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Assay of 6-thioguanine nucleotide, a major metabolite of azathioprine, 6-mercaptopurine and 6-thioguanine, in human red blood cells

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An assay for 6-thioguanine (6-TG) nucleotide, a major metabolite of the cytotoxic drugs azathioprine, 6-mercaptopurine and 6-thioguanine in human red blood cells (RBCs) has been developed. The metabolite was not detected in RBCs when azathioprine or 6-mercaptopurine was incubated with whole blood in-vitro. The assay for intracellular 6-TG nucleotide is specific and requires 8×10^8 RBCs (100 μl packed cells) for which the limit of sensitivity is 30 pmole 6-TG nucleotide. Pre-dose blood samples were obtained, 12 h after the last azathioprine dose, from 10 renal transplant recipients with stable functioning cadaver grafts on a total daily dose of 150 mg azathioprine. The mean 6-TG nucleotide concentration was 171 pmole/8×10^8 RBCs (s.d. = 84). The assay is also suitable for use in measuring 6-TG nucleotide in the RBCs of leukaemic children undergoing 6-mercaptopurine treatment.

Whilst measuring lymphocyte concentrations of 6-thioinosinic acid, an active metabolite of azathioprine, in kidney transplant recipients, by the method of Fletcher & Maddocks (1980), we observed that the main azathioprine metabolite in red blood cells (RBCs) was not 6-thioinosinic acid (6-mercaptopurine riboside 5′-phosphate) but a thionucleotide that released thioguanine on acid hydrolysis. This was not detected in RBCs when azathioprine or 6-mercaptopurine was incubated with whole blood in-vitro. Several studies point to the liver as a source of purines for RBC and other tissues, such as bone marrow cells, that require performed purines (Lajtha & Vane 1958; Pritchard et al 1975). RBCs also have a role in transporting purines to other organs (Henderson & Le Page 1959; Konishi & Ichihara 1979). We have developed an assay for intracellular thioguanine nucleotide and report studies on its concentration in the RBCs of renal transplant recipients taking azathioprine and in the RBCs of leukaemic patients treated with 6-mercaptopurine.

MATERIALS AND METHODS

Thioguanine (6-TG) nucleotide was extracted from RBCs by a modification of the thioinosinic acid assay of Fletcher & Maddocks (1980) and hydrolysed to the parent purine, 6-thioguanine, which was assayed fluorometrically (Finkel 1975; Dooley & Maddocks 1980). Toluene (glass distilled) was from Rathburn Chemicals and amyl alcohol (BDH, Analar) was added, to a final concentration of 170 mM, before use. Deionized glass distilled water was used throughout. The specifications of the instruments and glassware in the assay, and the preparation of all other reagents have been previously described (Fletcher & Maddocks 1980; Dooley & Maddocks 1980). During the development stages of the assay, when 6-thioguanosine 5′-phosphate standard was unavailable, RBCs containing 6-TG nucleotide, from a renal transplant recipient taking azathioprine, were used.

RBCs were obtained from whole blood which was centrifuged at 400 *g*, 20 °C for 10 min. The plasma and buffy coat were removed before the cells were washed twice in Hanks balanced salt solution. After each wash the cells were centrifuged at 150 g 20 °C for 10 min. The RBCs were counted (1:200 dilution in Hayem's fluid) and stored, at a concentration of approximately 8×10^8 cells per 100 μl at −20 °C.

For assay the RBCs were sonicated for 4 min, the thawed cells mixed well, 100 μl removed and added to 1 ml 0·1 M HCl in an acid washed, graduated test-tube. The volume was then made up to 2 ml with water. Tubes containing RBCs similarly processed but obtained from the venous blood of normal healthy volunteers were used as controls.

To the 2 ml sample was added 7 ml of a mixture of 0·1 M Tris/HCl buffer, pH 8·0, and 0·1 M phosphate buffer, pH 8·0 (7:1). This was mixed and then 1 ml of 0·5 M lanthanum chloride was added to precipitate the thionucleotides (Fletcher & Maddocks 1980). After thorough mixing the contents of the tubes were

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placed on ice for 10 min, the pH of the suspension being 6·7 (±0·3, n = 20). The mixture was then centrifuged at 2000 *g*, 10 °C for 10 min, the supernatant discarded and the precipitated 6-TG nucleotide resuspended in 0·5 ml 1·5 M H_2SO_4. After the volume had been adjusted to 1·5 ml with water the tubes were heated for 1 h at 100 °C in a Dri-Block (Techne Ltd), resulting in the complete hydrolysis of the nucleotide to the parent purine. At this point 6-TG standards were added to the control tubes which had been similarly treated and cooled. To each tube was then added 0·1 ml 10 mM dithiothreitol, 0·5 ml 5 M NaOH and water to a volume of 2 ml followed by 2 ml of 89 mM phenylmercuric acetate and 8 ml toluene. The tubes were then shaken for 10 min, ultrasonicated for 10 min and centrifuged at 900 *g* for 5 min. The toluene, 7 ml, was transferred to graduated conical test-tubes and back extracted with 0·5 ml 0·025 M HCl and, after shaking and centrifuging as before, most of the toluene was aspirated leaving only a small amount at the interface to the aqueous layer.

The aqueous solution, which contained the 6-TG, was oxidized at room temperature (20 °C) with 50 µl 2 mM potassium chromate and, after 2 min, the excess chromate ions were removed with 50 µl 21 mM sodium metabisulphate. The solution was then buffered to pH 10·1 with 1 ml of pH 10·45 sodium borate buffer and the relative fluorescence intensity measured at 410 nm exciting at 330 nm. The 6-TG nucleotide content of the RBCs was expressed as pmole of 6-TG produced after acid hydrolysis of the nucleotide present in 8×10^8 RBCs (pmol 6-TG nucleotide/8×10^8 RBCs).

For the study of the lanthanum precipitation of 6-TG nucleotide as a function of pH, a sample of RBCs from a renal transplant recipient receiving azathioprine was used. To a series of tubes containing 1·5 ml of water plus 100 µl of RBCs 1 M HCl was added in amounts varying from 0–200 µl. The volume was adjusted to 2 ml with water and the assay continued as before. After the addition of $LaCl_3$ the pH of each test-tube was measured using a microelectrode.

RESULTS

Precipitation of 6-TG nucleotide. The effect of pH on the recovery of 6-TG nucleotide from RBCs was studied and it was found that optimal, measurable, precipitation was achieved in solutions of pH 2·6–7·5. The thionucleotides from a renal transplant recipient's RBCs were precipitated at pH 6·7 and assayed, they contained 840 pmole 6-TG nucleotide/8×10^8 RBCs. These RBCs were diluted with control RBCs to give a range of 30–840 pmole 6-TG nucleotide/8×10^8 RBCs. Over this range 96% (±3%) of the 6-TG nucleotide contained within the RBCs was recovered.

Stability. (a) 6-TG in heating step. 6-TG standards, 30–3000 pmole, were added to tubes containing control red cells both before and after the acid hydrolysis step. No detectable loss of 6-TG was observed after 1 h at 100 °C in 0·5 M H_2SO_4. (b) Samples stored at −20 °C. When stored at −20 °C the RBC 6-TG nucleotide showed no detectable deterioration for 6 months.

6-TG nucleotide assay. There was a linear relationship between fluorescence and the concentration of 6-TG extracted from control RBCs over the range 30–3000 pmole/8×10^8 RBCs. The reproducibility of the extraction of 6-TG added to lanthanum precipitated, heated, control RBCs was determined in six separate duplicate assays over the range 30–3000 pmole 6-TG/8×10^8 RBCs. The extraction efficiency was 78·8% (s.d. = 6·4, n = 12). This was the same as the extraction efficiency of standard 6-TG added to control RBCs.

Whilst using the assay on a routine basis we observed a steady decrease in the extraction efficiency. This we traced to the purity of the water used in the assay. Deionized distilled water with a conductivity ≤4 mmhos results in a decreased extraction efficiency of 45–55%. The addition of dithiothreitol before the formation of the thioguanine phenyl mercury derivative was found to restore the extraction efficiency to 79%. The addition of dithiothreitol produced no apparent interference with the eventual formation of the phenyl mercury derivative.

Specificity of extraction. Thin layer chromatography (t.l.c.) on prepared cellose layers (Eastman) using two solvent systems (0·1 M HCl and isopropanol – methanol – water – ammonia, 60:20:20:1) allowed t.l.c. properties of the purine obtained from the purine nucleotide from patients RBCs to be compared with those of both standard 6-TG added to, and extracted from, control RBCs and standard 6-TG in 0·025 M HCl. The chromatograms were visualized by immersion in liquid nitrogen and irradiating with u.v. light (Maddocks & Davidson 1975). The spots visualized had the same R_F-value as the 6-TG standard (0·3 and 0·25 in the acidic and basic systems respectively at 4 °C). The green areas were marked, transfered into conical test-tubes and the material eluted with 500 µl 0·025 M HCl by agitation of the cellulose particles. After centrifuga-

tion, 900 *g*, 10 min, the resulting clear supernatant was oxidized and fluorescence scans obtained. The recovery of the applied standard 6-TG was 45%, as was the recovery of extracted standard 6-TG and the 6-TG extracted from lanthanum precipitated, heated RBCs of known thionucleotide content from patients. All three samples gave a typical 6-TG fluorescence scan.

Identity of 6-TG nucleotide. The t.l.c. properties of RBC 6-TG nucleotide from patients were compared against thioguanosine 5′-phosphate (thioguanosine 5′-diphosphate or triphosphate for comparison were not available). RBCs from patients (500 µl) were diluted 1:1 with water, as were control RBCs. The following standards (100 µl, 200 µM) were added to control cells: either (a) 6-thioguanosine 5′-phosphate, (b) 6-TG, (c) a 1:1 (v/v) mixture of 6-thioguanosine 5′-phosphate and 6-TG, (d) 6-thioguanosine, (e) 6-thioinosinic acid or (f) water. Extracts for t.l.c. were obtained by centrifuging the diluted RBCs in Centriflo ultrafiltration membrane cones (CF 25, Amicon). Centrifuging at 600 *g*, for 20 min, at 10 °C was sufficient to produce 300 µl of clear extract, any further centrifugation resulted in contamination of the sample by haemoglobin. T.l.c. was as detailed above using 50 µl of the extract in each of two solvent systems, 0·1 M HCl and 5% KH_2PO_4. The chromatograms were visualized as before, the results are in Table 1.

The 1:1 mixture of 6-thioguanosine 5′-phosphate and 6-TG added to RBCs was easily separated in each of the two solvent systems, but the standard 6-thioguanosine 5′-phosphate was contaminated with a small amount of 6-TG. The extract of RBCs from patients gave two spots one of which had the same R_F value as 6-thioguanosine 5′-phosphate, but the second which had a higher R_F value (0·54 in 5% KH_2PO_4; 0·65 in 0·1 M HCl) could not be identified. Acid hydrolysis of the ultrafiltration extract produced thioguanine, therefore the second spot could represent the diphosphate and/or triphosphate of 6-thioguanosine. No 6-TG was detected in the unhydrolysed extract.

In-vitro incubations. Fresh venous blood from normal healthy volunteers supplemented with glucose (25 mM) and phosphate (30 mM), was incubated in-vitro for 2 h at 37 °C in an atmosphere of 95% O_2/5% CO_2 with 10 µM azathioprine, 6-mercaptopurine or 6-TG. The RBCs were prepared as previously described and 100 µl amounts of the packed RBCs were assayed, in duplicate, for thioinosinic acid, 6-mercaptopurine, 6-TG nucleotide and 6-TG. Mercaptopurine was assayed as described in the thioinosinic acid assay of Fletcher & Maddocks (1980), 100 µl 10 mM dithiothreitol was added before the formation of the 6-mercaptopurine phenyl mercury derivative. The results are in Table 2. The azathioprine and 6-mercaptopurine incubations with RBC produced 6-thioinosinic acid (6-mercaptopurine riboside 5′-phosphate) only, no 6-TG nucleotide, 6-TG or free 6-mercaptopurine could be detected. The 6-TG incubation produced 6-TG nucleotide only, no free 6-TG or 6-thioinosinic acid could be detected, (lower limit of thioinosinic acid assay = 100 pmole/8 × 10^8 RBCs).

Table 1. The t.l.c. properties of RBC 6-TG nucleotide.

	Long wave u.v., R_F at 4 °C	
	5% KH_2PO_4	0·1 M HCl
TGMP[1] aqueous standard	1. Green 0·4	1. Green 0·49
	2. Green 0·1*	2. Green 0·11*
6-TG[2] aqueous standard	Green 0·1	Green 0·11
TGMP + 6-TG aqueous	1. Green 0·4	1. Green 0·49
standard	2. Green 0·1	2. Green 0·11
Patients' extract	1. Green 0·54	1. Green 0·65
	2. Green 0·34	2. Green 0·45
TGMP extract	1. Green 0·35	1. Green 0·46
	2. Green 0·09*	2. Green 0·1*
6-TG extract	Green 0·09	Green 0·09
TGMP + 6-TG extract	1. Green 0·21	1. Green 0·35
	2. Green 0·09	2. Green 0·09
Thioguanosine extract	Green 0·21	Green 0·38
Thioinosinic acid extract	Green 0·58	Blue 0·66
	short wave u.v., R_F at 4 °C	
All samples extracted from RBCs	quenching 0·81	quenching 0·81

1. 6-thioguanosine 5′ monophosphate.
2. 6-thioguanine.
* Spot just visible.

Table 2. Incubation of whole blood with 10 µM thiopurine—thionucleotide produced.

Purine (10 µM)	Time (h)	nmol thioinosinic acid/8 × 10^8 RBCs	nmol 6-TG nucleotide/ 8 × 10^8 RBCs
Azathioprine	2	1·92	none
	4	1·85	none
	6	2·01	none
6-Mercaptopurine	2	1·86	none
6-Thioguanine	2	none	1·42

Clinical applications. Ten renal transplant recipients with stable functioning cadaver grafts who had been taking 150 mg azathioprine daily for at least 6 months, had pre-dose blood samples: taken 12 h after the previous azathioprine dose. The mean 6-TG nucleotide level was 171 pmole/8 × 10^8 RBCs (s.d. = 84). No 6-TG was detected.

The RBC 6-TG nucleotide and 6-TG concentrations were determined in blood samples obtained

from three children with acute lymphocytic leukaemia taking 6-mercaptopurine in combination with methotrexate, vincristine and prednisone or prednisolone. These other cytotoxic drugs did not interfere with the assay. The 6-TG nucleotide levels are presented in Table 3. No 6-TG was detected.

Table 3. 6-TG nucleotide levels, after a 6-mercaptopurine oral dose, in children with acute lymphocytic leukaemia taking 6-mercaptopurine in combination with other cytotoxic drugs.

6-Mercaptopurine dose (mg)	Sex	Age	Surface area (m^2)	Time after dose (h)	pmole 6-TG nucleotide/ 8×10^8 RBCs
90	M	11	1·3	3	137
25	M	10	1·1	3	80
175	F	—	—	3	30

DISCUSSION

For the same dose of azathioprine a wide variation (range 72 to 330 pmole/8×10^8 RBCs) of 6-TG nucleotide was observed in the RBCs of renal transplant recipients with stable functioning grafts. Of the patients with acute lymphocytic leukaemia two of similar surface area, but taking different amounts of 6-mercaptopurine (25 or 90 mg), had RBC 6-TG nucleotide levels of 80 and 137 respectively whilst the third, on a higher 6-mercaptopurine dosage (175 mg) had much less nucleotide within the RBCs (30 pmole). This might be due to a lack of compliance, poor absorption or altered metabolism of 6-mercaptopurine.

All the patients' RBCs contained 6-TG nucleotides but in-vitro incubations using whole blood have shown that RBCs alone are unable to metabolize azathioprine or 6-mercaptopurine to 6-TG nucleotides. It could be tentatively concluded that the liver may play an important role in the ultimate appearance of 6-TG nucleotides in the RBC. The amount of 6-TG nucleotide within the RBC may be crucial in the exposure of certain tissues, particularly bone marrow, to active drug metabolites.

This assay is currently being used to assess the clinical significance of 6-TG nucleotide levels in renal transplant recipients taking azathioprine and in children with acute lymphocytic leukaemia undergoing combination therapy with 6-MP and other cytotoxic drugs (Herber et al 1982).

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REFERENCES

Dooley, R., Maddocks, J. L. (1980) Br. J. Clin. Pharmacol. 9: 77–82

Finkel, J. M. (1975) J. Pharm. Sci. 64: 121–122

Fletcher, L., Maddocks, J. L. (1980) Br. J. Clin. Pharmacol. 10: 287–292

Henderson, J. F., Le Page, G. A. (1959) J. Biol. Chem. 234: 3219–3223

Herber, S., Lennard, L., Lilleyman, J. S., Maddocks, J. L. (1982) Br. J. Cancer 46: 138–141

Konishi, Y., Ichihara, A. (1979) J. Biochem. 85: 295–301

Lajtha, L. G., Vane, J. R. (1958) Nature (London) 182: 191

Maddocks, J. L., Davidson, G. S. (1975) Br. J. Clin. Pharmacol. 2: 359–360

Pritchard, J. B., O'Connor, N., Oliver, J. M., Berlin, R. D. (1975) Am. J. Physiol. 229: 967–972