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Azathioprine suppresses the mixed lymphocyte reaction of patients with Lesch-Nyhan syndrome

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The mixed lymphocyte reaction of Lesch-Nyhan patients (HGPRT deficient) was used to study the immunosuppressive effects of azathioprine and 6-mercaptopurine (6-MP). Mitogen stimulated lymphocytes of these patients are highly resistant to azathioprine and 6-MP. When both stimulator and responder lymphocytes in the MLR were HGPRT deficient, azathioprine (36 μM) was much more inhibitory than 6-MP (100 μM). Azathioprine produced inhibition of 98.2% and 78.5% compared with the values of 63.9% and 30.6% for 6-MP. The difference in inhibitory activity between azathioprine and 6-MP was reduced when normal stimulator lymphocytes were cultured with HGPRT deficient responder lymphocytes in the MLR. These results provide very strong evidence that the nucleotide metabolites of azathioprine and 6-MP are unnecessary for immunosuppression. They also suggest that azathioprine and 6-MP interfere with antigenic triggering of the MLR.

Keywords azathioprine mixed lymphocyte reaction Lesch-Nyhan syndrome

Introduction

Azathioprine is one of the most commonly used immunosuppressive drugs, particularly in patients with organ transplants. However, it has the serious disadvantage of causing bone marrow toxicity which may be potentially fatal (Elion & Hitchings, 1975). Bone marrow toxicity is also associated with a greatly increased frequency of kidney transplant rejection. This is probably because azathioprine therapy has to be stopped or given in reduced dosage (Haesslein *et al.*, 1972; Oesterwitz *et al.*, 1978; Pollak *et al.*, 1980). For these reasons, the precise mechanism by which azathioprine causes immunosuppression is an important problem as it might be possible to separate its immunosuppressive from its bone marrow depressive effects.

Azathioprine is split to 6-mercaptopurine by red blood cells *in vivo* (Maddocks, 1979) and the latter is activated in cells to its nucleotide, 6-thioinosinic acid, by hypoxanthine-guanine phosphoribosyltransferase (HGPRT; E. C. 2.4.2.8). Thioinosinic acid is thought to inhibit purine nucleotide synthesis at the *de novo* and salvage pathway levels (Elion & Hitchings, 1975). Further, it can be metabolised to 6-thioguanine nucleotide and incorporated into DNA (Tidd & Paterson, 1974).

The critical site of azathioprine action for preventing cell division is unclear but there is strong evidence that nucleotide metabolites of 6-mercaptopurine are responsible for this effect. Thus, administration of azathioprine to patients with Lesch-Nyhan syndrome (HGPRT deficient) does not cause bone marrow toxicity as they are unable to form nucleotide metabolites of the drug (Nyhan *et al.*, 1968). Moreover, their lymphocytes (HGPRT deficient) when stimulated by phytohaemagglutinin (PHA) or pokeweed mitogen are highly resistant to azathioprine and 6-mercaptopurine (Allison *et al.*, 1977).

The immunosuppressive effect of azathioprine has been studied in detail using the human mixed lymphocyte reaction (MLR). Al-Safi & Maddocks (1983, 1984) found that it was more active at an early stage of the MLR when cell replication is at its lowest and is not inhibitory

when DNA synthesis is maximal. It was possible to completely reverse the inhibitory effect of 6-MP on the MLR with hypoxanthine and inosine, whereas these compounds did not prevent azathioprine inhibition of the MLR. It appears, therefore, that azathioprine and 6-MP inhibit the MLR by different mechanisms. Moreover, there was no evidence to suggest that azathioprine was interfering with purine metabolism. This view appears to be contradicted, however, by the absence of an inhibitory effect of azathioprine on PHA stimulated HGPRT deficient lymphocytes (Allison *et al.*, 1977). We considered that plant mitogen stimulation of lymphocytes is not a true immunological reaction as it does not involve antigenic triggering. Therefore we have studied the effects of azathioprine and 6-MP on the MLR, an antigenic triggered reaction, of patients with Lesch-Nyhan syndrome and report our findings.

Methods

Heparinized venous blood samples were obtained from three teenage boys with Lesch-Nyhan syndrome who were under the care of Dr Gillian McCarthy, Chailey Heritage, Sussex. They had all been previously investigated and their diagnoses confirmed by Dr R. W. E. Watts at the MRC Clinical Research Centre, Northwick Park, Middlesex. A control blood sample was obtained from a normal donor.

Lymphocytes were separated from 20 ml blood samples by treatment with dextran (MW 150 000) in normal saline and carbonyl iron powder (type SF, from G.A.F. Ltd, Manchester, England) before isolation on Ficoll-Metrizoate (Lymphoprep, Nyegaard AS, Oslo, Norway).

Stimulating cells were prepared with mitomycin-C (Sigma Chemical Co. Ltd, Dorset, England) as described by Bach & Voynow (1966). For culture, lymphocytes were suspended in RPMI 1640 (Flow Laboratories, Irvine, Scotland) prepared with glutamine 2 mM, HEPES buffer 20 mM, gentamicin 50 $\mu g\ ml^{-1}$ and amphotericin sodium desoxycholate (Fungizone) 2.5 $\mu g\ ml^{-1}$. They were cultured in triplicate in round bottomed microtitre plates (Flow Laboratories). Each well contained 200 μl, consisting of 50 μl each of: antigen responding lymphocytes (10^5), stimulating lymphocytes (10^5), inactivated pooled human AB serum diluted with culture medium to give a final concentration of 20% and the test drug solution or solvent diluted with culture medium.

Azathioprine and 6-MP stock solutions were freshly prepared by dissolving pure drug powder (Burroughs Wellcome, Crewe, UK) in 0.01 M sodium hydroxide, diluting with culture medium and sterilization by filtration through 0.22 μm sterile millipore filters.

Lymphocytes were incubated for 5 days at 37°C in a humidified desiccator flushed with a gas mixture of 5% CO_2 and 95% air.

The response was measured by the incorporation of tritiated thymidine (^{3}H-Tdr) into DNA.

Details of all these methods have been previously described (Al-Safi & Maddocks, 1983, 1984).

Results

As shown in Table 1, both azathioprine and 6-MP inhibited the MLR of patients with Lesch-Nyhan syndrome.

When both stimulator and responder lymphocytes in the MLR were HGPRT deficient, azathioprine (36 μM) was much more inhibitory than 6-MP (100 μM). Azathioprine produced inhibition of 98.2% and 78.5% compared with values of 63.9% and 30.6% for 6-MP.

When HGPRT deficient responder lymphocytes were cultured with stimulator lymphocytes from a normal subject, the difference in activity between azathioprine and 6-MP in inhibiting the MLR was reduced, with inhibitions of 68.4% and 61.2% respectively.

Discussion

Inhibition of the MLR by azathioprine and 6-MP, when both stimulator and responder cells lack HGPRT activity, provides very strong evidence that their nucleotide metabolites are not responsible for this immunosuppressive effect.

Even though the concentration of azathioprine used in these studies was only about a third of that of 6-MP, it was much more active in inhibiting the MLR of Lesch-Nyhan patients. In contrast, in the MLR of normal humans, azathioprine (36 μM) and 6-MP (100 μM) have similar inhibitory activity (Al-Safi & Maddocks, 1983). The difference in activity between the two drugs in inhibiting the MLR was reduced when Lesch-Nyhan responder lymphocytes were incubated with stimulator lymphocytes from a normal subject. This might be due to the normal cells providing HGPRT activity as a form of enzyme replacement therapy, although it is difficult to understand how the nucleotide metabolite of 6-MP would pass out of the normal lymphocyte and into the HGPRT deficient cell.

Table 1 Effects of azathioprine and 6-MP on the mixed lymphocyte reaction of Lesch-Nyhan patients. Lymphocytes were obtained from three Lesch-Nyhan patients (X, Y and Z) and from a normal blood donor (A).

	Median (n = 3) *(counts min^{-1})*			*% Inhibition*	
Reaction	*Control*	*Azathioprine (36 μM)*	*6-MP (100 μM)*	*Azathioprine*	*6-MP*
XXm	115	43	82	—	—
XYm	7,185	1,547	4,984	78.5	30.6
XZm	11,911	213	4,299	98.2	63.9
XAm	20,497	6,487	7,960	68.4	61.2

m = mitomycin-C treated lymphocytes.

As neither azathioprine nor 6-MP inhibit PHA stimulated Lesch-Nyhan lymphocytes, even at high concentrations of drug (Allison *et al.*, 1977), our results indicate that these drugs are acting at the stage of antigenic triggering of lymphocytes, possibly at the antigenic receptor on the cell membrane of the T-lymphocytes.

Hitchings (1967), Chalmers (1974) and Al-Safi & Maddocks (1984) have suggested that azathioprine might be acting as an alkylating agent. It is known to react readily with glutathione and cysteine giving rise to alkylated reaction products of 5-glutathionyl-1-methyl-4-nitroimidazole and 5-cysteinyl-1-methyl-4-nitroimidazole respectively (Chalmers, 1974). As the lymphocyte membrane is richly endowed with thiol groups (Mehrishi & Grassetti, 1969), possibly azathioprine alkylates these to produce inhibition of the MLR. This hypothesis would also explain the inhibition of attachment of sheep erythrocytes to human lymphocytes (rosette formation) by azathioprine (Bach *et al.*, 1969).

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