EFFECTS OF AZATHIOPRINE ON THE HUMAN MIXED LYMPHOCYTE REACTION (MLR)

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- 1 Azathioprine (36 μM) caused nearly complete and consistent inhibition of the mixed lymphocyte reaction (MLR).
- 2 The inhibitory effect of azathioprine (36 μM) on the MLR decreased with increasing delay of drug addition.
- 3 At 36 μM azathioprine was more inhibitory (34%) than 6-mercaptopurine.
- 4 The addition of red blood cells or glutathione decreased but did not abolish the difference in activity between azathioprine and 6-MP on the MLR.
- 5 The inhibitory effect of azathioprine or 6-MP on the human MLR was partially reversed after 24 h by washing.
- 6 Dithiothreitol (DTT) did not modify the reversibility of azathioprine inhibition by washing.
- 7 Restimulation of the washed azathioprine treated MLR cultures after 24 h by the same or different allogeneic stimulating lymphocytes had no effect.
- 8 The responding and stimulating lymphocytes were affected to the same extent by azathioprine.
- 9 These results suggest that azathioprine does not inhibit the MLR by deleting antigen stimulated lymphocytes. They would be more in keeping with an action on the lymphocyte membrane.

Introduction

Azathioprine is used universally in kidney transplantation yet its precise mode of action is still unknown (Maddocks, 1979). The human mixed lymphocyte reaction (MLR), which represents a transplantation reaction *in vitro*, has been used to investigate the immunosuppressive effects of azathioprine (Vas & Lowenstein, 1965; Skopinska *et al.*, 1968; Bach & Bach, 1972). Here we report some of our results of a detailed study of the effects of azathioprine on the MLR.

Part of this work was presented in the British Pharmacological Society Meeting at Strathclyde University (Al-Safi & Maddocks, 1982).

Methods

Lymphocyte separation

Lymphocytes were separated from heparinised venous blood samples (20 ml) obtained from normal healthy blood donors. Blood samples were treated with dextran (M.W. 150,000) in normal saline and carbonyl iron powder (type SF, G.A.F. Ltd,

Manchester, England), before isolation of lymphocytes on Ficoll-Diatrizoate (Histopaque – 1077, Sigma Chemical Co. Ltd., Dorset, England) (Boyum, 1968, 1976).

Stimulating cells

The stimulating cells were prepared with mitomycin-C (Sigma Chemical Co. Ltd) as described by Bach & Voynow, 1966).

Lymphocyte culture

Lymphocytes were suspended in RPMI 1640 (Flow Laboratories, Irvin, Scotland) prepared with glutamine (2 mM), Hepes buffer (20 mM) gentamicin (50 $\mu\text{g}/\text{ml}$) and amphotericin sodium desoxycholate (Fungizone) (2.5 $\mu\text{g}/\text{ml}$). They were cultured in triplicate in round bottomed microtitre plates (Flow Laboratories). Each well contained 200 μl , consisting of 50 μl each of 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 diluted with culture medium. Plates were incubated for five days at 37°C in a humidifier desiccator flushed with a gas mixture of 5% CO₂ and 95% air.

Washing technique

Lymphocytes were deposited by centrifuging the microtitre plates at 1000 rev/min (200 g) for 10 min using a GF-8 centrifuge (MSE Scientific Instruments, Crawley, England).

The supernatant of each well was withdrawn carefully with a 200P Gilson pipette (Anachem Ltd, Luton, England) with a sterile tip. To each well a 200 µl of washing fluid containing 20% AB serum was added. The microtitre plate was sealed with a plate sealer (Flow Laboratories), covered with its lid, vibrated on a plate shaker (Flow Laboratories) for 30 s and centrifuged as before. The process was repeated as necessary.

Measurement of DNA synthesis

[³H]-thymidine (1 µCi, sp. act. 24 Ci/mM, TRK 61, Radiochemicals Centre, Amersham, England) in 20 µl of culture medium was added to each well for the last 8 h on the fifth day. The cells were harvested, deposited on glass fibre filters and washed simultaneously with distilled water by the Skatron multiple cell harvester (Skatron, Norway, Flow Laboratories). The filters were dried in an oven at 60°C for 2 h and transferred into polypropylene mini-scintillation vials (LIP Ltd., Shipley, England) which were inside glass vials. To each mini-vial 3.5 ml of scintillation fluid (7.5 g PPO, 0.75 g POPOP and 2.5 l toluene) was added. Counting of radioactivity was performed by a liquid scintillation spectrometer (Intertechnique SL-30, Kontron Analytical, St Albans, England).

Drugs

Azathioprine (Imuran powder, Burroughs Wellcome, Berkhamstead, England) was dissolved in an equimolar solution of 0.01 M sodium hydroxide and diluted with phosphate buffer saline (PBS). The solution was prepared weekly.

6-MP (6-mercaptopurine hydrate powder, Sigma Chemicals Co. Ltd) was prepared freshly by dissolving the powder in boiling deionised water and diluted with PBS.

5-Mercapto-1-methyl-4-nitroimidazole was synthesised and checked for purity as described by Chalmers *et al.* (1967). It was dissolved in PBS.

DTT (Dithiothreitol, Sigma Chemicals Co. Ltd) was dissolved in PBS, divided into aliquots and stored at -20°C. The frozen solution was thawed before use.

Sterilization of the above solutions was done by passing them through 0.2 µm millipore filters.

Analysis of data

The mean counts/min and standard error of each triplicate was calculated. The difference in counts/min of the control or test MLR was calculated by subtracting the mean counts/min of the autologous MLR (AAM or BBm) from the mean counts/min of the allogeneic MLR (ABm or AmB) respectively. The difference in counts/min of the control MLR was considered as 100% uptake of [³H]-thymidine and the effect of drug was calculated accordingly. Student's paired *t*-test was used to assess statistical significance as the same lymphocytes were used both in test and control simultaneously.

Results

Effect on thymidine incorporation

Several doses of azathioprine were added to mixed lymphocyte cultures and tritiated thymidine incorporation measured 5 days later. It decreased with increasing doses of azathioprine (Figure 1) and at 36 µM the MLR was inhibited by 98%.

Figure 1 Effect of azathioprine on the MLR. *n* = number of pairs of test and control cultures. *NS, ***P* < 0.001, ****P* < 0.01

Reproducibility

Variation in inhibition of the MLR by azathioprine (36 µM) is shown in Table 1. In 193 cultures the mean inhibition of the MLR was 91% and the coefficient of variation was 11.6%.

Effect of delayed addition of azathioprine (36 µM) to the MLR

This resulted in decreasing inhibition with time (Table 2) but it was still statistically significant at 44%

Table 1 The distribution of the inhibitory effect (%) of azathioprine (36 μM) on the MLR of different individuals ($n = 193$)

Number of mixed lymphocyte cultures	% Inhibition range
5	56-65
19	66-75
32	76-85
40	86-95
97	96-105

after 72 h ($P < 0.001$). At 96 h there was no inhibition but at 112 h there was stimulation of the MLR by 33%. This was statistically significant, $P < 0.005$.

Are the effects of azathioprine due to 6-MP and/or 5-mercapto-1-methyl-4-nitroimidazole?

Azathioprine is rapidly metabolised *in vivo* by red blood cells to form 6-MP (Maddocks, 1979). Another product of this reaction, 5-mercapto-1-methyl-4-nitroimidazole had been isolated from human urine (Chalmers, 1974). To test for their inhibitory effects, several doses of 6-MP or 5-mercapto-1-methyl-4-nitroimidazole were added to the MLR. 5-Mercapto-1-methyl-4-nitroimidazole had no effect when used in concentrations of 18, 36, 72, 108 and 144 μM . However, with 6-MP inhibition increased with dose (Figure 2) although it was less effective than azathioprine. To inhibit the MLR by 90% 100 μM of 6-MP was necessary compared with 36 μM of azathioprine.

Table 2 Effect of adding azathioprine (36 μM) to the MLR after different intervals

Time (h)	Mean $\pm$ s.e. mean % inhibition	n	Significance P
0	95.5 $\pm$ 1.2	18	< 0.001
4	97.7 $\pm$ 0.6	7	< 0.01
12	95.0 $\pm$ 1.4	7	< 0.01
24	80.6 $\pm$ 2.3	18	< 0.001
48	62.6 $\pm$ 3.5	18	< 0.001
72	43.8 $\pm$ 5.3	11	< 0.001
96	4.0 $\pm$ 7.1	11	NS
112	-32.6 $\pm$ 9.4	11	< 0.005

Effect of red blood cells or glutathione on azathioprine inhibition of the MLR

The effects of azathioprine (36 μM) and 6-MP (36 μM) on the MLR were compared in the presence or absence of red blood cells (Table 3). Azathioprine

was more inhibitory than 6-MP in the absence or presence of red blood cells by 34% and 26% respectively. However, red blood cells reduced azathioprine inhibition by 25% which was statistically significant

Figure 2 Effect of 6-MP on the MLR.

n = number of pairs of test and control cultures.

* $P < 0.05$, ** $P < 0.02$, *** $P < 0.001$

($P < 0.001$) whereas with 6-MP the reduction in inhibition was 13% and statistically non-significant.

Glutathione at 1000 and 50 μM inhibited the MLR by 36% ($P < 0.025$) and 18% ($P < 0.05$) respectively, but at 36 μM had no effect (Figure 3). In the presence of glutathione at 1000, 50 and 36 μM , azathioprine (36 μM) was more inhibitory than 6-MP by 21% (NS) 53% ($P < 0.001$) and 10% ($P < 0.001$) respectively). Thus in the presence of 36 μM of glutathione, the difference in MLR inhibitory activity between azathioprine (36 μM) and 6-MP (36 μM) was reduced.

Effect of washing the azathioprine and 6-MP treated MLR

This was done after different incubation times and the results are shown in Table 4. There was statistically

Table 3 Comparison between azathioprine and 6-MP effects at (36 μM) on the MLR $\pm$ red blood cells

Mean $\pm$ s.e. mean % inhibition $\pm$ (n = 7)			
Without red blood cells	Without red blood cells	With red blood cells	With red blood cells
Azathioprine	6-MP	Azathioprine	6-MP
81.6 $\pm$ 2.6	54.3 $\pm$ 8.6	60.9 $\pm$ 2.2	44.7 $\pm$ 6.5
$P < 0.001$	$P < 0.01$	$P < 0.005$	$P < 0.02$
$P < 0.02$		$P < 0.05$

Figure 3 Effect of glutathione alone and together with azathioprine (36 μM) or 6-MP (36 μM) on the MLR. *n* = number of pairs of test and control cultures. G glutathione, Z azathioprine and M 6-MP

significant reversal of inhibition after 2, 6, 12 and 24 h with both drugs. Complete reversal occurred at 2 h with azathioprine and at 12 h with 6-MP. At 24 h, 43% of azathioprine inhibition and 78% of 6-MP inhibition was reversed.

Effect of washing with a thiol compound

Dithiothreitol was incorporated into the washing procedure as a thiol donor in an attempt to strip off drug possibly bound to thiol groups on the lymphocyte membrane. As shown in Table 5, there was no statistically significant difference in reversibility of azathioprine inhibition whether PBS or dithiothreitol was used in the washing.

Restimulation of washed lymphocytes

To determine whether azathioprine selectively inhibits antigen stimulated lymphocytes, the MLR cultures (after 24 h incubation and washing) were restimulated by the same or different allogeneic stimulating lymphocytes. As shown in Table 6, the

MLR cultures were still inhibited by 45% and 53% respectively, when restimulated by the same or different lymphocytes. Thus, neither the same nor different allogeneic stimulating lymphocytes reversed azathioprine inhibition of the MLR.

Effect on responding or stimulating lymphocytes

When responding or stimulating lymphocytes were incubated separately with azathioprine for 24 h, then washed before the MLR, inhibition was 44% and 39%, respectively. This difference was not statistically significant (Table 7).

Discussion

In these studies, azathioprine was shown to inhibit the MLR in a dose dependent manner with nearly complete and consistent inhibition at 36 μM .

The only detailed study of the effect of azathioprine on the MLR was reported by Bach & Bach (1972). They showed that low concentrations of azathioprine (0.036–3.6 μM) produced inconsistent effects and confirmed the results obtained by Vas & Lowenstein (1965). They also showed that the MLR was significantly inhibited by azathioprine at a non-cytotoxic concentration (36 μM) and complete inhibition was achieved at a cytotoxic concentration (360 μM).

Our investigation showed that the inhibitory effect of azathioprine (36 μM) decreased gradually with the time at which the drug was added to the MLR and at 96 h it had no effect. However, when azathioprine (36 μM) was added prior to labelling with tritiated thymidine, stimulation of DNA synthesis was achieved. The cause of this stimulatory effect is unclear. However, Bach & Bach (1972) showed that the azathioprine (36 μM) had no effect when added to the MLR after 48 h.

The disagreement between our results and those of Bach & Bach (1972) could possibly be due to some technical differences in the method used.

Table 4 Effect of washing the azathioprine or 6-MP treated MLR cultures after different intervals

Time (h)	n	Azathioprine (36 μM)		6-MP (36 μM)		% mean $\pm$ s.e. mean of reversal of inhibition	
		Without washing	With washing	Without washing	With washing	Azathioprine (36 μM)	6-MP (36 μM)
2	6	98.2 $\pm$ 1.0	-11.6 $\pm$ 14.6	61.9 $\pm$ 8.4	-21.5 $\pm$ 11.9	111.7 $\pm$ 14.5 (<i>P</i> < 0.001)	138.1 $\pm$ 26.4 (<i>P</i> < 0.005)
6	6	98.7 $\pm$ 0.7	12.5 $\pm$ 18.2	76.0 $\pm$ 2.0	-0.7 $\pm$ 6.1	87.7 $\pm$ 18.6 (<i>P</i> < 0.005)	101.1 $\pm$ 8.0 (<i>P</i> < 0.001)
12	9	95.0 $\pm$ 2.6	50.7 $\pm$ 6.7	39.3 $\pm$ 5.5	5.0 $\pm$ 7.5	47.2 $\pm$ 6.4 (<i>P</i> < 0.001)	104.3 $\pm$ 19.0 (<i>P</i> < 0.001)
24	7	78.6 $\pm$ 2.9	45.6 $\pm$ 5.7	30.4 $\pm$ 5.2	8.4 $\pm$ 5.0	42.7 $\pm$ 5.9 (<i>P</i> < 0.001)	77.5 $\pm$ 20.0 (<i>P</i> < 0.005)

Table 5 Effect of washing the azathioprine (36 μM) treated MLR cultures by phosphate buffer saline (PBS) or dithiothreitol (DTT) followed by culture medium

	% mean $\pm$ s.e. mean inhibition (n = 10)	
	Washing with 1 $\times$ PBS 2 $\times$ Culture medium	Washing with 1 $\times$ DTT (1 mM) 2 $\times$ Culture medium
Without washing		
83.5 $\pm$ 3.0	33.5 $\pm$ 5.6	38.2 $\pm$ 5.0
P < 0.001	P < 0.001	P < 0.001
	NS	

We have also demonstrated the superiority of azathioprine to 6-MP in inhibiting the MLR and thus confirming other *in vitro* (Bach *et al.*, 1969; Medzihardsky *et al.*, 1981) and *in vivo* (Calne, 1961) studies.

This difference in activity was decreased partially in the presence of thiol groups of reduced glutathione and red blood cells which are known to split azathioprine to 6-MP (Chalmers *et al.*, 1967; Miranda *et al.*, 1973; Maddocks, 1979).

The inhibitory effect of azathioprine was abolished completely (Elion *et al.*, 1961) or decreased to the level of 6-MP (Elion, 1967) by preadministration of glutathione or cysteine to animals.

The inability of 5-mercapto-1-methyl-4-nitroimidazole to inhibit the MLR excluded its possible role in explaining the superiority of azathioprine to 6-MP in inhibiting the MLR. 5-mercapto-1-methyl-4-nitroimidazole had only a slight inhibitory effect on protein synthesis of human lymphocytes (Smith & Forbes, 1970). The antibody response of mice to sheep erythrocytes was depressed by 5-chloro-1-methyl-4-nitroimidazole (Chalmers & Murray, 1972) and this inhibition was suggested to be due to the formation of 5-mercapto-1-methyl-4-nitroimidazole from the reaction of 5-chloro-1-methyl-4-nitroimidazole with glutathione or cysteine.

The partial reversal of azathioprine or 6-MP inhibition of the MLR after 24 h by washing could indicate development of a partial immune tolerance. Complete immune tolerance cannot be achieved clinically as stopping azathioprine therapy in renal transplant recipients is usually followed by kidney rejection (Zoller *et al.*, 1980).

Washing could possibly result in reversing azathioprine or 6-MP inhibition of the MLR at the extracellular and/or membrane levels(s) while the intracellular effects due to nucleotide metabolites are unlikely to be modified.

Reversal of azathioprine inhibition by washing has been demonstrated previously in PHA transformed lymphocytes (Schwartz *et al.*, 1969) and spontaneous rosette formation test (Bach & Dardenne, 1971).

Inability of dithiothreitol (DTT) to increase the reversibility of azathioprine inhibition might indicate that phosphate-buffered saline (PBS) and culture medium were sufficient to wash away azathioprine that was bound to the lymphocyte membrane. The intracellular inhibitory effects would not be modified due to the impermeability of the lymphocyte membrane to dithiothreitol (Ryzewski *et al.*, 1977). The choice of dithiothreitol for washing was based on its inability to cause any lymphocyte inhibition at the dose and time used (Kendall & Hutchins, 1978).

The lack of effect of restimulation of azathioprine treated MLR cultures after 24 h by the same or different allogeneic stimulating lymphocytes suggests that azathioprine effects are not selective and not restricted to the clones of lymphocytes first stimulated.

The partial inhibition of the MLR by azathioprine pre-treated stimulating or responding lymphocytes again confirms the non-selectivity in its effects. However, the partial inhibition of the MLR by azathioprine pretreated stimulating lymphocytes (incapable of DNA synthesis and cell division), suggests an effect on lymphocyte membrane or an antigen synthesis.

Schumacher *et al.* (1975), and Merryman *et al.*

Table 6 Effect of washing with culture medium $\times$ 2 and restimulation of MLR cultures exposed to azathioprine (36 μM) for 24 h, by the same or different allogeneic stimulating cells

Cells added	% Mean $\pm$ s.e. mean inhibition (n = 8)	Significance P
None	59.3 $\pm$ 7.7	< 0.001
Same stimulating cells	44.6 $\pm$ 7.5	< 0.005
Different stimulating cells	53.1 $\pm$ 7.5	< 0.001

Table 7 Effect of exposing the responding or stimulating cells to azathioprine (36 μ M) for the first 24 h followed by washing prior to the MLR

	% mean $\pm$ s.e. mean inhibition (n = 9)	Significance P
Without washing	99.2 $\pm$ 0.7	< 0.01
Washing of responding cells	44.4 $\pm$ 4.2	< 0.005
Washing of stimulating cells	39.2 $\pm$ 5.7	< 0.005

(1979), found that the thiol compound D-penicillamine inhibited the MLR and had no effect when incubated with the stimulating cells prior to the MLR. However, when the responding lymphocytes were incubated with D-penicillamine prior to the MLR there were conflicting results. Schumacher *et al.* (1975) found no effect while Merryman *et al.* (1979) showed a stimulatory effect on the MLR. The contradiction in these results is unclear.

In conclusion, azathioprine is most active at an early stage in the MLR when cell replication is lowest and is not inhibitory when DNA synthesis is at its maximum. It is non-selective and partially reversible in its effects. These results suggest that deletion of antigen stimulated lymphocytes is not its mechanism of action on the MLR.

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