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Effects of 2′,3′-Dideoxyinosine on *Toxoplasma gondii* Cysts in Mice

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The activity against *Toxoplasma gondii* of 2′,3′ dideoxyinosine (ddI), an anti-human immunodeficiency virus drug, was examined in an in vitro and in vivo study. Cell cultures infected with a strain known to cause chronic infections were used to show the dose-dependent effect of this drug compared with spiramycin and sulfadiazine. When a dose of 4 μg/ml was used, no infected THP-1 cells or parasites were found after 60 h of incubation. An electron-microscopic study confirmed that after 12 h at 1 μg/ml, the few parasites observed were severely altered. The treatment of chronically infected mice 3 months postinfection showed that a 30-day treatment with 2 mg of ddI/ml induced a significant reduction in the number of *T. gondii* cysts in the cerebral tissue. These cysts were not viable, as confirmed by immunofluorescence and reinfection experiments. These experiments suggest a possible role for ddI in the treatment of toxoplasmosis, and this possibility deserves further investigation.

Toxoplasma gondii, a coccidian protozoan, is an important pathogen in immunosuppressed patients, especially human immunodeficiency virus (HIV)-positive patients. Reactivation of cysts in tissue is a major pathogenic event and represents a life-threatening complication (15). Cerebral toxoplasmosis occurs in 3 to 40% of AIDS patients, depending on the prevalence of latent *T. gondii* in a given population (41). In the United States, as little as 3% and as much as 75% of different populations have serologic evidence of prior infection (35), whereas the overall prevalence in western Europe is higher and is approximately 50 to 75% in many areas (8, 33, 44) and in Danish AIDS patients the incidence is 8% (40). In recent years, the worldwide increase in the number of AIDS patients has prompted several groups of workers to look for an ideal treatment which would destroy the cysts. Cotrimoxazole (6), clarithromycin (2, 10, 36), clindamycin (4, 16), diclazuril (25), azithromycin (3, 13), pyrimethamine (24), and recently the new synthetic fluoronaphthyridone trovafloxacin (22) have already been tested.

Many drug combinations, such as trimethoprim-sulfamethoxazole (7), trimetrexate-leucovorin (27), pyrimethamine-sulfadiazine (1), pyrimethamine-dapsone (5, 11), pyrimethamine-clindamycin (9), clarithromycin-minocycline (23), rifabutin-atovaquone (38), and many others (39), have also been tested. These studies emphasize the parasiticidal effect of these drugs in addition to the anti-HIV treatment, establishing very heavy therapeutic arsenals. An anti-HIV drug active per se against *T. gondii* cysts in the brain could have a double potential activity, especially since this cerebral location presents considerable pharmacological difficulties for the development of antibradyzoite agents due to the amount of drug which effectively reaches the brain (29). In protozoan parasites, the purine pathway is a possible target for antiparasitic drugs (26). We thus chose to test a nucleoside analog, 2′,3′-dideoxyinosine (ddI), which has been used in HIV-positive patients with real success (28).

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The aim of this study was to assess ddI in vitro and in vivo. The effect of this drug on *T. gondii* cysts was analyzed histologically and microscopically.

MATERIALS AND METHODS

Parasites. *T. gondii* DUR was isolated from a child with subclinical congenital toxoplasmosis. After intraperitoneal injection, this strain was considered to have low virulence because it caused chronic infection in about 3 months. This avirulent strain was maintained in the laboratory by oral passage of cysts from the brains of infected mice.

Cell culture. THP-1, a human myelomonocytic cell line (European Collection of Animal Cell Cultures [ECACC] 88081201; Sophia-Antipolis, France) was used for *T. gondii* culture. These nonadherent cells were suspended in RPMI 1640 (DAP, Vogelgrun, France) supplemented with 2 mM glutamine, 100 U of penicillin/ml, 100 μg of streptomycin (Sigma, L'Isle d'Abeau, France)/ml, and 10% fetal calf serum (DAP). The numbers of THP-1 cells and tachyzoites were counted with a Malassez cell and adjusted to 10^5 cells/ml and 3×10^4 parasites/ml, respectively.

The parasitized cell suspension (300 μl) was distributed into each well of 12-well tissue culture plates (Falcon). After settlement of the cells, 200 μl of medium was aspirated and replaced by the same volume containing the dissolved drugs at concentrations ranging from 0.2 to 4 μg/ml.

All stock dilutions of drugs were made in sterile medium at a 10-mg/ml concentration. Working dilutions were freshly prepared for each experiment in a constant final volume of 300 μl. After 60 h at 37°C in a moist 5% CO_2–95% air atmosphere, the contents of each well was aspirated, placed in an Eppendorf microtube, and centrifuged at 32,000 × g for 2 min. A smear was made from the pellet and stained with Giemsa (20% [vol/vol]) in water. The percent infected cells was calculated after counting of 1,000 cells from triplicate wells. The percent growth inhibition was calculated with the following formula: 100 − [(% in treated wells × 100)/% in controls]. The cell viability was controlled microscopically to assess that the drugs were not toxic to the THP-1 cells.

Animals. Forty female OF1 mice 8 weeks of age (IFFA-CREDO, l'Abresle, France) were infected by gavage with cysts obtained from the brain of an infected mouse. The brain tissue was suspended in 2 ml of 0.9% NaCl and ground with a pestle and a mortar, and then the preparation was further homogenized by passage through a needle and syringe. The cyst concentration was determined by bright-field light microscopy. The cysts were diluted with sterile saline solution to a final concentration of 10 cysts/0.2 ml per mouse.

Drugs and treatment. Spiramycin, sulfadiazine, pyrimethamine, and clindamycin were bought from Sigma, L'Isle d'Abeau, France. ddI, in 25-mg tablets or as pure powder, was obtained from the Bristol-Myers Squibb Pharmaceutical Research Institute. Three months postinfection, the infected animals were divided into two groups of 20 each: untreated (or control) animals and ddI-treated animals.

Eight tablets were suspended in 100 ml of drinking water, which was offered ad libitum. This solution was made fresh every day. The treatment duration was 30 days. At the end of the treatment, the mice were anesthetized by diethyl ether inhalation.

The carotida was sectioned and blood samples were collected for serological

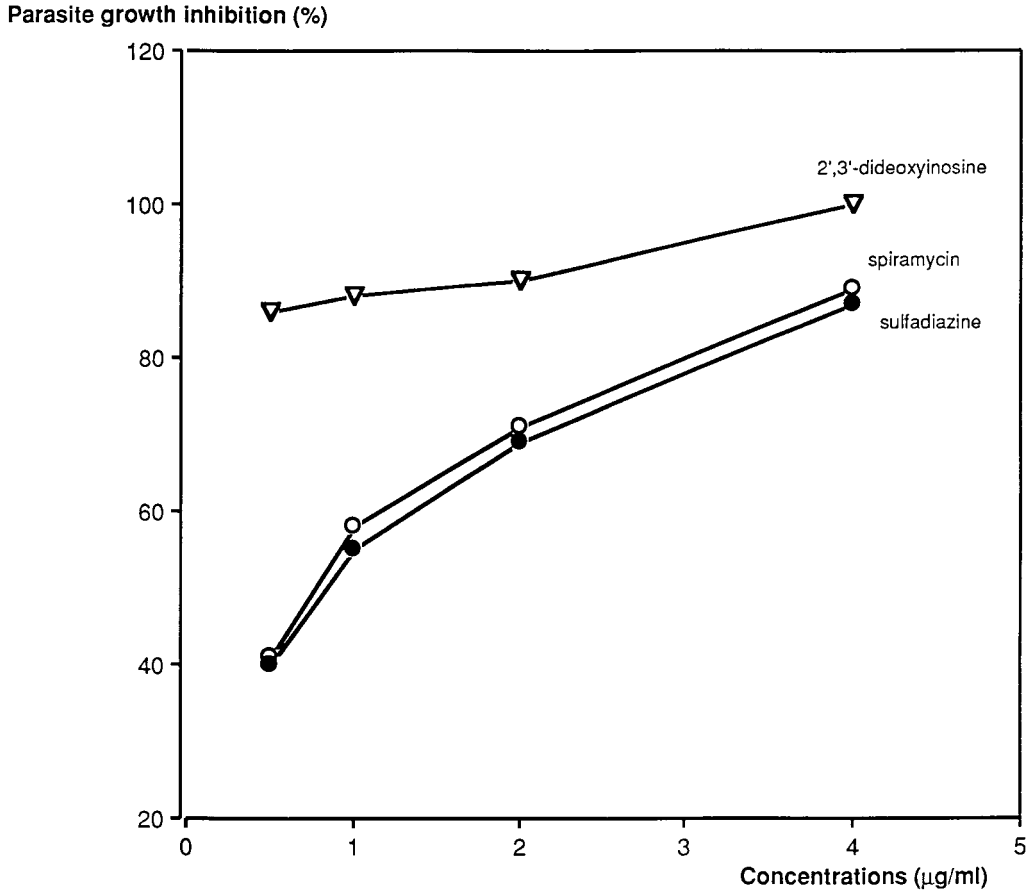

FIG. 1. In vitro effect of ddI on the growth of *T. gondii* compared to that of the reference drugs, spiramycin and sulfadiazine.

testing. The brain of each mouse was removed for microscopical and histological studies. A sample was collected to evaluate the number of cysts in treated and untreated mice. The tissue was mixed with phosphate-buffered saline (PBS) and ground as described above. The number of cysts in six samples of 20 μl each was determined for each brain.

Viability assay. The viability of the cysts recovered from the two groups of mice was tested by gavage of the brain homogenates to two groups of 10 naive 8-week-old OF1 mice each.

Three months postinoculation, these mice were sacrificed, the blood samples were collected for serological testing, and the occurrence of cysts was assessed by microscopic examination.

Indirect immunofluorescence. Immunofluorescence was performed to determine whether the treated mice produced antibodies to *T. gondii*. For this purpose, cryosections of control brain pieces were fixed with 100% methanol for 15 min and air dried. After blocking of the nonspecific binding with 0.5% bovine serum albumin (BSA) (wt/vol) in PBS for 30 min at 37°C and washings in PBS, the mouse sera were diluted 1/50 in PBS–0.5% BSA and were incubated for 30 min at 37°C. The reaction was visualized with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antiserum (Sigma) diluted 1/500 in 1/10,000 Evans blue-PBS.

The treatment efficacy was monitored by immunofluorescence as described above by using cryosections of the brain pieces from the control and treated mice except that tissue cysts were visualized with a rabbit antitoxoplasma antiserum (Virostat, Portland, Maine) diluted 1/500 followed by fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (1/500). A propidium iodide solution (1/50 mg/ml in PBS), which stains parasite nuclei red, was used to allow a better visualization of cysts and parasites.

Histology. For light microscopy, samples of parasitic lesions were incubated in Bouin's fixative, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections 4 μm thick were stained with Giemsa or hematoxylin-phloxine-safranin.

Electron microscopy. Electron microscopy was performed on culture samples which were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.5 for 2 h at 4°C. The samples were washed in cacodylate buffer and postfixed for 1 h at 4°C in 1% (wt/vol) osmium tetroxide. After three washes, the samples were dehydrated in a graded series of ethanol and embedded in Epon (Epox 812). Ultrathin sections were cut with a Reichert OMU_3 ultramicrotome, placed on 300-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX electron microscope.

RESULTS

In vitro tests. At the concentrations used (0.1 to 4 μg/ml), ddI had no effect on cell morphology, whereas 1 μg of clindamycin/ml modified the host cells' appearance. Conversely, clindamycin and pyrimethamine displayed a strong cell toxicity and a lower solubility, respectively, and were thus removed from the study. After a 60-h incubation, 1 μg of ddI/ml induced an 87 to 89% inhibition, comparable to the effects of spiramycin and sulfadiazine at 4 μg/ml (Fig. 1). As determined by comparison with controls, ddI seemed to induce the release of the tachyzoites into the medium at 0.2 and 0.5 μg/ml. At higher concentrations, the parasites disappeared and no parasitized THP-1 cell was observed.

The electron microscopy study showed that the tachyzoites were grouped (two or more) in control cultures (Fig. 2A). In the treated cultures, after 12 h of incubation with 1 μg of ddI/ml, few undamaged tachyzoites which had just invaded the cells were found, and no intact parasitophorous vacuole was observed (Fig. 2B and C). A few degenerating parasites, with loss of structure and disaggregation, were seen (Fig. 2D and E).

In vivo tests. In both groups, no mice died during the 30 days of treatment. The number of *T. gondii* cysts observed in the brains of mice treated with ddI was lower than that in the

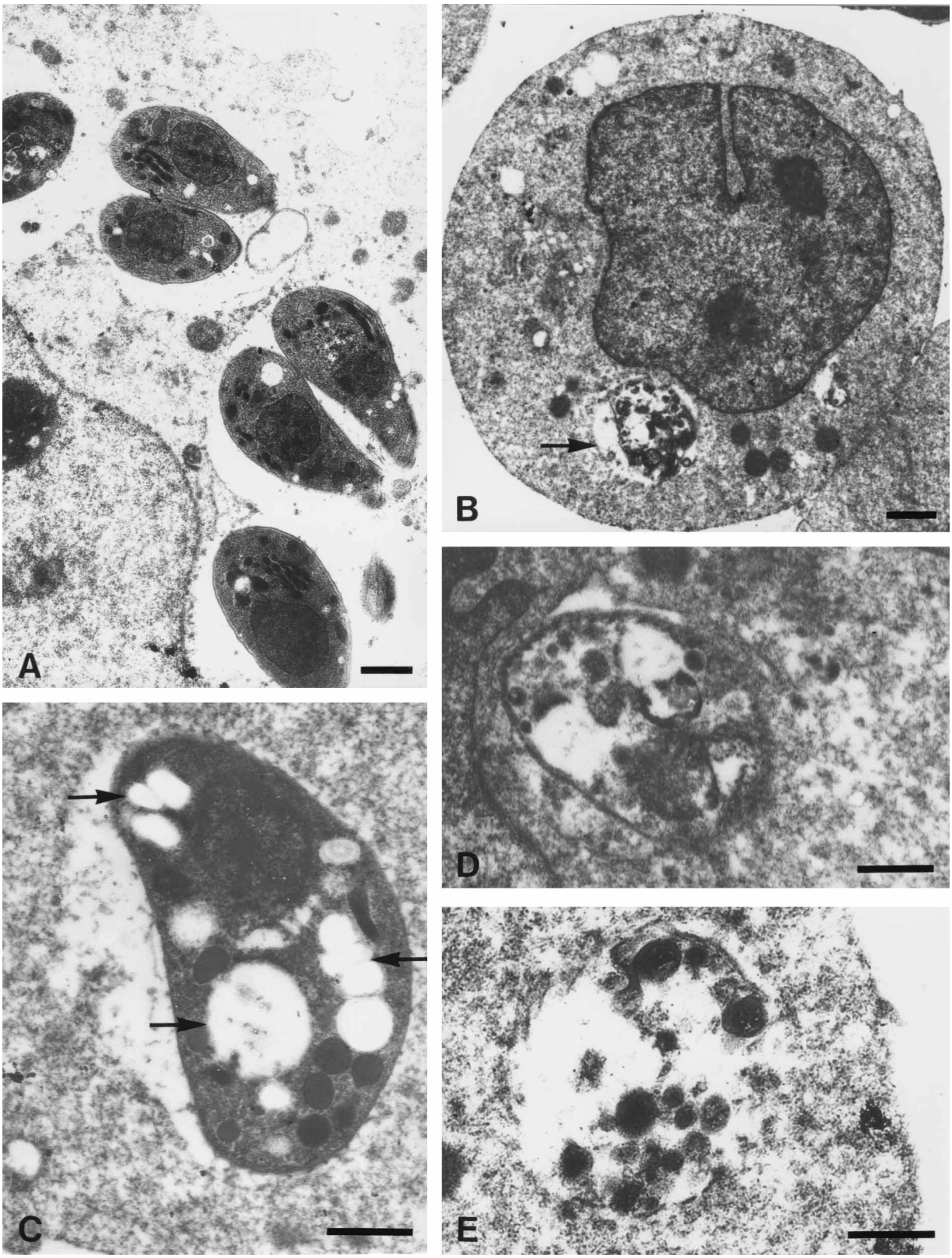

FIG. 2. Electron microscopy study of the in vitro effect of ddI on *T. gondii* in culture. Parasitized THP-1 cells were incubated with 1 μg of ddI/ml for 12 h at 37°C. (A) Control, parasitized THP-1 cell with several tachyzoites. Scale bar = 1 μm. (B) Treated cultures. Damaged parasites were observed (arrow). Scale bar = 1 μm. (C through E) The tachyzoites are damaged; several vacuoles appear in the tachyzoites (arrow). Scale bars = 0.5 μm.

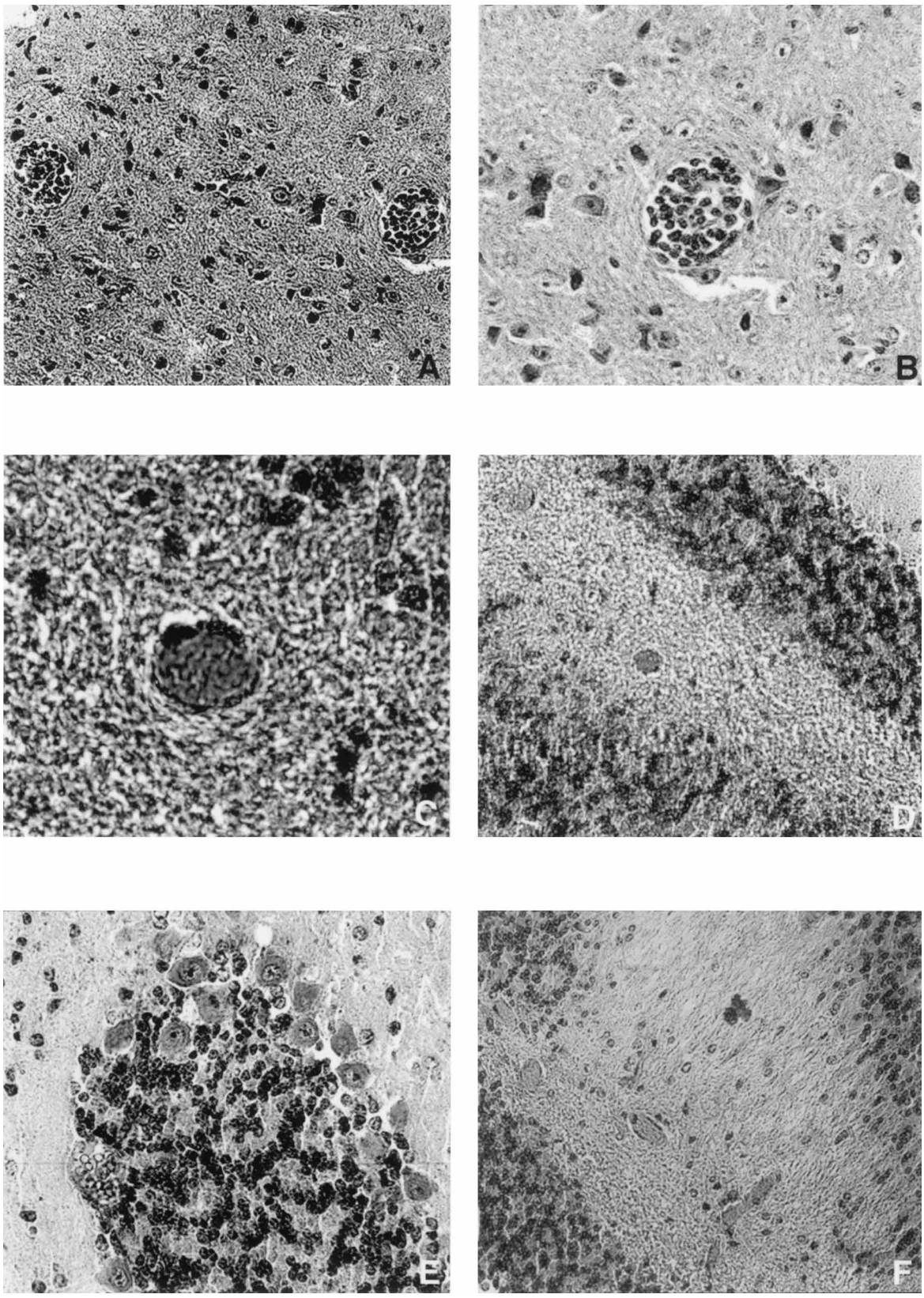

FIG. 3. Histological study of the OF1 mouse brain. (A and B) Controls (infected, untreated mice). Cysts were observed in the cerebral tissue. (C through F) Cerebral tissue of mice treated with ddI for 30 consecutive days. A few badly damaged cysts were observed, with disorganization of the tissue. Magnifications: ×625 (A), ×1,000 (B), ×2,500 (C), ×625 (D), ×1,000 (E), and ×625 (F).

controls, 100 ± 3 and 390 ± 5 cysts per brain, respectively. In control mice, the histological examination showed a modification of the cerebral tissue due to the presence of the parasite cysts. The trophozoites were free or grouped in the cerebral substance (Fig. 3A and B). In the treated animals, we observed only a few cysts which appeared dead, as well as a disorganization of the tissue and a necrosis process which do not seem to originate from a vascular source (Fig. 3C).

A defense mechanism of the neuroglia which induced a centrifugal cicatrization was also noted, but there was no inflammatory reaction in the cerebral tissue of controls and treated mice (Fig. 3D, E, and F).

In indirect immunofluorescence testing, the sera of infected treated mice showed a positive reaction comparable to the fluorescence obtained with the sera from control mice. The use of a commercial anti-*Toxoplasma* antibody showed that brain sections from treated animals had unlabeled cysts (not shown).

A reinfection experiment showed that secondary cysts were found in the animals infected with cysts from untreated mice, whereas naive mice infected with homogenates of brains collected from treated mice harbored no cysts.

DISCUSSION

In the United Kingdom, as in the United States, sulfonamide plus pyrimethamine is regarded as first-line therapy although the high incidence of toxicity necessitates administration of alternative regimens in a proportion of cases. There is an urgent need for the introduction of novel, effective agents for the prophylaxis, acute therapy, and maintenance treatment of toxoplasmosis in AIDS patients (18).

The in vitro culture experiments showed that low concentrations of ddI (0.5 μg/ml) administered for 60 h inhibited the growth of tachyzoites without damage to THP-1 cells. An extensive alteration of tachyzoites after only 12 h of incubation at 1 μg/ml was noted by electron microscopy. These doses were low compared to those of the other drugs tested with an avirulent strain of *Toxoplasma* (19).

In our study, the average dose absorbed by the animals was about 1 mg per day, and they received the drug continuously for 30 days. We can assume that our ddI solution (pH 7, room temperature) was stable during the experiments, since solutions were reported as stable above pH 6 for 8 h at 37°C (37). It has been reported that in mice, the concentration of ddI in the brain is less than 0.7 μg/ml for oral doses of 54.5 and 452 mg/kg of body weight (12).

The reported half-lives are 1 to 6 h for ddI and 8 to 24 h for ddATP (14), its active metabolite against HIV. In our experiments, it is not possible to know whether ddI or ddATP is the active form.

However, after 1 month of continuous treatment, the few cysts observed appeared extensively damaged microscopically and histologically, consistent with passage of the drug into the brain. The histological study showed that ddI did not trigger any inflammatory reaction and that it seemed to increase the efficiency of the neuroglia responsible for local protection, with destruction of the parasitized areas. Our hypothesis is that at first the parasites were damaged and then an important intervention of the neuroglia destroyed the infected areas. This is consistent with the results of the in vitro experiments, with a self-destruction of the parasites, but remains to be thoroughly studied.

We noted that in treated mice, there was a persistence of antitoxoplasma antibodies even though no intact cysts or trophozoites were seen in the cerebral tissue. Dead parasites may also induce an antibody response. These results were confirmed by the reinfection experiment with the brains of treated animals, which were negative, demonstrating that the parasites were eventually killed despite successful initial infection.

ddI is a potent inhibitor of HIV replication in human cells (30, 31) and is active against HIV in monocytes/macrophages in vitro (34). Currently, the treatment of cerebral toxoplasmosis in AIDS patients is mainly a combination of successive treatments, with the drug being used alone or in combination. These treatments seem to be very controversial, probably because of their very great complexity and the responses of the patients to different compounds (20, 42). In 1989, Israelski et al. (21) tested another antiviral agent, zidovudine (azidothymidine), on a virulent and an avirulent strain of *T. gondii* (RH and C56, respectively). They observed that zidovudine used alone in vitro had no discernible effect on the intracellular replication of the parasite and that there was no difference in vivo between control and treated animals when the drug was used alone or in combination with pyrimethamine.

Our present data are very encouraging. In these experiments, very low doses were used, compared to the doses used for AIDS treatment.

For adult, the oral dosage is 250 to 400 mg daily, and this could lead to protection of these patients against the reactivation of toxoplasmosis, which is currently of great importance for public health.

Currently, new forms such as liposomes are being tested to increase the pharmakokinetic properties of ddI (17) because of side effects such as neuropathy and pancreatitis (32, 43). These new forms could be also tested against *T. gondii.*

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