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The effects on polymorphonuclear leucocyte function of prednisolone and azathioprine *in vivo* and prednisolone, azathioprine and 6-mercaptopurine *in vitro*

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SUMMARY

The effects of prednisolone, azathioprine and 6-mercaptopurine on polymorphonuclear neutrophil chemotaxis, phagocytosis, and killing of *Staphylococcus aureus* and *Candida albicans* have been studied. In twenty patients with a functioning kidney graft, taking both azathioprine 2·5 mg/kg/day and prednisolone (mean dose 0·59 mg/kg/day; range 0·30–1·0 mg/kg/day), the polymorphonuclear function did not significantly differ from that in either twenty-two normal or eighteen uraemic controls. Addition of prednisolone, $1{\cdot}2 \times 10^{-5}$ M, azathioprine, $2{\cdot}1 \times 10^{-5}$ M, and 6-mercaptopurine, $2{\cdot}1 \times 10^{-5}$ M, using each drug alone, to normal human polymorphonuclear cells *in vitro* did not significantly alter their function.

It is concluded that prednisolone and azathioprine together *in vivo* and that both these drugs and 6-mercaptopurine singly *in vitro* have no significant deleterious effect on polymorphonuclear function and do not contribute, in this way, to the increased susceptibility of patients receiving these drugs to infection.

INTRODUCTION

Infection is a common complication following immunosuppression. The impairment of the antibody response and cell-mediated immunity in immunosuppressed patients contribute to this, and another possible cause is depressed polymorphonuclear leucocyte function. Defective polymorphs are a well recognized cause of infections, and we have therefore studied the effect of commonly used immunosuppressives on chemotaxis, phagocytosis and killing of staphylococci and candida by polymorphs.

We have adopted two approaches to this problem: (i) examining polymorph function *in vivo* in a group of patients with a kidney graft, receiving both prednisolone and azathioprine; and (ii) observing the individual effect on polymorph function *in vitro* of prednisolone, azathioprine and 6-mercaptopurine (6MP).

The latter was used since azathioprine is metabolized *in vivo* to 6MP, and this drug has received scant attention in *in vitro* experiments. Each drug was incubated with polymorphs alone, and with polymorphs in the presence of autologous whole blood, since the metabolic effects of drugs on cells may be altered by factors in the plasma.

MATERIALS AND METHODS

Patients. Twenty patients, aged 17–58 years, who had received a successful cadaver or live donor kidney transplant, were

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studied. Patients were not included if currently they had a recognized infection, were receiving antibiotics, were anaemic or hypophosphataemic. Nine of the patients were taking one or more antihypertensive drugs (hydrallazine, propranalol and prazosin), and eleven were taking diuretics (frusemide). All were on azathioprine, 2·5 mg/kg per day, and prednisolone orally in doses ranging from 0·30 to 1·0 mg/kg per day, the mean being 0·59 mg/kg/day. The serum creatinine of these patients ranged from 98–279 μmol/l, mean 149 μmol/l.

Controls and normals. Two control groups were used: (a) twenty-two normal, fit, laboratory, medical and nursing staff, aged 18–45 years, none of whom were taking drugs; and (b) eighteen uraemic patients, aged 18–63 years, were included as a control group since the renal function was not normal in the transplanted group. The uraemic patients were not on dialysis, and their serum creatinine levels ranged from 200–631 μmol/l, mean 399 μmol/l.

Fourteen were receiving antihypertensives and twelve diuretics (drugs as above). No patient with an infection or taking antibiotics was included. Furthermore, only patients with renal disease known to be not due to glomerulonephritis were included in this group, since glomerulonephritis may be associated with polymorphonuclear dysfunction (Gewurz *et al.*, 1967).

Polymorph separation. Venous blood containing 20 u/ml of preservative-free heparin (Evans Pharmaceuticals) was drawn between 9·00 a.m. and 11.00 a.m. and added to half its volume of 6% w/v dextran in 0·9% sodium chloride (Homodex). After 30 min at 37°C, the leucocyte-rich plasma was removed and washed twice with Hanks's buffered salt solution (HBSS) containing 5 u/ml heparin and 0·01% gelatin. The cell concentration was adjusted to 5×10^6/ml in HBSS, and cell viability checked by exclusion of trypan blue.

Incubation with drugs. Leucocytes were incubated with drugs either when the leucocytes were still present in the heparinized venous blood before the addition of dextran, or at the end of the separation procedure. The mixtures of polymorphs and drugs were incubated at 37°C for 30 min in a shaking waterbath simultaneously with control samples of polymorphs at the same stage of preparation without added drugs.

The separation of polymorphs incubated with drugs in venous blood was performed as above after incubation.

Chemotaxis. The portion of the chemotaxis chamber containing polymorphs was made from the outer casing of a 1·0 ml plastic syringe, with the lower end removed. A 3·0 μm pore size membrane filter (Millipore Ltd) was fixed to the lower end with non-toxic glue (Uhu, Jismar Ltd). During the chemotaxis experiments the syringe barrel was suspended by its shoulder in a plastic flat-bottomed tube, which served as the lower portion of the chemotaxis chamber.

A chemotactic stimulus was provided by *E. coli* lipopolysaccharide (Difco Ltd), 3·0 μg/ml in HBSS plus 10% fresh normal human serum; controls were measured by using HBSS alone in the lower chamber. 0·1 ml of polymorphs were placed inside the syringe barrel and incubated in a humidified atmosphere at 37°C for 30 min. The assessment of movement towards both chemotactic factor and the HBSS alone was made in triplicate.

Following the incubation the polymorphs were removed from the upper chamber, and the fluid in the lower chamber was replaced with 50% isopropyl alcohol (Fisons Ltd) to remove the filters. These were then fixed in 75% isopropyl alcohol, stained in haematoxylin and mounted in DPX.

Chemotaxis was measured by the leading front method and the results expressed as the difference between the distances travelled by the stimulated and unstimulated cells.

Bactericidal assay. 0·5 ml of polymorph suspension were added to 0·4 ml of 20% fresh NHS in HBSS and 0·1 ml of a washed overnight broth culture of Oxford *Staphylococcus aureus* (NCTC 6571) at 5×10^8/ml. Following incubation at 37°C for 20 min in a shaking waterbath, the samples were centrifuged at 1000 rev/min for 10 min, the supernatants discarded and the cells resuspended in 1·0 ml of HBSS with benzyl penicillin, 1000 u/ml, and streptomycin sulphate, 1000 μg/ml. Aliquots from each sample were washed in HBSS, lysed with 10 ml sterile distilled water and cultured on nutrient broth (Oxoid nutrient broth No. 2). The samples were incubated for a further 90 min at 37°C and then aliquots were assayed for surviving staphylococci. The numbers of viable bacteria from each aliquot were measured by colony counting at 18 hr. A bactericidal index was obtained by expressing the number of surviving bacteria at 90 min as a proportion of those cultured at 20 min.

Phagocytosis. 0·2 ml polymorphs with 0·1 ml 10% fresh NHS and 0·1 ml dead yeast particles (*Saccharomyces cerevisiae*) in distilled water at 10^9 yeast particles per ml were incubated in a shaking waterbath at 37°C for 30 min. The mixture was centrifuged at 500 rev/min for 5 min, the supernatant discarded, and the deposit washed in HBSS. Smears of the deposit were stained with May–Grunwald–Giemsa stain, counted for the number of yeast particles phagocytosed per 100 polymorphs, and the result expressed as the mean number of particles ingested per polymorph.

Candidacidal assay. A suspension of *C. albicans* was made from a 3-day-old broth culture (Sabouraud Liquid Medium, Oxoid Ltd) by washing the cells twice and resuspending them in HBBS at 10^7 organisms per ml. Their viability was checked by exclusion of methylene blue 0·1%; suspensions with more than 3% dead cells were discarded. 0·5 ml polymorphs were added to 0·25) ml fresh NHS and 0·25 ml cell suspension and incubated at 37°C for 60 min in a shaking waterbath. A control was provided by substituting HBSS alone for the polymorph suspension. 0·25 ml of 2·5% sodium deoxycholate (pH 8·7) was added to lyse the polymorphs, followed by 4·0 ml 0·01% methylene blue.

After centrifugation at 1500 rev/min for 15 min, the supernatant was discarded and the deposits placed on ice before counting the number of dead *C. albicans* per 300 cells. The percentage of dead cells in the control sample was subtracted from the test sample to give the percentage of candida killed by the polymorphs.

Drugs. Prednisolone sodium phosphate (Merck, Sharp & Dohme Ltd), azathioprine (Burroughs Wellcome & Co.) and 6-mercaptopurine (Burroughs Wellcome & Co.) were dissolved in sterile water and used in final concentrations equivalent to those achieved therapeutically.

TABLE I. Effect of prednisolone sodium phosphate, azathioprine and 6-mercaptopurine on polymorph function *in vitro*

			Incubation with heparinized blood											
			Chemotaxis			Phagocytosis			Bacterial killing			Candida killing		
Drug	*n*		Drug	No drug	Drug/ no drug	Drug	No drug	Drug/ no drug	Drug	No drug	Drug/ no drug	Drug	No drug	Drug/ no drug
Prednisolone	10	Mean	45·9	44·8	1·03	4·50	4·60	0·98	0·18	0·13	1·38	28·2	27·5	1·03
Sodium phosphate		s.e.	2·71	2·73	0·99	0·38	0·452	0·86	0·054	0·035	1·54	3·14	2·69	1·17
($1·2\times10^{-5}$ M)		*t*	0·663			0·318			1·146			0·542		
Azathioprine	10	Mean	47·0	42·4	1·11	4·90	5·00	0·98	0·32	0·33	0·97	27·5	27·0	1·02
($2·1\times10^{-5}$ M)		s.e.	2·53	3·87	0·65	0·30	0·351	0·87	0·082	0·090	0·91	3·05	2·64	1·15
		t	1·250			0·152			0·225			0·330		
6-Mercaptopurine	10	Mean	50·1	46·3	1·08	4·40	4·50	0·97	0·22	0·23	0·96	22·4	28·5	0·79
($2·1\times10^{-5}$ M)		s.e.	3·58	3·74	0·96	0·26	0·357	0·75	0·094	0·065	1·45	4·12	3·56	1·16
		t	1·458			0·338			0·041			1·182		

			Incubation with separated polymorphs											
			Chemotaxis			Phagocytosis			Bacterial killing			Candida killing		
Drug	*n*		Drug	No drug	Drug/ no drug	Drug	No drug	Drug/ no drug	Drug	No drug	Drug/ no drug	Drug	No drug	Drug/ no drug
Prednisolone	10	Mean	45·4	46·0	0·99	4·60	4·60	1·00	0·11	0·11	1·00	25·4	30·8	0·83
												($n=9$)	($n=9$)	
Sodium phosphate		s.e.	2·35	1·55	1·52	0·370	0·340	1·09	0·035	0·038	0·92	3·19	3·13	1·02
($1·2\times10^{-5}$ M)		*t*	0·347			0·165			0·952			0·346		
Azathioprine	10	Mean	38·9	42·7	0·91	4·40	4·70	0·94	0·24	0·22	1·09	29·5	29·0	1·02
($2·1\times10^{-5}$ M)		s.e.	2·23	2·41	0·93	0·259	0·224	1·16	0·078	0·066	1·18	2·89	3·45	0·89
		t	1·284			1·260			0·309			0·368		
6-Mercaptopurine	10	Mean	49·4	49·2	1·01	4·20	4·60	0·91	0·22	0·20	1·10	29·0	29·1	0·99
($2·1\times10^{-5}$ M)		s.e.	2·73	2·80	0·96	0·335	0·337	0·99	0·103	0·076	1·36	4·18	3·23	1·29
		t	0·068			1·768			0·494			0·057		

All measurements were made in duplicate, except those of chemotaxis which were made in triplicate and results recorded as the means. The paired *t*-test was used for statistical evaluation.

RESULTS

In vivo measurements (Fig. 1)

Polymorph function in the renal transplant group did not differ significantly from that in normals; there was also no significant difference between the transplant patients and uraemic patients.

In vitro experiments (Table 1)

The effect on polymorph function of incubating with drugs, either in the presence of heparinized blood or with the isolated cells alone, is shown for prednisolone ($1{\cdot}2\times10^{-5}$ M), for azathioprine ($2{\cdot}1\times10^{-5}$ M) and for 6MP ($2{\cdot}1\times10^{-5}$ M). In the doses used, these drugs produced no significant change in polymorph function ($P>0{\cdot}05$ in each experiment).

There were no differences in the distances travelled by unstimulated polymorphs between any of the groups of *in vivo* or *in vitro* studies (data not shown) in the chemotaxis experiments.

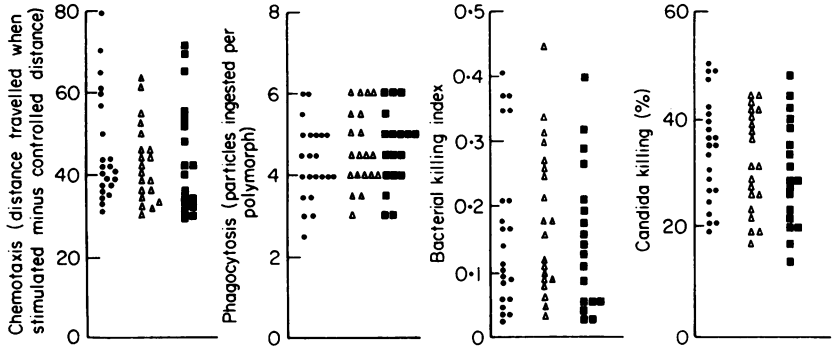

FIG. 1. Polymorph function *in vivo* in normals (●), patients with a renal graft taking prednisolone and azathioprine (△) and patients with uraemia (■).

DISCUSSION

We have shown that prednisolone and azathioprine combined *in vivo* and that each of these two drugs and 6MP singly *in vitro* have no detectable effect on neutrophil function at the doses used. It is, therefore, unlikely that these drugs contribute significantly, by depressing the neutrophil function, to the propensity of immunosuppressed patients to develop infections.

Other workers have found differing results when studying the effect of steroids on human polymorphs *in vitro*. Prednisolone itself has been examined in only one other study, in which chemotaxis alone was measured and found to be reduced by 50% at 10^{-7} M (Rivkin, Foschi & Rosen, 1976). Hydrocortisone and methylprednisolone have been more extensively investigated. Phagocytosis was found to be normal in the presence of hydrocortisone sodium succinate at $2{\cdot}2\times10^{-4}$ to 21×10^{-4} M (Olds *et al.*, 1974; Fuenfer, Olson & Polk, 1975; Mandell, Rubin & Hook, 1970), but reduced when the concentration was increased to $4{\cdot}2\times10^{-3}$ M (Olds *et al.*, 1974). Using the sodium phosphate ester of hydrocortisone at $1{\cdot}7\times10^{-4}$ M, Fuenfer *et al.* (1975) demonstrated reduced phagocytosis but normal bactericidal activity. Bacterial killing was found to be normal by Olds *et al.* (1974), but reduced by Fuenfer *et al.* (1975), using similar concentrations of hydrocortisone sodium succinate ($2{\cdot}2\times10^{-4}$ M and $1{\cdot}7\times10^{-4}$ M respectively), although again raising the concentration to high levels of 10^{-3} M (Olds *et al.*, 1974) and 2×10^{-3} M (Mandell *et al.*, 1970) brought about a reduction in this function. Methylprednisolone did not alter phagocytosis or bacterial killing, unless extremely high concentrations of $1{\cdot}3\times10^{-3}$ M were used (Olds *et al.*, 1974). Chemotaxis *in vitro* was diminished by hydrocortisone and methylprednisolone at concentrations of 10^{-5} M (Ward, 1971).

In summary, *in vitro* work has shown that steroids tend to have effects on polymorph function only at concentrations greater than those achieved *in vivo*, although some abnormalities have been detected at therapeutic concentrations. In the case of hydrocortisone, there seems to be some variation in effect, according to the particular ester in use. This may be due to the varying ability of each ester to enter cells. Leucocytes, for example, have been reported to be impermeable to the acetate ester (Rinehart *et al.*, 1974).

Steroids given alone to humans have not altered polymorph function. Methylprednisolone in doses up to 1000 mg intravenously had no effect on phagocytosis (Webel *et al.*, 1974; Olds *et al.*, 1974) or bacterial killing (Webel *et al.*, 1974), and Clarke *et al.* (1977) found normal bacterial killing in patients with ulcerative colitis in remission who were given prednisolone. Dexamethasone had no effect on polymorphonuclear chemotaxis, phagocytosis and the killing of bacteria or yeasts when administered as a single intravenous injection to normal volunteers (Glasser, Huestis & Jones, 1977).

Azathioprine has been studied *in vitro* in two other laboratories, but only its effect on chemotaxis was measured. Rivkin *et al.* (1976) found this to be reduced by 50%, although the concentration of drug at 7×10^{-5} M was three times that achieved *in vivo* at a dose of 2·5 mg/kg, whereas Ward (1971) found no diminution in chemotaxis at a concentration greater than 10^{-2} M. Azathioprine is metabolized *in vivo* to 6MP, but we found that this drug too was without effect *in vitro*; our observations on chemotaxis using 6MP have been confirmed by Ward (1971) using a very high concentration of greater than 10^{-2} M.

Pre-incubation of the polymorphs with the test drug in plasma, as opposed to Hanks's solution, did not affect our *in vitro* observations. We adopted these alternatives lest factors usually present in the plasma altered the drug or were required for its entry into the cell. Inevitably, heparin was present in the plasma incubations, although at a low concentration of 20 u/ml, and it is possible that this inhibited any action of the drug. Nonetheless, we considered this a closer approach to normal than incubating the drug with cells in the presence of serum.

Other *in vivo* studies in renal transplant recipients taking azathioprine and steroids together have provided results at variance with each other and with our own. Salant *et al.* (1976) found chemotaxis to be significantly diminished in a group of twenty-four patients receiving azathioprine, 50–200 mg/day, and prednisolone, 12·5–180 mg/day. However, within the group there was a wide variation of chemotaxis, some results being normal or even greater than normal as well as reduced; these values were unrelated to the steroid dose. The same authors, and McIntosh *et al.* (1976), studying a group of twenty-two patients, found phagocytosis to be unaffected, whereas bacterial killing was found to be normal and reduced respectively. McIntosh *et al.* (1976) used a consistent dose of azathioprine of 3·0 mg/kg and also employed anti-lymphocyte globulin (ALG) in fifteen of their patients, which may explain their different results, since Grogan & Smith (1975) detected reduced bacterial killing in a group of renal transplant patients who had received ALG, whereas this polymorph function was not affected in patients who had not received ALG.

Factors, other than immunosuppressive drugs, which can alter the polymorph function may have caused the inconsistent findings in these various groups of renal transplant patients. Subjects may have been receiving drugs such as antibiotics or have been infected or were in some way malnourished.

We conclude that the lack of clear-cut and consistent effects on neutrophil function by steroids and azathioprine at normal doses both *in vitro* and *in vivo* indicates that these drugs do not have a prime effect on neutrophils sufficient to contribute significantly to immunosuppressed patients' liability to infection.

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