EFFECTS OF THERAPEUTIC DRUGS ON LYMPHOCYTE TRANSFORMATION

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1 Over 40 non-steroidal drugs, routinely given on a long term therapeutic basis, were tested in lymphocyte transformation cultures at high therapeutic concentrations, or in serum cultures after short term oral drug administration. Lymphocyte transformation was assessed by measuring thymidine and 2-deoxyuridine incorporation into DNA.

2 When added *in vitro*, salicylate significantly inhibited thymidine (48%, $n = 35$) and deoxyuridine incorporation (20%, $n = 12$). Thymidine incorporation was specifically inhibited by phenylbutazone (39%, $n = 6$) and chlorpropamide (48%, $n = 5$). Specific inhibition of deoxyuridine incorporation, indicative of impaired lymphocyte folate metabolism, was obtained following the addition of pyrimethamine (51%, $n = 3$) and to some extent by carbimazole (30%, $n = 2$). In serum cultures, pyrimethamine inhibited deoxyuridine incorporation (30%, $n = 3$) and chloroquine inhibited the incorporation of both thymidine (37%, $n = 3$) and deoxyuridine (46%, $n = 3$).

3 Most classes of drugs used long term are unlikely to cause any significant clinical effect, by impairing lymphocyte function. Caution may be warranted when high doses of salicylate, phenylbutazone, chloroquine, pyrimethamine, chlorpropamide or combinations of these drugs are prescribed.

Introduction

Lymphocytes incubated with mitogens *in vitro* transform into blast cells, synthesize DNA and undergo cell division. The diverse metabolic changes associated with lymphocyte transformation, make it a unique model for studying the pharmacological action and toxicity of drugs on human cells. Use of the lymphocyte, in this respect, is particularly relevant as lymphocyte malfunction may become evident as one of the more serious of adverse drug reactions, immunosuppression (Sorrell & Forbes, 1975). On the other hand, drug enhancement of the lymphocyte transformation response is often cited as evidence for immunostimulation *in vitro* (Hadden *et al.*, 1976; Gifford *et al.*, 1980).

The effects of steroids on lymphocyte transformation have been well studied (Fauci, 1976; Ramer & Yu, 1978). Investigations with non-steroidal drugs have usually focussed on one drug, or on one pharmacological group of drugs, so that there is little comparative data relating to their effects on lymphocyte function. The wide range of culture conditions used in these studies, has often contributed to variable and sometimes contradictory reports (Williams &

Davidson, 1982). Extrapolation of *in vitro* test results to the clinical situation, has given rise to unnecessary concern over drug inhibition and over enthusiasm with regard to enhanced test responses. To assess the effects of drug therapy on lymphocyte transformation, we have investigated a large number of non-steroidal drugs in routine hospital use.

Methods

Culture medium

Eagles (MEM-10X concentration) medium (Wellcome) was reconstituted with sterile NaHCO_3 (20 mM), pyrogen-free water (May and Baker) or drug solution and supplemented with human serum. No antibiotics were used. HEPES buffer (1 M) was from Gibco and sterile 0.9% w/v NaCl from Travenol Laboratories. Purified phytohaemagglutinin (Wellcome) was used at a final concentration of 1 $\mu\text{g/ml}$. [Methyl- ^3H] thymidine, specific activity 15–20 Ci/mmol and 2-deoxy[6- ^3H] uridine (21 Ci/mmol)

were from the Radiochemical Centre, Amersham. Phytohaemagglutinin (PHA) was diluted with MEM and the radioisotopes with saline.

Drug solutions

The drugs selected for study were in routine hospital use, often prescribed for long term therapy and representative of many pharmacological groups. Maximum recommended values for serum or plasma therapeutic drug concentrations were obtained from the literature. An arbitrary value of 1 µg/ml was used in the absence of relevant data. The drugs donated by pharmaceutical companies and drug concentrations used in culture are listed in Table 1. The hydro-

chloride salts of amitriptyline, chlorpromazine, disopyramide, oxprenolol, propranolol, procainamide and trifluoperazine, the sulphate salts of guanethidine and chloroquine and sodium salts of salicylate and warfarin were used.

Solutions were made up and filtered (< 0.2 µm) on the day of use. Drugs insoluble in water were dissolved in alkali (PB, P, PH, A, SM, CH, F, IN), acid (R, PY), ethanol or propylene glycol (T, D, IN, E, SP, TH, C, DZ, DI). Diluted solutions produced no change in pH when added to culture medium (pH 7.2–7.4). Final concentrations of ethanol and propylene glycol were less than 0.05%. Rifampicin, phenylbutazone, methyl dopa and levo dopa slowly oxidise or hydrolyse in solution.

Table 1 Experimental drugs and drug concentrations

<i>Drug</i>	<i>Abbreviation</i>	<i>Concentration (µg/ml)</i>	<i>Manufacturer</i>
Allopurinol	A	400	Wellcome
Amitriptyline	AM	0.1	Roche
Carbimazole	CA	0.1	Nicholas
Chloroquine	CQ	1	May & Baker
Chlorpromazine	CP	0.75	May & Baker
Chlorpropamide	CH	250	Pfizer
Clofibrate	C	150	ICI
Clonidine	CL	0.01	Boehringer
Cotrimoxazole	COT (combination of sulphamethoxazole & trimethoprim)		
Diazepam	DZ	3	Roche
Digoxin	DI	0.002	Wellcome
Disopyramide	D	5	Roussel
Levodopa	LD	4	Roche
Ethacrynate	E	1	Merck Sharp & Dohme
Frusemide	F	30	Hoescht
Guanethidine	G	1	Ciba
Indomethacin	IN	8	Merck Sharp & Dohme
Isoniazid	I	15	Roche
Ketoprofen	K	8	May & Baker
Lithium carbonate	Li	14	Camden
Methyl dopa	MD	1	Merck Sharp & Dohme
Metoclopramide	MET	1	Beecham
Metronidazole	M	10	May & Baker
Oxprenolol	O	0.5	Ciba
Phenobarbitone	P	50	May & Baker
Phenylbutazone	PB	150	Geigy
Phenytoin	PH	25	Parke Davis
Potassium perchlorate	KClO ₄	1	Woodward
Practolol	PA	10	ICI
Procainamide	PR	16	Squibb
Prochlorperazine	PRO	0.5	May & Baker
Propranolol	PP	0.5	ICI
Pyrimethamine	PY	0.75	Wellcome
Rifampicin	R	20	Ciba
Salicylate	S	300	B.D.H.
Spironolactone	SP	0.05	Searle
Sulphamethoxazole	SM	90	Roche
Thiacetazone	TH	3	Smith & Nephew
Trifluoperazine	TR	0.5	Smith Kline & French
Trimethoprim	T	3.5	Roche
Warfarin	W	3	Duncan Flockhart

Table 2 Drug prescription

Drug	Dosage	Total dose
Allopurinol	100 mg 8 hourly (3 doses) + 200 mg	500 mg
Aspirin	600 mg 4 hourly (4 doses) + 1200 mg	3.6 g
Chloroquine	250 mg 8 hourly (3 doses)	750 mg
Clofibrate	500 mg 6 hourly (8 doses)	4 g
Co-trimoxazole	800 mg Sulphamethoxazole 3 doses 160 mg Trimethoprim 8 hourly	2.4 g 480 mg
Diazepam	5 mg 8 hourly (3 doses)	15 mg
Guanethidine	10 mg per day (2 doses)	20 mg
Indomethacin	25 mg 8 hourly (3 doses) + 50 mg	125 mg
Isoniazid	3–4 mg/kg per day (2 doses)	6–8 mg/kg
Ketoprofen	50 mg 8 hourly (3 doses) + 100 mg	250 mg
Phenobarbitone	30 mg 8 hourly (4 doses)	120 mg
Phenylbutazone	100 mg 8 hourly (3 doses) + 200 mg	500 mg
Phenytoin	100 mg 8 hourly (3 doses)	300 mg
Procainamide	250 mg 500 mg (2 doses) 8 hourly	1.25 g
Propranolol	40 mg 8 hourly (3 doses)	120 mg
Pyrimethamine	25 mg per day (2 doses)	50 mg

Drug therapy

Healthy volunteers, who were not receiving drug treatment, were given low or moderate oral doses of one drug (Table 2) over a 24 h period. Clofibrate was taken for 48 h.

Collection of blood samples

To minimise the effect of 'circadian rhythm' on transformation responses, blood was consistently taken in the morning (09.30–11.30 h). Blood for lymphocyte separation was placed in sterile containers with preservative-free heparin (20 units/ml). For serum cultures, blood (20 ml) was taken immediately before drug therapy and 2 h after ingestion of the last prescribed dose. Serum was separated from defibrinated blood, complement inactivated by heating at 56° for 30 min and stored at –20°C. This treatment did not affect the stability of the drugs studied in serum cultures.

Lymphocyte separation and pre-culture

Well washed mononuclear blood cells separated on Ficoll-Hypaque (Böyum, 1968) consisted primarily of lymphocytes, with an average of 16% monocytes. These were cultured overnight (2×10^6 /ml) in MEM with 5% human serum, in plastic petri dishes. Pre-incubated cells, obtained by gentle agitation of the culture medium, contained about 5% monocytes and were judged to be > 95% viable by phase microscopy.

Lymphocyte transformation studies

Lymphocytes were tested *in vitro* in the presence of high therapeutic drug concentrations (20% serum

cultures). Some drugs and their metabolites were tested in serum, obtained from subjects after short term therapy (98% serum). The culture conditions were extensively investigated in a previous study (Williams & Davidson, 1982) and gave optimum lymphocyte responses to PHA and maximum sensitivity to the inhibitory drug salicylate.

1. In vitro drug addition Drugs were tested in groups of 6–8 on lymphocytes from healthy subjects. Salicylate, an extensively investigated drug in lymphocyte transformation cultures (Crout *et al.*, 1975) was used as a reference drug in each group. A randomised culture plate design was used in all experiments, to eliminate any edge effect bias caused through evaporation. Incubation was carried out at 37°C in a humidified atmosphere containing CO₂.

Lymphocytes (10×10^6) were resuspended in 5 ml autologous serum. Aliquots (50 μ l) were placed in wells of a microtitre plate (1×10^5 /well) containing 150 μ l medium, with or without drug, and 50 μ l PHA. Thymidine (50 μ l, 0.05 μ Ci) was added to each well after 48 h and cultures were terminated after a further 5 h.

In another series of experiments, thymidine was compared with 2-deoxyuridine incorporation. This was to determine if the enhanced isotope incorporation, which occurred in the presence of drugs, was specific for thymidine. Isotopes were added as 10 μ l (0.5 μ Ci) and cultures were incubated with deoxyuridine for 24 h, because of the slower incorporation.

2. Cultures in serum from subjects receiving drug therapy The protocol adopted compared the effects of pre-treatment serum, and drug-containing serum, on normal and drug exposed lymphocytes from that subject. Lymphocytes were pre-incubated overnight in fresh complement inactivated serum. The follow-

ing day, 2×10^6 lymphocytes were resuspended in 2 ml serum from the second blood sample (containing drug) and in serum obtained from the previous day (cells from subjects receiving 2 days clofibrate therapy were incubated for this period before commencing the test). Lymphocytes in serum were distributed (200 μ l) into wells containing 5 μ l PHA (30 μ g/ml in 1 M HEPES buffer). After 48 h, 10 μ l of thymidine or deoxyuridine were added. Cultures were terminated after a further 5 h incubation. This procedure was repeated on the second day, by pre-incubating lymphocytes separated from the second blood sample overnight in serum from that sample (containing drug). This lymphocyte population was set up with PHA on the third day in pre-treatment serum and drug-containing serum.

Cell harvesting and processing of cultures

Cultures were harvested and processed using the millipore filter technique of Robbins *et al.* (1972) and double vial assembly for scintillation counting.

Presentation of results

Results are expressed as a percentage of the stimulated control value (culture without drug). Each point in Figures 1–6 represents the mean percentage change of six replicate cultures, for one subject. Deviation from the control response was tested for significance at the 5% level using Student's *t*-test.

Results

1. Lymphocyte transformation in the presence of high therapeutic drug concentrations (20% serum cultures)

The predominant response of transforming lymphocytes to therapeutic drug concentrations was enhanced isotope incorporation (Figures 1 and 2). Of the different drug types, the β -adrenoceptor blockers (O, PP, PA) consistently stimulated thymidine and deoxyuridine incorporation (Figure 1a).

Thymidine incorporation was considerably reduced ($P < 0.05$) by salicylate (48%, $n = 35$) chlorpropamide (48%, $n = 5$) and phenylbutazone (39%, $n = 6$). Suppression by salicylate was not consistent throughout the series of experiments and this was associated with the use of two different batches of PHA. Rifampicin (Figure 1c) significantly ($P < 0.05$) inhibited lymphocyte thymidine incorporation in 4 of 7 cultures (21%), sulphamethoxazole (Figure 2a) in 3 of 7 (26%) and allopurinol (Figure 2c) in 2 of 5 (35%). Phenytoin (37%) and co-trimoxazole (26%) inhibited thymidine incorporation in 1 of 6 cultures ($P < 0.05$). A number of other drugs produced low

Figure 1 [3 H]-thymidine (O) and [3 H]-deoxyuridine (●) incorporation by PHA stimulated lymphocytes in the presence of drugs added *in vitro*. Responses (counts/min) in control cultures without drugs—(a) (O) 34745 ($\pm$ 13394), (●) 13416 ($\pm$ 9907), (b) (O) 45763 ($\pm$ 24104), (●) 7755 ($\pm$ 987) and (c) (O) 49603 ($\pm$ 25215), (●) 55389 ($\pm$ 7743). EtOH ethanol, PG propylene glycol. For drug abbreviations see Table 1.

Figure 2 [^3H]-thymidine (○) and [^3H]-deoxyuridine (●) incorporation by PHA stimulated lymphocytes in the presence of drugs added *in vitro*. Responses (counts/min) in control cultures without drugs—(a) (○) 59538 (± 40709), (●) 56485 (± 12447), (b) (○) 46667 (± 23455), (●) 52096 (± 3613) and (c) (○) 52988 (± 23961), (●) 62624 (± 11555). For drug abbreviations see Table 1.

grade inhibition or moderate inhibition, in one of the series of test cultures.

There was often no correlation between the change in thymidine and deoxyuridine incorporation, in response to a particular drug. No suppression of deoxyuridine uptake by phenylbutazone or chlorpropamide was detected, while suppression by salicylate (20%, $n = 12$) was less than for thymidine. Rifampicin significantly ($P < 0.05$) inhibited deoxyuridine incorporation in all three cultures (18%), carbimazole (Figure 2c) in 2 of 4 cultures (30%) and chloroquine in one (39%). Suppression of deoxyuridine in the absence of thymidine suppression was noted with trimethoprim (Figure 2a) and was particularly evident in all three subjects with pyrimethamine (51%, $P < 0.05$).

2. Lymphocyte transformation in serum following drug therapy (98% serum cultures)

The effects of serum drug concentrations, achieved after short term medication, on lymphocyte transformation are shown in Figure 3. Each point denotes the average difference in the transformation response of lymphocytes in pre-treatment and post-treatment serum. In agreement with the results obtained in experiments with *in vitro* added drugs, isotope incorporation into DNA was usually enhanced in serum cultures containing drugs.

Serum from subjects who had taken chloroquine and pyrimethamine inhibited lymphocyte transformation. Chloroquine significantly ($P < 0.05$) inhibited thymidine (37%) and deoxyuridine incorporation (46%) in three subjects. Pyrimethamine specifically inhibited deoxyuridine incorporation (30%, $P < 0.05$). The inhibitory effects of these drugs were only observed with lymphocytes which had been obtained after drug therapy and further pre-incubated in drug-containing serum. These cells were exposed to drugs, *in vivo* and *in vitro*, for approximately 48 h after commencing the transformation test.

Discussion

Although transformation tests provide insight into the functioning of lymphocytes, they inadequately simulate conditions *in vivo*. Any artefacts generated in closed culture systems, through the manipulation of experimental parameters, are often permanent and enhance or antagonise the responses one intends to measure (Williams & Davidson, 1982). There has been considerable variation in the type of cell population used in transformation tests, ranging from whole blood cultures to extensively pure (99%) lymphocyte preparations. The mononuclear cell population in this study was pre-incubated, in order to reduce but not eliminate monocytes, to prevent

Figure 3 $[^3\text{H}]$ -thymidine ($\bigcirc\Delta$) and $[^3\text{H}]$ -deoxyuridine ($\blacktriangle$) incorporation by pre-treatment ($\bigcirc$) and post-treatment (Δ) lymphocytes stimulated with PHA, in drug-containing serum. Circles denote the difference in responses of pre-treatment lymphocytes from 3 ($n = 2$) subjects (pre-incubated in pre-treatment serum) in pre- and post-treatment autologous serum. Triangles denote the same changes for lymphocytes pre-incubated in post-treatment serum. $[^3\text{H}]$ -thymidine also measured in control subjects (no drug treatment $\bigcirc$, $n = 13$; Δ , $n = 9$). For drug abbreviations see Table 1.

monocytes from exerting a disproportionately suppressive effect through prostaglandin production. Prostaglandins accumulate to high levels in culture (Goodwin *et al.*, 1978) in contrast to their rapid removal *in vivo*.

Differences in methodology have contributed substantially to the inconsistencies apparent in published data, relating to the effects of drugs on lymphocyte transformation. The results from *in vitro* studies which have characterised a drug as an immunostimulant or immunosuppressant mean very little if the drug has been studied in isolation, as they may demonstrate properties which are common to the majority of drugs. In this study, a large number of therapeutic drugs were screened in one test system to assess their relative potency in comparison to a standard drug, salicylate. We acknowledge that one set of experimental conditions, though optimal, may be insufficient to detect the maximum inhibitory or stimulatory effects of all the drugs tested but the purpose of this screening test was to identify the drugs or drug metabolites requiring further investigation.

Simple inorganic ions, such as lithium and beryllium (Williams & Jones Williams, 1982) and a wide

variety of more complex organic molecules enhance nucleoside incorporation into lymphocytes stimulated with PHA. They may accomplish this by modifying the normal changes in intracellular cyclic nucleotide concentrations, induced by PHA. β -adrenoceptor blocking drugs which prevent increases in lymphocyte cyclic AMP following β -adrenergic receptor stimulation (Smith *et al.*, 1971) consistently enhanced isotope incorporation, in our experiments. Drugs showing this non specific effect on lymphocyte metabolism should clearly not be considered to have any specific immunostimulatory property.

Enhanced thymidine uptake may also result from a block in folate metabolism, with increased use of radioactive thymidine via the salvage pathway (Figure 4). Such an effect has been reported with methotrexate (Rozenszajn & Radnay, 1974). In the presence of anti-folate drugs, 2-deoxyuridine incorporation by lymphocytes should be inhibited. This was readily detected following *in vitro* addition of pyrimethamine and in transformation cultures in serum from subjects who had taken this drug. Suppression by trimethoprim, another antifolate, was not as marked. Our studies do not agree with the substan-

Figure 4 Pathways for deoxyuridine and thymidine incorporation into DNA, showing site of action of anti-folate drugs A.

tial inhibition of lymphocyte transformation reported for this drug by Gaylarde & Sarkany (1972). Rogers & Lietman (1976) similarly found therapeutic concentrations of co-trimoxazole, or its constituent drugs, inactive in this test.

Two other drugs which might have been expected to inhibit lymphocyte transformation, the anti-arrhythmic drug procainamide (Davies *et al.*, 1975) and the antidepressant lithium carbonate (Gupta & Crollini, 1975) had no effect. The inhibitory effect of chloroquine on lymphocyte function (Hurvitz & Hirschhorn, 1965; Panayi *et al.*, 1973) has been attributed to lysosome stabilisation (Weissman, 1964) and to direct interaction with DNA (Gaudin *et al.*, 1971).

The anti-tuberculous drug rifampicin and the anti-convulsants phenytoin and phenobarbitone are associated with impaired immune competence following therapy (Ruben *et al.*, 1973; Grob & Herold, 1972; Park & Brody, 1971). Suppressed lymphocyte transformation responses by therapeutic concentrations of these drugs have been reported (Nilsson, 1971; Dajani *et al.*, 1972; MacKinney & Vyas, 1973; Sorrell & Forbes, 1975). Our results suggest that phenytoin and phenobarbitone cause only moderate inhibition of thymidine uptake at 5×10^{-4} M, which is above their highest therapeutic serum concentrations. Suppression of transformation by high therapeutic concentrations of rifampicin, was also slight. To some extent, the discrepancy between our results and those of others may be due to the high ethanol concentrations (1%) used as solvent (MacKinney & Vyas, 1973). This level substantially inhibited our tests. Even with lower ethanol concentrations, one cannot exclude the possibility that the solvent may increase the susceptibility of lymphocytes, *in vitro*, to drugs.

The most potent drugs tested by *in vitro* addition were salicylate, chlorpropamide, phenylbutazone and allopurinol. While the inhibitory effects of sali-

cylate and phenylbutazone on transformation are well known (Anthony & Panusch, 1978; Panayi, 1975) chlorpropamide and allopurinol have not previously been investigated. Allopurinol achieves high serum concentrations (2.9×10^{-3} M) and is well tolerated, although there are reports of altered *de novo* purine synthesis and pyrimidine metabolism *in vivo* (Becker *et al.*, 1975). Suppression of thymidine incorporation into DNA *in vivo* would be compensated, to some extent, by use of the alternative pathway. Although the effects of drug combinations were not tested, the combined use of either aspirin or chlorpropamide with an anti-folate could possibly block both salvage and *de novo* pathways for DNA synthesis.

Our experiments showed that it was possible to measure lymphocyte transformation in culture medium consisting almost entirely of serum (98%). This modification made the test suitable for assessing directly the effects of drug therapy on lymphocyte function. Only serum from subjects who had taken chloroquine and pyrimethamine significantly inhibited lymphocyte transformation. Inhibition was greater in cultures containing post-treatment lymphocytes that had been pre-incubated in serum containing chloroquine and pyrimethamine, probably due to higher intracellular drug concentrations. The formation of an inhibitory drug metabolite, might explain why serum obtained following ingestion of chloroquine was more inhibitory than chloroquine added *in vitro*. In contrast, salicylate was more inhibitory when added *in vitro*. We attribute this to a higher *in vitro* salicylate concentration (300 $\mu\text{g/ml}$) than the serum salicylate levels achieved (highest levels 208 $\mu\text{l/ml}$).

In conclusion, the purpose of this study was to determine whether any of the classes of drugs, normally used therapeutically on a long term basis, could be shown to have a significant inhibitory effect on

lymphocyte transformation. Transformation was impaired by the anti-malarial drugs chloroquine and pyrimethamine and by the anti-inflammatory drugs salicylate and phenylbutazone. The inhibitory effect of chlorpropamide would merit further investigation.

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