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## Alterations of *Toxoplasma gondii* Induced by 2',3'-Dideoxyinosine In Vitro

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**ABSTRACT:** The time-course of action of the antiviral agent 2',3'-dideoxyinosine (ddI) against *Toxoplasma gondii* tachyzoites in vitro and its effects at the ultrastructural level were investigated. The very short latency of effect and high efficacy of ddI were evidenced by the fact that the drugs' effects on parasite growth occurred 2 hr after addition to the culture medium, and that an  $IL_{90}$  value of 0.5  $\mu\text{g/ml}$  was reached after 72 hr. Although without apparent effect on uninfected cells, ddI clearly acted on the intracellular parasites, which tended to disappear. Remaining tachyzoites were almost exclusively extracellularly located and often exhibited a clustering of mitochondria-like bodies and subsequent deep alterations of their plasma membranes. These results con-

firm previous findings and emphasize the potential usefulness of ddI in the management of cerebral toxoplasmosis, a major health problem in acquired immune deficiency syndrome patients.

Cerebral toxoplasmosis is a major health problem in acquired immune deficiency syndrome (AIDS) patients (Oksenhendler et al., 1994; Ammassari et al., 1996; Kaplan et al., 1996). At present, few drugs are efficacious against cerebral cysts, namely pyrimethamine (Winstanley et al., 1995; Klinker et al., 1996;

TABLE I. Kinetics of the in vitro inhibition of *Toxoplasma gondii* growth by ddI. A stock 10-mg/ml ddI solution was diluted in the culture medium. Results are the mean  $\pm$  SD of 5 different experiments.

Drug concentration ( $\mu\text{g/ml}$ )	Growth inhibition (%)				
	12 hr	24 hr	48 hr	60 hr	72 hr
0.05	29.7 $\pm$ 1.5	55.3 $\pm$ 1.5	60.7 $\pm$ 2.5	68.0 $\pm$ 3.0	72.7 $\pm$ 2.5
0.5	48.0 $\pm$ 3.0	58.3 $\pm$ 1.5	70.7 $\pm$ 3.1	81.0 $\pm$ 3.6	90.3 $\pm$ 2.5
1	55.0 $\pm$ 2.0	70.0 $\pm$ 2.0	78.7 $\pm$ 2.1	86.3 $\pm$ 3.1	100
2	57.7 $\pm$ 2.5	87.0 $\pm$ 2.0	87.7 $\pm$ 2.5	90.3 $\pm$ 2.5	100
4	93.0 $\pm$ 2.0	97.3 $\pm$ 2.1	99.0 $\pm$ 1.0	99.7 $\pm$ 0.6	100

Leport et al., 1996), azithromycin (Chang, 1996; Wiselka et al., 1996), atovaquone (Katlama et al., 1996) or drug combinations such as trimethoprim-sulfamethoxazole (Carr et al., 1992), clarithromycin-minocycline (Lacassin et al., 1995), doxycycline-pyrimethamine (Hagberg et al., 1993) pyrimethamine-dapsone (Derouin et al., 1991), pyrimethamine-clindamycin (Danne-mann et al., 1992), and pyrimethamine-azithromycin (Saba et al., 1993). In a previous study, we showed that 2',3'-dideoxyinosine (ddI), a nucleoside analog used as an antiviral agent (McGowan et al., 1990; Faulds and Brogden, 1992), was active against cerebral cysts of *Toxoplasma gondii* (Sarciron et al., 1997). An in vitro study was performed with several drug concentrations to determine the rate and the nature of the effect of this drug on *T. gondii*.

The DUR strain of *T. gondii* was isolated from tissues of a pregnant woman. This isolate is considered to be of low virulence because it causes chronic infection in mice, within about 3 mo. This avirulent strain was maintained in our laboratory by oral passage of cysts from the brains of infected mice.

The human myelomonocytic cell line THP-1 (ECACC number 88081201, Sophia-Antipolis, France) was used for the cultivation of *T. gondii*. These nonadherent cells were maintained in RPMI-1640 (DAP, Vogelgrun, France) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, L'Isle d'Abeau, France), and 10% fetal calf serum (DAP). At each passage, the number of THP-1 cells and tachyzoites was determined with a Malassez hemocytometer and adjusted to  $10^5$  cells/ml and  $3 \times 10^4$  parasites/ml, respectively.

Spiramycin and sulfadiazine were obtained from Sigma, and ddI was obtained as a bulk powder from Bristol-Myers Squibb Laboratories (Paris, France). All drug dilutions were made with the culture medium. One milliliter of parasitized cell suspension was distributed into each well of 6-well tissue culture plates. After settlement of the cells, 100 µl of medium was aspirated and replaced by the same volume containing the dissolved drugs at final concentrations ranging from 0.05 to 4 µg/ml. Positive controls were spiramycin and sulfadiazine. Working dilutions were freshly prepared for each experiment in a constant final volume of 1 ml. After different incubation times at 37 C in a moist 5% CO<sub>2</sub>-95% air atmosphere, the contents of each well were removed, placed in an Eppendorf microtube, and centrifuged at 15,000 rpm for 2 min. Viable cells were counted using ethidium bromide and acridine orange according to Suresh et al. (1994). The percentage of infected cells and the number of extracellular parasites were calculated after counting 1,000 cells from triplicate wells. Growth inhibition was calculated with the following formula:  $100 - (\% \text{ infected cells in treated wells} \times 100/\% \text{ in controls})$ . At least 5 different experiments were performed with each drug concentration in quadruplicate.

Culture samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.5 for 2 hr at 4 C. The samples were washed in cacodylate buffer and postfixated for 1 hr at 4 C in 1% (w/v) osmium tetroxide. After 3 washes, the samples were dehydrated in a graded series of ethanol and embedded in Epon (Epon 812). Ultrathin sections were cut with a Reichert OMU<sub>3</sub> ultramicrotome, placed on 300-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX electron microscope.

Concentration- and time-dependent toxicity of ddI on *T. gon-*

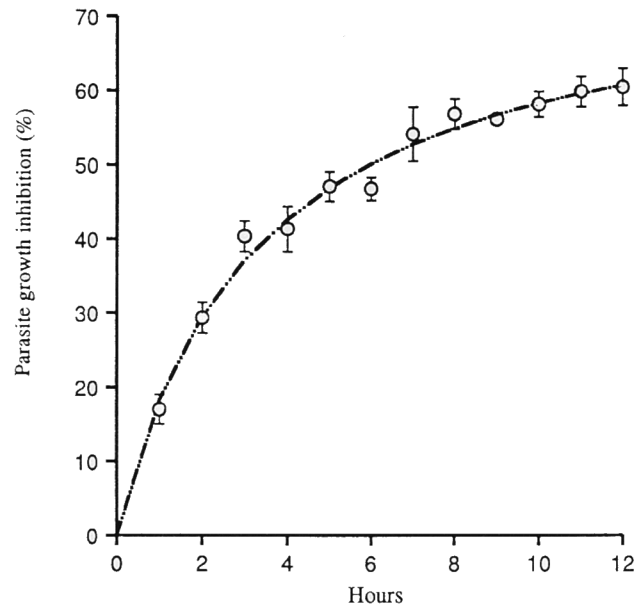


FIGURE 1. Initial effect of 2 µg/ml ddI on the growth of *Toxoplasma gondii*. Mean  $\pm$  SD of 5 different experiments.

ddi cultures is shown in Table I. Even at high concentrations, THP-1 cells were not apparently affected by this drug. After 24 hr, 50% inhibition of parasitic growth was obtained with ddI concentrations as low as 0.05 and 0.5 µg/ml, whereas complete inhibition was achieved at 72 hr with 1 µg/ml or more, a result not obtained with sulfadiazine and spiramycin (data not shown). After 12 hr, a progressive decrease in the number of intracellular parasites was observed and after 60 hr of incubation with ddI, even at lower concentrations, the location of the remaining parasites was mainly extracellular. Figure 1 shows the effect of 2 µg/ml ddI on the first 12 hr growth of *T. gondii* in THP-1 cells. It is important to note that this molecule induced the death of the tachyzoites from the very first hour of contact; at the same concentration, sulfadiazine and spiramycin needed 48 hr to achieve the inhibition that ddI induced after 2–3 hr. The electron micrographs studied provided evidence that detectable ultrastructural damage occurred after 6 hr (data not shown). As the incubation continued, most of the parasites were found outside the host cells undergoing a deep internal disorganization, followed by damage to the pellicle surrounding the tachyzoites (Fig. 2).

The aim of this study was to evaluate the time-course of action of ddI against *T. gondii* tachyzoites in vitro and to assess its effects at the ultrastructural level. The drug exhibited a very short latency of effect because the first effects on parasite growth could be seen as soon as 2 hr after its addition to the culture medium. Neither particular toxicity on THP-1 cells nor changes in the culture conditions were detected in the presence of ddI. Interestingly, whereas most of the parasites were intracellular in the control cultures and at the beginning of incubation, the number of cells harboring dividing tachyzoites dropped dramatically, whereas the extracellular location of the remaining parasites became predominant after 8 hr and almost exclusive after 24 hr of incubation with the drug. These extracellular *T. gondii* tachyzoites exhibited deep alterations in their inner structure, with subsequent damage to their outer membranes.

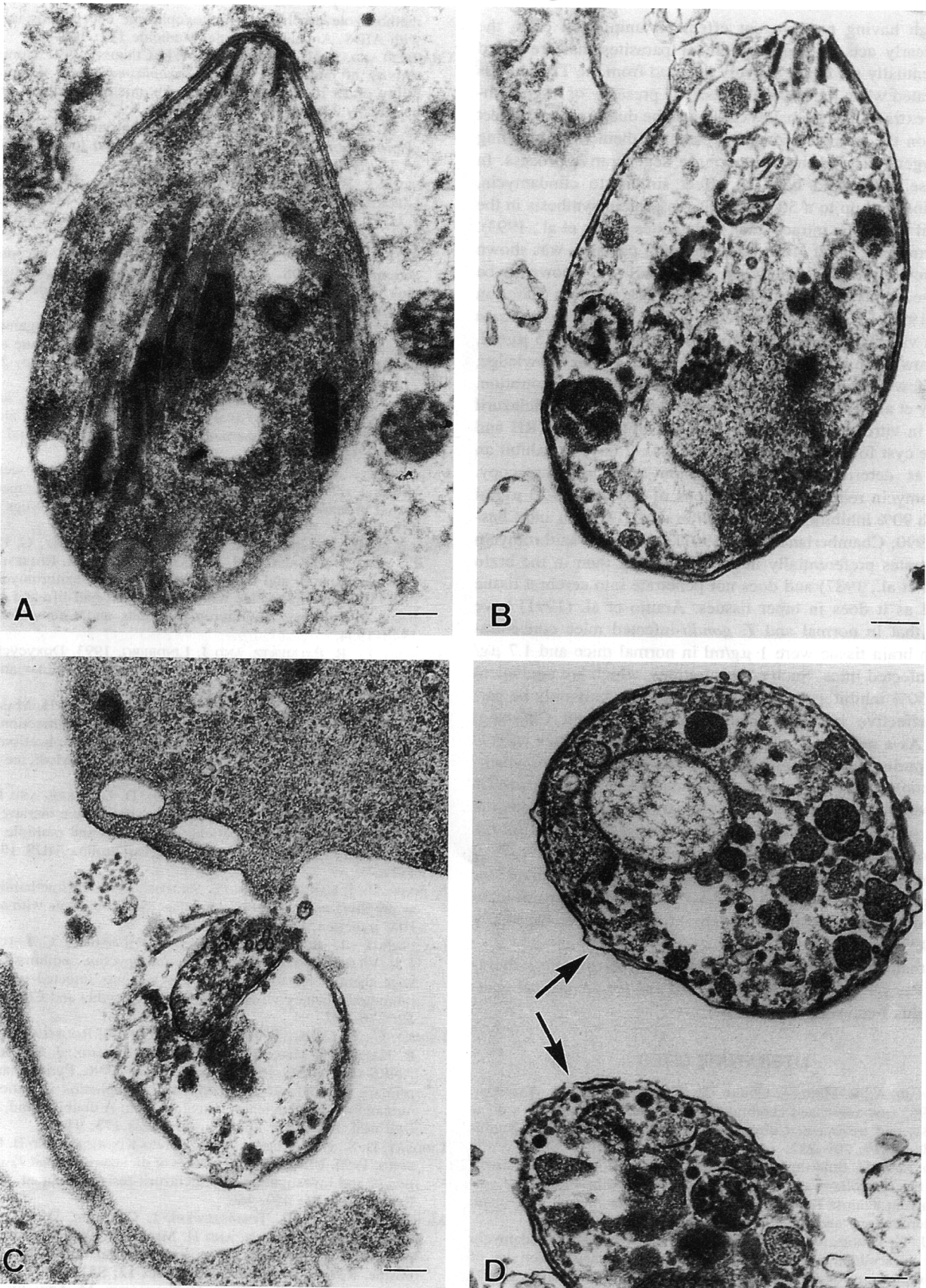


FIGURE 2. Sequential ultrastructural alterations caused by 2  $\mu\text{g/ml}$  ddI on free *Toxoplasma gondii* tachyzoites. **A.** Control, intracellular parasite. **B.** At 7 hr, a deep internal disorganization and an alteration of the outer cell membranes is visible. **C.** Extracellular degenerating tachyzoites are seen amidst healthy THP-1 cells (8 hr incubation). **D.** After 9 hr, most of the parasites are seen in an extracellular location. Note the disruption and blebs of the plasma membrane (arrows). Bar = 0.2  $\mu\text{m}$ .

Although having no apparent effect on uninfected cells, the drug clearly acts on the intracellular parasites that are killed and eventually are either actively released from the THP-1 cells or liberated when the host cell dies. The presence of large numbers of extracellular parasites could also be due to a direct effect of ddI on the free tachyzoites, preventing them from invading new target cells and thus triggering a degenerative process. In this case, ddI could have an effect similar to clindamycin, which induced up to a 50% decrease in protein synthesis in the free, but not in the intracellular, tachyzoites (Blais et al., 1993). Furthermore, a direct effect on developing parasites was shown in a previous *in vivo* study, in which ddI was shown to be effective against brain cysts and to reach brain tissues (Sarciron et al., 1997). A striking feature of ddI as an anti-*Toxoplasma* drug *in vitro* is its quick efficacy at low doses; with 0.5 µg/ml, 90% growth inhibition is reached after 72 hr. To our knowledge, only a few drugs are as active and rapid at this concentration. Lindsay et al. (1995) tested the anticoccidial molecule diclazuril on the *in vitro* development of 3 strains of *T. gondii* (RH and 2 tissue cyst formers). At 1 µg/ml, it took 2 days to exhibit an effect as determined by transmission electron microscopy. Azithromycin required concentrations of at least 10–20 µg/ml to reach 90% inhibition of *T. gondii* *in vitro* (Derouin and Chastang, 1990; Chamberland et al., 1991). Moreover, azithromycin concentrates preferentially in the lung rather than in the brain (Girard et al., 1987) and does not penetrate into cerebral tissue as well as it does in other tissues. Araujo et al. (1991) have shown that in normal and *T. gondii*-infected mice concentrations in brain tissue were 1 µg/ml in normal mice and 1.7 µg/ml in infected mice. Such concentrations, which are equivalent to the 50% inhibitory concentrations *in vitro*, may only be partially effective in acute infections (Derouin and Chastang, 1990). As a nucleoside analog, ddI probably interferes with *T. gondii* purine metabolism, but at present, it is not known whether the molecule itself or one of its metabolites, such as ddATP, is active. Further studies are underway to elucidate both the impact of ddI on this particular metabolic pathway and on the effect of chemotherapy on the cerebral tissue of the host.

These present *in vitro* results also confirm and extend our previous *in vivo* study and emphasize the potential usefulness of ddI in the management of cerebral toxoplasmosis, especially in AIDS patients.

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