (150 mg/kg for each injection). Treatments were administered for 10 days from day 1 with each antimicrobial agent alone or in combination and from day 4 with clindamycin and the pyrimethamine-sulfadiazine combination.

For each experiment, 80 to 82 mice were used; 30 control mice were not treated; 10 to 12 mice were treated and used for the determination of the mean survival time (group A); 40 treated mice were used for determination of parasitic loads (group B). At various intervals a group of five mice was sacrificed, and subcultures were performed. When the regimen was started on day 1 after infection, mice were examined on days 4, 6, 8, 10, 15, 20, and 30 after infection. When the regimen was started on day 4, mice were examined on days 6, 8, 10, 15, 20, and 30.

Sensitivity of the culture method. The sensitivity of the

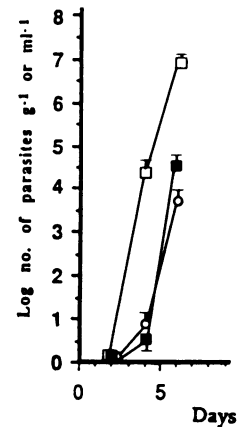


FIG. 1. Kinetics of parasitic loads in blood (O), lungs (□), and brain (■) in control mice infected on day 0 with 10⁴ tachyzoites of the RH strain. Each point represents the mean ± standard error of the mean for five mice.

culture method and the potential effect of the antimicrobial agents that were present in ground tissue were examined in a separate experiment. Five groups of five mice each were used; one group was not treated and four groups were treated for 3 days with pyrimethamine, sulfadiazine, clindamycin, and pyrimethamine combined with sulfadiazine by using the same posology described above. Mice were sacrificed, and then 10⁶ freshly harvested tachyzoites were added to each blood, lung, and brain preparation. Homogenates were prepared and then serially diluted, and 40 µl of each dilution was inoculated into tissue culture and then cultivated for 72 h. Cultures were examined for *T. gondii* by immunofluorescence as described above, and reciprocal titers were recorded for each organ. The sensitivity of the tissue culture method was estimated by comparing the final titer in the culture with the inoculum of 10⁶ parasites that was added to the preparations. The effect of the tissue preparation and the carry-over effect of each drug were examined by a two-way analysis of variance (17).

RESULTS

Determination of parasitic loads. Inoculation of serial dilutions of blood and organ homogenates into the cultures did not destroy the monolayers, even at the first dilution of 1/100. By immunofluorescence, parasitic foci were easily observed at a magnification of ×100; with heavily infected organs, these foci were confluent at the first dilution, but monolayers were preserved.

In the experiment in which 10⁶ tachyzoites were added to blood and tissue preparations, no significant difference of sensitivity was observed because of the organ factor ($P > 0.1$). Mean reciprocal titers were 5.49 ± 0.16 log units in blood, 5.46 ± 0.19 log units in lungs, and 5.43 ± 0.16 log units in brain. These results indicate that the tissue culture method allows for the demonstration of less than 10 parasites in these preparations. In mice that were treated for 3 days, no significant differences because of any treatment factor were observed for blood, lungs, and brain ($P > 0.1$).

Results of the different experiments with infected mice are given in Fig. 1 to 5. For each date of testing of controls, the logarithms ± standard errors of the mean of the parasitic load determined from the individual value from five group B mice are presented.

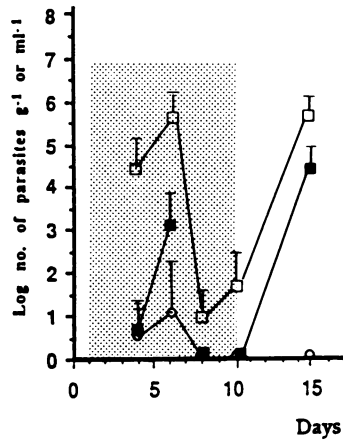


FIG. 2. Kinetics of parasitic loads in blood (○), lungs (□), and brain (■) in mice infected on day 0 with 10^4 tachyzoites of the RH strain and treated with pyrimethamine (18.5 mg/kg per day) for 10 days from day 1 after infection. Each point represents the mean \pm standard error of the mean for five mice. The shaded area represents the period of administration of antimicrobial agents.

Survival rates. The survival rates for the different group A mice (estimated by the Kaplan Meier product limit method) are presented in Fig. 6.

Kinetics of infection in untreated mice. The mean survival time was 7 days in group A mice (Fig. 6). Parasitic loads were determined in group B mice on days 2, 4, and 6 (Fig. 1); parasites were first detectable in lungs on day 2, and the parasitic load progressively increased throughout the follow-up period, with a maximum value of 7.1 ± 0.1 log units on day 6. Parasites were demonstrated in the brain on day 4. The parasitic load always remained at a lower level in brain compared with that in lungs (mean value, 4.7 ± 0.3 log units on day 6). Parasitemia was only detectable on day 4, with a maximum value of 3.8 ± 0.2 log units on day 6.

Effect of antimicrobial agents. When pyrimethamine was administered from day 1, 5 of 11 group A mice survived until day 30; death occurred between days 7 and 24 in 6 mice (Fig.

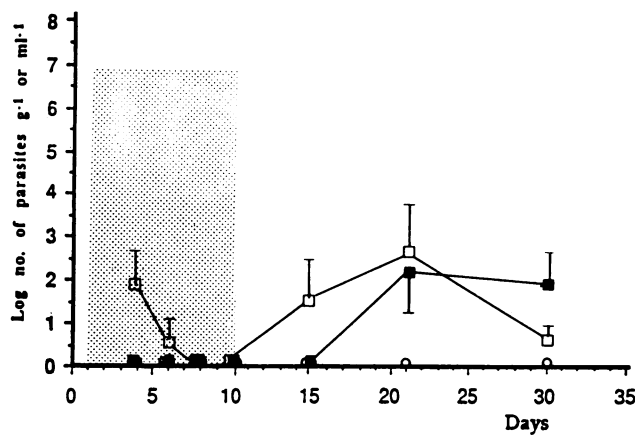


FIG. 3. Kinetics of parasitic loads in blood (○), lungs (□), and brain (■) in mice infected on day 0 with 10^4 tachyzoites of the RH strain and treated with sulfadiazine (375 mg/kg per day) for 10 days from day 1 after infection. Each point represents the mean \pm standard error of the mean for five mice. The shaded area represents the period of administration of antimicrobial agents.

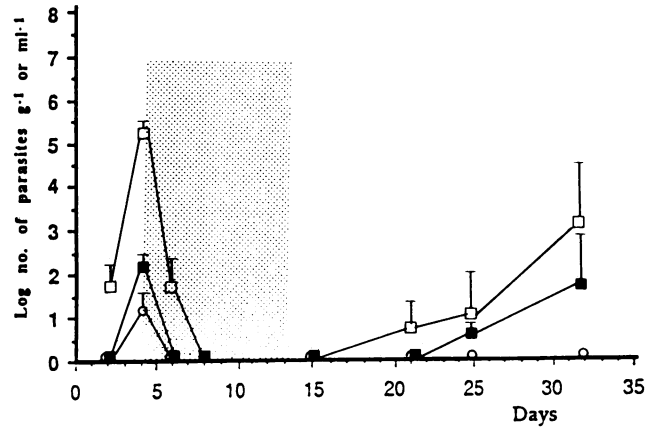


FIG. 4. Kinetics of parasitic loads in blood (○), lungs (□), and brain (■) in mice infected on day 0 with 10^4 tachyzoites of the RH strain and treated with pyrimethamine (18.5 mg/kg per day) and sulfadiazine (375 mg/kg per day) for 10 days from day 4 after infection. Each point represents the mean \pm standard error of the mean for five mice. The shaded area represents the period of administration of antimicrobial agents.

6). Parasitic loads determined in group B mice on days 4, 6, 8, 10, and 15 increased from days 4 to 6 in blood, brain, and lungs and then subsequently decreased on day 8 (Fig. 2). At that time, parasites could be demonstrated only in lungs of two mice. After the cessation of therapy, parasites were detectable in brain and lungs in all mice examined on day 15; parasitic loads were 4.7 ± 0.5 log units in brain and 5.8 ± 0.3 log units in lungs.

When sulfadiazine alone was administered from day 1, all group A mice had survived by day 30 after challenge (Fig. 6). Parasitic loads were determined in group B mice on days 4, 6, 8, 10, 15, 21, and 30 (Fig. 3). Parasites were detected in the lungs of four mice on day 4, with a mean value of 2.1 ± 0.6 log units, and then in lungs from one mouse on day 6, whereas brain and blood remained negative for parasites during that period. Parasites were undetectable on days 8 and 10. After the cessation of therapy, parasites were

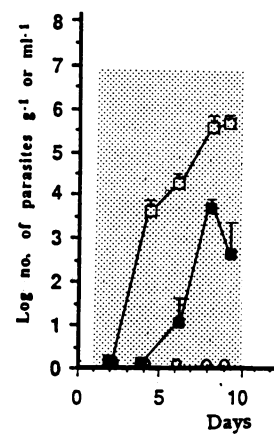


FIG. 5. Kinetics of parasitic loads in blood (○), lungs (□), and brain (■) in mice infected on day 0 with 10^4 tachyzoites of the RH strain and treated with clindamycin (300 mg/kg per day) for 10 days from day 1 after infection. Each point represents the mean \pm standard error of the mean for five mice. The shaded area represents the period of administration of antimicrobial agents.

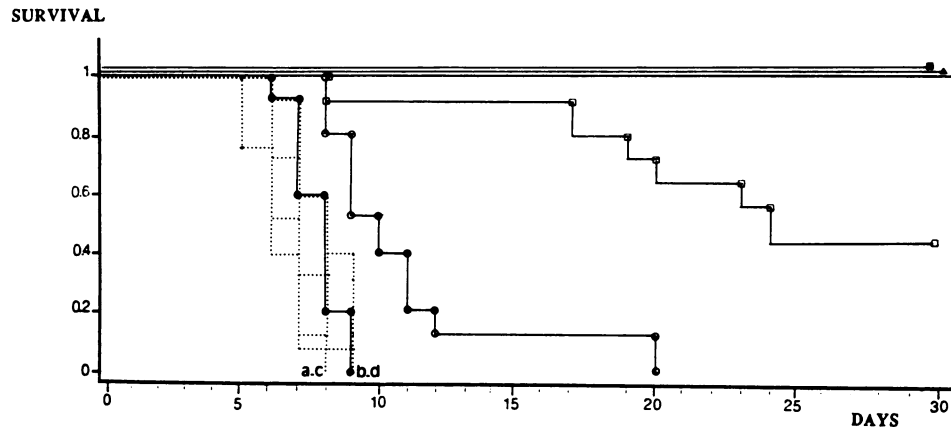


FIG. 6. Survival rates for control mice (dotted lines a to d) in four separate experiments and mice treated with sulfadiazine from day 1 (■), pyrimethamine from day 1 (□), clindamycin from day 1 (○) and day 4 (●), and pyrimethamine combined with sulfadiazine from day 1 (▲) and day 4 (△).

demonstrated in the lungs of two mice on day 15 and in brain and lungs of three mice on day 20; mean values progressively increased from days 10 to 20 and then decreased by day 30.

When pyrimethamine and sulfadiazine were administered in combination, all group A mice survived by day 30 after challenge (Fig. 6). Parasitic loads were determined in group B mice on days 2, 4, 6, 8, 15, 21, 25, and 32. When therapy was administered from day 1, parasites remained undetectable throughout the follow-up period (data not shown). In mice treated from days 4 to 13, parasites were eradicated on day 8 and remained undetectable until day 21. On day 21, parasites were found in the lungs of one mouse, and then on day 32 parasites were found in the lungs of three mice and in the brains of two mice, with increasing parasitic loads from days 21 to 32. Blood remained negative for parasites during this period (Fig. 4).

All group A mice treated with clindamycin on day 1 died within 20 days (Fig. 6). In group B mice, parasitic loads were determined on days 2, 4, 6, 8, and 9 (Fig. 5). Parasitemia was always undetectable, whereas the parasitic infection progressively involved lungs and brain tissues. On day 8, parasitic loads in lungs and brains were 5.5 ± 0.3 and 3.6 ± 0.2 log units, respectively. In mice treated from day 4, the mean survival time was 8 days (Fig. 6). Parasitic loads could be determined only on days 6 and 7 (data not shown). On day 6, parasitic loads in lungs and brains were 5.8 ± 0.1 and 3.8 ± 0.1 log units, respectively.

DISCUSSION

Numerous studies have examined the *in vivo* effect of antimicrobial agents in experimental toxoplasmosis, but discrepant results are often reported, mainly in relation to differences in the experimental procedures of drug administration and variations of the virulence of *Toxoplasma* strains according to the inoculum and mode of infection (2, 10, 13).

In this study, we chose to use a model of acute infection with a constantly highly virulent strain of *T. gondii* (RH strain) and a susceptible Swiss Webster mouse strain. The intraperitoneal route of administration with a large number of parasites (10^4 tachyzoites), which produces a constantly lethal infection, is also a guarantee of reproducibility for such a study. This mode of infection is not natural and differs from the reactivations which are observed in immunocompromised patients; however, we considered this model to be

relevant for the study of the effect of antimicrobial agents in this situation, as determinations of parasitic loads were performed in organs which are most commonly involved in severe toxoplasmosis in humans.

The subculture method was found to be much easier to perform than subinoculation to mice, since several hundred cultures can be performed simultaneously. This allowed the determination of parasitic loads over a wide range of dilutions of blood and organs. By immunofluorescence, parasitic foci were readily identified in the cultures for determination of endpoint dilution titers and parasitic loads. The sensitivity of this method for the demonstration of *T. gondii* was found to be less than 10 parasites in blood and tissue homogenates, with there being no effect of the antimicrobial agents that were present in the ground tissue. The repeated experiments in control mice showed that the kinetics of development of parasitic disease were reproducible under our experimental conditions and were characterized by an early and predominant lung involvement; this was followed by a more progressive increase of parasitic loads in brain. Blood parasitemia was constantly at a lower level, which indicated the early and predominant tissue affinity of the parasite.

The determination of parasitic loads in treated mice was more informative of the mode of action of antimicrobial agents than the determination of survival rates. When clindamycin was administered from day 1 after infection, the mean survival time was prolonged compared with that in control mice. The determination of parasitic loads in clindamycin-treated mice showed a more progressive increase of the parasitism in lungs and brains compared with those in the controls, whereas parasitemia was undetectable throughout the follow-up period. The disappearance of parasitemia in chronically infected mice treated with clindamycin has already been observed by Araujo and Remington (1); however, in our experiment, none of the mice treated from days 1 and 4 survived, which is in contrast to the lower mortality observed by other investigators in murine models of acute infection (1, 12, 16). Such differences may be related to the fact that in those studies, treatment was started immediately after infection, whereas we administered clindamycin 1 or 4 days after infection. Despite the early administration of clindamycin, Araujo and Remington (1) and Hofflin and Remington (12) noticed that parasites were not eradicated from the organs; our results also showed that clindamycin

does not inhibit the spread of infection in tissues and confirmed that this drug is more suppressive than curative. Since the pharmacokinetic data in mice show that this drug has a large diffusion and high concentrations in tissues, the poor efficacy in a model of acute infection is probably related to the low inhibitory effect of this drug on the parasite or to the fact that concentrations which have been found to be inhibitory *in vitro* (7) are not achieved in mice with daily administration of 300 mg/kg.

All mice treated with sulfadiazine (375 mg/kg per day) from day 1 survived and could be considered cured based on survival rates. Determination of parasitic loads showed an important decrease of lung involvement after the initiation of therapy, which indicated the early efficacy of this drug, but an increase in parasitic loads was noted at the first determination after the cessation of the treatment. Until the end of the follow-up period, mice were apparently healthy, but parasites were demonstrated at low levels in the brains and lungs of some mice 20 days after the cessation of therapy, whereas parasitemia was always undetectable.

When pyrimethamine was administered from day 1, the inhibitory effect was only demonstrable from day 6, indicating a latency in the *in vivo* effect of this drug. Parasitic loads decreased thereafter, but a relapse was constantly observed early after the cessation of therapy and there were high parasitic loads in brain and lungs, which may explain the delayed time of death in 40% of the mice.

Compared with sulfadiazine, pyrimethamine alone was found to have a lower efficacy in our model. This observation is surprising since *in vitro* studies have demonstrated that pyrimethamine is inhibitory at very low concentrations and is probably parasitocidal, whereas sulfadiazine is considered to be parasitostatic (5). However, these data can be interpreted in view of the pharmacokinetics of this drug in mice, in which pyrimethamine has a very short half-life (4.6 to 5.6 h) and accumulates in tissues within 7 days (4). This may explain the delay in response to therapy and the early occurrence of the relapses after the cessation of therapy. In contrast, the early efficacy of sulfadiazine could be related to its longer half-life in mice (11 h) and a large diffusion in tissues (3).

When pyrimethamine and sulfadiazine were administered in combination, 100% of mice survived, whether treatment was started at day 1 or 4, which confirms the high synergistic activities of these drugs (8, 9). When treatment was started early after infection, mice were considered cured, since parasites were always undetectable in blood, lungs, and brain. In contrast, when treatment was started on day 4, *i.e.*, when the parasitic infection already involved blood and organs, a dramatic decrease in the parasitic loads was observed, but relapses occurred after the cessation of therapy. The occurrences of such relapses has been reported previously by Eyles and Coleman (9) in mice treated with sulfadiazine and pyrimethamine. By determination of parasitic loads, we demonstrated that relapses occurred early after the cessation of therapy, even when mice were apparently healthy, and involved brain and lungs, whereas parasitemia was undetectable. In our model, the formation and *de novo* rupture of cysts cannot be proposed as an explanation for these relapses, since cysts are usually not obtained with the RH strain and cysts could not be formed within the short period of this study. Moreover, Swiss Webster mice are not immunocompromised and chronic infections are not reactivated spontaneously. The main explanation for the relapses that we observed in organs of mice treated with sulfadiazine, pyrimethamine, and combinations of the two

drugs is probably the escape of some parasites in organs in which inhibitory concentrations of drugs may not be achieved. This observation may justify the need for maintenance of long periods of therapy in humans and indicates that complementary studies are needed to better determine the tissue pharmacokinetics of these drugs in animals and humans in correlation with the distribution of *T. gondii* in tissues following an acute infection.

Finally, results of this study indicate that the kinetic study of parasitic loads in several organs of infected animals is a determinant for the assessment of drug efficacy since the evidence of parasitic relapse can be characterized by this method and may modify the usual therapy which can be proposed in humans.

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