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Pteroylpolyglutamate synthesis by lung- and culture-derived *Pneumocystis carinii*

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Abstract

Pneumocystis carinii synthesizes folates de novo from exogenous *p*-aminobenzoic acid (pABA). Lung-derived organisms take up [³H]pABA in vitro except in the presence of sulfamethoxazole. Supernatants from spinner-flask cultures take up [³H]pABA if they were inoculated with lungs from infected rats, but not if they were inoculated with lungs from uninfected rats. *P. carinii* folates consist primarily of pteroylpentaglutamates. *Plasmodium falciparum*, in contrast, contains primarily pteroyltetraglutamates. Culture-derived organisms synthesize folates at a four-fold higher specific activity than lung-derived organisms, possibly because they contain less contaminating lung debris. These data suggest that *P. carinii* remains metabolically active in culture for at least 4 days.

Keywords: *Pneumocystis carinii*; Folate; Opportunistic infection; Folylpolyglutamate; Sulfamethoxazole

1. Introduction

Antifolates, such as co-trimoxazole (sulfamethoxazole + trimethoprim), play critical roles in the prophylaxis and treatment of *Pneumocystis carinii* pneumonia [1]. These drugs act by inhibiting enzymes of the folic acid biosynthesis pathway. In most organisms, the endproducts of this pathway are pteroylpolyglutamates, which comprise various forms of folic acid with polyglutamate tails [2]. In this paper, we demonstrate that *P. carinii* form pteroylpolyglutamates from exogenous [³H]*p*-aminobenzoic acid (pABA).

2. Materials and methods

[3,5-³H]pABA (33.5 Ci mmol⁻¹) was purchased from Moravek Biochemical Inc. (Brea, CA). 7,8-Dihydroneopterin, *p*-aminobenzoyldiglutamate (pABA-glu₂), *p*-aminobenzoyltetraglutamate (pABA-glu₄) and *p*-aminobenzoylhexaglutamate (pABA-glu₆) were purchased from B. Schircks Laboratory (Wetzwill, Switzerland). RPMI 1640, Eagle's Minimum Essential Medium (MEM) and heat-inactivated fetal bovine serum were obtained from Gibco (Grand Island, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except where noted.

Plasmodium falciparum (strain FCR3) was cultivated in candle jars [3]. Cultures were preincubated

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Table 1
Uptake of [³H]pABA by *P. carinii* preparations

Experiment	Total uptake (pmol)	Specific activity (pmol (mg protein) ⁻¹)	Folate synthesis (pmol (mg protein) ⁻¹)
Lung-derived <i>P. carinii</i> ^a	0.38	3.3	2.3
Lung-derived <i>P. carinii</i> + sulfamethoxazole	0.007	0.07	
2-day culture-derived <i>P. carinii</i>	0.5	13.9	11.1
2-day controls	0.004	0.36	
4-day culture-derived <i>P. carinii</i>	1.24	13.9	8.9
4-day controls	0.01	2.1	

^a Average of two experiments.

for 12 h in folate- and pABA-free RPMI 1640 media, and then incubated for 24 h in the same medium plus dihydropterin (50 μM) and [³H]pABA (20 μCi). Parasites were then isolated by saponin lysis [4], and washed three times with phosphate-buffered saline (PBS).

Lung-derived *P. carinii* were partially purified from latently infected rats as described previously [5] and suspended in MEM containing 20% heat-inactivated fetal bovine serum. MEM contains no pABA. Culture-derived *P. carinii* were harvested from spinner flask cultures as described [6] and freed of host cells by low-speed centrifugation. As controls, supernatants were obtained in the same manner from spinner flasks which were inoculated with lung homogenates from uninfected rats. To suspensions of approximately 10⁷ organisms ml⁻¹, dihydropterin (50 μM, added from a 0.5 M stock solution in dimethylsulfoxide) and [³H]pABA (20 μCi) were added (with and without sulfamethoxazole, 1 μM) and then incubated for 6 h at 35°C in a 5% CO₂ atmosphere. Organisms were then pelleted by centrifugation at 1500 × g, and washed three times with cold PBS or Hank's buffer.

Pelleted organisms were then suspended in PBS and lysed by sonication using four treatments of 5 s

with a microprobe at maximum intensity (Sonifier Cell Disruptor, Heat Systems-Ultrasonics, Long Island, NY). Aliquots were taken and assayed for protein [7] and radioactivity in a Beckman LS 7000 Scintillation counter using Scintiverse BD scintillation fluid (Fisher Scientific, Fair Lawn, NJ). The remainder was stored at -70°C.

In order to assay pteroylpolyglutamates, cell lysates were thawed, heated to 100°C for 5 min, and cooled to 4°C. Precipitated protein was removed by centrifugation (12000 × g for 10 min) and the supernatant treated to remove the pterin ring [8].

3. Results

[³H]pABA is taken up by *P. carinii* in the absence of feeder cells (Table 1), as has been reported by others [9,10]. pABA is readily taken up by lung-derived *P. carinii*, except in the presence of sulfamethoxazole, which inhibits dihydropteroyl synthetase, the enzyme in *P. carinii* for which pABA is a substrate [10–13]. Supernatants from 2- and 4-day *P. carinii* cultures take up pABA at a four-fold higher specific activity than lung-derived organisms. pABA is not taken up by supernatants from control

Table 2
Distribution of polyglutamates (as percent of total) in *P. falciparum* and *P. carinii*

Experiment	pABA- glu ₁	pABA- glu ₂	pABA- glu ₃	pABA- glu ₄	pABA- glu ₅	pABA- glu ₆
<i>P. falciparum</i>	4.6	2	9.8	81.3	2.3	
Lung-derived <i>P. carinii</i> ^a	21.3	3.6	17.2	18.6	31.4	7.1
2-day cultured <i>P. carinii</i>	17	7.7	10.7	6.2	42.4	16
4-day cultured <i>P. carinii</i>	29.2	7.2	5.7	16.5	36.8	4.7

^a Average of two experiments.

cultures, which were inoculated with lungs from uninfected rats. Culture-derived *P. carinii* synthesize over four-fold more folates than do lung-derived organisms (Table 1). For both lung- and culture-derived organisms, > 60% of the [³H]pABA can be recovered as pABA-polyglutamates.

Since *P. falciparum* also incorporates exogenous [³H]pABA [14,15], we first carried out an analysis of the pteroylpolyglutamates synthesized by this organism. pABA-tetraglutamate represents approximately 80% of the pABA-glutamates (Table 2). Tetraglutamates are predominant, when parasites are incubated with pABA for only 6 h instead of 24, although representing only 45% of the total pteroylpolyglutamates (data not shown).

In contrast, both lung-derived and culture-derived *P. carinii* produce a more mixed population of polyglutamates with 31–42% pABA-pentaglutamate

and 17–30% monoglutamates (Fig. 1 and Table 2).

4. Discussion

The synthesis of pteroylpolyglutamates by *P. carinii* was previously reported by Comley et al. [10]. This study confirms that pteroylpolyglutamates are synthesized by this organisms, and that the predominant form are the pteroylpentaglutamates.

The identity of the major *P. carinii* folate as pteroylpentaglutamate can only be inferred, since pABA-glu₅ was not used as a standard. However, in six experiments, the predominant *P. carinii*-derived radioactive peak had retention times of 29.5–30.5 min, in between the retention times for pABA-glu₄ and pABA-glu₆, which were 27.2–28.4 min (*n* = 5)

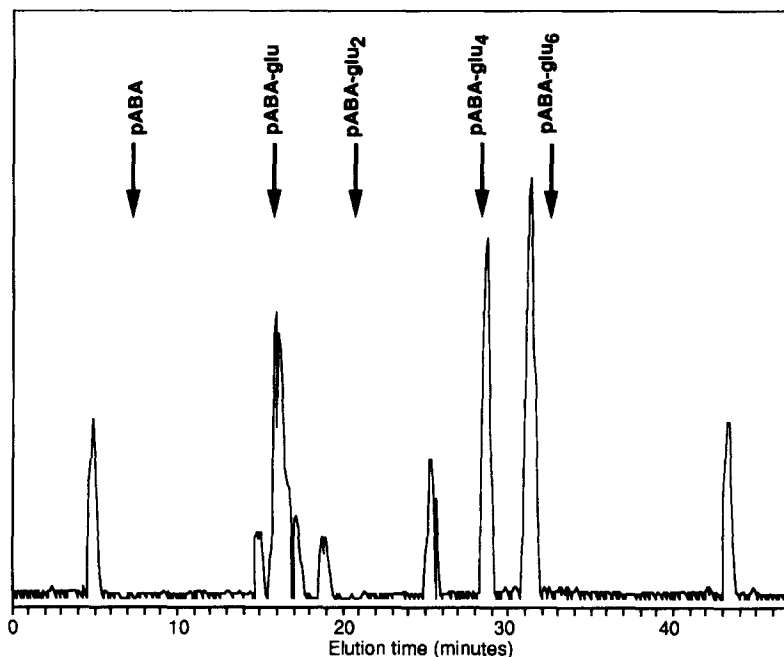


Fig. 1. High-performance liquid chromatography (HPLC) analysis of pABA-polyglutamates formed by lung-derived *P. carinii* after incubation with [³H]pABA. Pteroylpolyglutamates were converted into pABA-polyglutamates as described in the Materials and methods and then analysed on a Rainin Instrument HPLC (Woburn, MA) using a C18 hypersil 5 μ m column (4.6 \times 250 mm) (Phenomenex, Torrance, CA) by the method of Selhub [16], except that dithiothreitol was not used and a different gradient was used (Buffer B at 10% for 12 min, followed by a linear increase to 60% over the next 23 min). The radiolabelled eluate was monitored on a Flo-One radioactive detector (Radiomatic Instruments, Meriden, CT) and offset by 1.4 min to adjust for the time delay between this detector and the UV detector. Elution times of standards (monitored at 280 nm) are indicated by arrows.

and 31.0–32.2 min ($n = 5$), respectively. Thus, the *P. carinii* product elutes where pABA-glu₅ would be expected to elute.

In this study, *P. falciparum* was found to produce primarily pteroyltetraglutamates. Krungkrai and co-workers [17] have previously measured the polyglutamates of *P. falciparum* and found pentaglutamates to be the most common. There are three possible explanations for the different patterns observed. First, these investigators used the K1 strain, while FCR3 was used here, and there could be differences between strains. Second, Krungkrai and co-workers used HPLC to analyse pteroylpolyglutamates, while pABA-polyglutamates were analysed here. Third, there may have been differences in how folate-depleted the organisms were in the two studies; if so, *P. falciparum*, might resemble *Lactobacillus casei* whose polyglutamation status is dependent on the concentration of folate in the media (reviewed in [18]).

Culture-derived organisms take up [³H]pABA and produce pteroylpolyglutamates at 4 × higher specific activities than lung-derived organisms (Table 1). This is consistent with our previous observation that lung-derived organisms are contaminated with lung debris, and that cultivation of organisms in the presence of feeder cells removes this debris to yield purer organisms [5,6]. These data also confirm that *P. carinii* remains metabolically active when cultivated with feeder cells for at least 4 days.

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