Effects of Nucleic Acid Inhibitors on the Development of *Angiostrongylus costaricensis* in Vitro

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(Received 10 April 1998/Accepted 15 June 1998)

**ABSTRACT.** Nucleic acid biosynthesis of *Angiostrongylus costaricensis* was examined with various inhibitors; aminopterin (inhibitor of purine and pyrimidine *de novo* biosynthetic pathways), 8-azaguanine (specific inhibitor of purine salvage pathway) and PALA (specific inhibitor of pyrimidine *de novo* biosynthetic pathway) were applied in *in vitro* culture developing from the third stage larvae to young adult in chemically defined medium. It was suggested that *A. costaricensis* possessed functional purine and pyrimidine *de novo* biosynthetic pathways and also that they could utilize exogenous sources of purines and pyrimidines by salvage pathways for their development.

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**KEY WORDS:** *Angiostrongylus costaricensis*, *de novo* biosynthesis, nucleic acid biosynthesis.

In mammals, it is known that purine and pyrimidine nucleotides can be found by two different pathways, one the *de novo* biosynthetic pathway and the other the salvage reutilizing pathway. However, it was reported that most parasitic protozoa and *Schistosoma mansoni* were unable to synthesize the purine ring by *de novo* pathway [11, 17]. Accordingly, it was suggested that the source of preformed purine depended on the host, and that precursors were incorporated by salvage reutilizing pathway. However, there are only a few reports concerning the study of nucleic acid biosynthesis of parasitic helminths.

*Angiostrongylus costaricensis*, which causes abdominal angiostrongylosis, is known as an important human parasitic nematode first discovered by Morera and Cespedes [14]. In a previous report, third stage larvae of *A. costaricensis* were successfully cultured to the young adult stages using chemically defined Waymouth’s medium [6]. Thereafter, nutritional requirements were examined by deletion of single components from Waymouth’s medium, and ten amino acids, choline chloride, glucose and pyridoxine were shown to be essential for the parasite’s development [4, 5]. However, *A. costaricensis* did not require hypoxanthine the only nucleic acid precursor contained in Waymouth’s medium. This result suggested the existence of a purine *de novo* synthetic pathway in *A. costaricensis*. We herein describe further experiments using various inhibitors for nucleic acid biosynthesis of *A. costaricensis* under *in vitro* culture condition.

The third stage larvae of *A. costaricensis* were cultured in Waymouth’s medium MB752/1 (liquid) ( GibCO, New York: General Industries, Israel) for 2 weeks according to the methods described previously [6]. One hundred to 200 worms were cultured in a Leighton tube ( Weatone) containing 5 ml of culture medium. Hypoxanthine deleted Waymouth’s medium was prepared according to the method described by Hata [4]. Aminopterin (AP) (Sigma) and PALA (N-(phosphonacetyl)-L-aspartate) (kindly provided by the Second Department of Biochemistry, Chiba University School of Medicine) were dissolved in re-distilled water, making 42 µM and 10 µM of stock solutions, respectively. 8-azaguanine (AGU) (Sigma) was dissolved in hypoxanthine- and thymidine- free Waymouth’s medium, making 6.6 × 10^{-3} M of stock solution. These inhibitors were sterilized by filtration. They were then added to the Leighton tube containing 5 ml of culture medium and cultured for two weeks in 5% CO2 in air at 37°C. Thymidine (thymd) (Sigma), uridine (urid) (Sigma) and hypoxanthine (hypox) (Sigma) were dissolved in redistilled water, sterilized by filtration, and used for experiments, respectively.

Figure 1 shows the approximate sites of action of the various inhibitors on mammalian cells used in the present study [15].

**Purine nucleotide synthesis:** Figure 2 shows the effect of aminopterin, an inhibitor of *de novo* purine and pyrimidine syntheses, on the development of *A. costaricensis* in Waymouth’s medium. Waymouth’s medium was supplemented with thymidine to overcome the effect from the inhibition of pyrimidine synthesis by aminopterin. In media at concentrations of 0.042 µM of aminopterin or more, none of the worms developed to the young adult stage, although some developed to the late third stage at 0.042 µM. It was suggested that aminopterin inhibited the purine
**de novo** biosynthesis of *A. costaricensis*. However, when this aminopterin containing medium was supplemented with hypoxanthine (25 μg/ml), the worm development was recovered. Figure 3 shows the effect of addition of 8-azaguanine, inhibitor of purine salvage pathway, into aminopterin and hypoxanthine containing Waymouth’s medium. In this medium, the worm development became inhibited again, and none of the worms developed to the young adult stage. However, when aminopterin was deleted from this hypoxanthine- and 8-azaguanine-containing medium, the worm development was restored. These results suggested that *A. costaricensis* were able to synthesize purine by **de novo** pathway and also they were able to utilize an exogenous source of purine by salvage reutilizing pathway.

**Pyrimidine nucleotide synthesis:** Figure 4 shows the effect of PALA, a specific inhibitor of pyrimidine nucleotide biosynthesis, on the development of *A. costaricensis* in Waymouth’s medium with or without uridine. The addition of PALA at a concentration of 3 × 10^{-4} M completely prevented worm development to the young adult stage. However, when uridine (25 μg/ml) were added to the PALA-containing medium, worm development was recovered to the young adult. Figure 5 shows the effect of aminopterin on pyrimidine **de novo** biosynthesis and the development of the worms. To avoid an aminopterin-induced inhibition as seen in purine **de novo** biosynthesis, Waymouth’s medium was supplemented with hypoxanthine. The addition of aminopterin to Waymouth’s medium supplemented with hypoxanthine (25 μg/ml) then suppressed the worm development. However, when thymidine (25 μg/ml) was additionally supplemented, their development was recovered again. These results suggested that *A. costaricensis* was able to synthesize pyrimidine by **de novo** pathway and also utilized exogenous source of pyrimidines by salvage pathway.

The culture system for *A. costaricensis* in chemically defined medium [6] provides a tool for proving the existence of their nucleotide synthetic pathway. The present study suggested that *A. costaricensis* was able to synthesize purine and pyrimidine by **de novo** pathways, and also that they

**Fig. 2.** Effect of aminopterin on the development of *Angiostrongylus costaricensis* from third stage larvae to young adults in Waymouth’s medium supplemented with thymidine. % Young adult: percentage of young adult in cultured worms. AP: aminopterin. Hypox: hypoxanthine.

**Fig. 3.** Effect of 8-azaguanine on the development of *Angiostrongylus costaricensis* from third stage larvae to young adults in Waymouth’s medium supplemented with hypoxanthine. % Young adult: percentage of young adult in cultured worms. AP: aminopterin. Hypox: hypoxanthine. AGU: 8-azaguanine.

**Fig. 4.** Effect of PALA (N-(phosphonacetyl)-L-aspartate) on the development of *Angiostrongylus costaricensis* from third stage larvae to young adults. % Young adult: percentage of young adult in cultured worms. Urid: uridine.

**Fig. 5.** Effect of aminopterin on the development of *Angiostrongylus costaricensis* from third stage larvae to young adults in Waymouth’s medium supplemented with hypoxanthine. % Young adult: percentage of young adult in cultured worms. AP: aminopterin. Thymd: thimidine.
were able to utilize exogenous sources of purines and pyrimidines by salvage pathways for their development. However, to clarify the existence of these pathways, the future enzymatic studies might be necessary.

Concerning the nucleotide synthesis in parasites, all the parasitic protozoa studied lack the purine de novo synthetic pathway [7, 11]. Hence, they must take up free bases or nucleotides from the outside by salvage pathway. In parasitic helminths, there is little evidence that they synthesize purine and pyrimidine bases by de novo pathway from simple precursors. Senft et al. [17] reported that the adult stage of *Schistosoma mansoni* lacked functional nucleoside kinases and that they were incapable of de novo purine nucleotide synthesis. Their purine source depended on the salvage pathway. In the free living nematode, *Caenorhabditis briggsae*, and in the insect parasitic nematode *Neoaplectana glaseri*, it was suggested that they synthesized purines and pyrimidines by de novo pathways [9, 16]. In mammalian parasitic nematodes, Wong and Ko [19] reported that adult *Angiostrongylus cantonensis* obtained from lungs of rats possessed the capacity of utilizing the carbon atoms of glycine for purine ribonucleotide synthesis. This also suggests that adult worms of *A. cantonensis* can synthesize purine by de novo pathway. In *Brugia pahangi*, the uptake and incorporation of purine nucleic acid precursors, adenine, hypoxanthine and guanine were demonstrated [3].

Concerning the pyrimidine nucleotide synthesis, it is generally considered that many helminths possess both de novo and salvage pathways. Their existence was suggested in *Clonorchis sinensis*, *Paragonimus ohirai* [10], *Mesocestoides corti* [2], *Schistosoma japonicum* [8], *S. mansoni* [1, 12, 13], *Angiostrongylus cantonensis* [18]. The present study suggested that *A. costaricensis* also possessed a pyrimidine de novo synthetic pathway and salvage pathway. In *Brugia pahangi*, however, though a significant uptake of uracil was demonstrated, no evidence was obtained for the uptake and incorporation of thymine and cytosine [3]. They suggested that *B. pahangi* was unable to utilize these two pyrimidines.

ACKNOWLEDGMENT. The authors wish to thank Mr. Gerz A. for critically reading the manuscript.

REFERENCES