PHENYLALANINE DERIVATIVES ACTIVE AGAINST *TOXOPLASMA GONDII* BRAIN CYSTS IN MICE

SARCIRON M.E.*, WALCHSHOFER N.*, PARIS J.**, PETAVY A.F.*** & PEYRON F.***

Summary :

The action of phenylalanine derivatives against a cyst forming strain of *Toxoplasma gondii* was tested *in vitro* and *in vivo* in mice. These compounds were Phe-Phe-OMe (dipeptide methyl ester) 1 and its cyclized product, 3,6-dibenzyl-2,5-dioxopiperazine 2, Boc-L-Phe 3, L-Phe-OMe 4, Boc-L-Phe-L-Phe-OMe 5. After a 48 hr incubation *in vitro*, the compounds 3 and 5 induced a higher inhibition than the control molecule, pyrimethamine. In the *in vivo* studies, the compound 3 induced a 77 % decrease in the number of cerebral cysts, comparable to pyrimethamine. Compounds 1, 5 and 4 induced a decrease of about 63 % in the cyst number. A size reduction and an alteration of the wall of treated cysts were often noted. In a histological study, a reduction in cyst size without either inflammation or intervention of the nevroglial cells was observed. The present study provides evidence on the efficacy of phenylalanine derivatives and especially Boc-Phe 3, against *T.gondii* brain cysts in mice.

KEY WORDS : *Toxoplasma gondii*, brain cysts, *in vitro*, *in vivo*, phenylalanine derivatives.

Résumé : DÉRIVÉS DE LA PHÉNYLALANINE ACTIFS CONTRE LES KYSTES CÉRÉBRAUX DE *TOXOPLASMA GONDII* CHEZ LA SOURIS

Les effets de dérivés de la phénylalanine contre une souche kystogène de Toxoplasma gondii *ont été testés à la fois* in vitro *et* in vivo. *Ces composés sont L-Phe-L-Phe-OMe (dipeptide méthyl ester) 1 et son dérivé cyclisé 3,6-dibenzyl-2,5-dioxopipérazine 2, Boc-Phe 3, Phe-OMe 4, Boc-L-Phe-L-Phe-OMe 5.* In vitro *après 48 hr d'incubation les composés 3 et 5 entraînent une baisse importante du développement du parasite en culture comparés à une molécule de référence, la pyriméthamine.*

In vivo, *le composé 3 provoque une baisse de 77 % du nombre de kystes cérébraux. Les autres composés 1,5 et 4 entraînent une réduction d'environ 63 %. On note également une réduction de taille et une altération des membranes externes des kystes traités. Une étude histologique a montré les altérations induites par ces différents produits sur les kystes. Une réduction de la taille des kystes sans réaction inflammatoire des cellules de la névroglie est observée. Cette étude met en évidence l'efficacité de certains dérivés de la phénylalanine, en particulier Boc-Phe 3 sur les kystes cérébraux de* T.gondii *chez la souris.*

MOTS CLÉS : Toxoplasma gondii, *kystes cérébraux*, in vitro, in vivo, *dérivés de la phénylalanine.*

Reactivation of tissue cysts of *Toxoplasma gondii* is a major pathogenic event in the development of toxoplasmic encephalitis in AIDS patients (Oksenhendler *et al.*, 1994; Ammassari *et al.*, 1996; Kaplan *et al.*, 1996). To date, several drugs are studied in these polymedicine patients, since there is a need for active drugs with the smallest side effects. A few years ago, Rabinovitch (1989) reported the leishmanicidal activity of amino acid and peptide esters. This prompted us to evaluate the action of phenylalanine derivatives 1-5 (Fig. 1) against *T. gondii cysts* in mice brains. We thought that Phe-Phe-OMe 1, in relation to Bodor's studies (1977), could be considered as a pro-drug of L-Phe and L-Phe-OMe 4. In previous work we evidenced the action of compound 1 against the lamellar membrane of *E.multilocularis* metacestodes (Walchshofer *et al.*, 1993), this effect may be interesting in the case of a protozoan like *T. gondii*. That is why *in vitro* and *in vivo* studies on the antiprotozoan activity of 1-5 have been performed using pyrimethamine, a well known effective drug (Derouin & Chastang 1989), as a control. Boc-L-Phe-L-Phe-OMe 5 and Boc-L-Phe 3 instead of L-Phe were tested because Boc-derivatives may have a better biodisponibility than the corresponding free amino derivatives. Moreover, Rabinovitch (1989) reported that aminoacids are more active when they are *N*-protected.

MATERIALS AND METHODS

DRUGS

Pyrimethamine (Mw 248.5), Boc-Phe 3 (Mw 265), Phe-OMe, HCl 4 (Mw 215.5) were obtained from Sigma - Aldrich (L'Isle d'Abeau, France). Compounds 1 (Mw 362.5), 2 (Mw 294) and 5 (Mw 426)

* Pharmaceutical Department of Parasitology and Medical Mycology, 8, avenue Rockefeller, 69373 Lyon cedex 08, France.
** Laboratory of Medicinal Chemistry, Claude Bernard University Lyon.
*** Laboratory of Parasitology, Croix-Rousse Hospital Lyon, France. Correspondence: Dr M.E. Sarciron. Tel: +33 4 78 77 72 77 — Fax: +33 4 78 77 71 58 – e-mail: sarciron@cismsun.univ-lyon1.fr

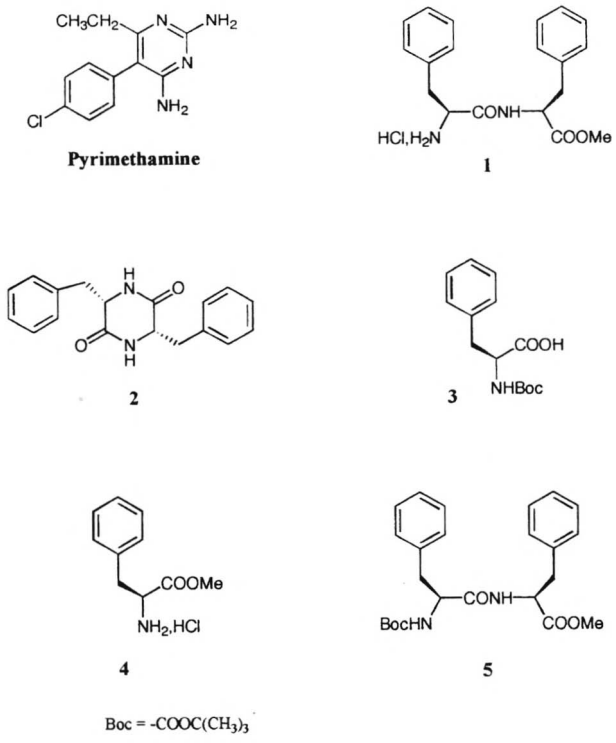

Fig. 1. – Formula of phenylalanine derivatives : dipeptide methyl ester 1; 3,6-dibenzyl-2,5-dioxopiperazine 2; Boc-L-Phe 3; L-Phe-OMe 4; Boc-L-Phe-L-Phe-OMe 5.

were synthetized as described by Walchshofer *et al.*, (1997).

T. GONDII ISOLATE

The DUR strain of *T.gondii* was isolated from the tissues of a pregnant woman. This avirulent strain was maintained in mice in our laboratory by oral passage of cysts from the brain of infected mice.

IN VITRO EXPERIMENTS

In vitro studies were carried out with the human myelomonocytic cell line THP-1 (ECACC number 88081201, Sophia-Antipolis, France). These non-adherent cells were maintained in RPMI 1640 (DAP, Vogelgrun, France) supplemented with 100 U/mL Penicillin; 100 µg/mL steptomycin (Sigma, L'Isle d'Abeau, France) and 10 % fetal calf serum (DAP, Vogelgrun, France). The number of THP-1 cells and tachyzoites were counted with a Malassez hemocytometer and adjusted to 10^5 cells/mL and 3.10^4 parasite/mL, respectively. Each tested molecule was diluted in the culture medium.

One mL of the parasitized cell suspension was distributed into each well of 6-well tissue culture plates (Falcon reference 3046, Becton Dickinson, Meylan, France). After settlement of the cells, 100 µL of medium were aspirated and replaced by the same volume containing the dissolved drugs, to a final concentration of 2 µg/mL. Pyrimethamine was used as a positive control. Working dilutions were freshly prepared for each experiment under a constant final volume of 1 mL. After incubation at 37 °C in a moist 5 % CO_2-95 % air atmosphere, the content of each well (1 ml) was removed, placed in an Eppendorf microtube and spun down at 15,000 rpm for two min. in a Beckman Avanti centrifuge.

The 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 of 1,000 cells from triplicate wells. The growth inhibition was calculated with the following formula: 100 - (% in treated wells × 100/% in controls). Five different experiments were performed with quadruplicates.

IN VIVO EXPERIMENTS

Sixty female OF1 mice of eight weeks of age (IFFA-CREDO-l'Abresle, France) were infected by gavage with cysts obtained from the brain of an infected mice. The brain tissue was suspended in 2 mL of 0.9 % NaCl and ground using a pestle and a mortar, then the preparation was further homogenized by passage through a needle and syringe. The cysts concentration was determined by bright field light microscopy . The cysts were diluted with sterile saline solution to a final concentration of ten cysts/0.2 mL per mouse. Three months post-infection, the infected animals were divided into six groups of ten each. Group 1: untreated mice; Group 2: pyrimethamine control group; Group 3-6: mice treated with one of the tested compounds (1, 3, 4, 5). Group 2-6 received per os three times a day the compound at the dose of 150 mg/kg/day under 0.2 mL volume per animal for five days.

The animals were euthanasied one week after the end of treatment. The brain of each mouse was taken out for cysts count, microscopical and histological studies. A sample was collected to evaluate the number of cysts in treated and untreated mice. The tissue was mixed with PBS (Phosphate Buffered Saline) and ground as above. The number of cysts in six samples of 20 µL each was determined for each brain.

LIGHT MICROSCOPY

The samples of brain sections were incubated in Bouin's fixative, dehydrated in a graded series of ethanol and embedded in paraffin. Sections of 4 µm were stained with Giemsa or HPS (hematoxylin, phloxine, safranine).

STATISTICS

The significance of differences was evaluated by Student's *t* test. P values ≤ 0.001 were considered significant.

RESULTS

In vitro, compounds 1, 2, 3, 4, 5 had a low solubility in the culture medium and a slower development of THP-1 cells was observed. The esters 1, 4 and 5 altered the cells to a small extent, whereas Boc-Phe 3 and dioxopiperazine 2 had no effect on them. Phe-Phe-OMe 1 had a very low solubility whatever the solvent used, which impaired the results analysis. Boc-Phe 3 seemed to be the most active molecule, and had a superior activity compared to pyrimethamine, the control molecule (Table I). After 48 hr of incubation, pyrimethamine induced a 45 % inhibition in parasite growth in cells ($p < 0.001$), whereas compounds 3, 5 and 4 induced an inhibition of 76, 68 ($p < 0.001$) and 20 % ($p = 0.1$), respectively. The number of free tachyzoites in the culture medium were decreased by 82 and 90 % with Boc-Phe and pyrimethamine, respectively. Unexpectedly, compound 2 induced a proliferation of the parasite.

The parasite's growth was more rapid than the growth of THP-1 cells in comparison with controls and consequently 2 was not tested *in vivo*.

In vivo studies. The mice tolerated the treatment of five days with the different compounds. Compound 3 induced a decrease of the number of cysts in cerebral tissue, comparable to the animals treated with pyrimethamine ($p < 0.001$) (Table II). Conversely to *in vitro* tests, the two Boc-derivatives 3 and 5 were not the most active. Whatever the molecule, a reduction of the size and often an alteration of the cyst walls, perhaps more marked with 3 were observed in phase contrast microscopy study (Fig. 2). The histological data confirmed these observations in phase contrast microscopy. A decrease in the size of cysts in treated mice compared to controls was noted, without reaction of the surrounding tissue (Fig. 3).

Compound	Number of parasitized cells × 10^4 12 hr	24 hr	48 hr
Controls	29 ± 0.5	31 ± 0.7	33 ± 0.8
Pyrimethamine	25 ± 0.6	24 ± 0.8	18 ± 0.2
1	23 ± 0.8	29 ± 0.8	25 ± 0.6
2	35 ± 0.9	36 ± 0.7	37 ± 0.4
3	27 ± 0.3	19 ± 0.8	8 ± 0.5
4	28 ± 0.7	29 ± 0.5	26 ± 0.4
5	27 ± 0.8	23 ± 0.8	10 ± 0.6

Table I. – Number of parasitized THP-1 cells by *Toxoplasma gondii*, cystic strain after incubation with the different molecules (2 µg/mL): dipeptide methyl ester 1; 3,6-dibenzyl-2,5-dioxopiperazine 2; Boc-L-Phe 3; L-Phe-OMe 4; Boc-L-Phe-L-Phe-OMe 5. The results are the mean ± S.E.

Compound	Number of cysts/brain	Inhibition (%)
Controls	650 ± 11.2	
Pyrimethamine	152 ± 5.7	77
1	213 ± 6.2	67
3	148 ± 7.3	77
4	250 ± 8.5	62
5	250 ± 7.9	62

Table II. – Number of *Toxoplasma gondii* cysts found in cerebral tissue from infected mice. They received for five consecutive days, an oral dose of 150 mg/kg/day of the different molecules. Results are the mean ± S.E.

DISCUSSION-CONCLUSION

In order to evaluate the efficacy of phenylalanine derivatives against a cystic strain of *T.gondii*, both *in vitro* and *in vivo* experiments were performed. All the molecules tested have comparable molecular masses. Although compound 1 was the most insoluble molecule *in vitro*, it was tested *in vivo* due to our previous results obtained against other parasites (Walchshofer *et al.*, 1993). The molecules 1, 4 and 5 altered the cells but esters are known to be toxic *in vitro* for monocytes and certain lymphoid cells (Rabinovitch 1989). Also, it is of importance that the amino groups of the most active compounds 3 and 5 are protected with a Boc (Butyl oxy carbonyl) moiety. We chose pyrimethamine as the control molecule because it is used in toxoplasmic encephalitis in HIV patients (Bachmeyer *et al.*, 1994; Leport *et al.*, 1996). The tested molecules had a certain efficacy against the parasite in animals despite the low dose (150 mg/kg/day) and the short treatment duration (five days) as compared to other studies. For example, Araujo *et al.*, (1992) used clarithromycin at the dose of 300 mg/kg/day for ten days. The treatment, beginning only 24 hr after infection, induced a significant reduction in the number of cysts in the brains of the infected mice which were killed eight weeks later. Also Khan *et al.*, (1996), have studied several quinolones (ciprofloxacin, temafloxacin, trovafloxacin, ofloxacin and tosufloxacin) at different doses 25, 50 100 mg/kg of body weight per day. The treatment was initiated 24 hr after infection as a single daily dose for ten days, but after the 30-day period all the mice were dead, meaning that these treatments were ineffective. Araujo *et al.*, (1997) showed an efficacity of ketolides antibiotics HMR 3647 and HMR 3004 both *in vitro* and *in vivo* in using a dose ranging from 15 to 40 mg/kg of body weight for ten days, treatment beginning three days after infection. Mice were examined for 30 days from the date of infection. In our study, we chose to initiate the treatment three-months after infection, when the cysts were well developped in the cerebral tissue, mimicking the common situation in Humans. These *in vivo* results showed that the tested drugs or their metabolites crossed the blood-

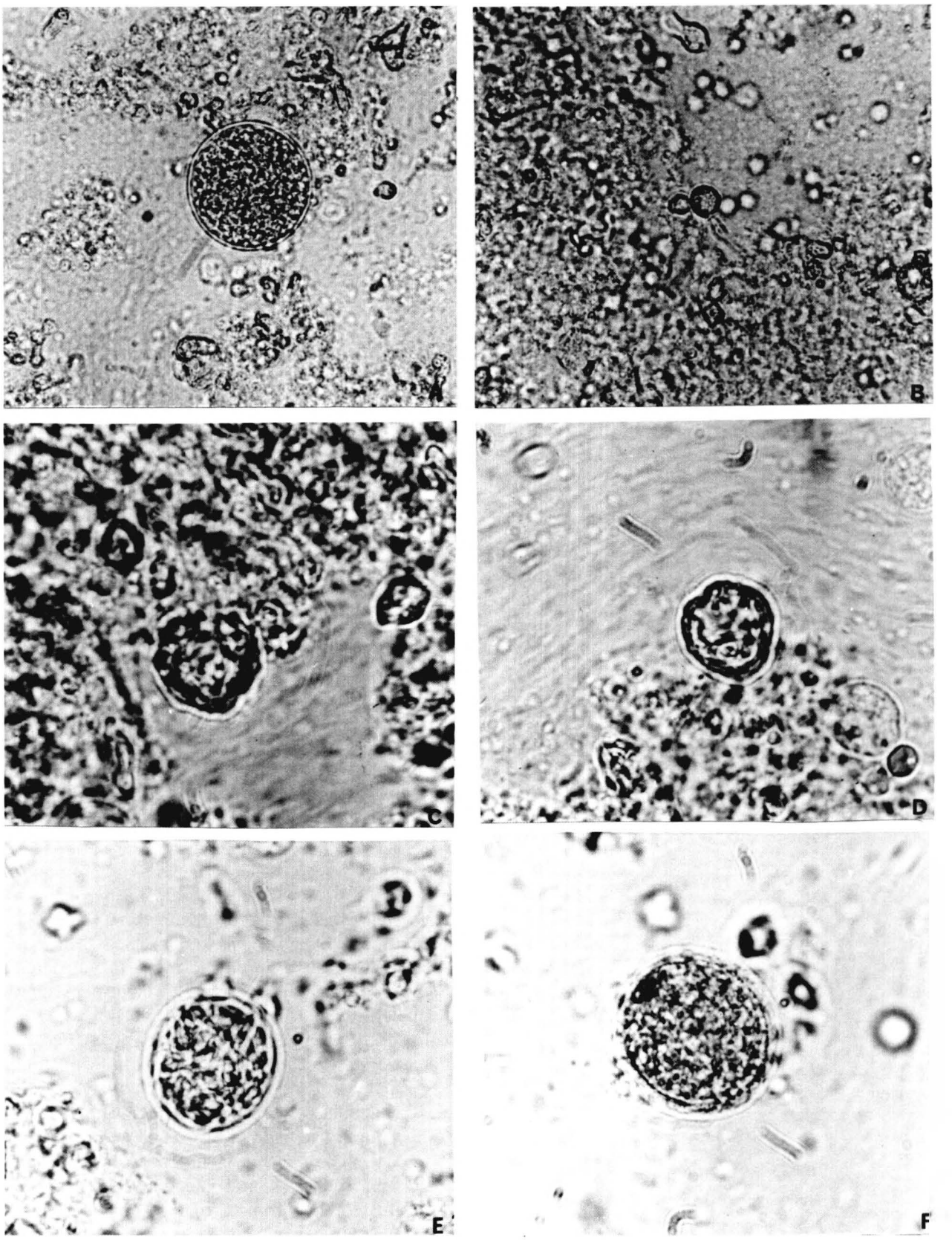

Fig. 2. – Phase contrast microscopy of cerebral cysts of *T. gondii* in control animal (A × 400) and after treatment with pyrimethamine (B × 400); there is a reduction in the size of the cysts compared to control; After Boc-Phe 3 treatment (C × 1,000), an alteration of the inner and outer membranes of the cysts was noted; 4 induced an alteration of tachyzoites (D × 1,000) with 5 and 1, disorganization and an alteration of tachyzoites were observed (E, F × 1,000).

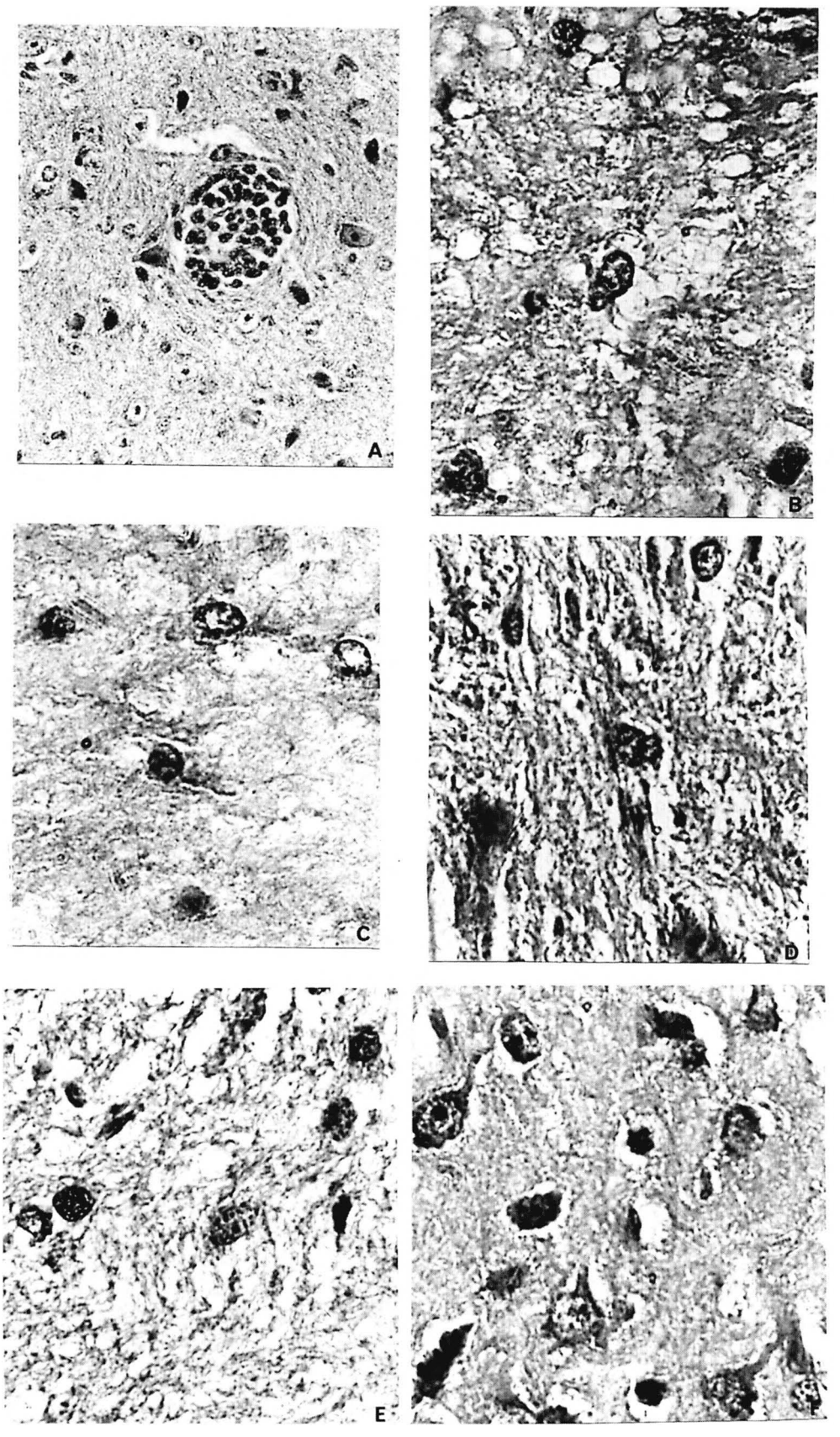

Fig. 3. – Histological study. (A) represents the cerebral tissue of an untreated mouse infected with *T.gondii* × 400. After the different treatments, a reduction in the size of cysts was observed without any immunological reaction of the surrounding cerebral tissue: pyrimethamine (B), Boc-Phe 3 (C), 4 (D), 5 (E), 1 (F) × 1,000.

brain barrier. In the histological study, the effects of the different treatments on the cerebral tissue, evidenced no immunologic reaction. In mice, a reduction in cysts size without inflammation or intervention of nevroglial cells was observed as previously reported with 2'-3'-dideoxyinosine (ddI) (Sarciron *et al.*, 1997). The present study evidences a certain efficacy of phenylalanine derivatives, especially Boc-Phe 3 whose molecular mass is comparable to that of pyrimethamine. Boc-L-Phe induced a reduction of the number of cerebral cysts as did pyrimethamine, but this molecule had a lower toxicity related to its structure. Indeed, some hematologic problems and rashs caused by pyrimethamine treatment in patients have been reported (Leport *et al.*, 1996). Our study is very interesting because yet today, the treatment of cerebral toxoplasmosis remains a great problem. To our knowledge it is the first study about the use of amino-acid against *T. gondii* both *in vitro* and *in vivo*. We presently extending our studies to other derivatives of phenylalanine in the purpose to select the the most effective molecules against this protozoa. However, the mode of action of a natural amino acid like L-phenylalanine against *T. gondii* remains to be discovered.

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