The Effect of Rifampin on *Toxoplasma gondii*[1] (35011)

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Recently a number of investigators have described the effectiveness of semisynthetic derivatives of rifamycin against mycobacteria (1, 2), trachoma (3–5), vaccinia and adenoviruses (6, 7), and gram-positive and gram-negative bacteria (8). One such derivative, rifampin,[2] appears to be a clinically useful antibiotic for certain bacterial infections (9, 10) and can be given orally. The drug appears to act by inhibition of RNA synthesis in intact bacteria (11), and it specifically inhibits the RNA polymerase reaction *in vitro* (12–15). Recent evidence indicates that rifampin inhibits the RNA polymerase reaction by acting on the enzyme rather than on the DNA template (7, 16, 17). At drug concentrations 100- to 1000-fold higher than those effective in bacteria, rifampin does not appear to inhibit RNA synthesis in mammalian cells or inhibit the *in vitro* mammalian RNA polymerase reaction (13, 15).

Toxoplasma gondii is an obligate intracellular protozoan which replicates within vertebrate cells and causes natural infection of many mammals, including man. The organism has been shown to infect and replicate efficiently in many mammalian cell types in tissue culture. Recently (18), we reported the isolation and preliminary characterization of the RNA of *T. gondii* and demonstrated that organisms free of mammalian cells were able to synthesize RNA. This system provided an opportunity to study effects of rifampin on toxoplasma.

Materials and Methods. Toxoplasma in L-cell cultures. The trophozoite form of *T. gondii* (RH strain) was obtained from the peritoneal exudate of female Swiss-Webster mice 48 and 72 hr after acute infection. The peritoneal fluid was collected into Earle's balanced salt solution (BSS) containing 10% fetal calf serum (FCS) and 10 units of heparin/ml. The preparation was then filtered through glass wool, centrifuged at 500*g* for 5 min at 4° and the sediment was resuspended and forced through a 27-gauge needle. The organisms were quantitated by counting on a Neubauer-Levy hemacytometer and appropriate dilutions in Eagle's minimum essential medium (MEM) were made prior to cell culture challenge.

The methods of cell culture and toxoplasma challenge were similar to those previously described (19). Monolayers of 1×10^5 L-cells were grown in MEM with 10% FCS overnight on 22-mm^2 coverslips and then infected with approximately 10–20 toxoplasma/L-cell. The toxoplasma were removed after 1 hr of adsorption at 37°, and the monolayers were washed once with BSS. The monolayers were then incubated with MEM with 10% FCS which contained various concentrations of rifampin. All experiments were carried out in an atmosphere of 5% CO_2 at 37°. At various time intervals, the monolayers were fixed in 9-aminoacridine hydrochloride in ethanol and stained with Giemsa stain.

RNA synthesis in toxoplasma. The proliferative form of toxoplasma was harvested as previously described (19) into BSS containing 100 units/ml of heparin. The exudate was forced through a 27-gauge needle, passed through glass wool and then filtered through sintered glass filters (Corning, medium coarse porosity 15–25 μ). With this method, large numbers of toxoplasma were obtained free from detectable cells other than small num-

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[2] Rifampin, formerly designated as rifampicin, and also referred to as rifamycin AMP.

bers of red blood cells (18). After filtration the organisms were centrifuged at 3000*g* for 10 min at 4° and than resuspended in MEM. Toxoplasma (6×10^7) were incubated in 3 ml of MEM with 10% dialyzed FCS, 5% dialyzed chick embryo extract and varying concentrations of rifampin at 37° for a total of 4 hr with continuous mixing with a magnetic stirring bar. At various time intervals, 30 μCi of ^{3}H-uridine (sp act 21 Ci/mmole) was added and tritium incorporation into RNA was measured as previously described (18).

Growth of L-cells in culture. Forty L-cell cultures were started with 1×10^5 cells/60-mm plastic tissue culture dish in 5 ml of MEM with 10% FCS. Rifampin was added to groups of 8 cultures at concentrations of 10, 20, 40, and 80 μg/ml. Each day for 4 days the cells from 2 dishes from each drug group and from cultures without rifampin were removed with trypsin and counted in a Coulter cell counter Model B.

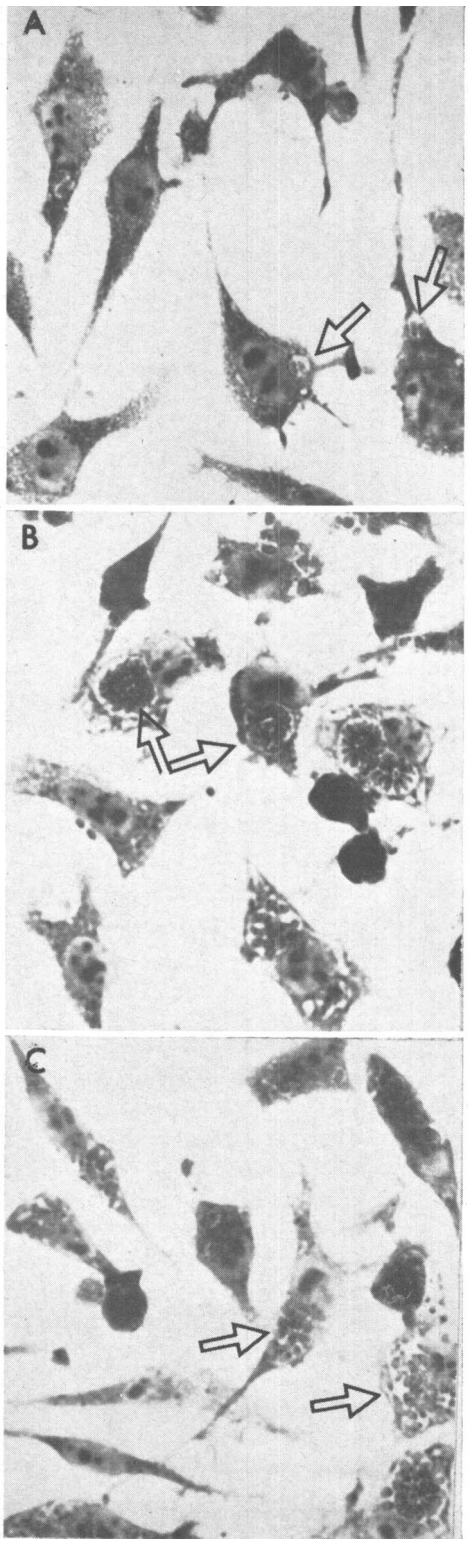
In vivo *experiments.* Rifamycin SV was added to powdered laboratory mouse food and blended thoroughly using 187.5 mg/100 g of meal or a 300 mg/kg of mouse weight dose based on approximately 4 g of food consumed by a mouse per day (20). After overnight starvation, mice were infected with an intraperitoneal dose of 2×10^4 toxoplasma of the RH strain (a dose resulting in death of 100% of normal mice by day 8 or 9). Twenty of the mice were immediately fed with food containing drug and another 10 were used as controls. Three mice not infected with toxoplasma were fed food containing drug to test toxicity. Rifampin was also fed to mice using the same procedures except a 200 mg/kg dose was used. In another feeding experiment rifampin at a dose of 500 mg/kg was administered orally by gavage immediately after challenging mice with toxoplasma. The animals were fasted for 6 hr prior to gavage to facilitate rapid absorption.

For intraperitoneal administration, rifamycin SV was dissolved in deionized H_2O and administered daily in concentrations of 10, 50, 100 and 200 mg/kg of mouse weight in a volume of 0.2 ml. The mice were infected with a dose of toxoplasma as described above 4 hr prior to the first inoculation of drug. Ten of the infected mice were not treated, and 10 were inoculated with 0.2 ml deionized H_2O daily. The 200 mg/kg dose of rifamycin SV was administered to 3 mice with no toxoplasma infection to test for toxicity. Rifampin was also administered intraperitoneally at a dose of 40 mg/kg.

TABLE I. Effect of Rifampin on Toxoplasma Multiplication in L-Cells.

Time after infection (hr)	Dose (μg)	No. of infected cells examined	No. of organisms initially invading cells[a]	Organisms (%) which multiplied		
				Undivided	Once	Twice or more
8	0	100	278	55	45	0.4
	20	103	330	62	38	0.4
	50	100	309	66	34	0
	75	104	199	65	35	0
	100	100	311	75	25	0
12	0	100	209	41	45	13
	20	100	230	36	56	8
	50	103	200	40	56	4.5
	75	100	213	56	42	2
	100	103	226	71	29	0
24	0	200	655	24	21	55
	20	201	508	21	25	55
	50	200	423	25	46	30
	75	203	392	24	69	7
	100	200	421	42	55	3

[a] Organisms which had multiplied within a vacuole were counted as one invading organism. The total number given represents the sum of these plus the total number of undivided parasites.



Results. Effect of rifampin on replication of toxoplasma in L-cells. To determine the effect of rifampin on the multiplication of toxoplasma, varying concentrations of the drug were added to L-cell monolayers following infection (Table I and Fig. 1). At 8 hr only 45% of invading toxoplasma in untreated cells had multiplied. That not all toxoplasma divide under similar conditions has been noted previously by Lycke and Lund (21). In cultures treated with a dose of 100 μg/ml, only 25% of organisms had divided by 8 hr. Organisms dividing more than once were only observed in the untreated cell monolayers and in monolayers treated with 20 μg/ml of rifampin. The 8 hr results shown in Table I are representative of those obtained in the 4 experiments performed and suggest that rifampin has an effect on the first division of toxoplasma.

At 12 and 24 hr, the inhibition of multiplication beyond the first division by rifampin can clearly be seen. In all experiments performed at 24 hr after infection, approximately 50% of organisms divided more than once in untreated cell monolayers, whereas less than 5% did so in monolayers treated with 100 μg/ml of rifampin. Doses of 50 and 75 μg/ml were also definitely inhibitory. A significant effect was not noted with 20 μg/ml at 24 hr and although this dose appeared to have some effect at 12 hr in the experiment in Table I, this was not observed in other experiments.

By 12 and 24 hr many cells in monolayers treated with 50 μg/ml or less had been destroyed. At these time periods, the percentage of organisms which had multiplied once probably reflects reinvasion of the monolayer by parasites released from disrupted cells rather than division of organisms from the original challenge. The higher percentage of organisms multiplying once in those monolayers treated with 75 and 100 μg/ml may also

FIG. 1. Effect of rifampin on toxoplasma multiplication in L-cells. Cultures are at 24 hr after infection with toxoplasma. A = treated with 100 μg of rifampin; B and C = untreated controls. Arrows in A point to organisms which have divided once; in B to rosettes of toxoplasma; in C to cytoplasm filled with multiplying organisms.

reflect newly infected cells. Figure 1 shows representative microscopic fields from a treated and an untreated monolayer 24 hr after infection. The remarkable difference in multiplication can be seen easily because of the large number of rosettes formed in the untreated cells.

Since toxoplasma multiplies only within cells and its multiplication undoubtedly depends on certain host cell functions, the inhibitory effect of rifampin on toxoplasma multiplication could be due either to an effect on toxoplasma itself and/or to an effect on the host cell. Experiments were performed to test the effects of rifampin on toxoplasma alone and on L-cells in culture.

Effect of rifampin on toxoplasma RNA synthesis. Since the only specific biochemical effect known for rifampin is inhibition of RNA synthesis in bacterial cells by inhibition of the RNA polymerase reaction, experiments were performed to determine whether rifampin inhibits RNA synthesis in extracellular toxoplasma. Toxoplasma, purified free of mouse peritoneal cells, were incubated with ^{3}H-uridine in the presence and absence

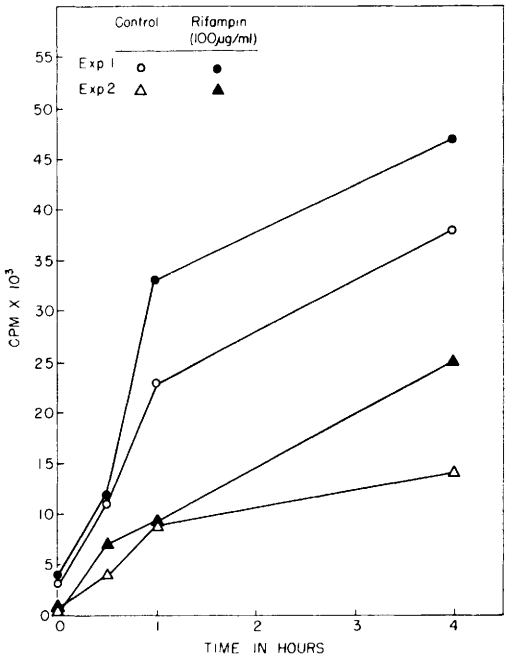


FIG. 2. Results of 2 experiments on the effect of dose (100 µg/ml) of rifampin on uptake of ^{3}H-uridine by cell-free toxoplasma (see text).

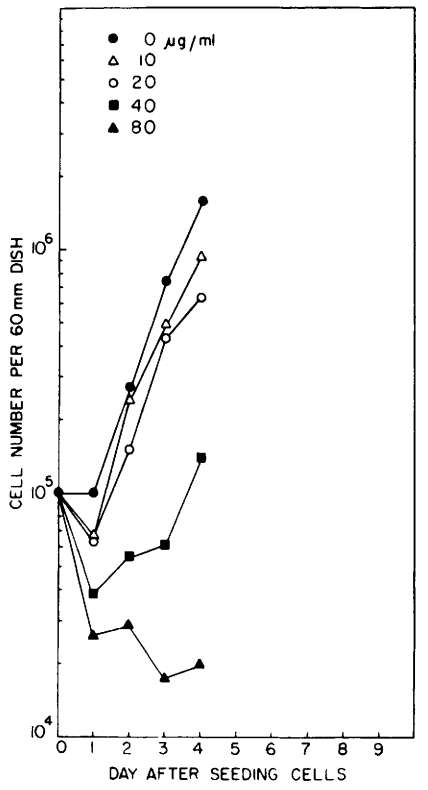


FIG. 3. Effect of various concentrations of rifampin on multiplication of L-cells.

of rifampin. Figure 2 shows that rifampin at 100 µg/ml had no apparent inhibitory effect on the rate of incorporation of ^{3}H-uridine into TCA precipitable RNA of toxoplasma over the 4 hr period. Similar results were obtained using 50 and 200 µg/ml of rifampin. In each of the 4 experiments performed, uridine uptake was definitely greater in rifampin-treated toxoplasma than in untreated organisms. The reason for this is unclear.

Effect of rifampin on L-cells in culture. To determine the effect of rifampin on L-cells, the rate of growth of L-cells in culture with different concentrations of rifampin was determined. Figure 3 shows that the cell number at least doubles each day in cultures without rifampin and with drug concentrations of 10 and 20 µg/ml there is only a slight reduction in the rate of cell growth. At 40 µg/ml, however, there is marked inhibition of cell growth and at 70 µg/ml the cell number in the cultures decreases progressively. Within 12 hr after adding 40 or 80 µg/ml

of rifampin to cultures, severe cytopathic changes in the cells were observed microscopically. Experiments with L-cells, similar to the ones with toxoplasma, to determine the effect of rifampin on ^{3}H-uridine incorporation into RNA showed that drug concentrations up to 100 μg/ml do not inhibit L-cell RNA synthesis confirming previous studies with other mammalian cells (13, 15). Thus rifampin exerts a significant toxic effect on L-cells at the same concentrations which inhibit toxoplasma multiplication. The enhanced uridine uptake noted in rifampin-treated toxoplasma was not observed in L-cells.

In vivo *studies.* To determine the effectiveness of rifampin and rifamyin SV in experimental toxoplasmosis, mice infected with the RH strain were fed laboratory chow in which these drugs were incorporated. In 2 experiments, a dose of 300 mg/kg/24 hr of rifamycin SV did not provide protection as measured by mortality or prolongation of time to death. The therapeutic effectiveness of rifampin against toxoplasma in mice was also investigated, since it is rapidly absorbed through the intestinal tract, whereas rifamycin SV is not. Rifampin, when administered in the diet at 200 mg/kg/24 hr or by gavage in 2 separate studies did not protect mice.

Parenteral therapy employing 10 to 200 mg/kg of rifamycin SV daily by the intraperitoneal route afforded no protection *in vivo* against toxoplasma. On day 8, all mice had died and intraperitoneal inoculations were discontinued in uninfected, treated control mice. Despite this, 2 of 3 mice receiving 200 mg/kg died on the 11th and 13th days, respectively, reflecting direct toxicity of rifamycin SV at this dose. Rifampin administered intraperitoneally did not protect mice in the one experiment performed.

Discussion. The results described above demonstrate the ability of rifampin to prevent multiplication of toxoplasma and thereby to protect cells from destruction by this parasite. The mechanism of inhibition of toxoplasma multiplication in L-cell cultures by rifampin is not clear.

Since rifampin was added to the L-cell cultures after toxoplasma infection had taken place, its action in this system appears to be intracellular. Our results indicate that RNA synthesis in cell-free toxoplasma is not inhibited by rifampin in concentrations more than 100 times that required to inhibit RNA synthesis in bacterial cells (11–15) and at the same concentrations which inhibit toxoplasma multiplication in L-cells. This suggests that general inhibition of toxoplasma RNA synthesis is not the mechanism by which rifampin inhibits toxoplasma multiplication in L-cell cultures. Our data do not exclude that rifampin might selectively inhibit synthesis of a minor fraction of toxoplasma RNA which is too small to be detected in our experiments and which is essential for toxoplasma multiplication. Such selective inhibition of RNA synthesis is not known to occur in sensitive bacterial cells. Similarly, we cannot exclude that toxoplasma RNA synthesis, during the organisms' multiplication cycle within L-cells, in some way becomes sensitive to rifampin, although this would seem unlikely if the same enzyme is involved in toxoplasma RNA synthesis under both conditions. We have not tested the effects of rifampin on other metabolic functions of purified toxoplasma.

Because the toxicity of rifampin for L-cells and its inhibition of toxoplasma multiplication occur at the same drug concentrations, it is possible that rifampin inhibits toxoplasma multiplication by its toxic effect on the L-cell. Inhibition of vaccinia and adenovirus (6, 7) replication in tissue culture occurs at similar high rifampin concentrations suggesting the possibility of a similar mechanism on host cells although an effect on the replicating parasites is not excluded. Although we have not elucidated the mechanism of rifampin toxicity on mammalian cells, the effect does not appear to be on specific inhibition of RNA synthesis and it occurs only at 100- to 1000-fold higher drug concentrations than required to inhibit RNA synthesis in sensitive bacterial cells. A recent report (5) suggests that rifampin at 100 μg/ml may alter protein synthesis in uninfected as well as in vaccinia virus-infected HeLa cells and that this effect is not related to inhibition of RNA synthesis. Moss and his colleagues (22) have reported that rifampin did not inhibit

vaccinia virus RNA polymerase and believe from their electron microscopy data that the primary effect of the drug is to interfere with the assembly of virus DNA and protein when the virus envelope is formed (23). This suggests that the action of rifampin in sensitive bacterial cells and in the mammalian cell–toxoplasma and vaccinia virus systems may be quite different.

Despite the marked effect against toxoplasma replication *in vitro* in mammalian cells cultures, administration of rifamycin SV or rifampin by the oral or intraperitoneal route did not confer protection against experimental toxoplasma infection in mice as measured by percentage mortality and time to death. The lack of efficacy *in vivo* most likely reflects the relatively high doses of rifampin which were necessary to limit multiplication of toxoplasma in tissue culture.

Rifampin in combination with drugs used in the treatment of toxoplasmosis (*e.g.*, sulfonamides and/or pyrimethamine) might enhance their effectiveness and allay emergence of bone marrow toxicity by allowing for use of smaller doses of pyrimethamine. That such synergism may occur is suggested by studies of the drug in murine tuberculosis (24).

The potential usefulness of this drug in combination with other antimicrobial agents in the treatment of toxoplasmosis in humans remains to be determined.

Summary. Rifampin, at concentrations of 50 μg/ml and greater, significantly inhibited multiplication of toxoplasma in L-cell cultures. Similar concentrations also inhibited growth of L-cells. No effect of rifampin was found on toxoplasma RNA synthesis in toxoplasma preparations free of mammalian cells or on RNA synthesis in L-cells. Mice which had received rifampin in high doses by the oral or intraperitoneal routes were not protected against lethal infection with toxoplasma.

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