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Culture of tissue-cyst forming strain of *Toxoplasma gondii* and the effect of cyclic AMP and pyrimidine salvage inhibitors

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Abstract: An *in vitro* culturing to examine the cyst stage of *Toxoplasma gondii* (ME49 strain) was investigated using murine peritoneal macrophages, and we also examined the effect of cAMP or DHFR inhibitors on the growth of bradyzoites. For experiments ICR mice were injected i.p. with 1,500 brain cysts. At 1, 3, 5 and 7 days, peritoneal exudates were isolated and then adherent peritoneal macrophages were cultured for 1, 3, 5 and 10 days. Growth pattern of bradyzoites was measured by [^{3}H]-uracil uptake assay and morphological pattern of pseudocysts formed in macrophages was observed with Giemsa stain. Mostly bradyzoites were observed in the macrophages extracted at 3 and 5 days post infection. After 3 days *in vitro*, a number of pseudocysts were formed in the macrophages and the size of pseudocysts was increased during further 5 and 10 days *in vitro* culture. cAMP stimulated the growth of bradyzoites when *in vivo* 3 and 5 days and then *in vitro* 5 and 10 days conditions were applied. In case of DHFR inhibitors, pyrimethamine produced a linearly decremental effect with a conc.-dependent mode but methotrexate was not effective against intracellular bradyzoites or pseudocysts in this system. It was suggested that cyst-forming strain of *T. gondii* (ME49 strain) could be maintained and cultivated *in vitro* by use of murine peritoneal macrophages. *In vivo* 3 and 5 days and then *in vitro* 5 and 10 days conditions appeared to be suitable for culturing of bradyzoites. cAMP and pyrimethamine had an effect of stimulation and inhibition on the growth of bradyzoite, respectively.

Key words: *Toxoplasma gondii*, tissue cyst, *in vitro* culture, pseudocyst, macrophages, cAMP, pyrimethamine, methotrexate

INTRODUCTION

Toxoplasmosis is a common and widespread parasitic infection of human and many species of warm-blooded animals which is caused by the obligate intracellular protozoa *Toxoplasma gondii*. Approximately 6 to 12 days after an initial infection with *T. gondii*, the rapidly dividing tachyzoites encysts into tissue cysts which preferentially locates in the central nervous system and striated muscle (Luft, 1989). Tissue cysts are usually considered as dormant or slowly metabolizing chronic stages (bradyzoite stage) of *T. gondii* (Frenkel & Escajadillo, 1987).

There has long been an interest in the cyst stage of *T. gondii* because of its major role in the transmission of toxoplasmosis to humans and the persistence of viable organisms in this

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stage throughout the life time of infected mammalian hosts (Frenkel, 1973). Recently, interest has been further increased by the important role of progressive toxoplasmosis originating from the rupture of cysts in patients with AIDS and other dysfunction syndromes (Luft & Remington, 1985). However, studies about cyst stages of *T. gondii* were little done because of absence of an *in vitro* culture system to maintain and analyze the cysts.

Therefore, in this study, we designed to establish the bradyzoite culture system for studying the cyst-forming condition using murine peritoneal macrophages. In addition, we examined the effect of cAMP and pyrimidine salvage inhibitor, especially DHFR (dihydrofolate reductase) inhibitor, on the growth of bradyzoites, which have been known to act on the tachyzoite form (Choi *et al.*, 1990; Youn *et al.*, 1990).

MATERIALS AND METHODS

Parasites: ME49 strain of *T. gondii* (kindly provided by Remington JS, Stanford University School of Medicine, Stanford, California, USA via Kobayashi A, Jikei University School of Medicine, Tokyo, Japan) was passaged in 5-6 wk old ICR mice by injection of 100 brain cysts into peritoneum per every 2 months.

Culture of peritoneal macrophages: For experiments ICR mice were injected i.p. with 1,500 brain cysts. At 1, 3, 5 and 7 days, peritoneal exudates were isolated. Resident peritoneal macrophages were harvested with Eagle's MEM supplemented with 10% fetal bovine serum (FBS). Peritoneal cells were plated on 96-well plates or 8-well chamber slides (NUNC). After 2 hr incubation at 37°C, non-adherent cells were removed by rinsing in EMEM. During *in vitro* cultivation, peritoneal macrophages were incubated in a humidified 95% air/5% CO_2 incubator. At 1, 3, 5 and 10 days, cells were harvested to measure the growth pattern or stained with Giemsa solution to observe a morphological pattern.

Measurement of growth of *T. gondii*: [5, 6-^{3}H]-uracil (10 μCi/well) was added for 48 hrs before harvest. Cells were harvested on a filter paper using cell harvester (Titer Tek Co.), and then incorporated radioisotopic activities were counted by a liquid scintillation counter (Kontron Co.). Cells in chamber slide were stained with Giemsa solution and observed under the light microscope. When the pseudocysts were observed, the number of pseudocysts were calculated by those of 10,000 macrophages and the size was measured.

Chemical treatments: Adenosine 3',5'-cyclic monophosphate (cAMP, Sigma Co.) was added at a final conc. of 0.5, 1, and 5 mM. DHFR inhibitors, 5-[4-chlorophenyl]-6-ethyl-2,4-pyrimidinediamine (PM, Sigma Co.) and methotrexate (MTX, Sigma Co.) were treated separately at a final conc. of 1, 10, and 100 μM.

RESULTS

Growth pattern of bradyzoites: Bradyzoites, grown in peritoneal cavities of mice for 1 day, incorporated [5,6-^{3}H]-uracil actively during *in vitro* culture afterward. Uracil uptake ratio to control of *in vitro* culture for 5 and 10 days were about 5 to 9 times, respectively (Fig. 1). Bradyzoites penetrated into macrophages were distributed randomly in the cytoplasms of macrophages. They existed as a free bradyzoite and no cyst-like form was observed. In 3 and 5 days *in vivo* conditions, uracil uptake ratio to control was slowly increased during *in vitro*

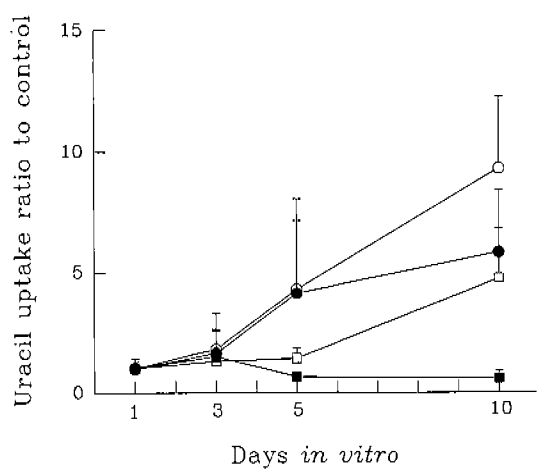

Fig. 1. Growth pattern of the bradyzoites by the time when macrophages were isolated from the peritoneal cavities of infected mice. ○: *in vivo* 1 day ●: *in vivo* 3 days □: *in vivo* 5 days ■: *in vivo* 7 days

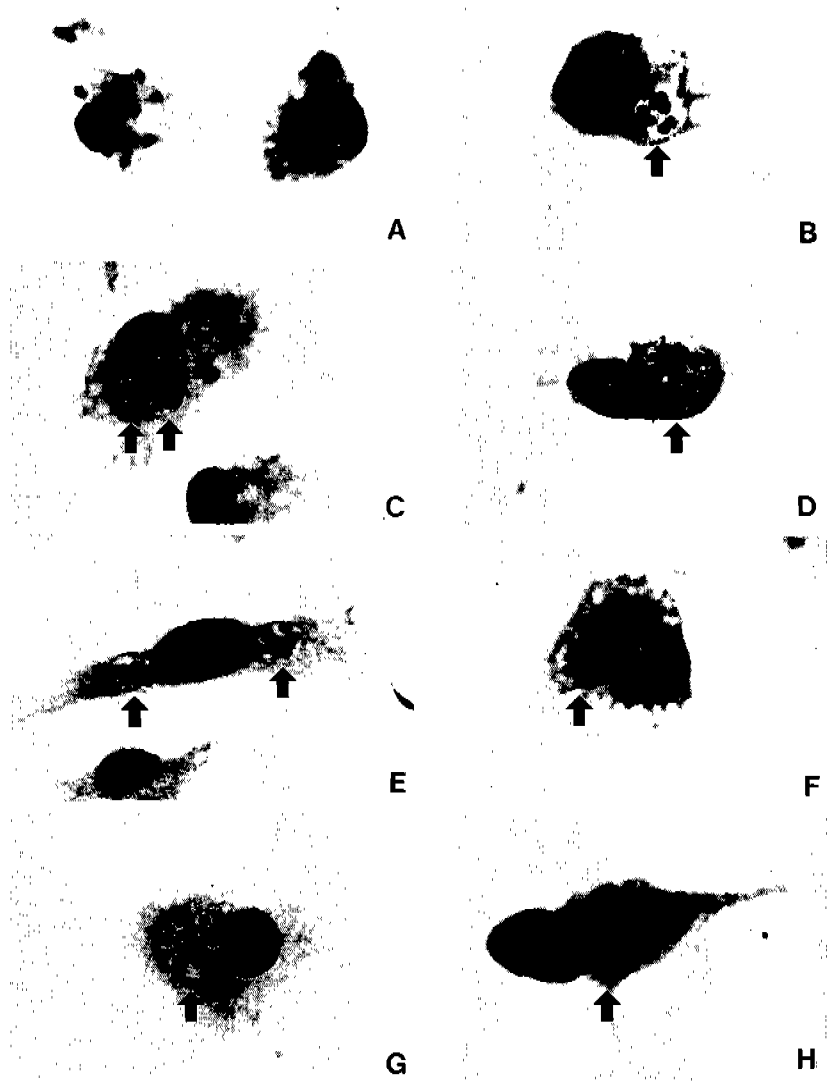

Fig. 2. Photographs of pseudocysts formed in the macrophages. **A, B:** *in vivo* 3 days and then *in vitro* 5 days. Free bradyzoites were also present in A. **C, D:** *in vivo* 3 days and then *in vitro* 10 days. Note 2 pseudocysts in the cytoplasm of a macrophage in C. **E, F:** *in vivo* 5 days and then *in vitro* 5 days. **G, H:** *in vivo* 5 days and then *in vitro* 10 days. Note many bradyzoites in the pseudocyst. Arrows indicate pseudocysts. Giemsa stain, × 1,000

cultivation (Fig. 1). Many large clumps of bradyzoites were observed in the cytoplasms of macrophages, 'pseudocysts' as well as small bradyzoite-filled vacuoles (Fig. 2). After 7 days *in vivo* culture, growth of bradyzoites remains basal level (Fig. 1). Free bradyzoites were rarely observed except a few pseudocysts. Bradyzoites were well grown at 1, 3 and 5 days *in vivo* conditions by judging from uracil uptake assay but most of pseudocysts were observed in 5 and 10 days *in vitro* culture of 3 and 5 days *in vivo* conditions. Therefore we choose the conditions of 3 and 5 days *in vivo* and then 5 and 10 days *in vitro* culture for further experiments.

The effect of cAMP: The effect of cAMP on the growth of bradyzoites was significant with a conc.-dependent mode in *in vivo* 3 days condition, both *in vitro* 5 and 10 days (Fig. 3). The number of developing pseudocysts in

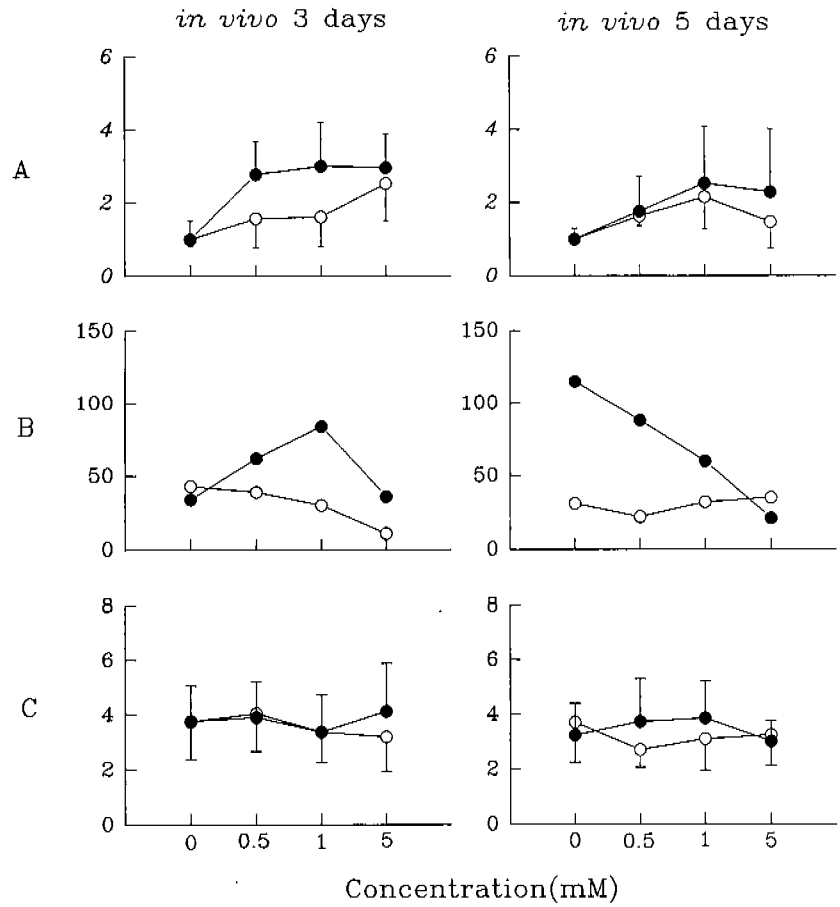

Fig. 3. Effects of cAMP on the growth of bradyzoites and pseudocysts. **A:** uracil uptake ratio to control, **B:** number of pseudocysts/10,000 host cells, **C:** size of pseudocysts (μm). ○: *in vitro* 5 days ●: *in vitro* 10 days

macrophages was increased up to 1 mM conc. of cAMP in *in vitro* 5 days but decreased in *in vitro* 10 days. In contrast, no remarkable changes were detected in the size of pseudocysts at any conc. of cAMP. *In ivivo* 5 days condition, conc.-dependent increasing pattern was seen except for conc. of 5 mM, *in vitro* 5 and 10 days both. The numbers of pseudocysts were decreased but the size was almost not changed. In case of increasing uracil uptake pattern and decreasing pattern in number of pseudocysts, many free bradyzoites were seen extracellularly (data not shown).

The effect of PM and MTX: PM, one of DHFR inhibitors, inhibited effectively the growth of bradyzoites, *in vivo* 3 and 5 days both (Fig. 4). In the uptake of uracil, decreasing pattern was seen with the treating conc. of PM, and also decreasing pattern of the number of pseudocysts. Especially, there were no pseudocysts above conc. of 10 μM. In contrast, the size of pseudocysts was almost unchanged. MTX in conc. from 1 to 100 μM showed no significant inhibition of uracil uptake by bradyzoites (Fig. 5). Similarly, no effect was noted in the number or size of pseudocysts (Fig. 5). Therefore, in this experimental system, MTX did not appear to be functioning as a DHFR inhibitor.

DISCUSSION

Culture of tachyzoites has been maintained in some cell lines for limited time. However, in our study, culture of bradyzoites of cyst

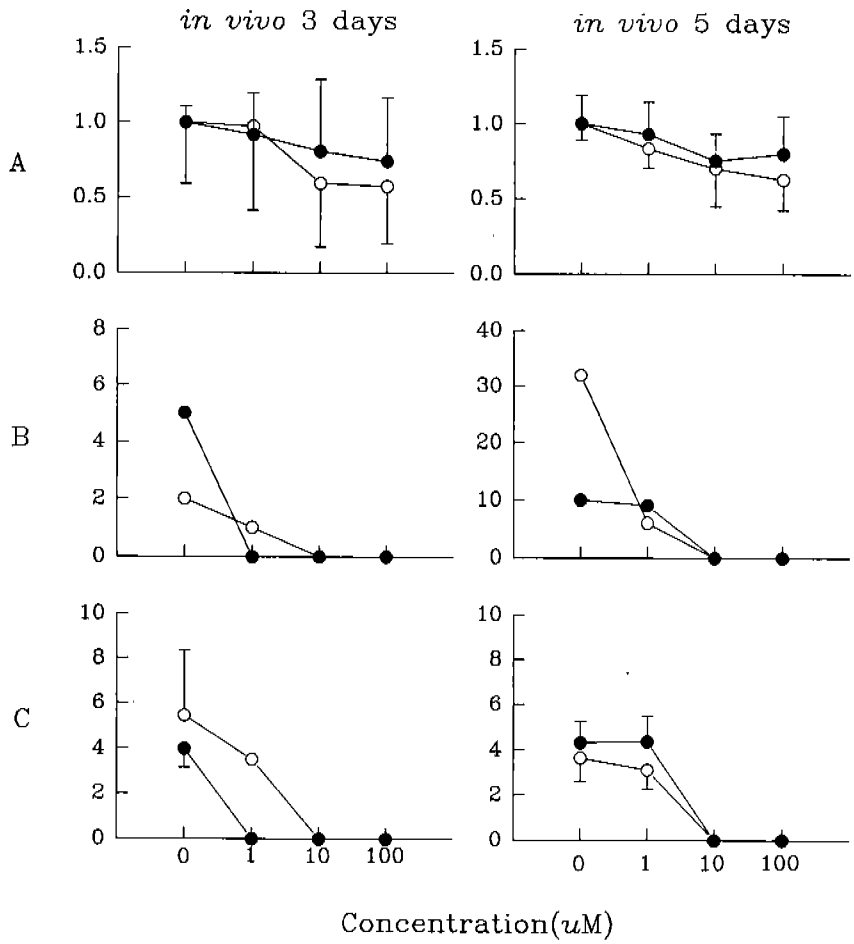

Fig. 4. Effects of PM on the growth of bradyzoites and pseudocysts. **A:** uracil uptake ratio to control, **B:** number of pseudocysts/10,000 host cells, **C:** size of pseudocysts (μm). ○: *in vitro* 5 days ●: *in vitro* 10 days

forming strain was tried and especially bradyzoite-infected cells *in vivo* were cultured *in vitro*. Our studies showed that cyst-forming strain of *T. gondii* (ME49 strain) can be maintained and cultivated *in vitro* by use of murine peritoneal macrophages. Dubey & Frenkel (1976) showed that cyst-like structures containing distinct PAS-positive granules were first seen in brain after 3 days of infection with tachyzoites and became numerous by 6 days, whereas Ferguson & Hutchison (1987) observed tissue cyst in brain after 11 days infection with cysts. On the other hand, tissue cysts were formed by inoculation of bradyzoites at 7 days post inoculation in BM cell cultures (Lindsay *et al.*, 1991).

In this system, *in vivo* 3 and 5 days and then *in vitro* 5 and 10 days appeared to be suitable for culture of bradyzoites to form pseudocyst. Cyst form was not observed after *in vitro* culture of 1 or 7 days *in vivo* cells. In case of 1 day, there were many free bradyzoites. It has been known that cyst formation is initiated, perhaps in response to some signal that occurs at the onset of the immune response (Luft, 1989). Active proliferation was still occuring at 1 day *in vivo* cells, which is thought to be occurred before the appearance of an early effective immune response. In host, another factors are believed to be involved in controlling of cyst development. Jones *et al.* (1986) reported that interferon-γ is not necessary for cyst formation *in vitro*, but it may allow cysts to remain for prolonged periods

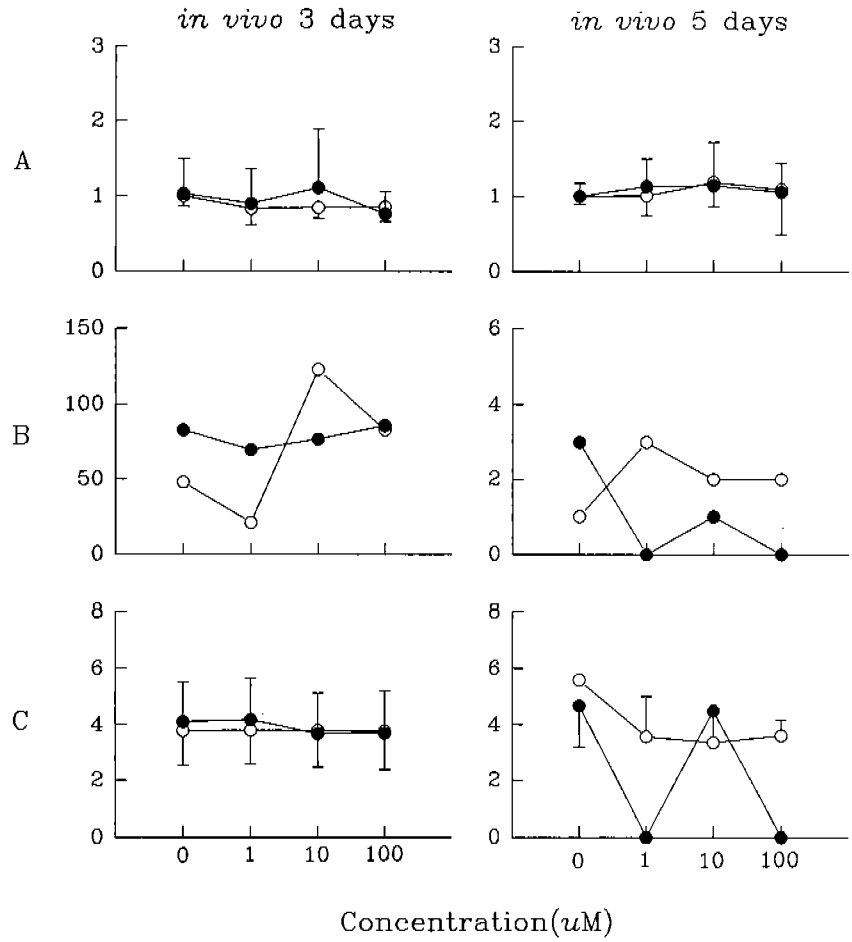

Fig. 5. Effects of MTX on the growth of bradyzoites and pseudocysts. **A:** uracil uptake ratio to control, **B:** number of pseudocysts/10,000 host cells, **C:** size of pseudocysts (μm). ○: *in vitro* 5 days ●: *in vitro* 10 days

without rupturing. Recent studies have shown that macrophages are involved in the inhibition of the cyst development during sporadic cyst rupture (Ferguson *et al.*, 1989) whereas $CD8^+$ T cells and class I major histocompatibility complex (MHC) genes intervene by regulating cyst numbers (Brown & McLeod, 1990). Cyst formation pattern after *in vitro* culture of *in vivo* 3 and 5 days cells were similar, but cyst form or free bradyzoites were rarely seen at *in vivo* 7 days. Cyst infected cells after 7 days post infection, cells might be transferred to any other preferential sites. However, we observed that intracellular 'pseudocyst' not extracellular true 'cyst' had a characteristic tissue cyst wall which surrounded the bradyzoites. Morphological differencies between pseudocyst and cyst will be further studied by electron microscopically.

cAMP has been known as a regulator of intracellular reactions. The differential balance of cAMP may result in activation of protein kinases (Smith *et al.*, 1981), transcription of specific genes (Nagamine & Reich, 1985) and changes in the cytoskeleton structure (Dedman *et al.*, 1979), which ultimately lead to morphogenetic cell alterations. cAMP stimulated the growth of bradyzoites. The numbers of pseudocysts were changed showing increasing or decreasing pattern according to infection or cultivation time. Factors to control the proliferation of bradyzoites or differentiation into cysts are unknown, but appeared to be associated with

the infection time course. Choi *et al.* (1990) previously reported that cAMP stimulated the growth of tachyzoites of *T. gondii* in HL-60 cells.

PM, well known as a DHFR inhibitor, produced a linearly decremental effect with a conc.-dependent mode. PM alone has marked efficacy against *T. gondii* and the combination of PM and sulfadiazine has synergistic activity (Mack & McLeod, 1984). PM was reported to have an effect on tachyzoite form (Youn *et al.*, 1990) as well as cyst form (Huskinson-Mark *et al.*, 1991). MTX, a potent DHFR inhibitor that acts in a fashion similar to PM, was not effective against intracellular bradyzoites or pseudocysts in this system. Bradyzoites may be resistant to chemotherapeutic agents that are effective against tachyzoites (Haverkos, 1987) or may have a DHFR resistant gene. Above results are consistent with a recent report that MTX failed to inhibit replication of *T. gondii* at conc. as high as 10^{-4} M (Allegra *et al.*, 1987). The inability of MTX to be transported across the parasite's cell membrane appeared to be responsible for its inactivity (Allegra *et al.*, 1987). Piritrexim, a lipid-soluble analogue of MTX, has recently been shown to be more potent than trimethoprim and PM inhibiting the DHFRs of *P. carinii* and *T. gondii* (Kovacs *et al.*, 1988). Effects of drugs on the cyst form of *T. gondii* also have been evaluated that cysts of *T. gondii* in the brains are reduced significantly by atovaquone (566C80)(Araujo *et al.*, 1993). Our observations suggested that *in vitro* culturing of bradyzoites might prove to be useful for studying of cyst-forming strain of *T. gondii* in a physiological aspect.

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=국문초록=

Toxoplasma gondii 약독주의 배양과 그 성장에 미치는 cyclic AMP와 pyrimidine salvage 억제제의 영향

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최원영, 박성경, 남호우, 김동진

Toxoplasma 약독주(ME49 주)의 배양계 확립을 위하여 뇌내 cyst를 마우스 복강에 주입하고 1, 3, 5 그리고 7일후 다시 얻어 배양기 부착성 세포를 배양하는 방법으로 ME49 주를 배양하였으며, ME49 주의 성장에 미치는 cAMP 및 DHFR 억제제의 영향을 관찰하였다. ME49 주로 감염된 대식세포의 형태학적 관찰은 Giemsa 염색방법을 이용하였고 성장정도는 [^{3}H]-uracil 표지량을 대조군에 대한 비로 나타내었다. 감염 3일 및 5일이 경과된 후에 채취한 복수의 대식세포에서 ME49 주의 bradyzoite가 주로 관찰되었으며, 배양기에서 3일 이상 경과된 후에는 pseudocyst를 형성하기 시작하였고, 5일, 10일이 경과되면서 pseudocyst의 크기가 증가하였다. cAMP를 농도별로 처리하였을 때 3일째와 5일째의 복수를 5일간 및 10일간 배양했을 때 농도의존적으로 성장을 촉진하였다. DHFR 억제제중 pyrimethamine의 경우 농도의존적인 성장억제효과를 나타냈고, methotrexate의 경우엔 ME49 주 bradyzoite의 성장에 영향을 미치지 않는 것으로 나타났다. 이상의 결과로 마우스 대식세포내에서 bradyzoite의 배양이 가능하고 그 배양조건은 3일째와 5일째 복수를 5일 이상 10일 정도 배양하는 것이 적당하며 cAMP 및 pyrimethamine이 bradyzoite의 성장을 각각 촉진 및 억제하는 것으로 나타났다.

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