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Purine metabolism in *Echinococcus multilocularis*

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Abstract

The activities of the enzymes in *Echinococcus multilocularis* metacestodes involved in purine salvage were studied by HPLC. As in most parasites, this cestode relies entirely on salvage of preformed bases and nucleosides for its purine requirement. Therefore, these enzymes may be targets for drugs in the chemotherapeutic treatment of diseases caused by this parasite. The animals used in this study were gerbils (*Meriones unguiculatus*). Enzyme activities from sera and hepatic tissue in control and infected animals were similar with the exception of adenine phosphoribosyltransferase which showed an activity 4-fold greater in the serum from control than in serum from infected animals. In the parasite, adenine and hypoxanthine-guanine phosphoribosyltransferases and adenosine deaminase had the highest activities. Therefore, in *E. multilocularis* metacestodes, this pathway seems to be important for the parasite's metabolism.

Keywords: *Ecinococcus multilocularis*; Cestoda; Metacestodes; *Meriones nguiculatus*; Metabolism; Purines; Salvage pathway; HPLC

1. Introduction

Alveolar hydatid disease, caused by the larval stage of the cestode *Echinococcus multilocularis* has a relatively high lethality, if remaining untreated, in humans in the Northern hemisphere [4,16]. This larval infection develops mostly in the liver. At present only a few molecules are available for the chemotherapy of this disease [3]. The absence of an effective drug treatment led us to study different metabolic pathways in this parasite [17,18] and to compare them with those of the host, in the hope that specific differences could be found to serve as a target for drug development [5]. We focused on purine biosynthetic pathway metabolism, which has been particularly studied in Protozoa such as *Plasmodium falciparum* [7]. In parasites, the salvage pathway seems to be the only route of purine synthesis [8,15].

The purpose of this study, was to compare the enzymatic activities of the principal enzymes of purine metabolic pathways in both control and infected animals. Enzymes of particular interest in purine salvage included: adenosine deaminase (ADA, EC.3.5.4.4), purine nucleoside phosphorylase (PNP, EC.2.4.2.1), guanase (EC.3.5.4.3), hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC.2.4.2.8), adenine phosphoribosyltransferase (APRT, EC.2.4.2.7) and xanthine oxidase (XO, EC.1.2.1.37) as shown in Fig. 1. The activities were measured in serum, hepatic tissue and in *E. multilocularis* metacestodes with the aim of showing both the importance of this pathway for the parasite and the possible changes caused by presence of the parasite in the host.

Abbreviations: ADA, adenosine deaminase; AMP, adenine 5′-monophosphate; APRT, adenine phosphoribosyltransferase; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; GTP, guanosine 5′-triphosphate; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; HPLC, high performance liquid chromatography; IMP, inosine 5′-monophosphate; KH_2PO_4, potassium dihydrogen phosphate; KOH, potassium hydroxide; $MgCl_2$, magnesium chloride; $MgSO_4$, magnesium sulfate; NaH_2PO_4, sodium dihydrogen phosphate; Na_2HPO_4, di-sodium hydrogen phosphate; NaOH, sodium hydroxide, $NH_4H_2PO_4$, ammonium dihydrogenphosphate; PNP, purine nucleoside phosphorylase; XO, xanthine oxidase.

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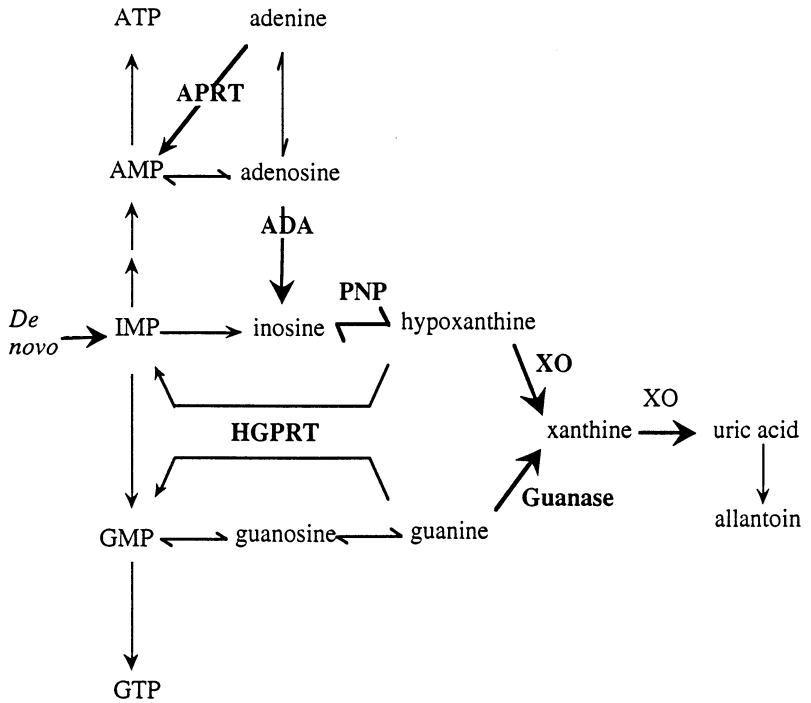

Fig. 1. The pathway of purine salvage and degradation. The bold lines indicate the activity of the enzymes studied. ADA, adenosine deaminase; APRT, adenine phosphoribosyltransferase; Guanase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; PNP, purine nucleoside phosphorylase; XO, xanthine oxidase.

2. Materials and methods

2.1. Parasites

The Savoie strain of *E. multilocularis* was maintained in 3-month old Mongolian gerbils (*Meriones unguiculatus*). A total of 14 gerbils were infected with ground metacestodes containing about 200 protoscoleces by intraperitoneal inoculation. The animals were anaesthetised by diethyl ether inhalation, 3-months post-infection. The carotid was sectioned for blood collection and metacestodes from the peritoneal cavity were collected and carefully separated from host tissue and washed with 0.9% NaCl. A total of 12 uninfected animals were used as controls.

2.2. Enzyme extraction procedure

Serum was removed after centrifugation at $3000 \times g$ for 10 min and filtered through 0.45 μm filters, diameter 13 mm. Metacestodes and hepatic tissue were ground in a buffer of 0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, 10 mM $MgSO_4$ pH 7.4, using a grinder (Polytron) for 5 min and centrifuged at $+4$°C for 1 h in a refrigerated Beckman centrifuge (Avanti 30, Rotor F 0650) at $12000 \times g$. Ground extracts were filtered through 0.45 μm filters, diameter 25 mm (Minisart, Sartorius). Samples were stored at -180°C. All enzyme assays involved measurement of product formation by HPLC.

2.2.1. HGPRT and APRT

HGPRT and APRT activities were measured simultaneously by determining the amounts of IMP and AMP produced after incubation of samples with adenine and hypoxanthine for 3 h. The samples were extracted in Tris–Mg^{2+} buffer, pH 7.4, containing 275 mM Tris, 25 mM $MgCl_2$ and 0.5 mg ml^{-1} BSA. The same buffer was used for incubation, but with the addition of 50 μM adenine and 50 μM hypoxanthine, as substrates for APRT and HGPRT, respectively, and 1 mM PRPP as the ribose donor [1]. Adenine and hypoxanthine were dissolved in 0.2 M KOH before dilution in the Tris buffer.

2.2.2. ADA

The ADA activity was estimated as the amount of inosine formed from adenosine as substrate. The samples were incubated with 750 μM adenosine for 1 h. The extraction mixture was 0.1 M sodium phosphate buffer, pH 7.4 with the addition of 0.5 mg ml^{-1} BSA. The adenosine stock solution was dissolved in 0.2 M KOH before subsequent dilution. The maximum enzyme activity was estimated for a range of adenosine concentrations (0.1–3 mM).

2.2.3. PNP

PNP activity was measured as the amount of hypoxanthine formed by samples incubated with 3 mM inosine for 70 min. Maximum activity was reached

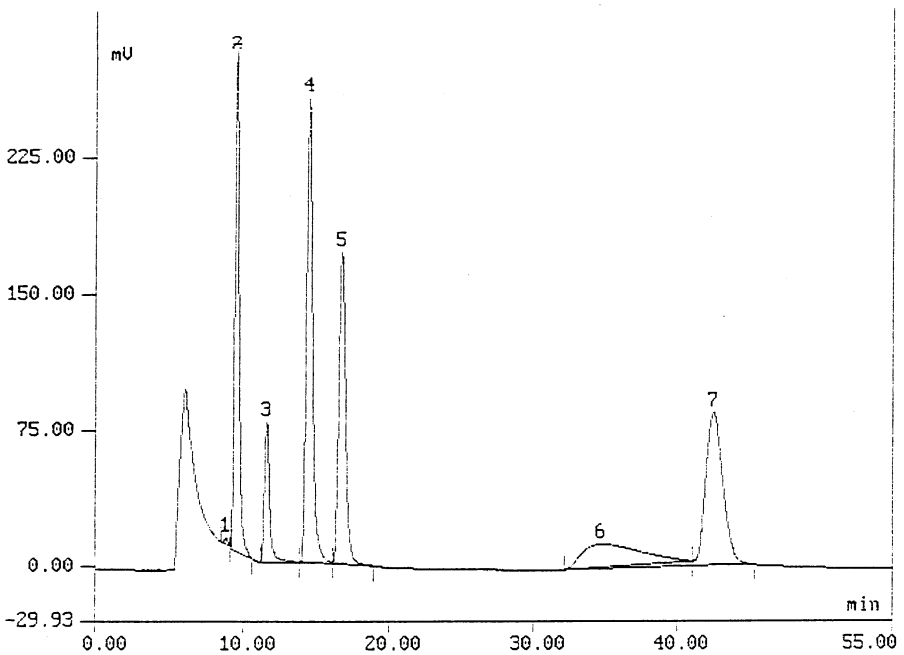

Fig. 2. Chromatogram of solution at 0.6 mM containing: GTP (1), IMP (2), uric acid (3), hypoxanthine (4), xanthine (5), adenine (6), and inosine (7). Mobile phase: 25 mM $NH_4H_2PO_4$, 1.5% methanol; pH 4.5; flow rate: 0.5 ml h^{-1}; 1 mM corresponding at 1×10^{-3} absorbance; detection wavelength 254 nm.

when inosine concentration was in the range 0.1–5 mM. The extraction mixture was the same as that used for the ADA assay.

2.2.4. Xanthine oxidase

The activity was measured as the formation of xanthine or uric acid, using hypoxanthine or xanthine as substrates, respectively. Incubation was carried out for 1 h. The extraction buffer was 0.01 M KH_2PO_4, 0.2 M NaOH, 0.2 mM EDTA pH 7.9–8. The samples were incubated in buffer supplemented with 0.05 mM hypoxanthine as substrate.

2.2.5. Guanase

Guanase activity was measured as the amount of xanthine formed after incubation for 1 h with 0.2 mM guanine. The extraction buffer was the same as that used for the ADA and PNP assays. All reactions were linear for at least 3 h [1].

2.3. Nucleotide analysis by HPLC

HPLC analysis was carried out on a Kontron 422 series system (Kontron Instruments, France). Reverse-phase chromatography was performed in an isocratic mode at room temperature. Each HPLC sample was made up by adding 30 μl of the reaction mixture to 270 μl of elution buffer. A 20 μl aliquot was injected into a Hypersil 5 ODS column (250×4.6 mm). The mobile phase was constituted with 25 mM $NH_4H_2PO_4$, pH 4.5 with the addition of 1.5% methanol. The elution buffer was prepared with double-distilled, deionized water and filtered through a 0.4 μm membrane filter. Elution was performed at a constant flow rate of 0.5 ml min^{-1} with the detector set at 254 nm and an absorbance sensitivity of 0.002 AFS (absorbance full scale).

Peak identification was performed by comparing retention times with those of freshly prepared standards and is shown in Fig. 2. Each assay was repeated three times.

2.4. Assay of protein

Bovine serum albumin served as the standard in the measurement of proteins. The amount of protein was determined by the Biorad protein assay DC method.

2.5. Reagents

All reagents used were of the highest grade available commercially: adenine, adenosine, hypoxanthine, guanine, inosine, GTP, IMP, AMP, uric acid were purchased from Sigma (L'Isle d'Abeau, France). Methanol was obtained from Carlo Erba (France). Hypersil 5 ODS column (250×4.6 mm^2) was obtained from Interchim (Montluçon, France).

3. Results

The mean weights of controls and infected animals were 65.6 ± 4.1 and 66.4 ± 6.7 mg, respectively.

Table 1
Specific activities of *E. multilocularis* enzymes of the purine salvage pathway

Enzymes	Control animals		Infected animals		
	Serum	Hepatic tissue	Serum	Hepatic tissue	Metacestode
Adenosine deaminase	7.5 ± 1.5	6.5 ± 1.4	5.2 ± 1.3	6.7 ± 0.8	8.8 ± 1.7
Purine nucleoside phosphorylase	4.6 ± 1.0	4.6 ± 1.5	3.1 ± 0.8	4.3 ± 0.3	3.5 ± 1.0
Guanase	2.3 ± 0.5	2.6 ± 0.1	1.7 ± 0.5	2.2 ± 0.1	3.1 ± 0.2
Xanthine oxidase	2.3 ± 0.5	2.8 ± 1.0	1.7 ± 0.5	2.5 ± 0.6	2.0 ± 0.5
Hypoxanthine-guanine phosphoribosyltransferase	1.3 ± 0.5	4.2 ± 1.9	1.8 ± 0.4	1.3 ± 0.1	7.4 ± 0.4
Adenine phosphoribosyltransferase	3.6 ± 1.8	3.5 ± 2.7	0.9 ± 0.5	3.2 ± 0.6	9.0 ± 1.6

Specific activities are expressed as nmol min^{-1} mg^{-1} protein. Mean $\pm$ S.D. of three measurements.

After 3-months of infection, it was noted that there was considerable variability in the 14 infected gerbils in respect to the growth of *E. multilocularis* metacestodes. Metacestode weights varied from 0.1 to 17 g. In control animals, protein concentrations for serum and for hepatic tissue were similar, 66 ± 8.3 and 75 ± 2.8 mg ml^{-1}, respectively. In infected animals, serum protein concentration (96 ± 1.4 mg ml^{-1}) was 50% greater than in controls. In hepatic tissue, the same protein concentration was seen in infected gerbils: 69 ± 1.8 mg ml^{-1}. In *E. multilocularis* metacestodes, protein concentration was 53 ± 7.9 mg ml^{-1}.

Specific activities of enzymes of the purine salvage pathway are summarized in Table 1. The specific activity of APRT was about 4-fold greater in the serum of controls than in infected animals. In hepatic tissue this enzymatic activity was increased and in metacestodes, the specific activity was higher than that found in the controls sera.

The specific enzymatic activities of ADA, PNP, guanase and XO of sera from controls, infected animals and metacestodes were similar. The specific activity of HGPRT in the sera of controls, infected gerbils and also in the hepatic tissue of parasitized animals were comparable. We noted an increase in this specific activity by a factor of about 2 for hepatic tissue of controls. In metacestode this specific activity was also important. The most important specific activities found in the parasite were APRT, ADA and HGPRT. These specific activities varied the most in hepatic tissue of infected gerbils in comparison with specific activities found in control animals.

4. Discussion

After 3-months of infection, *E. multilocularis* metacestode weights in gerbils varied significantly. Therefore, this parasite is developing more or less rapidly depending on the host. Gerbils have a different susceptibility towards the parasite. Yamashita has studied the susceptibility to this parasite between different hosts such as gerbils or white mice [20,21]. Similarly, Norman and Kagan, have also concluded that gerbils were the most useful model for *E. multilocularis* infestation, with a 3 month development, compared to 12 months in mice [14].

In *E. multilocularis* metacestodes, the highest specific activities of purine salvage pathway enzymes were found with APRT, HGPRT and ADA. Therefore, high concentrations of IMP, AMP and inosine indicated that the metabolic flux converges on the salvage pathway. Purine salvage pathways have also been studied in *Toxoplasma gondii*, the causative agent of congenital or cerebral toxoplasmosis [9] and significant differences exist between this Protozoan and *E. multilocularis* (Cestoda). In *T. gondii*, all enzymes activities were lower than those found in *E. multilocularis*. APRT activity was approximately 50-fold higher, HGPRT 15-fold and ADA of 440-fold. It is noteworthy that xanthine oxidase activity was detected in *E. multilocularis* metacestodes, an activity lacking in parasitic protozoans such as *Giardia* [13], *Plasmodium* [19], or *Leishmania* [10]. *E. multilocularis*, like many parasites, is dependent entirely on preformed bases and nucleosides for their nucleotide requirement. Thus the salvage of purine bases depends primarily on the phosphoribosyltransferases which catalyse the conversion of adenine, hypoxanthine and guanine to their respective nucleotides AMP, IMP and GMP in the presence of PRPP, a ribose donor. The results reported above are consistent with the fact that phosphoribosyltransferases seem to be key enzymes of purine salvage pathways. In many parasites, the enzymes of the purine salvage pathway have been used as chemotherapeutic targets [11].

Indeed, some of the enzymes of the system are able to accept purines analogues and to metabolize them into nucleotides; these analogues are used as metabolic inhibitors. In *Leishmania* [2] and in *Trypanosoma cruzi* [6], the allopurinol and allopurinol ribonucleoside, analogues of hypoxanthine and inosine, respectively, have been studied. Treatment of these parasites with allopurinol is promising because the parasite growth is blocked. In *E. multilocularis*, APRT, HGPRT and

ADA may be attractive targets for chemotherapeutic attack. It would now be interesting to study the enzymes of host tissue and *E. multilocularis* metacestodes in order to compare them and know if significant differences exist between parasitic Cestoda and humans, differences which could be potentially exploited in order to find specific inhibitors of these enzymes in the future.

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