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In vitro analysis of B lymphocyte function in uraemia

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SUMMARY

We have investigated the immune responses *in vitro* of uraemic patients undergoing regular haemodialysis or continuous ambulatory peritoneal dialysis. Twenty-five healthy subjects were also studied as controls. In uraemic patients, the number of T and B lymphocytes were within the normal range, but proliferative respones to phytohaemagglutinin (PHA) were impaired. Spontaneous immunoglobulin plaque forming cell (PFC) responses by peripheral blood mononuclear cells (PBMC) from uraemic patients were significantly lower than those of healthy subjects. The PFC response of uraemic PBMC to the T cell independent polyclonal B cell activator (PBA) Epstein–Barr virus (EBV) was comparable to the response of the healthy subjects, indicating that uraemic B cells are still capable of synthesizing immunoglobulin. Pokeweed mitogen (PWM) induced PFC responses of uraemic PBMC were also normal, whereas the response to another T cell dependent B cell activator, *Staphylococcus aureus* Cowan I (SAC), was very low. Addition of indomethacin to PWM- and SAC-activated cultures of uraemic PBMC enhanced the PFC response to SAC, but had little effect on the PWM response.

As full differentiation of B cells in response to SAC depends on helper T cells, we conclude that a defect in T lymphocyte function accounts for the reduced spontaneous and SAC induced production of immunoglobulin by uraemic PBMC. This defect may be mediated by an indomethacin-sensitive mechanism.

Keywords uraemia T cell B cell PFC PBA

INTRODUCTION

Uraemic patients have abnormalities of virtually every organ system and many studies have shown that the immune response is also impaired (Raskova *et al.*, 1984; Langhoff & Ladefoged, 1984). Clinically, this is supported by a high incidence of secondary malignancies (Matas *et al.*, 1975) as well as the infections which are a common cause of morbidity and death (Merrill, 1968). It has been known for some time that uraemic patients have suppressed cellular and humoral immune responses *in vivo* (Boulton-Jones *et al.* 1973) and, more recently, attention has been given to the study of lymphocyte function *in vitro* (Kunori *et al.*, 1980; Raska *et al.*, 1983). Although it has been shown that T lymphocyte function *in vitro* is impaired in uraemic patients (Huber *et al.*, 1969; Kauffman, Manzler & Phair, 1975), very little work has been done on the responses *in vitro* of human uraemic B cells. In particular, nothing is known about how uraemic B lymphocytes respond to PBA which differ in their dependence on T cells.

The aim of this study was to examine the immune response *in vitro* of uraemic patients with an emphasis on immunoglobulin production. The protein-A plaque forming cell (PFC) assay was used

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to examine the effect of uraemia on both T cell dependent and independent production of immunoglobulin and we have correlated these with other assays of lymphocyte number and function. We have also attempted to investigate the role of prostaglandins in the suppressed *in vitro* responses by uraemic PBMC.

PATIENTS AND METHODS

Subjects. Thirty-seven patients undergoing regular haemodialysis (4 h, three times a week) and 10 patients on continuous ambulatory peritoneal dialysis (CAPD) were studied, ranging in age from 18 to 63 years (mean ± s.e.m. 41 ± 2 years). The mean duration of dialysis before the study was 2·4 months, with a range from 1 to 13 months. Chronic renal failure was due to chronic glomerulonephritis (17 patients), chronic pyelonephritis (15 patients), polycystic kidney disease (five patients), essential hypertension (four patients), diabetes (four patients), and in the remaining two was of unknown aetiology. The mean serum creatinine before the study was 737 μmol/l with a range from 523 to 1134 (normal range: 62–120 μmol/l). None of the patients were receiving corticosteroids or other immunosuppressive drugs and none of them had evidence of systemic lupus erythematosus or other systemic immunological disease. Finally, none of the patients had received a blood transfusion. Twenty-five healthy subjects working in the Renal Unit and the Department of Bacteriology and Immunology were also studied sequentially as a control group. Their ages ranged from 20 to 55 years (mean ± s.e.m. 30 ± 1·5).

Separation of peripheral blood mononuclear cells (PBMC). PBMC were prepared from heparinized venous blood by separation over Ficoll-Hypaque and washed three times with RPMI 1640 (Gibco, Paisley, Scotland) before use.

Analysis of T and B lymphocyte numbers by immunofluorescence. T cell subsets were analysed by two-stage immunofluorescence using the monoclonal antibodies OKT3, OKT4, or OKT8 (Ortho Diagnostic Systems Ltd, High Wycombe, England). The number of B cells was measured by direct immunofluorescence for the presence of surface membrane immunoglobulin (sIg) using a fluorescein-conjugated antibody to human κ and λ chains. T3, T4, T8 and sIg positive cell counts were expressed as a proportion of the total lymphocyte count.

Proliferative responses to phytohaemagglutinin (PHA). PBMC ($0{\cdot}5 \times 10^6$ cells/ml) were resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (250 μg/ml) and L-glutamine (2 mmol, Gibco, Paisley, Scotland) and cultured in 0·1 ml in conical-bottomed microtitre plates (Titertek, Flow Laboratories) with 1/100 purified PHA (Wellcome, England) for 72 h at 37°C in 5% CO_2 in air in a humidified incubator. Eighteen hours before harvesting, the plates were pulsed with 50 μl of ^{14}C-Thymidine (Phoenix Pharmaceuticals, West Germany, 0·015 μCi/well) and cell bound DNA was then harvested using a multi-cell harvester (Titertek Cell Harvester, Skatron, Norway). Cell bound radioactivity was measured in a scintillation counter (Packard, United Technologies, USA). Cultures were performed in triplicate and the results are presented as the mean counts per minute (ct/min) per well.

PFC assay. The protein-A PFC assay was performed as described by Gronowicz, Coutinho & Melchers (1976) and modified by Bird & Britton (1979). Sheep red blood cells (SRBC, Flow Laboratories, UK) were washed six times in normal saline and were then coated with protein-A (Pharmacia, Uppsala, Sweden) by incubating one volume of SRBC with one volume of protein-A (0·5 mg/ml) and 10 volumes of chromic chloride ($2{\cdot}5 \times 10^{-4}$M, Analar, BDH Chemicals, Poole, England) for 45 min at 37°C with continuous rotation. After incubation, the protein-A coated SRBC were washed three times with Hank's Balanced Salt Solution (HBSS, Gibco, Paisley, Scotland) and used in plaque assays for up to 5 days after preparation. To perform the plaque assay, 25 μl protein-A-coated SRBC, 100 μl of lymphocyte suspension, 25 μl (1 : 30) rabbit antisera specific for the heavy chains of human IgA, IgG or IgM (Dako Immunoglobulins, Copenhagen, Denmark) and 25 μl (1 : 4) of SRBC absorbed guinea pig complement (Sera-lab, England) diluted in HBSS were added to 750 μl of agar supplemented with DEAE-dextran (2 mg/ml, Pharmacia, Uppsala, Sweden) and 1% polyethylene glycol 6000 (PEG 6000, Sigma, St Louis, USA) kept in a 46°C water

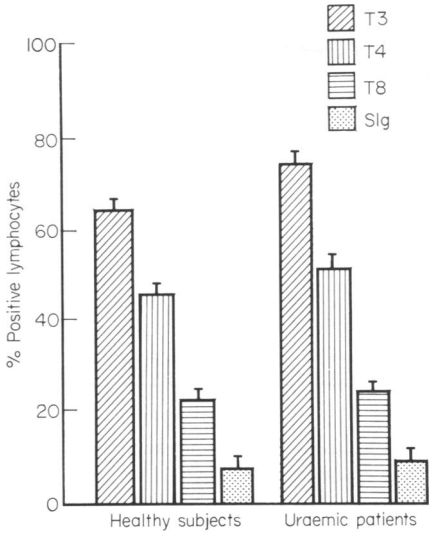

Fig. 1. Proportions of T lymphocyte populations and B cells in peripheral blood of healthy subjects and patients with uraemia. Results are mean + 1 s.e. of the percentage of the total lymphocyte count. B cells were measured by the presence of surface immunoglobulin light chains (SIg).

bath. After mixing in a vortex mixer, three 200 μl drops were pipetted into a plastic 90 mm Petri dish (Sterilin, Teddington, England) and a 24 mm × 32 mm coverslip immediately placed on top of each drop. The plates were then incubated for 8 h at 37°C in a 5% in air in a humidified incubator. Potential PFC were identified by naked eye as holes in the agar and the presence of a central lymphoid cell at the centre of haemolytic spots was confirmed by microscopy. These plaques were counted and the results were expressed as the mean PFC count (of triplicate determinations) per million plated cells.

Production of immunoglobulin in response to PBA. PBMC were cultured in the presence of pokeweed mitogen (PWM) (Gibco, Paisley, Scotland), *Staphylococcus aureus* Cowan Strain I (SAC), or Epstein–Barr virus (EBV). SAC was prepared following the procedure described by Forsgren, Svedjelund & Wigzell (1976). Supernatant from the B95-8 marmoset cell line was used as source of EBV (Dr A. Campbell, Department of Biochemistry, University of Glasgow). Briefly, $0{\cdot}5 \times 10^6$ PBMC/ml were cultured for 6 days in plastic tubes (12 mm × 75 mm, Sterilin, Teddington, England) before being washed twice in RPMI 1640 and assayed for immunoglobulin secreting cells using the plaque assay as described above. PBMC were cultured in the presence of 1/100 PWM, or 1/1000 SAC or were exposed to EBV by suspending 1×10^6 cells in 0·2 ml of virus containing supernatant and incubating the cell pellet for 60 min at 37°C (Bird & Britton, 1979).

Indomethacin. Indomethacin (Sigma, Poole, Dorset, UK) was first dissolved in 0·15 M Tris-buffer (pH = 8·8) and then made up in RPMI 1640 (1 μg/ml) before addition to PWM and SAC cultures.

Statistical analysis. Statistical analysis was carried out using Student's unpaired *t*-test or the Signed Rank test. Results were expressed as the mean ± s.e.m.

RESULTS

T and B lymphocyte studies. Total white cell counts and lymphocyte counts of uraemic patients were both within the normal range (data not shown). The results from B and T lymphocyte studies performed in 10 patients with uraemia and 20 healthy subjects were expressed as percentage of the total lymphocyte count and are presented in Fig. 1. Patients with uraemia had a normal proportion

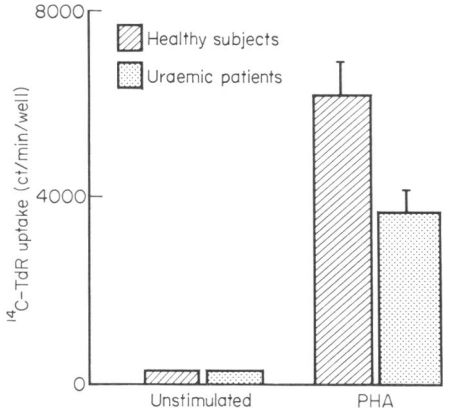

Fig. 2. Proliferative responses to PHA by PBMC from healthy subjects or patients with uraemia. Results shown are the mean ct/min well + 1 s.e.m. in 72 h cultures.

of T cells as measured by the monoclonal antibody OKT3 and the percentage of OKT8$^+$ and OKT4$^+$ cells were also not significantly different from those found in healthy controls. Uraemic patients also had a normal proportion of B cells, as measured by the presence of surface immunoglobulin light chains.

Proliferative responses of T lymphocyts in vitro. PBMC from 15 healthy controls and 17 uraemic patients were stimulated with PHA and their proliferative responses assessed on day 3. It can be seen that the responses to PHA were significantly lower in uraemic patients compared with controls (Fig. 2, $P < 0{\cdot}0001$).

Spontaneous production of immunoglobulin by uraemic patients. The spontaneous secretion of IgA, IgG, and IgM by freshly harvested uraemic and control cells was investigated by measuring plaque formation over 8 h in the absence of mitogens. As shown in Fig. 3A, uraemic patients had very low levels of all immunoglobulin classes, with IgA- and IgG-PFC numbers around 25%–30% of levels found in controls ($P < 0{\cdot}001$). IgM-PFC were also suppressed, although this was not as marked as with IgA and IgG (40% of control levels, $P < 0{\cdot}05$). Unstimulated uraemic PBMC produced more IgG than IgA, whereas the opposite pattern was observed in the control group. However, this difference between IgA- and IgG-PFC counts in the uraemic group failed to reach statistical significance.

Production of immunoglobulin in response to PBA. The ability of uraemic PBMC to produce immunoglobulin in response to polyclonal B cell activators was studied by counting immunoglobulin secreting cells in cultures stimulated with PWM, SAC or EBV.

The results of the PWM induced cultures are presented in Fig. 3B and it can be seen that IgA, IgG, and IgM-PFC counts were not significantly different from those in healthy controls. In addition, both groups showed higher IgG responses than either IgM or IgA. The PWM-induced PFC responses of haemodialysis patients did not differ significantly from those of the CAPD patients (data not shown).

In contrast to PWM, uraemic patients had significantly lower IgA, IgG and IgM responses to SAC compared with the control group (Fig. 3C $P < 0{\cdot}02$, $P < 0{\cdot}001$, $P < 0{\cdot}001$, respectively). This was most pronounced for IgG and IgM responses and, unlike controls, the IgG response of uraemics to SAC was no higher than the IgA and IgM responses, with very similar numbers of PFC being found for all classes.

When the responses of control and uraemic PBMC to EBV were studied, equivalent PFC responses were found in the two groups, with only IgM-PFC responses being slightly but not significantly lower in uraemic subjects (Fig. 3D).

Effect of indomethacin on production of immunoglobulin in vitro. Chronic uraemia has been shown to suppress mitogen responses of rat spleen cells via an adherent cell (Alevy, Slavin &

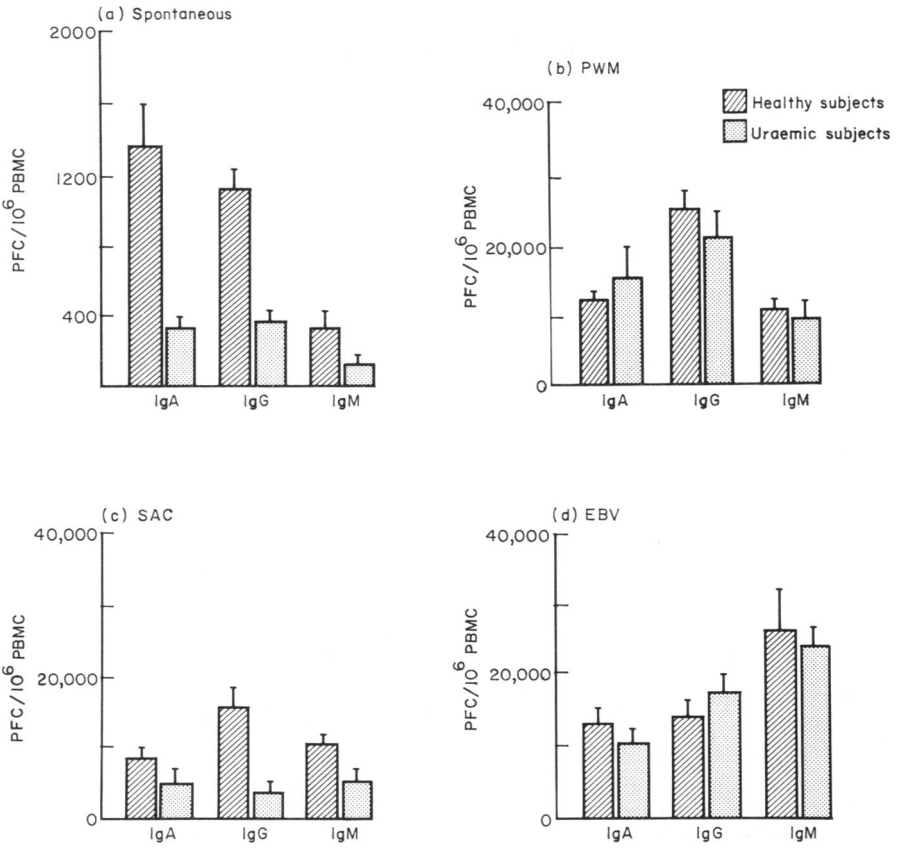

Fig. 3. Immunoglobulin production *in vitro* by PBMC from healthy subjects or patients with uraemia. Spontaneous production of IgA, IgG and IgM (A) and responses to PWM (B), SAC (C) and EBV (D) measured by the protein A plaque forming cell assay. Results shown are mean PFC/10^6 PBMC + 1 s.e.m. obtained after 6 days culture with mitogens.

Table 1. The effect of indomethacin on PWM and SAC induced production of immunoglobulin by normal PBMC (mean IgG-PFC/10^6 cells of triplicate determinations from six healthy subjects; the percentage values represent the proportional change after adding indomethacin)

Subject	PWM	PWM+IND	% Change	SAC	SAC+IND	% Change
1	31735	32134	+1%	15614	18732	+20%
2	21051	22211	+6%	12670	17330	+37%
3	18766	16957	−10%	14935	12201	−19%
4	15785	14902	−6%	21337	26681	+26%
5	33151	35692	+8%	13494	16435	+22%
6	28581	29345	+3%	14313	18625	+30%
Mean+ s.e.m.	24844+ 2964	25206+ 3454	+0·3+3%	15393+ 1261	18334+ 1932	+19+8%

IND, indomethacin.

Table 2. The effect of indomethacin on PWM and SAC induced-production of immunoglobulin by uraemic PBMC (mean IgG-PFC/10^6 cells of triplicate determinations from 11 uraemic patients; the percentage values represent the proportional change after adding indomethacin)

Patient	PWM	PWM+IND	% Change	SAC	SAC+IND	% Change
1	4775	5995	+26%	21506	18500	−14%
2	925	1486	+60%	1088	1660	+52%
3	16958	3557	−79%	1156	3303	+185%
4	4162	2569	−39%	9250	12950	+40%
5	4008	6801	+69%	1798	4162	+131%
6	1541	1982	+28%	1632	2522	+54%
7	14230	12331	−13%	1206	1360	+8%
8	22460	13056	−42%	12065	6727	−44%
9	10551	12662	+20%	5088	4201	−18%
10	40831	52505	+29%	3816	8174	+114%
11	30649	19955	−35%	2520	8055	+219%
Mean± s.e.m.	13735+ 3946	12081+ 4282	+2±14%	5556+ 1941	6510+ 1596	+66±26%

IND, indomethacin.

Hutcheson, 1981). As it seemed possible that this suppression was mediated by prostaglandins, we studied the role of prostaglandins in regulating *in vitro* production of immunoglobulin by uraemic PBMC, by adding indomethacin to cultures stimulated with PWM or SAC.

Table 1 shows that indomethacin had no overall effect on PWM-induced production of immunoglobulin by control PBMC. While there was a increased IgG-PFC response to SAC with indomethacin in five of the six control PBMC, this effect was relatively small (Table 1). When indomethacin was added to PWM cultures of uraemic PBMC, a variable effect was seen. In six of 11 patients there was an increased IgG-PFC response which ranged from 20% to 69% above the background levels while in the remaining five cases a decrease occurred (Table 2). In contrast, when indomethacin was added to cultures stimulated with SAC, eight of 11 uraemic patients showed an increased IgG-PFC response which was frequently very marked, with up to 219% enhancement, although a decreased response was seen in three cases (Table 2).

While the overall effect of indomethacin on uraemic cells was not statistically significant, it appears that the PBMC of uraemic patients are more sensitive to enhancement by indomethacin than those of controls.

DISCUSSION

In this study we have found that both T cell subset and B cell proportions were normal in previously non-transfused uraemic patients. This contrasts with recent reports of low helper T cell numbers in uraemia (Lortan *et al.*, 1982; Raska *et al.*, 1983), but our results may be a result of our studying a non-transfused population (Bender *et al.*, 1984). As there is now clear evidence that blood transfusion results in depression of the immune response (Kahan, 1984), we have studied only patients who have never been transfused so that any abnormalities are likely to be due to the uraemia rather than blood transfusion. Such an influence may well have gone unrecognized in some of the previous studies in this field.

The low PHA responses in our uraemic patients confirm a number of previous reports of depressed proliferative responses to T cell mitogens (Huber *et al.*, 1969; Kauffman *et al.*, 1975; Holdsworth *et al.*, 1978; Kunori *et al.*, 1980; Alevy & Slavin, 1981) and indicate a functional rather than a numerical T cell defect.

The spontaneous production of immunoglobulin by uraemic PBMC was impaired and this impairment was more pronounced for the IgA and IgG classes, although IgM responses were also significantly lower than normal. This selective pattern has not been reported before and, indeed, our findings contradict previous reports which did not detect low spontaneous immunoglobulin production by uraemic PBMC (Horsburgh, Hall & Wood, 1983; Satomi *et al.*, 1983). Although we cannot explain this discrepancy, the low number of spontaneous PFC which we observed suggested a defect in B cell differentiation. We therefore attempted to identify the nature of this defect using T dependent and independent polyclonal B cell activators.

EBV-induced stimulation of B cells occurs in the absence of T cells (Bird & Britton, 1979; Bird *et al.*, 1981) and our uraemic patients had normal PFC responses to this activator, indicating that, in uraemia, B cells probably retain a normal potential to secrete immunoglobulin. PFC responses to the T cell dependent activator, PWM, were also normal in uraemic patients, suggesting that helper T cells are present and are capable of being stimulated by PWM. Although surprising in view of a defective proliferative response to PHA, this apparent discrepancy may reflect the fact that helper T cells do not require cell division in order to exhibit helper activity (Keightley, Cooper & Lawton, 1976). In contrast to these findings, SAC-induced PFC responses were impaired in uraemia, confirming and extending an earlier report by Kunori *et al.* (1980). While SAC stimulates B cells directly via an interaction between its cell wall protein and immunoglobulin on the B cell surface, and the resulting proliferative response is T cell-independent, full differentiation of the B cell is dependent on T cell help (Falkoff, Zhu & Fauci, 1982). However, unlike PWM, SAC does not stimulate T cells directly (Greaves & Janossy, 1972) and responses to SAC may require preactivated helper T cells. This supports the concept that the defective response to SAC in uraemic patients is due to abnormal pre-activation of helper T cells *in vivo*. Although we cannot exclude a role for suppressor T cells in the defective response to SAC, this seems unlikely as SAC is little influenced by suppressor T cells, whereas responses to PWM should be profoundly affected by increased suppressor T cell activity (Pryjma *et al.*, 1980). That impaired helper T cell activity is responsible for the immune defects in uraemia is also supported by the finding that exogenous IL-2 can restore the abnormally low PHA responses by uraemic PBMC (Langhoff *et al.*, 1985).

An explanation for this helper T cell defect may come from the finding that in uraemia the SAC response was partially restored by indomethacin in most patients. In contrast, neither control responses to SAC, nor any type of PWM response were altered by indomethacin. This finding is supported by the fact that prostaglandin E_2 (PGE_2) inhibits B cell proliferation in response to SAC but not in response to PWM, probably by inhibiting production of B cell differentiation factor (BCDF) (Thompson, Jelinek & Lipsky, 1984). It has also been reported that prostaglandins suppress IL-2 production in man (Rappaport & Dodge, 1982). As IL-2 receptors are expressed on activated B cells (Mingari *et al.*, 1984; Muraguchi *et al.*, 1985), the low spontaneous and SAC responses in our study could be due either to inhibition of IL-2 or BCDF production by release of prostaglandins during uraemia. Inhibition of IL-2 production by PGE might also explain the depressed responses to PHA by uraemic PBMC and it would be of interest to examine directly the effects of prostaglandins and IL-2 on spontaneous and SAC induced plaque formation by uraemic PBMC.

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