Effects of Drug Therapy on *Toxoplasma* Cysts in an Animal Model of Acute and Chronic Disease

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**Purpose.** To evaluate the effects of drug therapy on the clinical course of acute acquired *Toxoplasma* retinochoroiditis and on the number of *Toxoplasma* cysts present in the brain and ocular tissues in the hamster animal model.

**Methods.** The Syrian golden hamster animal model of *Toxoplasma* retinochoroiditis was used. In acute disease, systemically administered atovaquone was compared with conventional therapies (pyrimethamine combined with sulfadiazine; clindamycin; and spiramycin). The clinical course of the ocular disease was determined with retinal examination and photography of the fundus. The number of *Toxoplasma* cysts remaining after treatment was evaluated in aliquots of brain homogenate and in retinal tissue. The effect of atovaquone on cerebral *Toxoplasma* cyst count was also studied in chronic disease.

**Results.** None of the drugs administered altered the course of the acute disease, judged by clinical examination. Atovaquone alone significantly reduced the number of cerebral *Toxoplasma* cysts after acute disease. Atovaquone also significantly reduced the cerebral *Toxoplasma* cyst count in chronic disease.

**Conclusions.** Tissue cysts are believed to be responsible for reactivation of *Toxoplasma* retinochoroiditis. Atovaquone has the potential to reduce the risk of recurrent disease. (Invest Ophthalmol Vis Sci. 1998;39:1171-1175)

Ocular toxoplasmosis is the most common cause of human retinochoroiditis and accounts for as much as 55% of all cases worldwide. Treatment of the disease presents a problem to ophthalmologists, and there is much debate about the most effective treatment regimen. Clinical experience has shown that the current treatment does not seem to alter the natural progression of the disease, and there is no evidence that the available drugs are effective against the tissue cysts that are believed to be responsible for the disease’s recurrence. Evaluation of these drugs in the treatment of cysts in patients is complicated by ethical considerations and retinal biopsy cannot be justified in this condition.

The Syrian golden hamster has recently been described as a reliable animal model of *Toxoplasma* retinochoroiditis. This model is used in the present study to evaluate and compare the effect of currently available drugs on the clinical course of acute acquired *Toxoplasma* retinochoroiditis and on the number of *Toxoplasma* cysts remaining in the cerebral tissues after infection. Atovaquone, a new drug that is effective against the tachyzoite and the tissue cysts of *Toxoplasma* gondii in vivo and in vitro, was evaluated in acute and chronic infections. Atovaquone is a hydroxynaphthoquinone, trans-2[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthalenedione, with an empiric formula C_{22}H_{19}O_{3}Cl.

Hydroxynaphthoquinones were first developed (The Wellcome Foundation, Beckenham, Kent, UK) as antimalarial drugs when shortage of quinine during World War II prompted search for alternative preparations. The early drugs were ineffective in human disease because of poor absorption and rapid metabolism, but continued research resulted in the synthesis of atovaquone in the 1980s. The drug proved more active against strains of *Plasmodium falciparum* in vitro and in vivo. Unlike the earlier hydroxynaphthoquinones, atovaquone did not undergo rapid hepatic metabolism. The drug was subsequently shown to be active against the related parasite *T. gondii*. The hydroxynaphthoquinones are potent inhibitors of the mitochondrial electron transport chain, competing with the electron carrier ubiquinone at the ubiquinone-cytochrome c reductase region (complex III).

**Materials and Methods**

**Maintenance of Toxoplasma Stocks**

The avirulent ME49 cyst-forming strain of *T. gondii* maintained in mouse brain was kindly supplied by Jack Remington, Palo Alto Medical Research Foundation, (Palo Alto, CA). For maintenance of the stocks, a chronically infected mouse was killed, and its brain tissue was gently homogenized with 1 ml phosphate-buffered saline, by mortar and pestle. A 0.2-ml aliquot of this suspension was inoculated intraperitoneally onto each of the adult BALB/c mice (weight, 22–24 mg), which were used for maintenance of the stocks.

**Experimental Animals**

Adult female outbred Syrian golden hamsters (B and K Universal; The Field Station, Grimston, Aldborough, Hull, UK), each...
weighing 100 g to 120 g, were used in the experiment. Animals were kept in single cages at an average room temperature of 20°C in natural light. They were fed pellets (HMB, Special Diet Services, Witham, UK) and drank tap water ad libitum from a drinking bottle. All hamsters had normal findings on physical and ophthalmic examination and were free of serum antibodies to T. gondii at the beginning of the experiment.

All procedures used were in accordance with the Animal (Scientific Procedures) Act of 1986 and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antimicrobial Agents

The drugs used were pyrimethamine (Sigma, St. Louis, MO), sulfadiazine sodium (May and Baker, Dagenham, UK), clindamycin phosphate (Upjohn, Crawley, UK), vancomycin (Eli Lilly, Basingstoke, UK), spiramycin (kindly supplied by Celso Reis, Gerencia de Pesquisas Medicas, Rhodiafarm, Sao Paulo, Brazil), and atovaquone (The Wellcome Foundation).

Induction of Toxoplasmosis in the Hamster Animal Model

A suspension of the ME49 T. gondii strain was prepared from the infected stock mouse 6 weeks after the inoculation of the mouse with the parasite. According to the protocols described previously, each hamster was inoculated intraperitoneally with 0.2 ml mouse brain homogenate on day 0 of the experiment.

Clinical Course

Retinal examination with an indirect ophthalmoscope and a 30-diopter lens was performed on all hamsters on days 0, 7, 10, and 14 and weekly thereafter. Fundus photographs were taken with a fundus camera. Full pupillary dilation was achieved with one drop each of tropicamide 1% (Alcon Laboratories, Herts, UK) and phenylephrine 2.5% (Smith and Nephew Medical, Hull, UK) instilled 30 minutes before the examination.

Treatment Protocol for Acute Infection

At the first clinical signs of retinal disease, the hamsters were randomly assigned to one of five groups of 10 animals, and treatment was administered in the drinking water according to the following dosage regimens: group 1, untreated control; group 2, treated with 15 mg/l pyrimethamine and 1000 mg/l sulfadiazine; group 3, treated with 400 mg/l clindamycin; group 4, treated with 2000 mg/l spiramycin; and group 5, treated with 476 mg/l atovaquone.

The drug dose was calculated from the human treatment regimen correlating to milligrams per kilogram of body weight in the hamsters. The daily human treatment regimens were as follows: 50 mg pyrimethamine, 4 g sulfadiazine, 1200 mg clindamycin, 3 g spiramycin, and 3 g atovaquone. The 8-ml average daily fluid consumption of hamsters kept under laboratory conditions was used to calculate the concentration of drug supplied in the drinking water.

In a previous small pilot study, diarrhea developed in one half of five hamsters treated with clindamycin, and they died within 24 hours. The addition of 170 mg/l vancomycin (calculated from the human treatment regimen of 1 g/day) to the drinking water in this treatment group prevented further side effects. Vancomycin is not known to have anti-Toxoplasma activity. The solubility of atovaquone was improved with 50 ml/l chemophore and 50 ml/l acetone (provided by V. Andrews, Pharmacy Department, Moorfields Eye Hospital, London, UK) and the final concentration was 160 μg/ml, measured by immunoassay (performed by Martin French, Wellcome Research Laboratory, Beckenham, UK). The average daily number of milliliters of water drunk by each animal was measured and the drug supply changed weekly. After a 4-week treatment course, all hamsters received fresh drinking water (no drug) for an additional 2 weeks. The hamsters were then killed in a carbon dioxide chamber, and their brains and eyes were removed. The fresh brain was used immediately for cerebral cyst counts, and the eyes were fixed in 10% formaldehyde for histologic examination.

Treatment Protocol for Chronic Infection

The study group consisted of 16 hamsters that had chronic, inactive toxoplasmosis (diagnosed by retinal examination) 12 weeks after inoculation. Eight hamsters were treated with atovaquone at the same concentration as in the previous experiment. The treatment period was 4 weeks, in keeping with the usual treatment period in human disease. Eight untreated animals served as control subjects. After 4 weeks, all the animals were killed, and their brains and eyes were removed. The fresh brain was used immediately for cerebral cyst counts, and the eyes were fixed in 10% formaldehyde for histologic examination.

Evaluation of the Effect of Treatment

Cerebral Cyst Counts. The brain of each hamster was cut along the sagittal plane, and one half was homogenized in 1 ml phosphate-buffered saline, ground by mortar and pestle. A 0.025-ml aliquot of homogenate was placed on a microscope slide and mounted with a coverslip (24 mm X 24 mm). The number of Toxoplasma cysts were counted in four 0.025-ml aliquots from each hamster by light microscopy (magnification, X100). To calculate the total number of cysts in the whole brain, the sum of the number of cysts counted in the four aliquots was multiplied by 20. Cyst counts in the brain after the different treatment protocols were compared using Student's t-test and one-way analysis of variance (ANOVA).

Retinal Cyst Count. Retinal cysts were counted in chronic infection only. Both eyes from each hamster were fixed in 10% formal saline and embedded in paraffin. Thick sections (0.3 μm) were cut from three levels. One section from each level was stained with hematoxylin and eosin and examined for Toxoplasma cysts by light microscopy (magnification, X100). The number of cysts in 48 sections from each group (one section from each of the three levels from both eyes of each animal) were compared. The relatively small sample did not provide sufficient evidence of normally distributed data. Therefore, nonparametric equivalents of the t-tests and of ANOVA (Mann-Whitney and Kruskal-Wallis tests) were also used to confirm the results, although an assumption of normally distributed cyst counts was not crucial to the validity of the t-tests (the sampling distribution of the difference between the means is expected to be normal, even when the data have a skewed distribution).
RESULTS

Drug Administration

There was no significant difference in the volume of water drunk by each hamster in any of the different groups when measured on a weekly basis. The average daily water consumption was 8 ml (range, 5-10 ml), as expected. 0

Clinical Course

The ocular signs were in keeping with the course of the disease, as described previously. The first signs of ocular disease were seen at 10 or 14 days after inoculation. Multiple small, white, well-demarcated lesions were noted, mainly at the posterior pole, without significant vitreitis. By 14 days after inoculation, Toxoplasma lesions were observed in both eyes of all hamsters. These lesions progressively enlarged and lost their sharp borders. At 6 weeks, the lesions reached their peak of inflammatory activity; thereafter, signs of regression were noted. At 12 weeks, all lesions were determined to be completely inactive by clinical examination in all animals.

There was no difference in the clinical course of active Toxoplasma retinal lesions between treatment groups or when compared with the course in control animals, when observed with indirect ophthalmoscopy or when assessed photographically (Figs. 1, 2). No hamster exhibited signs of systemic Toxoplasma infection during the course of the experiment. One hamster in the control group died 6 weeks after inoculation. The cause of death was undetermined.

Cerebral Cyst Counts

Acute Disease. The mean number of cerebral cysts per whole brain of each animal in the five groups is compared in Table 1. Groups 1 to 4 had similar mean cyst counts. To avoid a large number of t-tests, these groups were compared using a single significance test, one-way ANOVA. The atovaquone group had a markedly lower mean cyst count, which and was compared by t-test with the count in each of the other groups.

There was no significant difference in the number of cerebral Toxoplasma cysts between groups 1 through 4 (ANOVA test for equality of means, P = 0.48; Levene test for equality of variance, P = 0.76). The atovaquone-treated group had a significantly lower mean number of cerebral cysts than the control group or either of the other three treatment groups (Fig. 3).

Chronic Disease. The mean number of cysts per sample was 0.87 in the atovaquone-treated group (17.4 per whole brain) and 15.9 in the control group (318 per whole brain). The difference was significant (P < 0.01). The nonparametric Mann-Whitney and Kruskal-Wallis significance tests confirmed the results obtained from the t-test and ANOVA. The highest significance was P = 0.002 (Mann-Whitney test) and in the comparison of groups 1 through 4, P = 0.53 (Kruskal-Wallis test).

Retinal Cyst Count

There were two cysts in 48 sections from treated eyes compared with five cysts in 48 sections from control eyes. These numbers are too small for meaningful statistical analysis.

DISCUSSION

The results of this study show that none of the drugs used altered the clinical course of acute, acquired Toxoplasma retinochoroiditis in the hamster animal model. The finding of no significant difference between groups 1 through 4 does not necessarily indicate that there was no treatment effect. The study had little chance of detecting small or moderate differences as significant. In comparing any two means, the sample size provided a power of approximately 0.8 (80% chance) to
detect a significant difference \((P < 0.05)\), only if the true (expected) difference between the mean cyst counts was substantial—that is, 9 or higher.

Atovaquone was the only drug to affect the number of cerebral \textit{Toxoplasma} cysts. It caused a significant reduction (90%) in acute disease (Fig. 3). Atovaquone also caused a significant reduction in cerebral cyst count when used in chronic disease. In acute disease, the treatment was started at the first signs of retinal lesions (10-14 days after inoculation). Because tissue cysts have been described as early as 7 days after inoculation in the animal model,\(^{15,16}\) it is uncertain whether the drug was effective against the tachyzoites or the tissue cysts. The study of the effect of atovaquone in chronic, inactive disease attempted to answer this question. In chronic infection, the \textit{Toxoplasma} organisms are expected to be in the latent, cystic form, and the effect of drug therapy on this stage of the life cycle may be determined. In this situation, atovaquone caused a significant reduction in the number of \textit{Toxoplasma} cysts in the cerebral tissue. Previous studies have shown that 90% of total body cyst load is contained in the cerebral tissue.\(^{17}\) \textit{Toxoplasma} cysts are only rarely noted in the ocular tissue,\(^{8,18-20}\) perhaps because of the smaller mass of neural tissue in the eye.\(^{17}\) This was confirmed in our model in which only five cysts were counted in 48 sections from eyes with untreated \textit{Toxoplasma} retinochoroiditis. Although atovaquone caused a reduction in the number of cysts found in the retinal tissue when compared with the number in control animals, the numbers involved are very small. Much larger groups of animals would be required for meaningful statistical analysis. For this reason, we elected to count the number of cysts in the cerebral tissue. However, it is unknown whether there is a correlation between the number of \textit{Toxoplasma} cysts in the brain and the number of cysts in the eye. Additional study is required to resolve this question.

It is likely that reactivation of ocular toxoplasmosis is caused by several factors. Rupture of the tissue cysts with infection of retinal cells by liberated organisms is believed to play a major role in the pathogenesis of recurrent disease,\(^{21,22}\) but immunologic factors may also be involved in the breakdown of the cysts and the reactivation of the parasites.\(^{18,23}\) Regardless of the relative contribution by each of these mechanisms, reduction in the number of cysts in the retinal tissue should reduce the risk of disease recurrence.

It is possible that atovaquone is effective against the tachyzoite and the encysted form of the \textit{Toxoplasma} parasite. By destroying free tachyzoites, fewer viable organisms are left for subsequent cyst formation. Thus, atovaquone may become the drug of choice for the treatment of acute episodes. New regimens with prolonged or repeated courses of treatment may be suggested, to achieve reduction of the cyst load and thereby

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**Figure 3.** Mean number and 95% confidence limits of \textit{Toxoplasma} cerebral cysts per animal in the treatment groups for acute disease. Pyr, pyrimethamine; Sulfa, sulfadiazine.

**Table 1.** Toxoplasma Cysts

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number of Animals</th>
<th>Number of Cysts</th>
</tr>
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<tbody>
<tr>
<td>Pyr. + Sulpha.</td>
<td>10</td>
<td>8.4 ± 1.93</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>12.89 ± 2.91</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>10</td>
<td>11.3 ± 2.2</td>
</tr>
<tr>
<td>Atovaquone</td>
<td>10</td>
<td>1.5 ± .52</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>10</td>
<td>8.9 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
reduce the risk of recurrent disease in the future—especially important in the immunosuppressed patient. Further studies of the efficacy this drug are merited.

CONCLUSIONS

None of the drugs studied altered the clinical course of the ocular lesions. Atovaquone was the only drug to affect the number of cerebral Toxoplasma cysts in acute disease. Atovaquone also caused a significant reduction of cerebral Toxoplasma cysts in chronic disease. Tissue cysts are believed to be responsible for disease reactivation, and this drug may reduce the risk of disease recurrence.

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References


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