

# Comparative effects of cotrimoxazole (trimethoprim-sulphamethoxazole), pyrimethamine-sulphadiazine and spiramycin during avirulent infection with *Toxoplasma gondii* (Beverley strain) in mice

B.T. Nguyen & S. Stadtsbaeder

Service de Microbiologie, Cliniques Universitaires Saint-Luc, 10/1752, Université Catholique de Louvain, 1200 Brussels, Belgium

- 1 The antitoxoplasm effects of cotrimoxazole (Ctx), spiramycin (Spir) and pyrimethamine-sulphadiazine (Pmm-Sdz) were compared during both proliferative and chronic phases of infection of mice with the Beverley (Bev) strain of *Toxoplasma gondii* of low virulence.
- 2 The therapeutic efficacy of the drugs was determined according to the following criteria: (i) specific antibody response; (ii) acquired resistance to lethal challenge with the virulent RH strain of *Toxoplasma*; and (iii) persistence of parasites in tissues (brain, liver, spleen) of treated mice.
- 3 The results indicated that Ctx, like Pmm-Sdz, had a greater effect than Spir upon toxoplasma organisms during the proliferative phase of infection. In contrast, none of the three drugs tested was active against tissue cysts in chronically infected mice.

## Introduction

Previous studies have shown the efficacy of cotrimoxazole (Ctx) against *T. gondii* both *in vitro* in tissue cultures of a variety of mammalian cells (human blood monocytes, HeLa cells, mouse tumour cells and peritoneal macrophages) and *in vivo* in mice (Sander & Midvedt, 1970; Stadtsbaeder & Calvin-Préval, 1973; Nguyen & Stadtsbaeder, 1975; 1978; Nguyen, Stadtsbaeder & Horvat, 1978; Grossman, Krahenbuhl & Remington, 1978). All the observations have been made with the highly virulent RH strain of toxoplasma which causes an acute and rapidly lethal infection in mice.

In humans, toxoplasmosis is usually a mild disease leading to a chronic phase with persistence of cysts in the host tissues. Therefore, it seems of interest to study the antitoxoplasm activity of Ctx under experimental conditions comparable to those encountered naturally in man. For this reason, we have used the Beverley (Bev) strain of toxoplasma which causes an infection in mice (the pathology of which is very similar to that observed in man) of a latent or subclinical nature with encystment in tissues during the chronic phase.

In the present study, the therapeutic efficacy of Ctx, Spiramycin (Spir) and pyrimethamine sulphadiazine (Pmm-Sdz) during both evolutive and

chronic phases of *Toxoplasma* infection in mice is compared.

## Methods

### Mice

NMRI female mice (25 to 27 g, 8 to 10 weeks of age) were obtained from Animalerie Centrale de l'Université Catholique de Louvain, 1200 Brussels, Belgium.

### *Toxoplasma*

Both the avirulent Bev and the virulent RH strains of *Toxoplasma gondii* were used. These two strains have been maintained for several years in the laboratory by regular mouse passage as previously described (Stadtsbaeder, Nguyen & Calvin-Préval, 1975).

### Avirulent infection

Brains from at least 3 mice infected 6 weeks previously with Bev strain of *Toxoplasma* were forced

through a 19G needle in 5 ml saline until complete homogenization of the brains was achieved. Smears were made to check the presence of *Toxoplasma* cysts. The homogenate was further diluted in a proportion of 1 brain/5 ml saline. A 0.5 ml volume of the brain suspension was inoculated subcutaneously (s.c.) into each mouse. This inoculum was sufficient to afford complete immunity to normal mice (Stadtsbaeder & Nguyen, 1977).

#### Virulent challenge

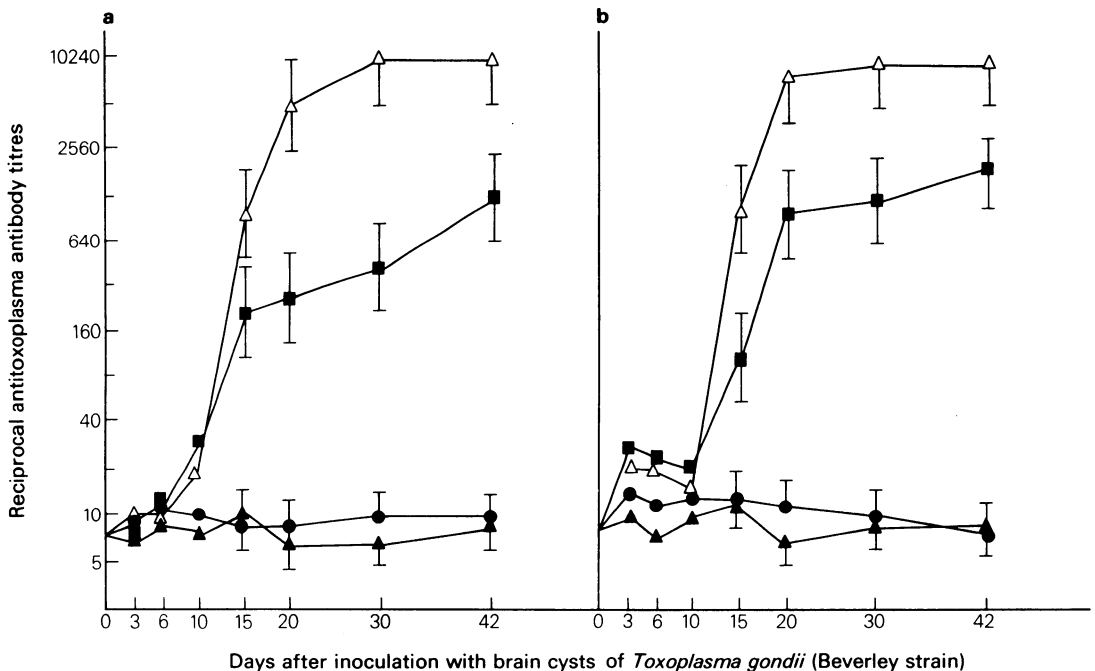
Trophozoites of the virulent RH strain of *Toxoplasma* were obtained as described elsewhere (Stadtsbaeder *et al.*, 1975). Briefly, the parasite-rich peritoneal exudate was induced in mice by inoculating intraperitoneally (i.p.)  $10^7$  trophozoites in 1 ml saline. At 48 h after infection, the parasites were harvested by washing out the peritoneal cavity with 2 ml saline. The *Toxoplasma* organisms were washed once with saline before use. The challenge inoculum ( $1.5 \times 10^5$  parasites) in a volume of 0.5 ml saline was injected i.p. into each mouse tested. Survivors at 30 days were considered resistant.

#### Drugs

Commercial tablets of Ctx and Spir were used. Pure Pmm and Sdz in powder form were kindly supplied by Wellcome Belgium.

#### Treatment

Treatment was started either immediately after avirulent infection (evolutive phase) or 15 days later (chronic phase) and continued for 42 days. For practical reasons, the drugs were added to drinking water at the following concentrations: Ctx = 980 mg%, Pmm + Sdz = 5 + 250 mg% and Spir = 1 g%. These doses have been used successfully in our previous studies for treatment of experimental acute toxoplasmosis in mice (Stadtsbaeder & Calvin-Préval, 1973). The volume of water consumed by each group of mice was recorded every two days throughout the treatment period. Each mouse consumed approximately  $2.5 \pm 0.5$  ml per day. Accordingly, the approximate mean concentrations of drugs taken daily by each mouse (mean weight = 26 g) were as follows: Ctx =  $923 \text{ mg kg}^{-1}$ , Pmm + Sdz =  $4.8 + 240 \text{ mg kg}^{-1}$  and Spir =  $962 \text{ mg kg}^{-1}$ .



**Figure 1** Kinetics of antitoxoplasma antibodies in mice after inoculation with Bev strain of *T. gondii* and either left untreated ( $\Delta$ ) or treated with cotrimoxazole ( $\bullet$ ), pyrimethamine-sulphadiazine ( $\blacktriangle$ ) or spiramycin ( $\blacksquare$ ) immediately after inoculation. Sera were tested by the indirect haemagglutination (a) or the indirect immunofluorescence (b) test. Each point represents the reciprocal of mean antibody titre with standard error bars from at least six individual specimens.

### Assessment of immune responses

At the end of the treatment period, half of the animals from each group were challenged with virulent RH toxoplasma to determine their degree of acquired resistance. The other animals were killed. Blood was collected by cardiac puncture. Antitoxoplasma antibodies were titrated on each serum individually with both indirect immunofluorescence (IF) and indirect haemagglutination (IHA) tests. Reagents for serological tests were purchased from Bio-Mérieux (France). Polyspecific goat anti-mouse immunoglobulins conjugated with fluorescein isothiocyanate (Nordic) were used at a dilution of 1/50 in phosphate buffered saline, pH 7.4.

### Tissue subinoculation

Persistence of toxoplasma organisms in tissues of mice was detected by subinoculation into fresh mice. The brain, spleen and liver were removed and then homogenized separately in saline (1 organ/5 ml). Tissue homogenization was performed under maximum sterile conditions using sterilized materials. Antibiotics with no known toxoplasmicidal activity (50 units penicillin and 50 µg streptomycin per ml) were incorporated in the emulsions to avoid bacterial contaminations. The brain emulsion was injected s.c. in a volume of 0.5 ml per mouse. The spleen and liver suspensions were injected i.p. in a volume of 1 ml per mouse. Resistance to virulent challenge and presence of antitoxoplasma antibodies in the subinoculated mice were determined 42 days later with the techniques described above.

## Results

### Effects of drugs during proliferative phase of infection

In the first set of experiments, we evaluated the effects of different drugs during the proliferative phase of toxoplasmic infection in the mice. For this purpose, the animals received treatment immediately after inoculation with Bev toxoplasma.

The results of serological tests performed during the course of treatment are shown in Figure 1. In mice inoculated but not treated (Bev group), specific antibodies rose rapidly from the 10th day and remained at high titres thereafter. The evolution of antibodies in mice treated with Spir (Bev + Spir group) paralleled that of the untreated Bev group. However, the antibody titres were significantly lower ( $P < 0.001$ ) suggesting a partial inhibiting effect of the drug upon the parasites. In contrast, there was no detectable rise in antitoxoplasma antibodies in the groups of mice treated either with Ctx (Bev + Ctx

**Table 1** Effects of drugs on acquired resistance to virulent challenge with *Toxoplasma gondii* RH strain in mice previously inoculated with *Toxoplasma gondii* (Bev strain)

Group <sup>a</sup>	Survival after virulent challenge <sup>b</sup>	
	Experiment I	Experiment II
Bev	6/6 <sup>c</sup> (100)	6/6 (100)
Bev + Spir	3/6 ( 50)	4/6 ( 67)
Bev + Ctx	0/6 ( 0)	0/6 ( 0)
Bev + Pmm-Sdz	0/6 ( 0)	0/6 ( 0)
Uninoculated	0/6 ( 0)	0/6 ( 0)

Spir = Spiramycin; Ctx = cotrimoxazole; Pmm-Sdz = pyrimethamine-sulphadiazine.

<sup>a</sup>Treatment was started immediately after Bev inoculation and continued for 6 weeks.

<sup>b</sup>Resistance to virulent challenge with *T. gondii* RH ( $1.5 \times 10^5$  trophozoites per mouse) was determined at the end of the treatment period (42 days).

<sup>c</sup>Number of survivors at 30 days/Number challenged. Figures in parentheses indicate percentage survival.

group) or with Pmm-Sdz (Bev + Pmm-Sdz group); in these two groups, the antibody titres remained at low levels comparable to those found in the uninoculated control group.

Assessment of resistance to lethal challenge with virulent RH strain of *T. gondii* (Table 1) performed at the end of the treatment period (42 days after Bev inoculation) showed that 100% of the mice in the Bev group survived. The rate of survival in the Bev + Spir group was approximately 50%; that of Bev + Ctx or Bev + Pmm-Sdz group was 0% indicating a complete absence of acquired immunity to challenge in these animals.

Results of tissue subinoculation from treated and untreated Bev-infected mice into fresh mice are given in Table 2. Antibody production and RH-resistance were positive in 100% of the mice subinoculated with brain emulsion from untreated Bev group. Similar results were obtained after subinoculation of brain emulsion from Bev + Spir group. In contrast, all the animals subinoculated with brain emulsion from Bev + Ctx or from Bev + Pmm-Sdz group remained serologically negative and did not resist RH challenge. The results of spleen and liver subinoculations were less clear, probably because of the lower frequency of infection in these organs than in the brain in the mouse (Beverley & Henry, 1971). Nevertheless, spleen and liver subinoculations from some of the Spir-treated but from none of the Ctx and Pmm-Sdz-treated mice gave positive results.

### Effects of drugs during chronic phase of infection

In a second set of experiments, we compared the

**Table 2** Results of subinoculation into fresh mice of tissues from treated and untreated mice in the proliferative phase of infection with *Toxoplasma gondii* (Bev strain)

Groups from which tissues were obtained <sup>c</sup> :	No seropositive mice <sup>a</sup> after subinoculation with:			No RH-resistant mice <sup>b</sup> after subinoculation with:		
	brain	liver	spleen	brain	liver	spleen
Bev	12/12 (9102)	5/12 (2304)	6/12 (4480)	12/12	3/12	3/12
Bev + Spir	12/12 (5973)	3/12 (1706)	3/12 (2133)	10/12	1/12	2/12
Bev + Ctx	0/12 (<10)	0/12 (<10)	0/12 (<10)	0/12	0/12	0/12
Bev + Pmm-Sdz	0/12 (<10)	0/12 (<10)	0/12 (<10)	0/12	0/12	0/12
Uninoculated	0/12 (<10)	0/12 (<10)	0/12 (<10)	0/12	0/12	0/12

Abbreviations as in Table 1

<sup>a</sup>Number of mice with a positive immunofluorescence test/Number subinoculated. Figures in parentheses indicate reciprocal of mean antibody titre in seropositive mice.

<sup>b</sup>Resistance to virulent challenge with *T. gondii* RH ( $1.5 \times 10^5$  trichozoites per mouse) was determined 42 days after tissue subinoculation. Results are expressed as number of survivors at 30 days/Number challenged.

<sup>c</sup>Tissues were collected from each group of mice 6 weeks after treatment which was started immediately after Bev inoculation.

effects of the different drugs on the persistence of cysts in mice bearing a chronic infection. For this purpose, treatment was not started until 15 days after Bev inoculation. Lainson (1958) reported that tissue encystment began from the 8th day following inoculation with toxoplasma strains of low virulence.

The results in Table 3 indicate that the different drugs used in this study did not affect the immune status already acquired by the mice during the pre-treatment period. Indeed, specific antibody titres and RH-resistance remained at high levels after 42 days of treatment with any of the drugs.

The effects of drugs on the persistence of cysts are

**Table 3** Effects of drugs on the immune status of mice chronically infected with *Toxoplasma gondii* (Bev strain)

Group <sup>a</sup>	Antibody titres <sup>b</sup>	Resistance to RH challenge <sup>b</sup>
Bev	5120–10240 <sup>c</sup> (100)	12/12 <sup>d</sup>
Bev + Spir	5120–10240 (100)	12/12
Bev + Ctx	2560–10240 (100)	11/12
Bev + Pmm-Sdz	2560–10240 (100)	12/12
Uninoculated	<10	0/12

Abbreviations as in Table 1.

<sup>a</sup>Treatment with different drugs was started 15 days after inoculation with Bev toxoplasma.

<sup>b</sup>Antitoxoplasma antibodies and resistance to lethal challenge with RH toxoplasma was determined after 42 days of treatment.

<sup>c</sup>Range of reciprocal antibody titres determined by indirect immunofluorescence test. Figures in parentheses indicate percent seropositive.

<sup>d</sup>Number of survivors at 30 days/Number challenged with  $1.5 \times 10^5$  trophozoites of *T. gondii* RH.

shown in Table 4. Serological and RH-resistance data from subinoculated mice show that none of the three drugs tested here had succeeded in eradicating tissue cysts and more particularly, brain cysts from mice chronically infected with the Bev strain of toxoplasma. In fact, whichever drug had been given all or almost all of the mice subinoculated with brain emulsions became serologically positive and resistant to RH challenge. The parasites were less often recovered after subinoculation of spleens or livers probably for the reasons given above. However, there were no significant differences ( $P > 0.05$ ) in the subinoculation results between treated and untreated groups.

## Discussion

After inoculation into fresh mice of brain cysts from mice chronically infected with toxoplasma strains of low virulence, the parasites undergo a short period of proliferation (8 to 11 days). During this period, the parasites disseminate throughout the host body before definitive encystment in the tissues. The first phase is termed pseudocystic phase and the second one, the cystic phase (Lainson, 1958). With the Bev strain, the pseudocystic phase is rarely lethal for the mice. Our results have shown that Ctx, like Pmm-Sdz, was very active against toxoplasma during this phase. Indeed, mice treated immediately after inoculation with Ctx or with Pmm-Sdz failed to develop either antitoxoplasma antibodies or immunity after challenge with the virulent RH strain of toxoplasma. Furthermore, subinoculations of brain, spleen and liver from these treated mice into fresh mice were all negative. These observations suggest a rapid eradication of the parasites from mice treated with Ctx or with Pmm-Sdz. Under similar experimental condi-

**Table 4** Results of subinoculation into fresh mice of tissues from treated and untreated mice in the chronic phase of infection with *Toxoplasma gondii* (Bev strain)

Groups from which tissues were obtained <sup>c</sup> :	No seropositive mice <sup>a</sup> after subinoculation with:			No RH-resistant mice <sup>b</sup> after subinoculation with:		
	brain	liver	spleen	brain	liver	spleen
Bev	12/12 (9813)	5/12 (2048)	5/12 (4096)	11/12	6/12	3/12
Bev + Spir	12/12 (8960)	3/12 (2133)	4/12 (3840)	11/12	4/12	5/12
Bev + Ctx	11/12 (8145)	4/12 (1120)	2/12 (1920)	12/12	2/12	3/12
Bev + Pmm-Sdz	12/12 (8533)	3/12 (1920)	4/12 (1920)	10/12	4/12	2/12
Uninoculated	0/12 (<10)	0/12 (<10)	0/12 (<10)	0/12	0/12	0/12

Abbreviations and a, b and c: see footnotes to Tables 1 and 2, except that treatment was started 15 days after Bev inoculation.

tions, Spir was less effective. This antibiotic controlled to a certain extent the development of infection but failed to eradicate the parasites from the mice. This was substantiated by the fact that specific antibodies were observed in all the treated animals but in lower titres than in the untreated controls. On the other hand, the brains of treated animals, when subinoculated were positive in 100% of cases. The partial efficacy of Spir against toxoplasma observed in the present study is in accordance with previous studies showing that Spir delayed the growth of *T. gondii* RH trophozoites within cell cultures without eliminating the infection (Nguyen & Stadtsbaeder, 1975; Nguyen *et al.*, 1978). Similarly, Spir only afforded partial protection against experimental acute toxoplasmosis in mice (Garin & Eyles, 1958; Stadtsbaeder & Calvin-Préval, 1973).

Persistence of toxoplasma cysts within the host tissues probably contributes to the maintenance of immunity against reinfection. On the other hand, the presence of cysts within the tissues may under certain circumstances represent a potential danger of reactivation of infection. This situation is particularly critical for immunocompromised patients and infants suffering from congenital toxoplasmosis. The most severe lesions are generally located in the eye, brain and heart muscle where cysts are abundant (Frenkel, 1974). Since present chemotherapeutic agents have no effects upon toxoplasma cysts, it is of interest to test the activity of Ctx against this particular form of the parasite. Results described in this study indicated that Ctx, like Pmm-Sdz and Spir, failed to eradicate tissue cysts from chronically infected mice. This was particularly obvious with cerebral cysts, since brain subinoculations were positive in 100% of cases. Nevertheless, with the present subinoculation technique, we were unable to determine if there was a substantial reduction in the number of cysts in the treated organs. On the basis of histological observations, Beverley, Freeman, Henry & Whelan (1973) reported that Spir and Pmm-Sdz inhibited growth of cysts in the mice without destroying the parasites.

The lack of efficacy of Ctx upon toxoplasma cysts in mice may have at least three possible explanations. Firstly, it has been reported that trimethoprim (Tmp) and sulphamethoxazole (Smz), the two components of Ctx, are both inhibitors of bacterial folate metabolism (Bushby & Hitchings, 1968). Tmp interferes with the conversion of folic acid to folinic acid by inhibiting the dihydrofolate reductase enzyme whereas Smz competes with para-aminobenzoic acid in its conversion to folic acid. Thus, the combined action of Tmp and Smz results in the inhibition of bacterial nucleic acid and protein synthesis. Although it has not been studied in *Toxoplasma*, the mode of action of Ctx against this protozoan may be related to that observed in bacteria since both types of microorganisms possess the same folate pathway (Frenkel & Hitchings, 1957).

Little is known about the metabolic activities of *Toxoplasma* trophozoites versus bradyzoites. The mean generation time of trophozoites does not exceed 6 h (Sourander, Lycke & Lund, 1960; Bommer, Hofling & Heunert, 1969; Jones, Len & Hirsch, 1975); that of bradyzoites may reach 3 weeks (Beverley, 1958) suggesting lower metabolic activities in the latter than in the former form. Because of the specific antimetabolite property of Ctx, one would therefore expect Ctx to be more effective against fast-growing trophozoites than against dormant intracystic bradyzoites. This assumption is supported by results from previous studies showing that Pmm, a synthetic analogue of Tmp, was active only against multiplying trophozoites of RH strain of *T. gondii* (Cook & Jacobs, 1958) most probably by interfering at first with DNA synthesis of toxoplasma (Sheffield & Melton, 1975). With regard to the above considerations, it would be of value to verify further the anticystic activity of Ctx in mice with a longer period of treatment.

Secondly, bradyzoites are enclosed within a cyst wall (Frenkel, 1974b) which may protect the parasites from the lethal action of Ctx. Further evidence is needed to verify this hypothesis.

Finally, the anatomical localization of toxoplasma cysts in the host organism, more particularly with regard to cerebral cysts, against which the poor capacity of Ctx to cross the blood-brain barrier (Fowle, 1973) is important, must probably be taken into account.

In conclusion, results presented in this study confirm the efficacy of Ctx during the proliferation phase of *Toxoplasma* infection in mice. This drug combination exhibited a more radical effect than Spir; its

efficacy was similar to that of Pmm-Sdz. Therefore, the use of Ctx in treatment of acute toxoplasmosis in humans deserves consideration, especially as this drug is less toxic than Pmm-sulphonamide combinations for man (Frisch, 1973). Resistance of *Toxoplasma* cysts to the present drug régimes, and in particular to Ctx, remains a serious problem to be resolved.

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