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Effects of pyrimidine salvage inhibitors on uracil incorporation of *Toxoplasma gondii*

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Abstract: Metabolic inhibitors which act in the process of pyrimidine salvage influenced on the uracil incorporation into nucleic acids of *Toxoplasma*. Inhibitors of dihydrofolate reductase, pyrimethamine and methotrexate, and inhibitors of thymidylate synthase, fluoro-uridine, fluoro-dUMP and fluoro-uracil, diminished isotopic uracil uptake in dose-dependent manners. Azauridine which suppresses *de novo* pyrimidine biosynthesis did not affect the salvage even in a relatively high dose. These results suggested that the activation of uracil salvage should be closely related with the function of TMP biosynthetic enzymes.

The pattern of thymidine uptake had no differences between control HL-60 cells and *Toxoplasma* infected cells, which did not reflect the specific proliferation of *Toxoplasma*. It can be exploited to characterize the effects of various compounds related with the proliferation of *Toxoplasma*, especially its DNA synthesis.

Key words: *Toxoplasma gondii*, uracil salvage, dihydrofolate reductase, thymidylate synthase TMP biosynthesis.

INTRODUCTION

Parasites differ most markedly by the absence or modification of metabolic pathways that are present in the mammalian host. In general parasites are metabolically lazy and rely on the metabolism of the host for the supply of prefabricated components such as purine bases, fatty acids and amino acids (Fairlamb, 1989).

Most parasitic protozoa appear to be unable to synthesize the purine ring *de novo*. *Toxoplasma gondii*(Schwartzman and Pfefferkorn, 1982), *Trypanosoma cruzi*, *Leishmania donovani* (Wang *et al.*, 1983), *Plasmodium lophurae*, *Eimeria tenella*(Wang and Simashikevich, 1981) and *Trichomonas vaginalis*(Wang, 1983; Wang *et al.*, 1983) depend on the specific network of salvage pathways to satisfy their requirements for purine bases. *De novo* pyrimidine biosynthesis, on the other hand, takes place in most parasitic protozoa(Hammond *et al.*, 1981).

Besides the *de novo* pathway of pyrimidine, *Toxoplasma* was able to incorporate uracil into its nucleic acids through the specific uracil salvage, while the host cell incorporated uracil at the basal level(Pfefferkorn and Pfefferkorn, 1977a; Kim and Choi, 1989). It was suggested that *Toxoplasma* have greater activities of uridine phosphorylase and uracil phosphoribosyltransferase to build UMP from uracil base than the mammalian cells(Pfefferkorn and Pfefferkorn, 1977b; Pfefferkorn, 1978). In order to incorporate UMP into DNA molecule we thought that enzymes of TMP biosynthesis such as dihydrofolate reductase, thymidylate synthase and serine hydroxymethyltransferase should play an important role in this process(Zubay, 1983). Here we examined the influences of metabolic inhibitors which have been known to act in the

processes of pyrimidine salvage and *de novo* metabolism on uracil incorporation into *Toxoplasma.*

MATERIALS AND METHODS

Chemicals and reagents

5-Fluoro-2′-deoxyuridine-5′-monophosphate (fluoro-dUMP), 5-fluoro-uridine, 6-azauridine, pyrimethamine and methotrexate(amethopterin) were purchased from Sigma Chemical Co. and 5-fluorouracil from Roche Laboratories. [5, 6-^{3}H]-uracil (37.1 Ci/mmol) and [methyl-^{3}H]-thymidine (6.7 Ci/mmol) were purchased from New England Nuclear.

Culture conditions

Virulent tachyzoites of RH strain of *Toxoplasma* were maintained and propagated by continuous passage in ICR mice and were purified from the peritoneal exudate (Kim and Choi, 1989; Choi *et al.*, 1988). HL-60 cells were maintained in Earle's MEM supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin and 10 mM 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.

Measurement of proliferation of *Toxoplasma*

5×10^5 cells/ml of HL-60 cells were co-cultured with the same number of tachyzoites of *Toxoplasma* in 96-well plate for 24 hr. And then [5, 6-^{3}H]-uracil (12 μCi/well) and [methyl-^{3}H]-thymidine (1 μCi/well) were added separately and incubated for additional 2 hr. HL-60 cells grow in suspension culture so can be harvested in a 96-well plate directly and simply. Cells were ruptured and nucleic acids were harvested onto a filter paper using cell harvester (Titertek Co.) by osmotic and mechanical pressure. Radioactivity incorporated into total nucleic acids was measured in a liquid scintillation counter (Kontron Co.).

RESULTS

We first examined the changes of isotopic uracil incorporation into the nucleic acids(RNA and DNA) of *Toxoplasma* under various concentrations of pyrimethamine and methotrexate which have been known as the inhibitors of dihydrofolate reductase. Both the compounds influenced on uracil uptake of *Toxoplasma* in a dose-dependent manner(Fig. 1) while the host cells incorporated uracil continuously at the basal level. Uracil incorporation into *Toxoplasma* was more affected by pyrimethamine rather than methotrexate at the concentrations over 10 μM. We also measured isotopic thymidine incorporation into the DNA of host cell and *Toxoplasma.* Thymidine uptake increased until the concentration of compounds reached 10 μM and then decreased in the higher concentrations, although the patterns of uptake had no difference between control HL-60 cells and *Toxoplasma*-infected cells(Fig. 2), which did not reflect the specific

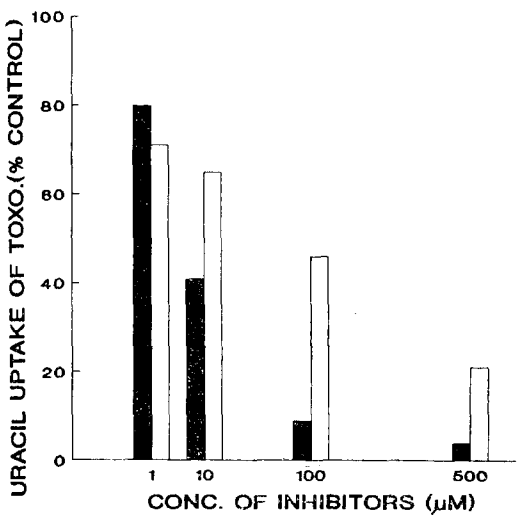

Fig. 1. Inhibition of uracil incorporation by dihydrofolate reductase inhibitors in *Toxoplasma gondii.* Various concentrations of inhibitors, pyrimethamine (■) and methotrexate (□) during the time of co-culture were treated. Effects of inhibitors were described as a percent ratio of isotopic uracil uptake to control which was not treated.

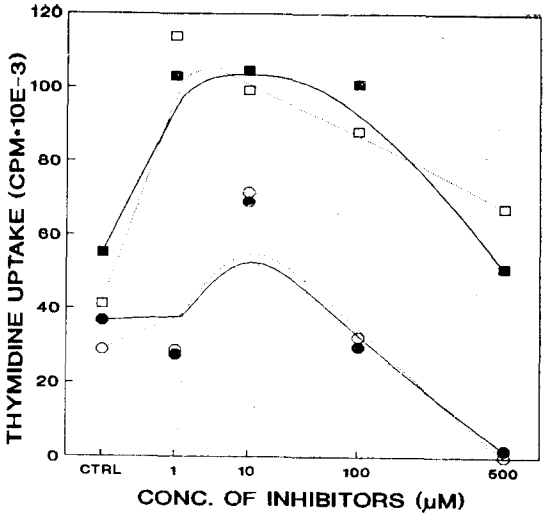

Fig. 2. Effects of pyrimethamine (•, ○) and methotrexate (▪, □) on thymidine incorporation in *Toxoplasma*-infected HL-60 cells (—) and HL-60 cells alone (···).

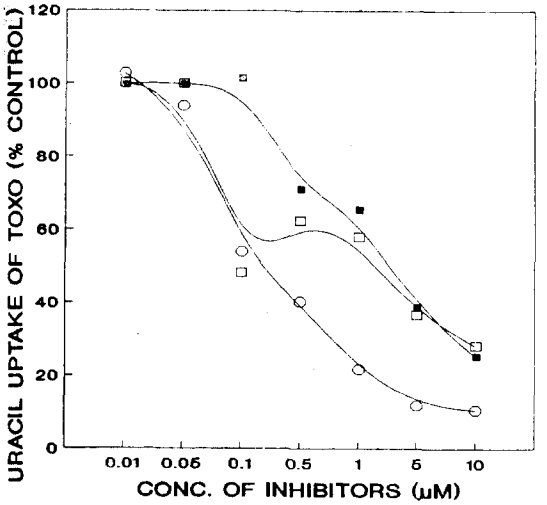

Fig. 3. Inhibition of uracil incorporation by thymidylate synthase inhibitors in *Toxoplasma gondii*. Various concentrations of inhibitors, fluoro-uridine (—▪—), fluoro-dUMP (—□—) and fluorouracil (—○—) were treated. Effects of inhibitors were described as a percent ratio of isotopic uracil uptake to control which was not treated.

proliferation of *Toxoplasma* (Morgan and Canning, 1974).

Fluoro-uridine, fluoro-dUMP and fluoro-uracil are inhibitors of thymidylate synthase (TS) as a dUMP analogs. Uracil incorporation into nucleic acids were also decreased by these chemicals in a dose-dependent mode. Fig. 3 showed that *Toxoplasma* was more sensitive to fluoro-uracil and fluoro-dUMP than to fluoro-uridine. Fluoro-uridine and fluoro-uracil are known to be metabolized into fluoro-dUMP within the cytoplasm by the enzymes of *Toxoplasma* (Pfefferkorn and Pfefferkorn, 1977a). Therefore, fluoro-uracil was likely to compete with isotopic uracil in the early pathway to the formation of dUMP, presumably by uracil phosphoribosyltransferase.

To investigate whether the decrease of uracil incorporation resulted from the real inhibitory effects of thymidylate synthase and dihydrofolate reductase we also treated chemicals above mentioned 2 hr pulsely after 24 hr-co-culture with the concentrations which diminished the uracil incorporation to less than 50 percent of the control groups. Table 1 showed there was not striking difference between the cumulative and the pulse treatment of chemicals except for that of pyrimethamine and fluoro-uridine. Fluoro-uridine should be metabolized within the cytoplasm of *Toxoplasma* for its action as a dUMP analog. Pyrimethamine was likely to have some other effects which were related with cytotoxicity. Sheffield and Melton (1975) reported that pyrimethamine also influenced the process of endodyogeny and caused striking morphological change such as rounded body shape and fragmented nucleus. This suggested that thymidylate synthase and dihydrofolate reductase played a important role in the uracil incorporation of *Toxoplasma*.

Azauridine is known to inhibit orotate monophosphate(OMP) decarboxylase which converts OMP into dUMP in the *de novo* synthesis of pyrimidine. This compound did not affect the uracil incorporation even in a relatively high dose (Fig. 4). We suggested two possibilities to explain these results. First, azauridine could not fit well to *Toxoplasma* enzyme, which did not play a role as a substrate, and the second, azauridine might activate the uracil salvage to cope with the shut-down of UMP resulted from its action to inhibit OMP decarboxylase.

Table 1. Acute effects of inhibitors with the concentration which diminish the uracil incorporation to 50% of control group

Inhibitors (conc.)	Inhibition of uracil incorporation (% control)
fluoro-uridine (0.7 μM)	23.3%
fluoro-dUMP (0.7 μM)	34.8%
fluoro-uracil (0.1 μM)	48.6%
pyrimethamine (5 μM)	14.0%
methotrexate (50 μM)	55.8%

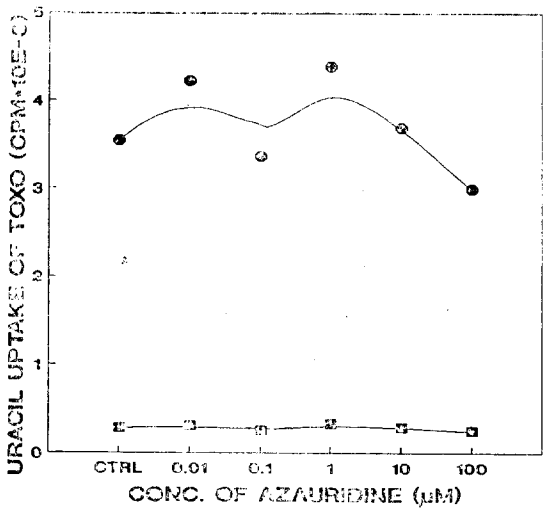

Fig. 4. Effects of azauridine on uracil incorporation of *Toxoplasma gondii* (—•—) and HL-60 cells (—▪—).

DISCUSSION

As to the differential incorporation of uracil into intracellular proliferating *Toxoplasma* rather than host cells, Pfefferkorn (1977) pointed out an active uracil phosphoribosyltransferase and 40-fold greater activity of uridine phosphorylase in *Toxoplasma* instead of uridine kinase in host cell salvage. It was also reported that trypanosomatids could synthesize UMP not only by the *de novo* pathway but also by the uracil salvage in which uracil phosphoribosyltransferase might be participated(Hammond and Gutteridge, 1982 & 1984). We thought that activation of uracil salvage in parasitic protozoa was closely related with the function of dihydrofolate reductase. Generally protozoan dihydrofolate reductase was reported to have a higher molecular weight than that of mammalian dihydrofolate reductase as revealed in *Plasmodium*, *Trypanosoma*, *Crithidia*(Ferone and Roland, 1980) and *Eimeria tenella* (Wang *et al.*, 1975). Especially in *Plasmodium falciparum* (Bzik *et al.*, 1987) and Trypanosomatids (Conderre *et al.*, 1983), both the dihydrofolate reductase and thymidylate synthase activities often resides on a single bifunctional homodimeric polypeptide with a high molecular weight. Presumably this arrangement would provide advantages to the cells in view of the efficiency of gene expression and metabolism.

Methotrexate showed less inhibitory effect than pyrimethamine on the incorporation of uracil into *Toxoplasma*(Fig. 1). Parasitic dihydrofolate reductase was known to be even more sensitive to pyrimethamine. There was a report that methotrexate promote the growth of *Crithidia fasciculata* at the same concentration as does folate. This suggested that methotrexate sometimes be metabolized to active compounds which have no inhibitory effect on the reducing activity (Hammond and Gutteridge, 1984). Compared with drug sensitivity and uracil salvage the nature of dihydrofolate reductase in *Toxoplasma* will be interesting in view of evolutionary divergence of the protozoan parasite.

The fact that *Toxoplasma* incorporated much more uracil into its nucleic acids selectively than host cells provides a rapid and simple measurement for DNA and RNA synthesis of *Toxoplasma*. It can be exploited to characterize the effects of various compounds related with the proliferation of *Toxoplasma* especially the DNA synthesis. Besides, *Toxoplasma* does not incorporate exogenous thymidine into its nucleic acids by some mechanism such as membrane impermeability to thymidine nucleotide, specificity of thymidine kinase or deficiency of thymidine kinase (Pfefferkorn and Pfefferkorn, 1977a). The third case was not agreeable according to the partial assay we performed in the *Toxoplasma*-inoculated mouse peritoneal exudate(data not shown). Although *Toxoplasma* must be

cultured *in vitro* with host cells, we could observe the effects of various compounds acting on DNA synthesis of parasite and host *i.e.*, by uracil in *Toxoplasma* and by thymidine in host cells.

Protozoan proliferation may be particularly sensitive to inhibitory effects of purine analogs for the absence of *de novo* purine synthesis (Schwartzman and Pfefferkorn, 1982). Therefore allopurinol, ribosyl allopurinol and formycin B inhibit the growth of several species of *Leishmania* and *Trypanosoma* at the concentrations of non-toxic to mammalian cells(North and Wyler, 1987). In addition to purine analogs uracil analogs which block the uracil salvage such as emimycin can be used in the therapy of toxoplasmosis(Pfefferkorn *et al.*, 1989).

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=국문초록=

*Toxoplasma gondii*의 활성화된 uracil 도입 과정에 미치는 pyrimidine 대사 억제제의 영향

가톨릭의대 기생충학교실

윤지혜 • 남호우 • 김동진 • 최원영

Pyrimidine salvage 과정 및 *de novo* 합성과정에 작용하는 억제제들이 *Toxoplasma gondii*의 특이한 uracil 도입 과정에 미치는 영향을 방사능을 표지한 uracil과 thymidine을 사용하여 검토하였다. Dihydrofolate reductase (DHFR)에 작용하는 억제제인 methotrexate, pyrimethamine 그리고 thymidylate synthase(TS)의 경쟁적 억제제인 fluoro-uridine, fluoro-dUMP, fluoro-uracil을 각각 처리한 경우, 그 농도에 비례적으로 uracil 표지가 감소하였다. OMP decarboxylase에 작용하는 억제제인 azauridine에 대해서는 100 μM 농도 이상에서도 uracil 표지량에 변화가 거의 없었다. 본 결과로부터 *Toxoplasma*가 uracil을 DNA로 도입할 때 dihydrofolate reductase와 thymidylate synthase가 관여하는 TMP biosynthesis 과정이 중요함을 알 수 있었고, uracil salvage 과정이 있는 다른 기생성 원충의 경우와 비교했을 때 *Toxoplasma*에 있어서도 효율적인 다기능의 DHFR-TS system의 존재를 예상할 수 있었다.

Thymidine 도입의 양상은 모든 경우에 있어 HL-60 세포만의 경우와 *Toxoplasma*가 함께 배양된 HL-60 세포에서 차이가 없었다. 이로부터 thymidine은 *Toxoplasma* 성장을 반영하지 않음을 알 수 있었다. *Toxoplasma*의 특이한 uracil 표지와 *Toxoplasma*에 배타적인 thymidine 표지는 기생 원충과 숙주세포의 성장, DNA 합성 정도 및 이들 과정에 미치는 제제들의 영향을 쉽고 간단하게 연구할 수 있는 방법이 된다.

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