ASSAY OF THIOINOSINIC ACID, AN ACTIVE METABOLITE OF AZATHIOPRINE, IN HUMAN LYMPHOCYTES

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1 A specific assay for the measurement of thioinosinic acid, in human lymphocytes, has been developed with a sensitivity of 50 ng of thioinosinic acid per 5×10^6 lymphocytes.

2 Thioinosinic acid is precipitated from purified lymphocytes as the lanthanum salt. Acid hydrolysis results in the formation of 6-mercaptopurine which, when converted into its phenyl mercury derivative, can be easily extracted into toluene. Back-extraction of the toluene layer with 0.1N HCl regenerates 6-mercaptopurine which is then assayed fluorometrically.

3 Blood samples were taken from renal transplant recipients 3 h after an oral dose of 50 mg azathioprine. The results from 5 patients gave a range of 54 to 173 ng of thioinosinic acid per 5×10^6 lymphocytes, with a mean of 110 ng.

4 In an *in vitro* incubation of azathioprine, 1mM with fresh human blood, 160 and 180 ng of thioinosinic acid per 5×10^6 lymphocytes was formed after 0.5 h and 5 h respectively.

5 The assay is suitable for the study of the kinetics of thioinosinic acid formation in lymphocytes of patients with kidney transplants. It could also prove useful in the study of thioinosinic acid formation in leukaemia patients undergoing 6-mercaptopurine treatment.

Introduction

Azathioprine the (1-methyl-4-nitro-5-imidazolyl) derivative of 6-mercaptopurine, is one of the most widely used agents in human immunosuppressive therapy. In spite of several years of clinical use its precise mechanism of immunosuppression has so far eluded definition, although many loci of action are known (Hitchings & Elion, 1972).

Azathioprine and 6-mercaptopurine (6-MP) clearly suppress cell-borne immunity, as shown by their effects on prolonging the survival of renal allografts (Calne, 1967; Hamburger, Crosnier, Dormont & Bach, 1972). However, azathioprine is inconsistent in its effect as an immunosuppressive and anti-rejection drug. Dosage is monitored by observing the depression of the peripheral white cell count, which is in itself a toxic effect of the drug.

A number of studies have shown that the biological and biochemical effects of azathioprine are attributable to its cleavage to 6-MP and the subsequent anabolic transformation of the free purine to its ribonucleotide, thioinosinic acid, and other thionucleotides (Brockman, 1963; Elion & Hitchings, 1965; Elion, 1967). The conversion of 6-MP into thioinosinic acid by hypoxanthine guanine phosphoribosyl transferase (HGPRT) is considered essential for the inhibitory action of azathioprine by the finding that, in children with the Lesch-Nyhan syndrome, lymphocytes stimulated by

phytohaemagglutinin are unaffected by 6-MP (Allison, Hovi, Watts & Webster, 1975). Moreover, azathioprine administration has no inhibitory effect on purine synthesis and does not cause haematological toxicity in these patients.

In man, the cleavage of azathioprine, to form 6-MP, occurs very rapidly in the blood. (Maddocks, 1979a). Whole blood is required for this cleavage, plasma alone is unable to metabolize azathioprine. It is postulated that the erythrocytes are the main site of the splitting of azathioprine to 6-MP by a non-enzymatic thiol cleavage mediated by sulphhydryl compounds, (Miranda, De Beacham, Creagh & Elion, 1973; Maddocks, 1979b). Further metabolism of 6-MP, to its active nucleotide metabolites, is intracellular. Since nucleotides do not transverse cell membranes without dephosphorylation, they do not circulate in plasma. To date, it has not been possible to devise methods sufficiently sensitive to measure them in the cells of patients under treatment (Elion & Hitchings, 1975).

Nucleotide metabolites are central to the action of azathioprine, thus it is possible that immunosuppressive therapy may be optimized, and systemic toxicity avoided, by measuring their intracellular concentrations. This paper describes the development of a sensitive method for the isolation and assay of thioinosinic acid from lymphocytes.

Thus, for the first time, thionucleotide concentrations in cells can be investigated clinically on a routine basis.

Methods

Principle of assay

Thioinosinic acid (6-mercaptopurine riboside-5'-phosphate) is a nucleotide with a 6-substituted purine ring, a structural analogue of inosinic acid (inosine monophosphate). In this assay the phosphate and -SH groups of thioinosinic acid are utilized in its extraction from lymphocytes.

After the rupture of the lymphocyte cell membrane, the cell contents are liberated. At this stage the phosphate group of thioinosinic acid is used to precipitate the nucleotide as the lanthanum salt (Bakay, Telfer & Nyhan, 1969). Thus, thioinosinic acid is removed from the lymphocyte along with other nucleotides present in the solution. The precipitated thionucleotide is resuspended in sulphuric acid. The parent thiopurine, 6-mercaptopurine, is produced from the thionucleotide by acid hydrolysis.

6-mercaptopurine is removed from the cooled solution using a novel extraction procedure which involves conversion of 6-MP into a phenyl mercury derivative (Maddocks, 1979a). The phenyl mercury derivative is much less polar than the parent drug and is readily extracted into toluene. The 6-MP is recovered by back extracting the organic layer with 0.1M HCl. The resulting aqueous solution of 6-MP is then assayed fluorometrically after oxidizing the thiopurine to the purine-6-sulphonate.

Experimental instrumentation

An Aminco-Bowman spectrophotofluorimeter with an X-Y recorder (Bryans) was employed for fluorescence measurements. The sensitivity of the spectrophotofluorimeter was increased by using an off axis ellipsoidal condensing system, which enables increased resolution of the excitation spectrum, and by inserting stainless steel mirrors into the cell compartment, which increase the light path through the sample solution. All measurements were made with excitation and emission slit widths of 0.5 mm and 1.0 mm respectively. All other slits were at maximum width. Samples were measured in fused quartz cuvettes of 170 μ l maximum volume, internal diameter 3 mm square and height 20 mm.

All pH measurements were taken on a Phillips PW 9409 digital pH meter.

Reagents

Azathioprine and 6-MP were gifts from Burroughs Wellcome and Company. 6-mercaptopurine riboside-5'-phosphate (thioinosinic acid) was obtained as the barium salt from Sigma. All other thiopurine derivatives were also obtained from Sigma.

A solution of phenyl mercury acetate (BDH) 89mm, was prepared by dissolving the compound in boiling distilled deionized water and kept in a dark bottle. A fresh solution was prepared weekly.

Toluene (BDH) was washed, with 1M NaOH and 0.5M H_2SO_4 and redistilled. The fraction boiling between 108 and 110°C was collected. Before use 170mm amyl alcohol (AnalaR, BDH) was added to the toluene.

Sodium metabisulphite (BDH), 21 mm, was prepared daily. All other reagents were BDH AnalaR grade.

Volumes of reagents, up to 5 ml, were delivered using Gilson automatic pipettes. For volumes over 5 ml Oxford pipettors were used.

Buffers and stock solutions

A 0.1 M Tris (hydroxymethyl) aminomethane buffer was prepared and adjusted to pH 8.0 with 1M HCl. A 0.1M solution of potassium dihydrogen orthophosphate was adjusted to pH 8.0 with 1M KOH. Before use 7 volumes of Tris buffer were mixed with 1 volume of phosphate buffer.

Stock solutions of both 6-MP and thioinosinic acid, 100 μ g/ml, were prepared in 0.1M HCl. The thioinosinic acid stock solution was prepared by dissolving 10 mg in 10 ml of 0.1M NaOH. Water (50 ml) was then added followed by 11 ml of 1M HCl. The volume was then made up to 100 ml. The stock solution of 6-MP was prepared similarly (Maddocks, 1979). These stock solutions were kept at 4°C in dark stoppered bottles. No deterioration was observed when the stock solution was kept for a period of 3 months under these conditions. All dilutions of the stock standards were made daily in 0.1M HCl.

Glassware

All glassware used was treated with nitric acid to remove any fluorescent material. Overnight soaking in 30% nitric acid was sufficient for reagent bottles and most test-tubes. However, tubes used in the acid hydrolysis step were soaked in a 5% solution of Decon 90 overnight, rinsed in tap water and deionized distilled water. These tubes were then placed in a concentrated nitric acid bath and the acid heated until fuming for 30 min and, after cooling, rinsed in deionized distilled water.

For the preparation of lymphocytes all glassware

used was silicone-coated using a 2% solution of dimethyldichlorosilane in 1,1,1-trichloroethane. The coated glassware was dried in the oven for one hour and then rinsed in deionized distilled water before use.

Extraction and determination of thioinosinic acid levels in lymphocytes

Preparation of lymphocytes Control lymphocytes were obtained from the venous blood of normal healthy volunteers. Aliquots (10 ml) of blood were also obtained from patients undergoing azathioprine therapy. The lymphocytes were prepared from whole blood by a modification of the method originally described by Boyum (1968).

Whole blood (10 ml) was diluted with 14 ml phosphate buffered saline (PBS) and 8 ml aliquots were carefully layered onto 3 ml Histopaque in a 16 × 100 mm conical bottom test-tube. The tubes were centrifuged as 400 g, 20°C for exactly 30 min. The plasma was removed to within 0.5 ml of the lymphocyte band. The lymphocytes were carefully removed from the interface and washed twice in 5 volumes of PBS. After each wash the tubes were centrifuged at 100 g, 20°C for 10 min to reduce platelet contamination. Viability of the lymphocytes was determined by trypan blue staining and cell-counting on duplicate samples. Sterilin disposable test-tubes were used throughout this preparation.

The lymphocytes obtained from 10 ml of blood were adjusted to a concentration of 1.0×10^7 /ml in PBS. The prepared lymphocytes, in 0.5 aliquots were frozen in liquid nitrogen and placed in a freezer at -20°C. Patient samples were stored until a sufficient number were obtained to assay.

Precipitation of thioinosinic acid The conditions necessary for the quantitative precipitation of nucleotides with lanthanum chloride were described by Bakay *et al.* (1969). The method below is a slight modification of that.

The lymphocytes were removed from the freezer and allowed to thaw. Trypan blue staining showed that none of the lymphocytes was viable. The examination of a sample under a microscope showed that the freeze-thaw procedure was sufficient to rupture the lymphocyte membrane.

A 0.5 ml aliquot of lymphocytes was placed in an acid-washed, 10 ml graduated test-tube and the volume was made up to 2 ml with 0.1M HCl. At this stage thioinosinic acid standards were placed in the assay. A stock solution of thioinosinic acid in 0.1M HCl was diluted in 0.1 M HCl and 1 ml of the appropriate dilution added to 0.5 ml of control lymphocytes. Again the volume was made up to 2 ml with 0.1M HCl.

To a sample volume of 2 ml was added 7 ml of a 7 : 1 mixture of 0.1M Tris buffer, pH 8.0, and 0.1M phosphate buffer, pH 8.0. After gentle mixing 1 ml 0.5M lanthanum chloride was added, the tubes mixed and placed on ice for 10 min. At this point the pH inside the test-tube was about 6.0 and a dense white precipitate of lanthanum phosphate was immediately visible, this caused the co-precipitation of the nucleotides contained in the sample. Because the lanthanum phosphate precipitate is a heavy substance, it sedimented quantitatively (Bakay *et al.*, 1969) at a relatively low centrifugal force. A firmly packed white pellet was produced by centrifuging at 2,700g, 10°C, for 20 min in an MSE Coolspin centrifuge, the supernatant was discarded.

Acid hydrolysis The precipitated thiopurine was resuspended in 0.5 ml 1.5M H₂SO₄ and the volumes adjusted to 1.5 ml with water. The test-tubes were then heated for 1 h at 100°C in a Dri-Block (Techne Ltd). These conditions result in the complete hydrolysis of the nucleotide to the parent purine.

Extraction of 6-MP At this stage 6-MP standards were included with the cooled tubes. To the sample solution 0.5 ml 5M NaOH was added and the volume was made up to 2 ml with water. In the lanthanum containing tubes a white precipitate of lanthanum hydroxide formed which in no way interfered with the extraction of 6-MP. To the alkaline solution of 6-MP 1 ml of 89 mM phenyl mercury acetate (PMA) and 8 ml toluene, containing 170 mM amyl alcohol, was added and the stoppered tubes shaken on a mechanical shaker, at approximately 280 cycles min⁻¹, for 10 min. The tubes were then centrifuged for 3 min at 900g in an MSE bench centrifuge, and 7 ml toluene was transferred to a graduated conical test-tube. The toluene, containing the 6-MP phenyl mercury complex, was back extracted with 0.3 ml 0.1M HCl. The phenyl mercury chloride remained in the toluene whilst the free 6-MP passes into the aqueous phase. The tubes were shaken and centrifuged as before. After centrifugation almost all the toluene was aspirated leaving only a small amount at the interface to the aqueous layer.

Oxidation The aqueous phase, containing 6-MP, was oxidized by the addition of 50 µl 2 mM potassium chromate. After 2 min the excess chromate ions were removed by the addition of 50 µl 21 mM sodium metabisulphite. Sodium hydroxide (5M, 10 µl) was then added to each tube before centrifugation at 900 g for 3 min. After each addition the tubes were mixed. In an alkaline solution the maximum fluorescence of the resulting purine-6-sulphonate was obtained and the relative fluorescence of the samples and standards were measured at 398 nm with an excitation wavelength of 288 nm.

Figure 1 Effect of pH on the precipitation of thioinosinic acid.

Figure 2 Standard curve for the concentration of thioinosinic acid (ng) in 5×10^6 lymphocytes. The interassay mean deviation = 8%.

Results

Thioinosinic acid assay

The pH of the solution is the most critical factor for the quantitative precipitation of nucleotides (Bakay *et al.*, 1969) so the precipitation of thioinosinic acid was studied as a function of pH. To a series of tubes, containing 0.5 ml of lysed lymphocytes, was added 1 ml 500 ng/ml thioinosinic acid, prepared from the stock solution in deionized distilled water. HCl (1M) was then added in amounts varying from 0–500 μ l. The volume was adjusted to 2 ml with water and the precipitation continued as before. After the addition of lanthanum chloride the pH of each test-tube was measured using a micro-electrode. Figure 1 shows the precipitation of thioinosinic acid as a function of pH. Optimal precipitation was achieved in solutions of pH 3.5–7.0 with a phosphate carrier content of 20 mM and a lanthanum content of 50 mM. Under these conditions 97% ($\pm 2\%$) of added thioinosinic acid was precipitated over a range of 50–2,000 ng/ml.

The acid hydrolysis of thioinosinic acid solutions, both aqueous and those containing 5×10^6 lysed lymphocytes, were compared against equimolar 6-MP solutions taken through the hydrolysis step before PMA extraction. 100% conversion of the purine was observed after 1 h at 100°C.

There was a linear relationship between fluorescence and the concentration of thioinosinic acid over the range of 50–2,000 ng/ 5×10^6 lymphocytes. 50 ng was designated the lower limit of the standard curve (Figure 2), which was defined as the reading twice that of the blank.

The reproducibility of the assay was determined at the thioinosinic acid concentrations of 50, 100, 200, 300, 500, 1000 and 2000 ng/ 5×10^6 lymphocytes in six separate assays, each in duplicate. In total 12 samples at each concentration were measured. The percentage deviation of each result from the mean fluorescence intensity for each concentration was calculated. The interassay mean deviation, for the whole group, was 8%. The intra-assay mean deviation was determined by replicate analysis of 12 samples of 500 ng thioinosinic acid per 5×10^6 lymphocytes. The mean deviation was 3%. The extraction efficiency of the 6-MP formed from thioinosinic acid was 63%. This was the same as the extraction efficiency of standard 6-MP.

Specificity

It was initially required to confirm that the 6-MP produced from the thioinosinic acid, extracted from control lymphocytes, had the same excitation and

emission maxima as the standard 6-MP solution. Thioinosinic acid was added to and subsequently isolated from lymphocytes. The spectral characteristics of the isolated thiopurine were identical to those observed in the standard, unextracted, oxidized 6-MP. Thus, no artifacts were produced by the isolation procedure.

The addition of physiological sulphhydryl compounds (cysteine and glutathione) and purines (adenine, hypoxanthine, xanthine and uric acid) to aqueous solutions of both thioinosinic acid and thioinosinic acid added to lysed lymphocytes, did not interfere with the assay. Likewise, the addition of the azathioprine metabolites 6-methylmercaptapurine and 6-methylmercaptapurine riboside, at any stage during the extraction procedure, did not interfere with the assay. To test the specificity of the initial precipitation reaction a vast excess of 6-MP (10 $\mu\text{g/ml}$) was added to lysed control lymphocytes in the initial precipitation reaction. The firmly packed pellet produced by centrifugation contained no 6-MP. An excitation-emission scan performed at the end of the assay corresponded exactly to the scan performed on the blank tube, which initially contained lysed lymphocytes in 0.1M HCl.

Thin layer chromatography (t.l.c.) on prepared cellulose layers (Eastman) was carried out using two solvent systems, 0.1M HCl and isopropanol:methanol:water:ammonia (60:20:20:1). Firstly, the t.l.c. properties of the extracted drug, obtained from the addition of thioinosinic acid to lysed lymphocytes, were compared to those of standard 6-MP. The chromatograms were visualized by immersion in liquid nitrogen and irradiating them with u.v. light (Maddocks & Davidson, 1975). The spots visualized had the same R_F values as the 6-MP standards (0.49 and 0.4 in the acidic and basic systems respectively) and no interfering substances were present. The spots were marked with pencil and scraped into conical test-tubes. HCl (0.1M, 300 μl) was added and the thiopurine eluted by agitation of the cellulose particles. The tubes were centrifuged at 900 g. The resulting clear supernatant was removed and oxidized as usual. The recovery of the applied standard 6-MP solution was 50%. The recovery of the extracted 6-MP, obtained from the addition of thioinosinic acid to lysed control lymphocytes, was also 50% of the value originally measured by the assay procedure.

An *in vitro* incubation of fresh human blood with 1mM azathioprine was performed. Blood (40ml), containing glucose (25 mM) and phosphate (50 mM), was incubated with azathioprine at a final concentration of 1mM. The sample, final volume 50 ml, was placed in a conical silicone-coated flask in a shaking water bath and incubated at 37°C in an atmosphere of 95% O_2 /5% CO_2 . After 0.5 h and 5 h, 25 ml samples were removed and the lymphocytes

isolated. The cells were counted and trypan blue staining showed > 95% viability.

The thioinosinic acid assay was then performed on 0.5 ml aliquots of the lymphocytes containing 5×10^6 cells. Quadruplicate samples at each time interval were processed. This enabled duplicate samples to be oxidized and the excitation-emission scans recorded. Duplicate samples of unoxidized extracted 6-MP were also applied to t.l.c. plates with 6-MP standards and 6-MP extracted standards, which had been obtained initially from the addition of thioinosinic acid to lysed control lymphocytes, and run in two different solvent systems.

The excitation-emission scans produced from both the 0.5 h and 5 h samples could be directly superimposed on those of the oxidized 6-MP standard, showing an excitation maxima at 288 nm and emission maxima at 398 nm. The results of five experiments gave 160 ng thioinosinic acid/ 5×10^6 lymphocytes after 0.5 h incubation and 181 ng thioinosinic acid/ 5×10^6 lymphocytes after 5 h. The mean deviation, expressed as a percentage of the mean, was 7.8% and 6% respectively. The developed chromatograms showed spots in the same region as the 6-MP standards, no interfering substances were present. The spots were eluted with 0.1M HCl as described previously. 50% of the drug applied to the t.l.c. plate was recovered by this elution procedure. The recovery was the same for all the 6-MP spots.

A 20 ml venous blood sample was taken from a renal transplant patient, 3 h after an oral dose of 50 mg of azathioprine, and the lymphocytes isolated. The thioinosinic acid assay was performed on triplicate samples of lysed lymphocytes. Thioinosinic acid standards were included. Excitation-emission scans were performed on the oxidized product and the maxima coincided with those of the oxidized standard 6-MP. The patient's lymphocytes contained 104 ng of thioinosinic acid/ 5×10^6 lymphocytes. The third sample was subjected to t.l.c. analysis in two different solvent systems, as before. The R_F value of the spot visualized corresponded to that of the 6-MP standard. No interfering substances were present. Elution of all the spots, from the t.l.c. plate, gave a 50% recovery in every case. Similarly, blood samples were taken from renal transplant recipients 3 h after an oral dose of 50 ng azathioprine. The results from five patients gave a range of 54 to 173 ng thioinosinic acid/ 5×10^6 lymphocytes, with a mean of 110 ng.

Discussion

Thioinosinic acid is recognized as a major metabolite of both azathioprine and 6-mercaptopurine and, as such, has been isolated and identified. Separation and detection has been done by such techniques as ion-exchange chromatography and chemical analysis

(Paterson, 1959) and by thin-layer chromatography (Bennet & Allen, 1971), DEAE-Sephadex chromatography (Caldwell, 1969) and HPLC (Nelson, Bugge, Kransny & Zimmerman, 1973) of radioactive metabolites. However, these previous attempts, lacking sensitivity, and in some cases, specificity, are of little value in studying the kinetics of thioinosinic acid, especially in human lymphocytes.

The assay procedure reported may be easily performed in any laboratory possessing an adequate spectrophotofluorimeter. Using a standard, unmodified, Aminco Bowman spectrophotofluorimeter the lower limit of the standard curve was 70 ng of thioinosinic acid, per 5×10^6 lymphocytes, compared to 50 ng using a modified spectrophotofluorimeter. The thioinosinic acid level in the

lymphocytes contained in a sample of peripheral blood may be determined within a day. Conversely, lymphocyte samples may be safely stored at -20°C for weeks. The accumulated samples may then be assayed simultaneously in large batches. Not only can kinetic studies be done on patients taking therapeutic doses of azathioprine but, coupled with the assays for azathioprine and 6-mercaptopurine (Maddocks, 1979) already available in this laboratory it is now possible to study in depth the pharmacokinetics of this important immunosuppressive drug.

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