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Host immune status in uraemia

II. SERUM FACTORS AND LYMPHOCYTE TRANSFORMATION

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SUMMARY

A model of experimentally induced uraemia has been used to study the effect of serum from uraemic rats on the immune responsiveness of thymus-derived (T) lymphocytes. Splenic lymphocytes from normal or uraemic animals responded to mitogenic stimulation with concanavalin A to a similar degree when cultured in a tissue culture medium containing the maximum non-toxic concentration of normal or uraemic serum in the culture system (3%). Serum from uraemic animals, however, had an immunosuppressive effect if the serum was first dialysed for 24 hr before being added to the tissue culture medium. When an alternative vessel was used which allowed the concentration of serum in the medium to be increased to 10%, serum from severely uraemic animals markedly suppressed the capacity of lymphocytes from normal animals to respond to Con A. Thus while serum from uraemic animals can be shown to be immunosuppressive, the results of the experiments are influenced by the conditions *in vitro*. The type of culture vessel and the concentration of serum in the culture medium are particularly critical determinants. It is likely that variations in laboratory procedures have contributed to the differences of opinion on the effect of serum from uraemic individuals on lymphocyte function.

INTRODUCTION

In a previous study we have shown that splenic lymphocytes from uraemic rats responded to mitogenic stimulation, *in vitro*, to the same degree as sham-operated rats (Miller & Stewart, 1980). The immune responsiveness of the intact uraemic animals, however, was depressed suggesting the presence of a humoral factor in the serum of uraemic animals. Determination of the precise nature of the immune deficit in uraemia is a matter of importance and in the present experiments the influence of serum from uraemic animals on the mitogenic responsiveness of lymphocytes cultured *in vitro* has been investigated. Under the conditions of the *in vitro* analysis, the optimum non-toxic concentration of normal serum in the culture medium was 3%. Splenic lymphocytes from normal or uraemic animals cultured in medium containing the optimum concentration of normal or uraemic serum responded to mitogenic stimulation to a similar degree. However, when the vessel was modified to allow the concentration of serum in the medium to be increased to 10%, serum from severely uraemic animals markedly suppressed the capacity of normal lymphocytes to respond to Con A. Thus the differing conditions of analysis used in related studies may account for the varying conclusions reached regarding the effect of serum from uraemic individuals on lymphocyte function.

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MATERIALS AND METHODS

Animal strain

Adult female rats (F_1), the progeny of an inbred strain of DA and AS2 rats were used in all these experiments.

Induction of uraemia

A reproducible state of stable uraemia was induced by the controlled resection of the renal parenchyma. Two levels of uraemia, termed 'moderate' and 'severe' were produced. Details of the procedure have been given in an accompanying paper (Miller & Stewart, 1980) and described fully elsewhere (Ormrod & Miller, 1980).

Thymus-derived (T) lymphocyte-specific mitogens

Concanavalin A (0·5 mg) (Con A; Sigma Cat. No. 2010; Sigma Chemical Company, St Louis, Missouri) was dissolved in 1 ml of phosphate-buffered saline (PBS). A fresh solution was prepared for each culture.

In vitro analysis of the mitogenic response to T lymphocyte-specific mitogens

Roswell Park Memorial Institute medium (RPMI 1640; GIBCO Diagnostics, Madison, Wisconsin) was used in all the experiments. Glass-distilled water deionized using Milli Q apparatus (Millipore Corporation, Massachusetts) was used to reconstitute the medium. The Milli Q apparatus was housed in a cool room at 4°C and a timer-switch circulated water for 3 min every 2 hr. Each litre of culture medium contained 70 ml of 2·8% sodium bicarbonate which was added to maintain the culture at pH 7·2 in an atmosphere of 10%CO_2 in air. The medium also contained 100 μg/ml of both penicillin and streptomycin. Rat serum obtained from normal animals was heat-inactivated and added to the medium to give a final concentration of 3% rat serum in RPMI. This preparation formed the complete medium.

Lymphoid cells were obtained from spleens removed under sterile conditions into ice-cold RPMI 1640. Each spleen was held with forceps and teased with a dental excavator to produce a single-cell suspension that was then allowed to stand for 5 min to enable cell clumps and debris to sediment. The cell suspension was then pipetted into a second tube, centrifuged at 400 ***g*** for 5 min and finally resuspended in the complete tissue culture medium (RPMI 1640). The mitogenic responsiveness of splenic lymphocytes from uraemic animals was analysed using tissue culture tubes (Falcon Cat. No. 3033; Oxnard, California) and Marbrook culture vessels (Marbrook, 1967). In the standard test-tube culture, $2{\cdot}5 \times 10^6$ splenic lymphocytes were cultured in 3 ml of complete medium. Five microlitres of the 0·5 mg/ml solution of Con A was added to each ml of culture medium. In the standard Marbrook culture, 5×10^6 splenic lymphocytes in 1 ml of complete medium were suspended on a dialysis membrane in the insert-tube of a Marbrook culture vessel which contained 10 ml of RPMI 1640 medium in the external reservoir. Twenty microlitres of the 0·5 mg/ml solution of Con A was added to the contents of each 'insert-tube.' Non-stimulated cultures containing splenocytes without Con A served as a control for each cell suspension assayed in both culture systems. All cultures were held for 90 hr at 37°C in a humid atmosphere of 10% CO_2 in air. One microcurie of ^{3}H-thymidine, 2 Ci/mmol, (Radiochemical Centre, Amersham, England) was added to each culture 16 hr before harvesting. The amount of ^{3}H-thymidine incorporated into cellular DNA was determined after terminating the culture by the addition of distilled water followed by filtration of each culture through a Whatman GFC 2·5-cm glass fibre filter (Whatman Incorporated, Clifton, New Hampshire) held in a machined metal block. The contents of the test-tube were rinsed with distilled water and finally with 1 and 5% trichloroacetic acid to precipitate macromolecules retained in the fibreglass filters. Filters were transferred to glass vials for ^{3}H-thymidine counting and dried at 37°C for 24 hr. Ten millilitres of scintillation fluid was then added and the vials were counted in a Beckman model B liquid scintillation spectrometer (Beckman Instruments Incorporated, Fullerton, California). Mitogenic stimulation of splenocytes from normal animals resulted in a ^{3}H-thymidine incorporation of approximately 250,000 d.p.m./10^6 lymphocytes after the addition of 1μCi of ^{3}H-thymidine. In unstimulated cultures, less than 10,000 d.p.m./10^6 lymphocytes were recorded.

Confidence limits of the culture method
These details have been given in an accompanying paper (Miller & Stewart, 1980).

RESULTS

Effect of normal serum on the maximum mitogenic response in culture
Cultures of splenic lymphocytes from a pool of normal animals were established in tissue culture medium containing increasing amounts of normal serum from 1–5%. Optimum responses to mitogenic stimulation with Con A were found when the tissue culture medium contained 3% of serum. Higher concentrations were inhibitory (Fig. 1).

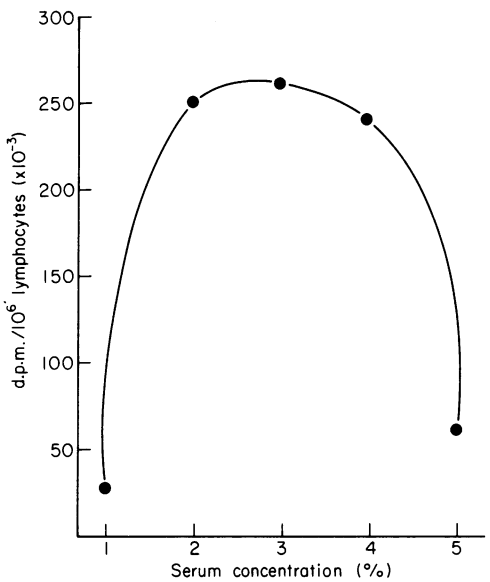

Fig. 1. Effect of normal rat serum on mitogenic responsiveness (Con A). Splenic lymphocytes ($2{\cdot}5 \times 10^6$) from normal animals were cultured in tissue culture medium containing increasing amounts of serum. Each point is the mean amount of ^{3}H-thymidine incorporated by cultures of splenic lymphocytes.

Effect of serum from uraemic animals on the mitogenic responsiveness of splenic lymphocytes
The mitogenic responsiveness of lymphocytes from normal, sham-operated and uraemic animals, cultured in normal and uraemic serum was determined. A concentration of 3% serum in tissue culture medium was used in all the experiments and the analysis of each serum sample was carried out with a range of cells in culture from $2{\cdot}5$ to $0{\cdot}3 \times 10^6$. The results are shown in Fig. 2. Serum from sham-operated animals, added to the tissue culture medium, did not affect the mitogenic responsiveness of normal lymphocytes when compared with normal serum. Cells from sham-operated animals showed a moderately depressed response at the highest cell concentration in both normal and sham serum but comparable or even elevated responses at lower cell concentrations. Serum from moderately uraemic animals did not adversely affect normal lymphocytes and, in fact, the mitogenic responsiveness of the normal cells was increased when cultured in the presence of uraemic serum. The response of lymphocytes from moderately uraemic animals was comparable in normal and uraemic serum. Similar results were obtained when serum from severely uraemic animals was used in culture. Normal splenic lymphocytes responded equally well in tissue culture medium containing normal or uraemic serum and the responsiveness of uraemic lymphocytes, although reduced, was not influenced by the source of serum in the culture medium.

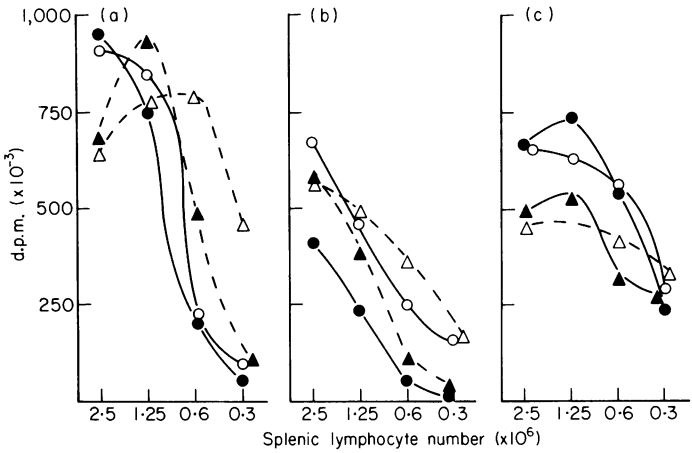

Fig. 2. Effect of serum from sham-operated and uraemic animals on mitogenic responsiveness of splenic lymphocytes from normal and uraemic animals. (a) (●——●) Lymphocytes – normal, serum – normal; (○——○) lymphocytes – normal, serum – sham; (▲- - - -▲) lymphocytes – sham, serum – normal; (△- - - -△) lymphocytes – sham, serum – sham. (b) (●——●) Lymphocytes – normal, serum – normal; (○——○) lymphocytes – normal, serum – moderate uraemia; (▲- - - -▲) lymphocytes – moderate uraemia, serum – normal; (△- - - -△) lymphocytes – moderate uraemia, serum – moderate uraemia. (c) (●——●) Lymphocytes – normal, serum – normal; (○——○) lymphocytes – normal, serum – severe uraemia; (▲——▲) lymphocytes – severe uraemia, serum – normal; (△- - - -△) lymphocytes – severe uraemia, serum – severe uraemia. Each point is the mean amount of ^{3}H-thymidine incorporated by cultures of splenic lymphocytes carried out in triplicate.

Delayed addition of mitogen to the culture

Under the conditions of the standard culture the stimulatory effect of mitogen added at the beginning of the culture may have overridden any potential inhibitory activity that serum from uraemic animals may have had on lymphocyte function. Accordingly a duplicate series of cultures of splenic lymphocytes from normal, sham-operated and uraemic animals were established. Con A was added at the commencement of the culture to one series and after the culture had been established for 24 hr to the other. The results (Table 1) have shown that even when the addition of mitogen to the culture is delayed it was not possible to demonstrate an inhibitory effect by serum from uraemic animals.

Enhancement of serum-suppressive activity by dialysis

Experiments by other investigators (Nelson & Penrose, 1973) suggested that the depression of mitogenic responsiveness attributed to factors in uraemic serum might be enhanced if serum was

Table 1. Effect of the delayed addition of mitogen

	Addition of mitogen		
Source of serum	No delay (a)	24-hr delay (b)	b/a x 100
Normal animals	332,635*	324,376	98
Sham-operated group	313,852	311,485	99
Moderately uraemic group	322,317	308,147	96
Severely uraemic group	311,309	246,710	79

* Mitogenic response of splenic lymphocytes from normal animals to Con A (d.p.m./10^6 lymphocytes).

Each point is the mean amount of ^{3}H-thymidine incorporated by cultures carried out in triplicate.

Table 2. Dialysis enhances the immunosuppressive activity of serum

Serum source	Non-dialysed serum	Dialysed serum*	$\frac{\text{Dialysed}}{\text{non-dialysed}} \times 100$
Normal animals	699.855†	502.246	72
Sham-operated group	729.170	580.282	80
Moderately uraemic group	639.804	362.186	57
Severely uraemic group	568.446	275.950	49

* Pooled serum from normal, sham-operated and uraemic animals was dialysed at 4°C for 24 hr against three changes of serum-free tissue culture medium.

† Mitogenic response of splenic lymphocytes from normal animals to Con A (d.p.m./10^6 lymphocytes). Each point is the mean amount of ^{3}H-thymidine incorporated by cultures carried out in triplicate.

dialysed before being added to the tissue culture medium. Pooled serum from control, sham-operated and uraemic animals was dialysed at 4°C for 24 hr against three changes of serum-free tissue culture medium. Dialysis did increase the ability of serum to block the response of lymphocytes to mitogenic stimulation (Table 2). The incorporation of dialysed serum from moderately and severely uraemic animals reduced the immune responsiveness of normal splenic lymphocytes to 57 and 49% respectively of the mitogenic response of lymphocytes cultured in medium containing non-dialysed serum.

Effect of serum on mitogenic responsiveness: analysis by an alternative method

One limitation of the tissue culture tube as a vessel for determining the effect of uraemic serum on mitogenic responsiveness was that a concentration of normal serum above 3% in the medium inhibited the splenic lymphocyte response from normal animals to Con A. An alternative culture system has been described by Marbrook (1967) that allows the use of serum concentrations in culture up to 10% before any inhibiting effects are noted (Miller & Creaghe, 1975). Further experiments were carried out in which splenic lymphocytes from normal animals were cultured in these vessels. Serum from normal, sham-operated and severely uraemic animals was added to tissue culture medium to give a concentration of 10%. The mitogenic responses of lymphocytes were mildly suppressed by serum from moderately uraemic animals but serum from severely uraemic animals markedly depressed the capacity of normal lymphocytes to respond to Con A (Fig. 3).

DISCUSSION

Depression of the cell-mediated immune response *in vivo* in the intact uraemic host has been reported consistently (Dammin, Couch & Murray, 1957; Morrison, Manness & Tawes, 1963; Souhami, Smith & Bradfield, 1973) although the immune responsiveness of isolated lymphocyte suspensions may be normal (Johnston & Slavin, 1976). Several explanations accounting for the depressed cell-mediated immune responses in uraemia have been proposed and include the lymphopenia associated with uraemia (Wilson, Kirkpatrick & Talmage, 1965), vitamin B_6 deficiency (Dobbelstein *et al.*, 1974), concomitant antimicrobial therapy (Munster *et al.*, 1977; Tarnawski & Batko, 1973) and the activation of cellular immune suppressor mechanisms. Soluble factors with immunosuppressive activity have been searched for in the serum of uraemic patients (Hanicki *et al.*, 1976; Harris *et al.*, 1972; Slavin & Fitch, 1971) but as yet no single compound or combination of compounds has been able to mimic the immunosuppressive effects noted *in vivo*.

Opinions also vary considerably on the effect of serum from uraemic individuals on lymphocyte function. Some investigators have concluded that such serum may depress the immune function of normal animals (Nelson & Penrose, 1973; Newberry & Sanford, 1971; Silk, 1967) while others have

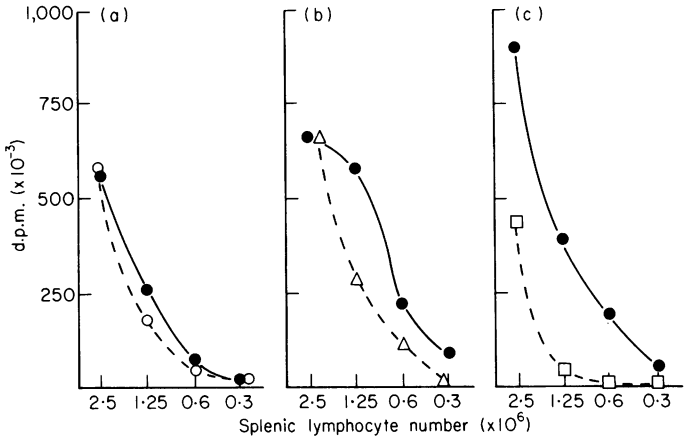

Fig. 3. Use of Marbrook culture vessels to allow increased concentration of serum to be added to the culture medium. Splenic lymphocytes from normal animals were cultured in the presence of serum from normal, sham-operated and uraemic animals. (a) (●——●) Serum – normal; (o– – – –o) serum – sham; (b) (●——●) Serum – normal; (△– – – –△) serum – moderate uraemia; (c) (●——●) serum – normal; (□– – – –□) serum – severe uraemia. Each point is the mean amount of ^{3}H-thymidine incorporated by cultures of splenic lymphocytes carried out in triplicate.

reported contrary results (Kasakura & Lowenstein, 1967). There is even the suggestion that the lymphocytes may respond better to mitogenic stimulation with Con A when cultured in the serum from uraemic patients (Birkeland, 1976).

The difficulties inherent in studying immunosuppressive factors in serum of patients with renal failure of differing aetiology and with varying degrees of uraemia prompted the present study. Under the conditions of the experiment it was possible to analyse serum from groups of animals with defined degrees of renal failure without the variables associated with clinical studies. The experiments have shown that tissue culture conditions are important determinants of the effects that serum from uraemic animals may have on lymphocyte function *in vitro*. Maximum tolerable amounts of serum from uraemic animals added to tissue culture medium did not depress the immune function of normal lymphocytes cultured in a plastic test-tube tissue culture system. Similar results were obtained when lymphocytes were incubated with uraemic serum in tissue culture medium 24 hr before mitogen was added. The effects of any suppressive factors were therefore not masked by the addition of Con A at the commencement of the culture. Serum concentrations above 3% in tissue culture medium could not be used in the test-tube system as even normal serum in culture medium in excess of that figure depressed mitogenic responsiveness.

An immunosuppressive component of uraemic serum could be demonstrated under two sets of conditions. In the first, dialysis of serum from uraemic animals for 24 hr prior to its addition to the culture medium was found to enhance the activity of a component with immunosuppressive activity. The results are in agreement with those of Nelson & Penrose (1973). No attempt was made to identify the dialysable material inhibiting the activity of the immunosuppressive component but our results, and the wide range of ^{3}H-thymidine incorporation found for lymphocytes cultured in different sources of 'normal' serum suggest that dialysable anti-inhibitors may also exist in normal serum. The other set of conditions under which immunosuppressive activity of uraemic serum could be demonstrated involved the use of a double-walled culture vessel which allowed the concentration of serum in the tissue culture medium to be raised from 3 to 10%. Under these circumstances the mitogenic responsiveness of normal splenic lymphocytes was markedly depressed when serum from uraemic animals was incorporated into the medium.

The demonstration of a humoral immunosuppressive factor in renal failure could have far-reaching implications for patient management. *In vitro* analyses such as those described in this report offer the best means of determining such activity, but before conclusions can be drawn or

extrapolations made, the conditions in the assay system need to be fully considered. The effect of serum from uraemic or normal animals on lymphocyte function *in vitro* is the nett outcome of supportive and inhibitory factors. Plasma or serum incorporated into tissue culture medium represents a small proportion of the total medium and there are limitations on the ability of a serum containing synthetic medium to mimic the effect of humoral factors, *in vivo*, on lymphocyte function. The present results have shown that the outcome of an investigation may be clearly influenced by the conditions *in vitro* and, as a minimum requirement, future experiments should include data establishing the effect of varying concentrations of serum on lymphocyte function. The failure to do so in the past may have contributed to the differences of opinion in the literature on this topic.

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