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An ultrastructural study of the effect of treatment with atovaquone in brains of mice chronically infected with the ME49 strain of *Toxoplasma gondii*

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Summary. The morphological effects of drug treatment with atovaquone in the brains of mice chronically infected with *Toxoplasma gondii* was examined by light and electron microscopy. As early as 1 and 2 weeks of treatment there appeared to be fewer tissue cysts compared to untreated controls and this reduction was more significant after 4 weeks treatment. There also appeared to be a decrease in the number of inflammatory nodules and the severity of the meningitis. Ultrastructurally, the cysts of both treated and control animals were located within host cells. There was a marked increase in both the number of cysts with lysed bradyzoites and the number of degenerate bradyzoites after 4 weeks treatment. It is probable that the drug is more active against the metabolically active immature bradyzoites than the mature organisms. Drug treatment does not appear to result in rupture of tissue cysts or release of *Toxoplasma* antigens since there is a reduction rather than an increase in the inflammatory response. This drug may be useful in treating chronic toxoplasmosis since it appears to be active against the bradyzoites reducing the parasite burden (cyst number) without initiating a destructive inflammatory response.

Keywords: chronic toxoplasmosis, treatment, atovaquone, ultrastructure

Toxoplasma gondii has emerged as a major opportunistic pathogen of immunocompromised individuals, particularly those infected with human immunodeficiency virus (Navia *et al*, 1987). The majority of these life-threatening infections are due to recrudescence of the parasite in chronically infected patients. The synergistic combination of pyrimethamine and a sulphonamide is effective in treating these symptomatic cases where it is active against the proliferative phase of the parasite. Unfortunately, these drugs appear to be ineffective against the cyst form of the parasite. This ineffectiveness is clearly shown by patients with AIDS who have been treated for toxoplasmic encephalitis and who frequently relapse following discontinuation of the combination even after many months of therapy (Luft & Remington 1992). Thus, there is a critical need to find less toxic drugs and drugs which would be active against the chronic stages of the parasite. Atovaquone, initially

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Table 1. Comparison of *Toxoplasma* tissue cyst and inflammatory nodule density, cyst size and the proportion of cysts with dividing or degenerate bradyzoites after 4 weeks of treatment

		Light microscopy				Electron microscopy*	
	No. of mice	Total cysts/ half-brain†	Tissue cysts/ mm^2†	Nodules/ mm^2	Average diameter of cysts (μm)†	Percentage of cysts with dividing zoites	Percentage of cysts with degenerating bradyzoites
Control	5	2054 ± 1157	5.33 ± 0.96	0.2	47 ± 14.6	40	0
Treated	5	420 ± 179	0.53 ± 0.08	0.03	30 ± 9.40	0	56

* Based on exmination of 17 cysts from treated and 31 cysts from control mice.
† Results are mean ± s.e.m.

developed as an antimalarial, has been shown to be active against opportunistic infections (Hudson *et al.* 1991). In recent studies it has shown remarkable in-vitro and in-vivo activity against both the proliferative (tachyzoite) and tissue cyst stages of *T. gondii* (Araujo *et al.* 1991; 1992; 1993).

In the present report, we have extended these studies to an ultrastructural examination of the brains of chronically infected mice treated with atovaquone to identify the changes in the brain and tissue cysts associated with drug treatment.

Materials and methods

Mice

Inbred female CBA/Ca mice of approximately 8 weeks of age were used.

Toxoplasma gondii

Tissue cysts of the strain ME49 were obtained from the brains of chronically infected mice (Huskinson-Mark *et al.* 1991).

Treatment with atovaquone

In an initial experiment, six mice were infected by intraperitoneal inoculation of 10 tissue cysts. Four weeks post-infection, the mice were randomly divided into two groups of three. One group was treated with 200 mg/kg/day atovaquone administered by gavage, while the control group received phosphate buffered saline (PBS) by the same route. One mouse from each group was autopsied after 1, 2 and 4 weeks of treatment. In a second experiment, ten mice were similarly infected and divided into two groups. The treated and control groups received similar regimes of atovaquone or PBS as in the first experiment for 4 weeks, then both groups were autopsied and the brains examined.

Autopsy

At autopsy the mice were sacrificed by CO_2 narcosis and the brain immediately removed. It was divided longitudinally into two equal halves. One half was used to determine the total number of tissue cysts present by counting the number of cysts as described previously (Huskinson-Mark *et al.* 1991). The other half was processed for ultrastructural examination. The brain was placed directly in the primary fixative, 4% glutaraldehyde in 0.1 M phosphate buffer, and cut into 1-mm cubes to allow penetration of the fixative. The samples were post-fixed in osmium tetroxide, dehydrated in ethanol, treated with propylene oxide and embedded in epoxy resin (E Mix) (Ferguson & Hutchison 1987a). Sections 1 μm thick were stained with Azure A prior to light microscopic examination. These sections were used to quantify the number of tissue cysts and inflammatory lesions. Thin sections were cut from selected areas and stained with uranyl acetate and lead citrate prior to examination in a Jeol 100 CX electron microscope.

Results

The untreated control mice all exhibited similar pathological changes within the brain. Details of the changes in the brain associated with chronic infection with the ME49 strain of *T. gondii* are described elsewhere (Suzuki *et al.* 1989). In the present study the brains of control mice infected for 8 weeks contain numerous tissue cysts plus a number of microglial or inflammatory nodules (Table 1). All the mice exhibited moderate to severe meningitis and there was extensive cuffing of the blood vessels by inflammatory cells. This cuffing of blood vessels was widespread throughout the brain and did not appear to be localized to areas with tissue cysts. The majority of inflammatory cells were plasma cells with lower numbers of lymphocytes and macrophages.

In the initial experiment, the brains of mice treated with

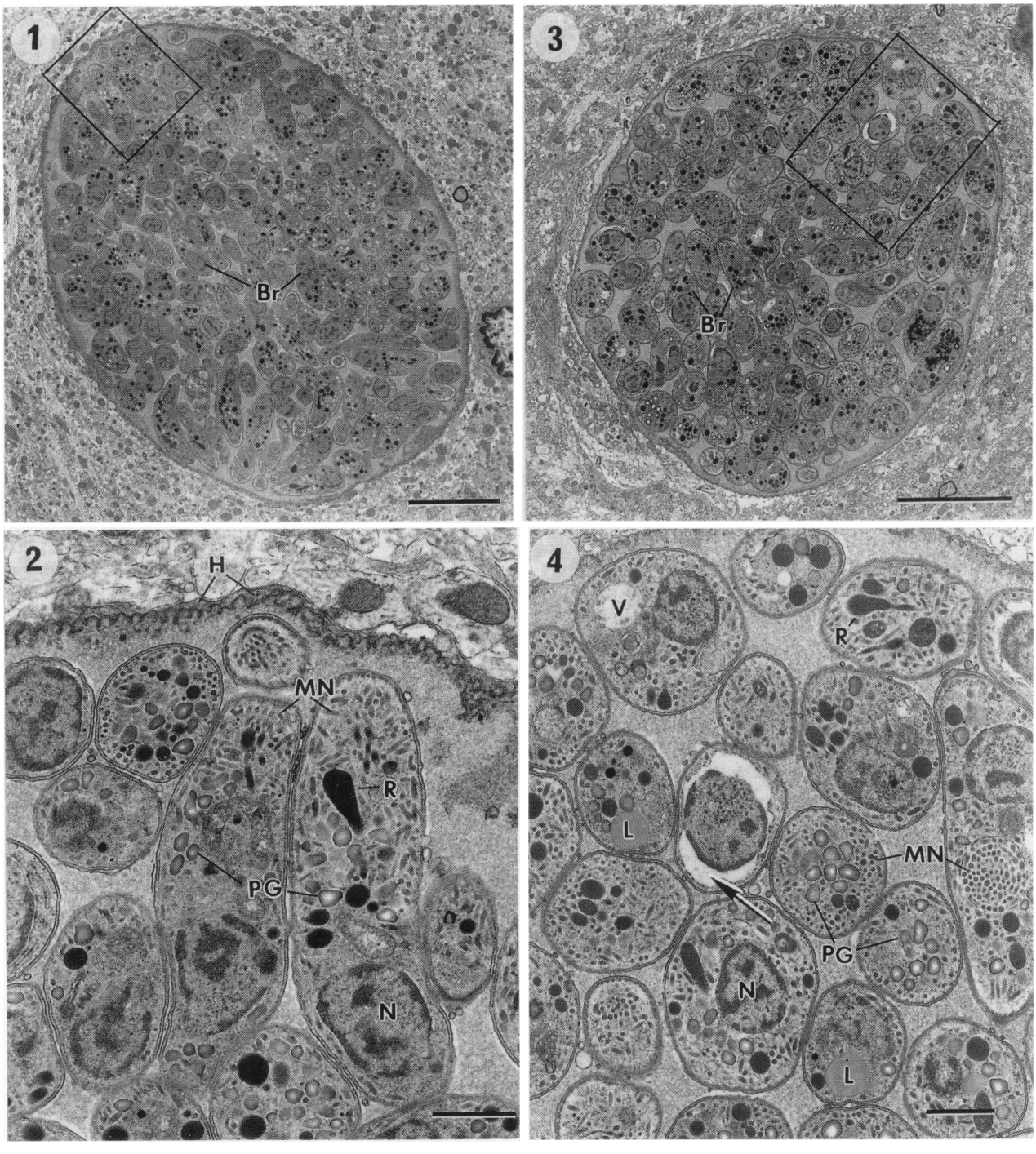
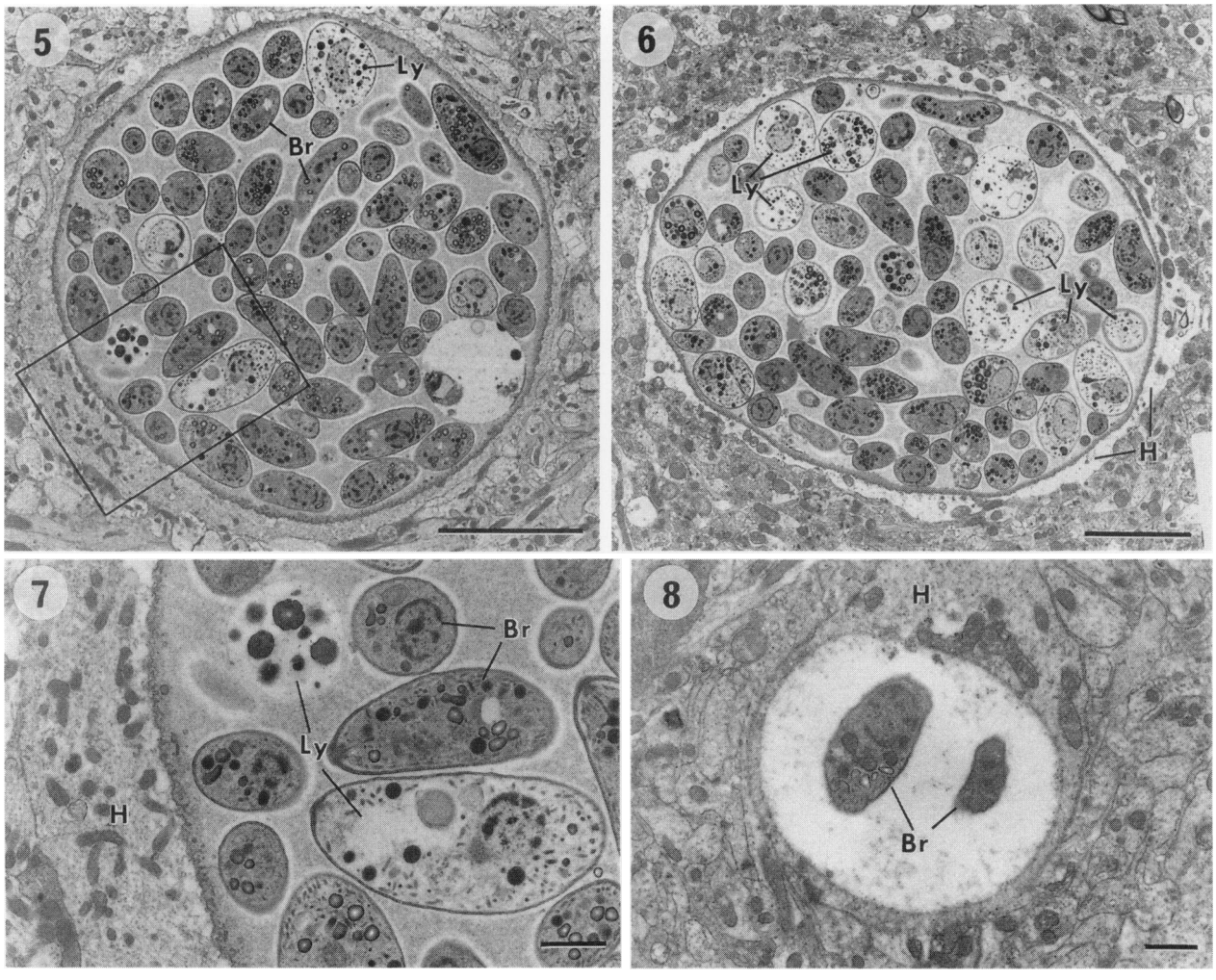


Figure 1. Low-power electron micrograph of a tissue cyst containing mature bradyzoites (Br). Control animal, 8 weeks post infection, untreated. Bar is 5 μm. **Figure 2.** Detail from the enclosed area in Figure 1 showing the bradyzoites with their characteristic organelles of nucleus (N), rhoptries (R), micronemes (MN) and polysaccharide granules (PG). Note the thin rim of host cell cytoplasm (HC) surrounding the cyst. Bar is 1 μm. **Figure 3.** Low-power electron micrograph of a tissue cyst filled with intact-looking bradyzoites (Br). One week of treatment. Bar is 5 μm. **Figure 4.** Detail of the enclosed area in Figure 3 showing the bradyzoites with their characteristic organelles of nucleus (N), rhoptries (R), micronemes (MN), and polysaccharide granules (PG). Note the abnormal presence of lipid droplets (L) and vacuoles (V) and the dilatation of the nuclear membrane (arrow) in certain organisms. One week treatment. Bar is 1 μm.



Figures 5 and 6. Micrographs of tissue cysts after 4 weeks of treatment showing variable numbers of lysed organisms (Ly) admixed with intact bradyzoites (Br). H-host cell cytoplasm. Bars are 5 μm. **Figure 7.** Detail of the enclosed area in Figure 5 showing the host cell cytoplasm (H) surrounding the tissue cyst. Note the lucent appearing cytoplasm and disruption of the organelles in the lysed organisms (Ly) compared to the intact bradyzoites (Br). Bar is 1 μm. **Figure 8.** Small tissue cyst within an intact host cell (H) containing electron-lucent ground substance and two bradyzoites (Br). Four weeks treatment. Bar is 1 μm.

atovaquone for one or two weeks contained fewer tissue cysts than did brains of controls and this was more marked after 4 weeks treatment. This significant reduction in tissue cyst numbers after 4 weeks of treatment was confirmed in the second experiment (Table 1). At this stage, cysts were difficult to find and required examination of multiple sections through the brains. This reduction in cyst numbers in treated compared to controls was confirmed in the total cyst count of the other half of the brains (Table 1). In addition, the average size of the tissue cysts in the treated mice was markedly smaller than in the controls (Table 1). There was also a decrease in the number of inflammatory nodules compared to the controls (Table 1). In addition, although the treated mice still exhibited meningitis and cuffing of blood vessels, it appeared to be less marked than in the untreated controls.

An ultrastructural study of 31 tissue cysts from the untreated control brains showed the cysts to be located within intact host cells (Figures 1 and 2). Certain tissue cysts contained only mature bradyzoites which were characterized by their crescentic shape with a basally located nucleus and the apical cytoplasm packed with numerous micronemes, rhoptries, dense bodies and

polysaccharide granules (Ferguson & Hutchison 1987b). Other cysts contained both mature and immature organisms. In approximately 40% of the cysts there was evidence of the immature organisms multiplying by endodyogeny (Table 1). Within the cysts examined, there was no evidence of bradyzoite degeneration.

Due to the low number of cysts, a great deal of searching through multiple sections was required to enable the ultrastructural examination of 17 tissue cysts within the treated groups. All the cysts observed had intact cyst walls and were located within viable host cells (Figures 5–8). This host/parasite relationship was similar to that seen in controls although a number of cysts were enclosed by a wider band of host cell cytoplasm than that seen in untreated mice (Figure 7). There was no evidence of an inflammatory cell reaction around any of the cysts including those containing numerous degenerate bradyzoites. After one week of treatment the cysts initially appear similar to untreated controls (cf. Figures 1 and 3). However, a detailed ultrastructural analysis showed that in certain cysts a proportion of the bradyzoites contained centrally located lipid droplets and electron-lucent cytoplasmic vacuoles not seen in controls (cf. Figures 2 and 4). At 2 weeks of treatment, a few of the cysts contained lysed degenerate bradyzoites. By 4 weeks of treatment there was an increase to 56%in the number of cysts with degenerate bradyzoites (Table 1) and the number of lysed organisms within the cysts had also increased (Figures 5 and 6). These lysed organisms contain electron-lucent cytoplasm with remnants of the nucleus, polysaccharide granules and apical organelles (Figure 7). However, in all cases, a number of apparently viable mature bradyzoites were present although no intact immature or multiplying bradyzoites were observed in treated animals (Figures 5–7). An additional finding in treated mice was a few very small cysts with only two or three mature bradyzoites (Figure 8). Such cysts were not observed in untreated mice.

Discussion

The drug atovaquone has been shown to have antimalarial properties, acting on the parasite mitochondrial electron transport chain (Hudson *et al.* 1991). It has recently been shown that this drug may also be active against both proliferative and chronic stages of *Toxoplasma* (Araujo *et al.* 1991; 1992; 1993). In the present morphological study of the brains of chronically infected mice, drug treatment appears to reduce significantly the parasite burden but had failed to eradicate the parasite after 4 weeks of treatment. The reduction in number of inflammatory nodules associated with treatment may result from the action of atovaquone on the proliferative form (tachyzoite) which can be responsible for certain of these lesions. It would also point to cyst eradication not involving cyst rupture or release of *Toxoplasma* antigens. This is because of the observation that rare cyst rupture in untreated animals results in a marked inflammatory cell response resulting in nodule formation (Ferguson *et al.* 1989). In the Panamanian night monkey (*Aotus lemurins*), there is a high incidence of cyst rupture with the development of numerous microglial nodules which led to the proposal that inflammatory nodules in chronic *T. gondii* infections represent tombstones to cyst rupture (Frenkel & Escajadillo 1987). This lack of inflammatory cell response to cysts in treated animals is consistent with the ultrastructural observation of the retention of cysts within their viable host cell.

An ultrastructural examination of the low number of cysts in treated animals showed that a large proportion of them contained degenerate lysed bradyzoites which were not observed in untreated controls. In contrast, the cysts of untreated animals had a proportion of immature and dividing bradyzoites which were not present in treated animals. It is possible that the drug is more effective against metabolically active immature bradyzoites than against the fully differentiated organisms. Thus, drug treatment appears to result in individual bradyzoite death with the products of bradyzoite lysis being reabsorbed by the host cell. It is possible that this results in a reduction in size and final absorption of the cyst by the host cell. This would be consistent with the observation that the average size of the cysts was smaller in the treated mice. The shrinkage of the cysts could explain the more extensive area of host cell cytoplasm around the cysts. The small cysts observed in the treated animals could represent an advanced stage in this process. This could provide a mechanism to explain the absence of an increasing inflammatory response associated with the decrease in cyst numbers, within the brains of treated animals.

Treatment of mice with toxoplasmic encephalitis (TE) with doses of atovaquone of 200 mg/kg/day for up to 12 weeks resulted in significant reduction in the numbers of *T. gondii* cysts and in an almost complete clearance of the inflammation in their brains (Araujo *et al.* 1992). Further experiments in our laboratory revealed that prolonged administration of doses of atovaquone as low as 5 mg/kg/day were sufficient to significantly reduce the inflammatory response in brains of mice with TE (F.G. Araujo & T.S. Remington, unpublished observations).

The activity of atovaquone in the treatment of disseminated acute murine toxoplasmosis, was markedly augmented when the drug was used in combination with

pyrimethamine or sulphadiazine (Araujo *et al.* 1993). These combinations, however, have not yet been examined in murine TE.

From the ultrastructural observations it would appear that the drug is more active against the immature than mature bradyzoites. The findings for the ME49 strain in which many cysts contain immature bradyzoites may be unrepresentative of the effects on strains of *T. gondii* in which the vast majority of cysts in chronic infections contain only mature bradyzoites (Ferguson & Hutchison 1987b). To answer this question would require the examination of chronic infections using other strains of *T. gondii*.

In conclusion, it would appear that atovaquone is a useful drug for treatment of chronic toxoplasmosis since it markedly reduces the parasite burden (number of cysts) without stimulating a damaging inflammatory response within the brain.

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