Journal of Chromatography, 553 (1991) 205–210
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2119

Purine and pyrimidine compounds in murine peritoneal macrophages cultured *in vitro*

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ABSTRACT

Extracts of murine peritoneal macrophages were analysed by ion-pair reversed-phase high-performance liquid chromatography during incubation at 37°C *in vitro*. Four-step gradient elution was applied to an ODS column (250 × 4.6 mm I.D.) at a flow-rate of 1.3 ml/min, allowing the separation of hypoxanthine, inosine, guanosine, adenosine, IMP, CDP, AMP, GDP, UDP, ADP, CTP, GTP, UTP and ATP within 50 min. Samples of $0.4 \cdot 10^6$–$0.5 \cdot 10^6$ cells were washed twice with RPMI 1640 medium and extracted with perchloric acid. Nucleotide concentrations of murine peritoneal macrophages did not change during incubation for 4 days *in vitro*.

INTRODUCTION

Purine and pyrimidine compounds are of major importance for a multitude of cellular functions. Synthesis and catabolism of nucleotides are well understood and reviewed [1–3]. Extensive metabolic studies have been performed on liver, heart, and red blood cells. However, only a limited number of investigations have been made on nucleotide metabolism in white blood cells, although there are relationships between nucleotide metabolism and several specific functions of these cells, such as the following: phagocytosis is accompaied by a decline in ATP [4]; the activation of lymphocytes is promoted by ATP [5]; guanine nucleotides activate the O_2^--generating NADPH-dependent oxidase [6] and adenosine inhibits O_2^- generation [7]; an ecto-5′-nucleotidase cleaves external AMP to adenosine, which is then transported into the macrophage by a purine nucleoside carrier [8]; and the activity of this enzyme characterizes functionally different cell populations [9]. A genetic defect of adenosine deaminase disturbs lymphocyte proliferation and causes immunodeficiency [10].

A thorough study of the nucleotide profile in lymphocytes and macrophages has not been published. This work was aimed at the determination of a wide range of purine metabolites in macrophages by gradient ion-pair reversed-phase high-per-

0021-9673/91/$03.50

formance liquid chromatography (HPLC), allowing the separation of purine nucleotides, nucleosides and nucleobases in a single run.

EXPERIMENTAL

Macrophage culture

A 2-ml volume of 10% proteose peptone (Difco Labs., Detroit, MI, U.S.A.) was injected into the peritoneal cavities of male mice (age 2 months). After 4 days, 10 ml of RPMI 1640 (Flow Labs., Irvine, U.K.) were injected into the peritoneal cavity and the suspended macrophages were aspirated. The cells were washed twice with that medium. Then $10 \cdot 10^6$ cells were suspended in RPMI 1640 containing 10% foetal calf serum. The suspension was placed in plastic culture dishes of 35 mm diameter (Terumo, Tokyo, Japan). The cells were allowed to adhere at 37°C under 5% carbon dioxide during a 3-h incubation. The non-adhering cells were removed by rinsing twice with RPMI 1640. The incubation was continued and after 24 h the medium was exchanged [11]. At this time and at the fourth day of incubation aliquots were sampled for nucleotide analysis.

Sample extraction

Macrophages were scraped off, washed twice with RPMI medium and nucleotides were immediately extracted with 6% perchloric acid; after centrifugation, the supernatant was neutralized with potassium hydroxide. The samples were cleaned from insoluble potassium chlorate by centrifugation at 900 *g* for 3 min and in addition by percolation through a 0.2-μm membrane filter (Sartorius, Germany).

HPLC equipment

The HPLC system consisted of equipment from Waters Assoc. (Milford, MA, U.S.A.), with two Model 510 HPLC pumps, an automated gradient controller, a programmable multi-wavelength detector (peak identification by UV absorbance measurement at 254 and 280 nm), a Model 745 data module and Rheodyne Model 7125 injector with a 50-μl sample loop.

Chromatographic conditions

Buffer A [10 m*M* $NH_4H_2PO_4$ (Merck, Darmstadt, Germany) + 2 m*M* tetrabutylammonium phosphate (PIC Reagent A; Waters Assoc.)] and buffer B [buffer A containing 20% (v/v) acetonitrile (LiChrosolv, Merck)] were used. The flow-rate was 1.3 ml/min. A Supelcosil ODS column (250 × 4.6 mm I.D.) was applied.

The following gradient was used: 5 min isocratic with 100% buffer A, followed by a 12-min linear gradient up to 80% B, then 30 min isocratic with 80% B–20% A followed by a 2-min linear gradient to 100% B, returning in 5 min to 100% buffer A.

Quantification and peak identification

In perchloric acid extracts, recoveries of 81 ± 12% for ATP and 76 ± 14% for hypoxanthine were obtained. The recoveries for the other compounds were between these values. Different amounts of each standard compound were injected and the linearity of the calibration graph for the particular concentration range was checked (nucleosides, nucleobases, 50–500 pmol; nucleotides, 200–1500 pmol). Peak identi-

fication was performed by coelution of the macrophage extracts with known standards.

RESULTS AND DISCUSSION

Ion-pair reversed-phase separation

Ion-pair methods were used initially to enhance the selectivity in solvent extraction and later they were introduced to improve reversed-phase LC separations [12]. The ion-pair reversed-phase (IP-RP) mode is applied nowadays to separate bases and acids, pharmaceuticals, amino acids, peptides, proteins and nucleic acids and for chiral separations [12,13]. Nucleotides interact with cationic ion-pair reagents owing to their ionic properties and in this way a good retention of these compounds on C_{18} columns is possible. In the experiments performed here, tetrabutylammonium phosphate was chosen. The pH of the buffers was 5.8. Hypoxanthine, inosine, guanosine, adenosine, IMP, CDP, AMP, GDP, UDP, ADP, CTP, GTP, UTP and ATP eluted as shown in Fig. 1. c-AMP elutes after ATP (not shown in Fig. 1). Similar IP-RP separations of nucleotide standards have been reported [14].

Nucleotide concentrations in murine peritoneal macrophages

Murine peritoneal macrophages were cultivated for 4 days at 37°C. Perchloric acid extracts were analysed by the IP-RP gradient separation described at the first and fourth days. Fig. 2 shows the corresponding elution profile of an extract obtained after

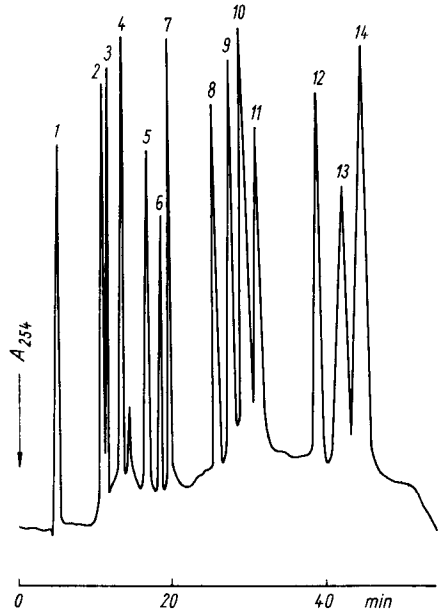

Fig. 1. IP-RP separation of nucleotide, nucleoside and nucleobase standards (400 pmol each). Detection at 254 nm. Column, Supelcosil ODS (250 × 4.6 mm I.D.); flow-rate, 1.3 ml/min. Peaks: 1 = hypoxanthine; 2 = inosine; 3 = guanosine; 4 = adenosine; 5 = IMP; 6 = CDP; 7 = AMP; 8 = GDP; 9 = UDP; 10 = ADP; 11 = CTP; 12 = GTP; 13 = UTP; 14 = ATP.

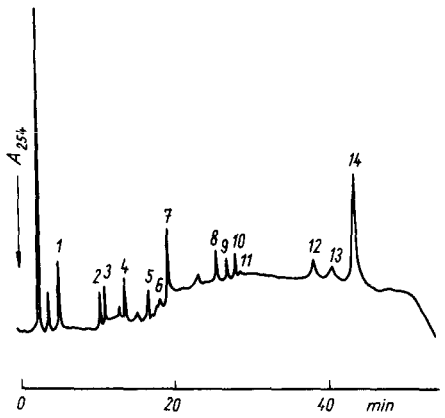

Fig. 2. Chromatographic elution profile of an extract of murine peritoneal macrophages after incubation *in vitro* for 4 days. $0.4 \cdot 10^6$ cells were used for the extraction.

in vitro cultivation for 4 days. Nucleotide concentrations were not significantly changed during incubation (Table I), indicating that the metabolic integrity during incubation was maintained. This is in accordance with data on unstimulated granulocytes cultivated for 3 days [15]. RPMI 1640 is known to be a good storage

TABLE I

NUCLEOTIDE CONCENTRATIONS IN EXTRACTS OF MURINE PERITONEAL MACROPHAGES

Values are given in pmol per 10^6 cells (mean ± S.E.M., $n = 5$). Macrophages harvested from the peritoneal cavity of 2-month-old mice treated with 10% proteose peptone were plated in culture dishes in RPMI 1640 medium with 10% foetal calf serum. Non-adhering cells were removed by rinsing with culture medium after incubation for 24 h. The incubation was performed at 37°C under 5% CO_2. Samples were scraped off from the dishes, washed twice by rinsing with RPMI medium and were immediately extracted with 6% perchloric acid.

Nucleotide	Time of cultivation	
	1 day	4 days
Hypoxanthine	11.3 ± 3.2	13.2 ± 5.4
Inosine	4.3 ± 0.7	5.1 ± 1.2
Guanosine	4.6 ± 0.5	4.9 ± 0.8
Adenosine	2.8 ± 0.9	3.1 ± 1.2
IMP	3.1 ± 1.1	3.0 ± 1.1
CDP	2.5 ± 1.6	2.0 ± 1.5
AMP	22.5 ± 6.5	23.5 ± 7.1
GDP	61 ± 12	62 ± 16
UDP	32 ± 18	33 ± 19
ADP	55 ± 21	51 ± 22
CTP	3.8 ± 0.9	3.0 ± 1.8
GTP	27 ± 9	26 ± 12
UTP	18 ± 8	18 ± 11
ATP	1398 ± 172	1314 ± 235

TABLE II

NUCLEOTIDE CONCENTRATIONS IN LYMPHOMA CELLS, LYMPHOCYTES, POLYMORPHONUCLEAR LEUCOCYTES AND MACROPHAGES

Values are given in pmol per 10^6 cells.

Nucleotide	Cells							
	Lymphoma L5178Y [3]	Lymphoma K 562 [17]	Lymphocytes			Lymphocytes (man) [19]	Polymorpho nuclear (rat) [20]	Peritoneal macrophage (mouse) (this work)
			Man [18]	Pig [18]	Rat [18]			
ATP	2600	557	855	1107	237	826	830	1398
ADP		73.5	302	475	95		131	55
AMP		54	43	43	70		28	22.5
Ado		10.6					4.3	2.8
GTP	700	169.5	182	205	80	200		27
GDP		50.5	53	67	32			61
Guo								4.6
UTP		126.7	142	96	53	120		18
UDP		12.4	20	15	24			32
IMP		162					46	31
Ino							9	4.3
Hyp		25					21	11.3
CTP			39	34	27	83		3.8
CDP			12	16	11			2.5

medium also for neutrophils [16]. During phagocytosis the ATP concentration decreases and the nucleotide metabolism deteriorates [4].

In Table II, concentrations of ribonucleotides in white blood cells are surveyed.

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