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The effect of cyclic AMP on the growth of *Toxoplasma gondii in vitro**

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Abstract: To assess the role of cAMP on the growth and proliferation of *Toxoplasma* in HL-60 cells we tested the effect of exogenous cAMP and cAMP analogues to the co-culture system of *Toxoplasma* and HL-60 cells. cAMP, dbcAMP, and br-cAMP stimulated the growth of *Toxoplasma* at a specific concentration, i.e., 10^0 mM, 10^0 mM, and 10^{-1} mM, respectively. There were differences in growth induction kinetics and in the rate of promotion. These results were further verified by treating the co-culture with adenylate cyclase activator, pNHppG, cAMP phosphodiesterase activators, imidazole and A23187, and cAMP phosphodiesterase inhibitors, IBMX, compound 48/80, and theophylline, separately. When the cytosolic cAMP levels increased by the reagents mentioned above, *Toxoplasma* in the cytoplasm of HL-60 cells stimulated to proliferate more rapidly with concentration-dependent modes compared to the control, and *vice versa*. It is suggested that some mechanisms are activated by the high levels of cAMP in the cytoplasm, which result in the stimulation of *Toxoplasma* proliferation.

Key words: *Toxoplasma gondii*, *in vitro* culture, cAMP, adenylate cyclase, cAMP phosphodiesterase

INTRODUCTION

To study the mechanisms that mediate cell division and differentiation, it is necessary to identify specific molecules that may be responsible for the intracellular signaling mechanisms that trigger cell growth in response to changes in the environment. Cyclic AMP has been known as a second messenger and plays a dominant role in the regulation of eukaryotic growth(Strickler and Patton, 1975; Gomes *et al.*, 1980; Pilkis *et al.*, 1982; El-Maghrabi and Pilkis, 1984). The relative amounts of this molecule in the cell can change according to the environment by the balance of enzymatic activities of synthetic and degradative, enabling the organisms to adapt to new conditions. The differential balance of cAMP may result in activation of protein kinases (Smith *et al.*, 1981), transcription of specific genes(Nagamine and Reich, 1985; Oyama and Blumberg, 1986), and changes in the cytoskeleton structure (Dedman *et al.*, 1979), which ultimately lead to morphogenetic cell alterations.

Toxoplasma gondii, the protozoan causative of toxoplasmosis (Levine, 1977), has the limitations of its dividing activities within the boundary of eukaryotic cells from various tissues and species of mammals and birds (Choi *et al.*, 1987). To establish a more simple and stable culture system of *Toxoplasma in vitro*, we have

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attempted to culture with various cells and cell lines, and could introduce HL-60 which was derived from a patient of promyelocytic leukaemia (Collins *et al.*, 1977) as a host cell (Choi *et al.*, 1988; Kim and Choi, 1989). After the invasion to host cell *Toxoplasma* interacts with host in several fashions, the transmission of signals to growth and proliferation is one of these. In the process of signaling between *Toxoplasma* and host cells cAMP may exert as a messenger which mediates the interrelationships in the cytoplasm of HL-60, i.e., changes in the concentration of cAMP of host cell cytoplasm control the growth and division of *Toxoplasma*, or cAMP permeated directly to the cytoplasm of *Toxoplasma* function as a messenger to growth, or both.

Therefore, we will define the role of cAMP on the growth and proliferation of *Toxoplasma* in HL-60 cells by adding cAMP or cAMP analogues exogenously and by modulating the enzymes which regulate the concentration of cAMP in this co-culture system.

MATERIALS AND METHODS

Chemicals

All chemicals treated, adenosine-3′, 5′-cyclic monophosphate(cAMP), N^6, 2′-0-dibutyryladenosine-3′, 5′-cyclic monophosphate (dbcAMP), 8-bromoadenosine 3′, 5′-cyclic monophosphate (br-cAMP), epinephrine, cholera toxin, guanylyl imidodiphosphate(pNHppG), imidazole, A23187, 3-isobutyl-1-methylxantine (IBMX), compound 48/80, and theophylline were purchased from Sigma Chemical Co..

Parasites:

Virulent tachyzoites of RH strain of *Toxoplasma* were maintained and propagated by continuous passage in ICR mice, and were purified from the peritoneal exudate with saline.

Cell Culture:

HL-60 cells were maintained in Earle's minimum essential medium (EMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM N-2-hydroxyethylpiperazine-N-2′-ethane sulfonic acid (HEPES), pH 7.4. Cells were grown at 37 °C in a humidified 95% air/5% CO_2 atmosphere. Cells were subcultured weekly at a seeding density of 3×10^5 cells/ml.

Growth of *Toxoplasma* in HL-60 Cells

Growth of *Toxoplasma* in the cytoplasm of HL-60 cells were detected by counting the radioactivity of [5, 6-^{3}H]-uracil which was labeled preferentially to the dividing *Toxoplasma* rather than to host cells (Pfefferkorn and Pfefferkorn, 1977; Kim and Choi, 1989). One hundred μl of HL-60 cells of 5×10^5 cells/ml alone and HL-60 cells mixed with the same number of *Toxoplasma* were seeded in 96-well plates(NUNC). And then various concentrations of reagents were added to each well and incubated for 60 hr. At 12 hr-interval, cells were incubated with 1 μCi/well of [5, 6-^{3}H]-uracil (NEN) for 2 hr, and then harvested on a filter paper using cell harvester(Titer Tek. Co.) to count the radioactivity in a liquid scintillation counter(Kontron).

In addition, [methyl-^{3}H]-thymidine uptake to the cells were also tested with the above mentioned method whether the reagents treated might play roles on the nucleic acid metabolism of the host cells.

RESULTS

Control Growth of *Toxoplasma* in HL-60 Cells

When [5, 6-^{3}H]-uracil was treated pulsely for 2 hr at every 12 hr during the time course of experiment, it was absorbed preferentially to dividing *Toxoplasma* in the cytoplasm of HL-60 cells rather than HL-60 cells alone(Fig. 1). The absorption rate increased rapidly until 48 hr and decreased thereafter.

cAMP and Growth of *Toxoplasma*

It was first tested the role of exogenous cAMP and cAMP analogues on the growth of *Toxoplasma*. The results presented in Fig. 2, 3, and 4 show the growth of *Toxoplasma* incubated

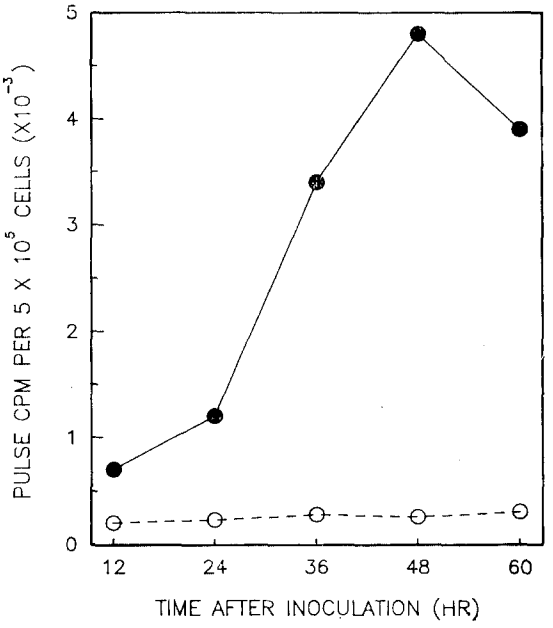

Fig. 1. Kinetics of uptake of [^{3}H]-uracil into infected (•—•) and uninfected (○--○) HL-60 cells with *Toxoplasma*. Cells were incubated with ^{3}H-uracil for 2 hr pulsely at 12 hr interval.

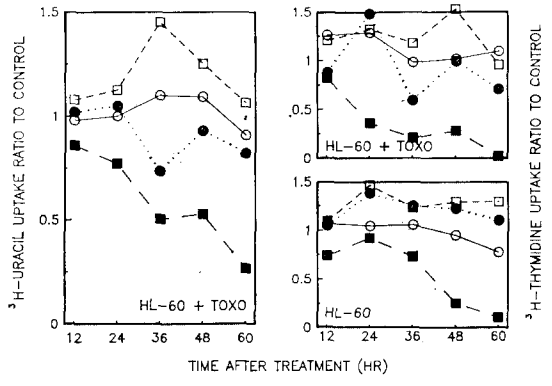

Fig. 2. Effect of cAMP on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of cAMP, 0.5 mM (○—○), 1 mM(□--□), 5 mM (•···•), and 10 mM (▪--▪) treated at the time of inoculation of *Toxoplasma*, and the cells were labeled with ^{3}H-uracil(left) and ^{3}H-thymidine(right) for 2 hr. Effect of cAMP was described as a ratio of ^{3}H-uracil uptake to control which was not treated.

with various concentrations and durations of cAMP, dbcAMP, and br-cAMP, respectively. The effect of cAMP and cAMP analogues was very obvious but the stimulatory phenomena

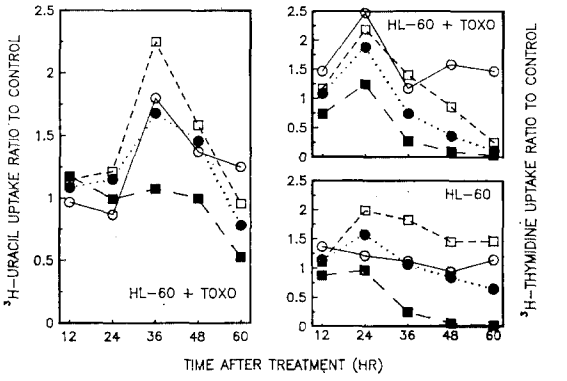

Fig. 3. Effect of dbcAMP on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of dbcAMP, 0.5 mM (○—○), 1 mM (□--□), 5 mM (•···•), and 10 mM (▪--▪) were treated at the time of inoculation of *Toxoplasma*. Refer to the legend of Fig. 2.

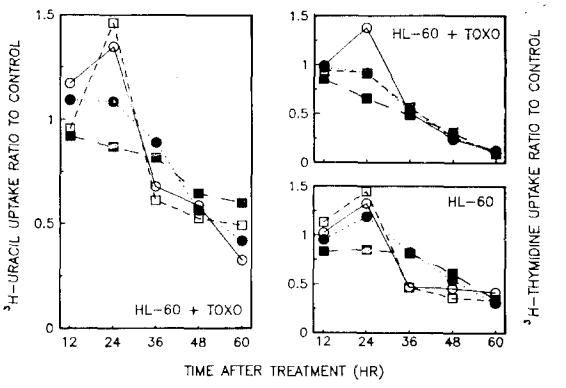

Fig. 4. Effect of 8-br-cAMP on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of 8-br-cAMP, 0.05 mM(○—○), 0.1 mM (□--□), 0.5 mM (•···•), and 1 mM (▪--▪) were treated at the time of inoculation of *Toxoplasma*. Refer to the legend of Fig. 2.

were different among these reagents in kinetics and concentrations of reagents added. In the case of cAMP treatment, the concentration that stimulated the growth of *Toxoplasma* but not inhibited host cell proliferation indexed by the uptake of ^{3}H-thymidine was 1 mM, *i.e.*, 10° mM, and the effect was pronounced by about 1.5-fold at 36 hr, whereas higher concentration than 1 mM inhibited the growth of *Toxoplasma* oppositely that might result from potency of cAMP to differentiate the host cells terminally(Fig. 2). The stimulatory concentration of dbcAMP was

0.5 to 5 mM, *i.e.*, 10° mM, and the effect was bigger than that of cAMP of about 2.3-fold at 36 hr, and higher concentration suppressed the growth of *Toxoplasma* by exerting to the differentiation of the host cells(Fig. 3). In addition, br-cAMP also stimulated the growth of *Toxoplasma* but the concentration of br-cAMP was 50 μM to 0.1 mM, *i.e.*, 10^{-1} mM, and kinetics of promotion appeared to be about 1.5-fold at the time of early incubation of 24 hr as well, and higher concentration also constrained the growth of *Toxoplasma*(Fig. 4).

Adenylate Cyclase and Growth of *Toxoplasma*

To conform the stimulation of the growth of *Toxoplasma* by cAMP, it was tested the effects of the adenylate cyclase activators which activate this enzyme to elevate the cytosolic cAMP level. Of these, epinephrine (Sevilla *et al.*, 1976), cholera toxin(Gill and Meren, 1978) and pNHppG(Pfeuffer and Helmreich, 1975) were analyzed to promote the growth of *Toxoplasma*. However, the effects of epinephrine and cholera toxin of various concentrations were very little compared to control(data not shown). But, the more specific adenylate cyclase activator pNHppG enhanced the proliferation of *Toxoplasma*. As described in Fig. 5, various concentrations of pNHppG of 10 μM to 0.5 mM promo ted the growth of *Toxoplasma* by about 1.5- to 2.3-fold at early incubation time of 24 hr. At these concentrations host cells were also affected to increase the dividing activity.

cAMP Phosphodiesterase and Growth of *Toxoplasma*

It was also investigated whether the modulation cAMP phosphodiesterase activity played a role in the growth of *Toxoplasma*. cAMP phosphodiesterase activators which lowered cytosolic cAMP level such as A23187 and imidazole inhibited the growth whereas inhibitors which raised the level of cytosolic cAMP such as IBMX, compound 48/80, and theophylline led to a faster kinetics of growth of *Toxoplasma*.

Order of 100 μg/ml A23187 decreased the growth of *Toxoplasma* gradually to below 50%

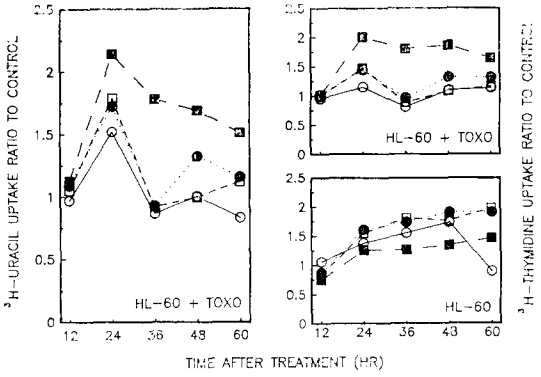

Fig. 5. Effect of guanylyl imidodiphosphate(pNHppG; adenylate cyclase activator) on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of pNHppG, 0.01 mM (o—o), 0.05 mM (□--□), 0.1 mM (•···•), and 0.5 mM (■--■) were treated at the time of inoculation of *Toxoplasma*. Refer to the legend of Fig. 2.

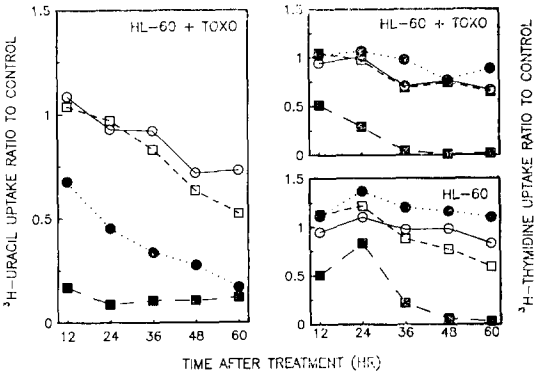

Fig. 6. Effect of A23187(cAMP phosphodiesterase activator) on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of A 23187, 0.5 μg/ml (o—o), 1.0 μg/ml (□--□), 5.0 μg/ml (•···•), and 10 μg/ml (■--■) were treated at the time of inoculation of *Toxoplasma*. Refer to the legend of Fig. 2.

to control during the experimental time span as shown in Fig. 6. And 100 mM of imidazole also inhibited the growth of *Toxoplasma* with the similar kinetics to those of A23187(Fig. 7) without reducing the host cell proliferation.

As represented in Fig. 8, 9, and 10, cAMP phosphodiesterase inhibitors which inactivated the enzyme with consequent increase in the levels of cytosolic cAMP, resulting in the

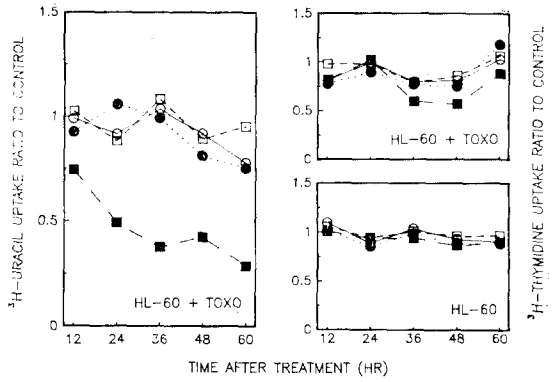

Fig. 7. Effect of imidazole(cAMP) phosphodiesterase activator) on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of imidazole, 0.1 mM (○—○), 0.5 mM (□--□), 1.0 mM (●···●), and 5 mM (■--■) were treated at the time of inoculation of *Toxoplasma*. Refer to the legend of Fig. 2.

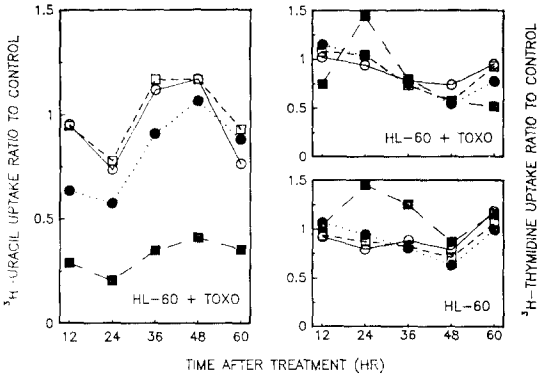

Fig. 8. Effect of IBMX (cAMP phosphodiesterase inhibitor) on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of IBMX, 0.1 mM (○—○), 0.5 mM (□--□), 1.0 mM (●···●), and 5.0 mM(■--■) were treated at the time of inoculation of *Toxoplasma*. Refer to the legend of Fig. 2.

stimulation of the growth of *Toxoplasma*. Although IBMX(Oliveira *et al.*, 1984) inhibited a little the growth of *Toxoplasma* in early times of incubation but promoted the growth in late incubation times of 36 to 48 hr at concentrations of 0.1 mM and 0.5 mM. When more higher concentrations than 1 mM were applied, the growth of *Toxoplasma* was inhibited in early times of incubation whereas did not interfere the host cell proliferation (Fig. 8). Under the

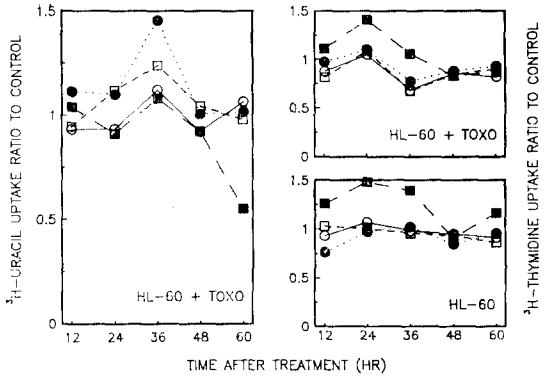

Fig. 9. Effect of theophylline(cAMP phosphodiesterase inhibitor) on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of theophylline, 0.1 mM (○—○), 0.5 mM (□--□), 1.0 mM(●···●), and 5 mM (■--■) were treated at the time of inoculation of *Toxoplasma*. Refer to the legend of Fig. 2.

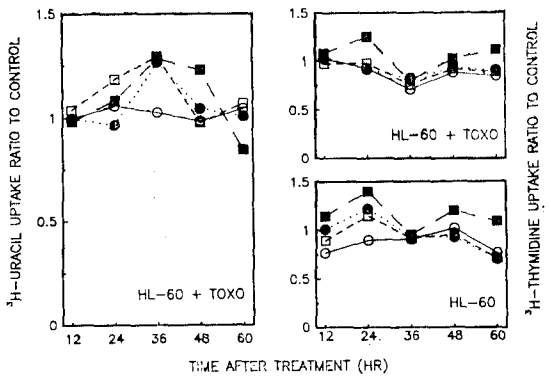

Fig. 10. Effect of compound 48/80(cAMP phosphodiesterase inhibitor) on the growth of *Toxoplasma* in HL-60 cells. Various concentrations of compound 48/80, 0.1 mg/ml (○—○), 0.5 mg/ml(□--□), 1.0 mg/ml(●···●), and 5 mg/ml(■--■) were treated at the time of inoculation of *Toxoplasma*. Refer to the legend of Fig. 2.

concentrations of non-toxic to cells, 0.1 mM to 1 mM, theophylline promoted the growth of *Toxoplasma*(Fig. 9). And various concentrations of compound 48/80, which was a calmodulin inhibitor played a role in inactivation of cAMP phosphodiesterase, also increased the growth of *Toxoplasma* during the experimental time course without interruption of host cell division(Fig. 10).

DISCUSSION

Our results have shown that cAMP was involved in the triggering and modulation of the growth of *Toxoplasma*, since either the addition of cAMP and cAMP analogues, adenylate cyclase activators, or cAMP phosphodiesterase inhibitors stimulated the growth of *Toxoplasma*. The stimulatory effect of increased cytosolic cAMP levels was reflected in the kinetics of the growth of *Toxoplasma* induction as compared to controls to which no reagents were treated.

A direct evidence for the role of cAMP in triggering the growth of *Toxoplasma* was the fact that cAMP (Fig. 2), dbcAMP (Fig. 3), and br-cAMP (Fig. 4) induced the growth of *Toxoplasma*. There were differences not only in *Toxoplasma* growth induction kinetics but also in the concentration depending on the cAMP analogues treated. This is probably resulted from differential permeability of the membranes. The more permeable br-cAMP (Harris *et al.*, 1975) resulted in higher effect on the growth of *Toxoplasma*. And over the concentrations inspired to promote the growth of *Toxoplasma* inhibited reversely the growth, which was suggested to stimulate the differentiation of host HL-60 cells to inhibit or kill the cytoplasmic *Toxoplasma*(Choi *et al.*, 1988). It was identified that the modulation of enzymes of synthetic (Fig. 5) and degradative (Fig. 6∼10) which restricted the cytosolic cAMP also affected the growth of *Toxoplasma* as well, with kinetics of concentration-dependent fashion until the concentrations reached to those of cytotoxic or harmful to cells as presented by ^{3}H-thymidine incorporation to co-culture system and HL-60 cells alone.

It was not clear whether the effect of stimulation resulted from the transmission of signals produced in the cytoplasm of host HL-60 cells or from the signals that was made in the cytoplasm of *Toxoplasma* after the direct transportation of reagents across the membranes of host cell and *Toxoplasma* directly. But it was likely that cAMP and cAMP analogues stimulated the growth of *Toxoplasma* as a dose-independent mode, therefore it is suggested that some processes were performed in the cytoplasm of host cells which ultimately participated in the host-parasite relationships.

As yet however, the precise role of cAMP in the onset of *Toxoplasma* growth remained to be investigated. A cAMP-dependent protein kinase (Smith *et al.*, 1981) has been recently described in *Trypanosoma cruzi*(Ulloa *et al.*, 1986). Alternatively, cAMP might play a role in the regulation of the expression of some genes as in other eukaryotes(Nagamine and Reich, 1985). In agreement of these two possibilities, it was very likely that the effect of cAMP on the growth of *Toxoplasma* was intracellular since the stimulation was directly related to the permeability of the cell to the analogues (Fig. 2∼4). Nevertheless, it is not at present ruled out the possibility of cAMP cell-surface receptor as in the case of *Dictiostelium* (Oyama and Blumberg, 1986). The observation that the nonhydrolyzable GTP analogue pNHppG(Rodbell, 1980) stimulated the growth of *Toxoplasma* (Fig. 5) suggested that a GTPase activity was involved in the transmission of signals to grow, therefore some mechanisms might be active to transmit the signals via cAMP cell-surface receptor of outer cell membrane associated GTPase inside the membrane.

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=국문초록=

Cyclic AMP 대사가 *Toxoplasma gondii*의 체외 배양에 미치는 영향

가톨릭의대 기생충병연구소
최원영 • 남호우 • 윤지혜 • 김동진 • 김운규 • 김운식

세포내 cAMP의 농도 변화가 *Toxoplasma*의 성장 및 증식에 미치는 영향을 검토하기 위하여 직접 cAMP와 cAMP의 analogue를 첨가하거나 간접적으로 세포내 cAMP의 합성이나 분해를 담당한 효소들의 활성을 변화시킴으로써 cAMP의 농도를 조절하여 그 효과를 측정하였다. HL-60 세포를 숙주 세포로 사용하여 동수의 *Toxoplasma*를 첨가하여 배양하였으며, 처리 효과는 배양 세포계에서 *Toxoplasma*에만 특이하게 표지되는 ^{3}H-uracil과 *Toxoplasma* 및 HL-60 세포에 공통으로 표지되는 ^{3}H-thymidine을 각각 매 12시간마다 2시간씩 배양하여 그 표지량을 측정하여 비교 분석하였다.

직접 cAMP와 cAMP의 analogue인 dbcAMP 및 br-cAMP를 첨가하였을 때, 각각 특이한 농도에서, 즉 1 mM, 0.5~5 mM 및 0.1 mM에서 *Toxoplasma*의 성장 및 증식을 향상시켰다. 이때, 성장 및 증식을 유도하는 시기 및 그 증가도에서도 차이가 있는 것으로 나타났다.

이 결과들은 세포내 cAMP의 농도를 증가시키도록 cAMP의 합성 및 분해에 관여하는 효소들의 활성을 변화시켰을 때에도 같은 양상으로 나타났다. cAMP의 합성 효소인 adenylate cyclase의 활성제인 pNHppG, cAMP의 분해 효소인 cAMP phosphodiesterase의 활성제인 A23187과 imidazole 및 억제제인 IBMX, compound 48/80 및 theophylline을 각각 처리하였는데, 세포내 cAMP의 농도가 증가되었을 때에는 *Toxoplasma*의 성장 및 증식을 향상시켰으나, cAMP의 농도를 감소시켰을 때에는 억제하였다. 이 때 배양계에 독성을 일으키는 농도 이하에서 농도 의존성의 경향을 보였으며, 유도 시기 및 그 증가도에는 차이가 있었다. 따라서 세포 내에서 향상된 수준의 cAMP가 어떤 기전을 활성화시키며, 그 결과 세포질에서의 *Toxoplasma*의 성장 및 증식을 자극하게 된다는 것을 시사하였다.

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