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Interleukin 2 synthesis in the presence of steroids: a model of steroid resistance

K. B. WALKER, JULIA M. POTTER*, A. K. HOUSE *University Department of Surgery, and *Department of Pharmacology, Queen Elizabeth II Medical Centre, Nedlands, Perth, Western Australia*

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SUMMARY

In this study interleukin 2 (IL-2) synthesis by human lymphocytes in the presence and absence of prednisolone in a group of normal subjects has been assessed. An association between suppression *in vitro* of induced phytohaemagglutinin-blastogenesis by prednisolone and synthesis of IL-2 was found. Those subjects whose lymphocytes are identified as steroid-resistant have significantly higher IL-2 activity in the supernatants of both steroid and non-steroid treated lymphocyte cultures than steroid sensitive subjects. The addition of exogenous IL-2 was found to ablate the suppressive effects of steroids on lymphocyte blastogenesis. These results suggest that significantly greater activity of IL-2 in the culture supernatants of steroid resistant subjects may represent a mechanism for glucocorticoid resistance *in vitro* and help explain the relationship between increased loss of grafts and steroid resistance in renal allograft recipients.

Keywords prednisolone interleukin 2 graft rejection transplantation

INTRODUCTION

Mitogen induced blastogenesis of peripheral blood lymphocytes has been used as an indicator of immune responsiveness for many years (Kozower, Veatch & Kaplan, 1974). Recently it has become evident that lymphocytes generate many cell products that play a vital role in the response of the lymphocyte to mitogen or antigen. One of these, interleukin 2 (IL-2), a product of T helper ($OKT4^+$) lymphocytes, is an absolute requirement for the proliferation of lymphocytes (Lachman & Maizel, 1983). Glucocorticosteroids have been shown to inhibit lymphocyte proliferation *in vitro* by specific suppression of IL-2 synthesis (Gillis, Crabtree & Smith, 1979a, b).

Recently, a lymphoproliferative assay has been used to characterize the steroid response *in vitro* of individuals to prednisolone in a normal population. Thus subjects who are resistant to the suppressive effects of prednisolone on lymphocyte blastogenesis *in vitro* have been identified (Walker, Potter & House, 1985). There is an association between increased graft failure in renal dialysis patients and resistance to prednisolone suppression *in vitro* of mitogen-induced blastogenesis (House, Potter & Walker, 1986). To determine if this steroid response *in vitro* is related to IL-2 synthesis, parallel studies of IL-2 production by mitogen-stimulated peripheral blood lymphocytes have been undertaken. These studies have also assessed the effects of prednisolone on IL-2 synthesis by normal lymphocytes.

Correspondence: Dr J.M. Potter, Department of Pharmacology, The Queen Elizabeth II Medical Centre, Nedlands, Perth, Western Australia 6009.

METHODS

Subjects. Normal subjects drawn from hospital and laboratory staff volunteers (eight male, four female: age range 20–47 years) were studied. No subjects were on medication or had any evident disease.

Interleukin 2 assay. The production of IL-2 by lymphocytes stimulated by phytohaemagglutinin (PHA) was assessed by an IL-2 specific lymphoproliferative assay modified from that of Gillis *et al.* (1978). IL-2 dependent target cells were prepared from peripheral blood lymphocytes isolated from normal volunteers using Ficoll-Paque after the method of Böyum (1968). They were cultured at 1×10^6 cells/ml in RPMI 1640 (Flow Labs, Aust.) supplemented with 1% heat-inactivated fetal calf serum (HI-FCS) (CSL, Aust.), 1% penicillin/streptomycin (PS) (Flow Labs, Aust.) and 15 μg/ml PHA (Difco, USA). This suspension was incubated for 72 h at 37°C in a 5% CO_2 atmosphere, then diluted 1 : 2 with lymphocyte conditioned medium (LCM) (cell-free medium from lymphocyte cultures stimulated with PHA for 48 h) and incubated a further 24 h. The cells were harvested, washed once in phosphate buffered saline, pH 7·4 (PBS) and resuspended at $0{\cdot}5 \times 10^6$ cells/ml in culture medium diluted 1 : 1 with LCM. Incubation was continued, with further additions of LCM every 24 to 48 h, subculturing when cell numbers demanded. Twelve days after starting the cell culture the lymphocytes, now responsive to IL-2 and not to PHA, were harvested and used as target cells in the IL-2 lymphoproliferative assay.

Mitogen-induced IL-2 synthesis by peripheral blood lymphocytes was determined as follows: peripheral blood lymphocytes separated from heparinized whole blood as described above were resuspended at 1×10^6 cells/ml in RPMI 1640 supplemented with 1% HI-FCS and 1% PS. A 4 ml volume of cell suspension was cultured in the presence of prednisolone (Steraloids, USA) and PHA diluted in RPMI 1640 to give final concentrations of prednisolone from 0 to 2·8 μmole/l and PHA of 15 μg/ml. Cultures were carried out in six-well 'Linbro' microculture plates (Flow Labs, Aust.) and incubated at 37°C in a 5% CO_2 humidified atmosphere for 48 h, the cell-free culture supernatants then collected and stored at −20°C until assayed.

Before assay the supernatants were dialysed at 4°C overnight using 10,000 mol. wt. cutoff dialysis tubing (Union Carbide, USA) against a 1000 times volume of sterile PBS (pH 7·4) to remove prednisolone. The culture supernatants, in parallel with a high activity standard, were serially diluted in duplicate in RPMI 1640 supplemented with 1% HI-FCS and 1% PS in a 96-well microcytotoxicity tray (Flow Labs, Aust.). The IL-2 dependent target cells (at 1×10^6 cells/ml in 100 μl) were added and the tray incubated for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The cultures were pulse labelled with 1 μCi tritiated thymidine (The Radiochemical Centre, Amersham, UK) 4–6 h before termination of the assay. Cultures were then harvested with a multiple sample automated cell harvester (Titertek, Flow Laboratories, Australia). Quantification of IL-2 activity was achieved by assessing the relative activity of each IL-2-containing supernatant against the high activity standard using probit analysis as described by Gillis *et al.* (1978). The high activity standard used in this assay was obtained by desalting and freeze-drying approximately 1 litre LCM and resuspending in 1/10th original volume RPMI 1640. This standard was given a value of 10 IL-2 units per ml of culture supernatant after the method of Gillis *et al.* (1978).

In vitro sensitivity assay. Assessment of the sensitivity *in vitro* of peripheral blood lymphocytes to prednisolone was assessed after the method of Walker *et al.* (1985). Isolated peripheral blood lymphocytes at a concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 20% HI-FCS and 1% PS were cultured in flat-bottomed microcytotoxicity trays with PHA at a final concentration of 15 μg/ml and prednisolone (0–278 μmole/l). Cultures were incubated for 72 h at 37°C in a 5% CO_2 humidified atmosphere, pulse-labelled for 4–6 h with 1 μCi of ^{3}H-thymidine, then harvested as previously described. Suppression of lymphocyte blastogenesis was expressed as the concentration of prednisolone producing 50% inhibition (ID_{50}), calculated by probit analysis of the percent inhibition vs prednisolone dose response curve (Walker *et al.*, 1985).

The effect of exogenous IL-2 on an individual's response to prednisolone in the steroid sensitivity assay was assessed by the addition of 100 μl of LCM to all wells of the standard steroid sensitivity assay described above.

Statistical analysis. Statistical analysis was by non-parametric techniques using the Statistical Package for the Social Sciences (SPSS, version 9) (Nie *et al.*, 1975; Hull & Nie, 1981).

RESULTS

The response of normal lymphocytes to prednisolone *in vitro* varied greatly, with two apparently distinct populations being identified (Fig. 1). In one group, prednisolone significantly suppressed lymphocyte blastogenesis at low (up to 0·28 μmole/l) concentrations, whilst in the other group, a similar concentration of prednisolone was found to have a negligible effect (Fig. 1). The individual's response has been expressed as the 50% inhibition dose (ID_{50}) of prednisolone (Walker *et al.*, 1985). Those subjects with an ID_{50} of greater than or equal to 1 μmole/l prednisolone have been arbitrarily defined as steroid resistant. It can be seen in Table 1 that the ID_{50} values of those subjects defined as steroid-sensitive are substantially lower than 1 μmole/l, whilst the steroid resistant subjects have ID_{50} values of much greater than 1 μmole/l, reflecting the division of responses in this normal

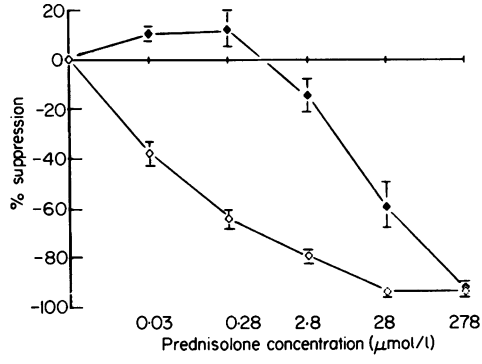

Fig. 1. Normal population response in steroid sensitivity assay: suppression of lymphocyte blastogenesis. This figure represents the mean ± s.e.m. of responses described in Table 1. (♦) Steroid resistant (three subjects); (◊) steroid sensitive (nine subjects).

healthy population as shown in Fig. 1. In four of the subjects, measurements have been repeated on fresh lymphocytes collected several months apart. Whilst the ID_{50} values showed some variation, the cells were consistently sensitive or resistant. The ID_{50} of the lymphocytes in the steroid sensitivity assay was not related to the overall proliferative response (^{3}H-thymidine uptake) ($r = 0{\cdot}465$, $n = 12$, $P > 0{\cdot}1$) or stimulation index ($r = 0{\cdot}002$, $n = 12$, $P > 0{\cdot}5$) (Table 1).

The specificity and sensitivity of the target cells in the IL-2 assay are shown in Fig. 2. Gillis *et al.* (1978) defined IL-2 activity from a $\log_2$ dilution series as 10 units per ml being that concentration producing 50% of maximum blastogenesis in the target cells. It is necessary therefore to ensure that the IL-2 standard medium used in the assay produces maximal blastogenic response in the target cells. This was true for the assay used in this study and is shown in Fig. 2 by the sustained (plateau) response of the cells to the high activity standard over the first few dilutions. The test supernatants, one of which is shown in Fig. 2, were serially diluted in parallel and produced a lesser blastogenic response. Varying PHA concentrations did not alter target cell blastogenesis confirming target lymphocyte IL-2 dependence.

Synthesis of IL-2 in response to PHA challenge was found to vary greatly between individuals, with steroid resistant subjects apparently synthesizing significantly greater amounts of IL-2 than the steroid sensitive individuals ($P < 0{\cdot}05$, Table 1). The synthesis of IL-2 was not related to the proliferative response of the lymphocytes as described by overall response (^{3}H-thymidine uptake) ($r = 0{\cdot}192$, $n = 12$, $P < 0{\cdot}4$) or stimulation index ($r = -0{\cdot}035$, $n = 12$, $P < 0{\cdot}5$). Prednisolone (at a

Table 1. Steroid sensitivity and IL-2 synthesis in lymphocytes from normal subjects

Subject	Sex	ID_{50} (μmole/l)	Thymidine incorporation*		SI†	Interleukin 2	
			Background	PHA		*De novo*‡	2·8 μmole/l§
Steroid sensitive subjects							
M.A.	M	0·31	1208	39297	31·5	1·5	0·07
P.L.	M	0·07	616	107767	173·9	0·10	0·01
M.M.	F	0·36	331	79660	239·6	2·0	0·09
		0·28	475	70772	147·9	2·2	0·7
		0·07	1650	28685	16·4	2·7	1·0
J.M.P.	F	0·006	1253	40294	31·2	0·4	0·2
		0·04	676	50476	73·7	0·5	NA
P.H.	M	0·020	996	18806	17·9	0¶	0¶
P.R.	M	0·07	1823	48662	25·7	0·45	0·13
H.S.	F	0·09	763	53479	69·1	0·18	0·01
		0·09	523	174960	333·0	1·6	0·25
G.S.	M	0·002	1523	17611	10·6	0·27	0·4
N.S.	M	0·03	586	59762	101·0	0¶	0¶
Geometric mean‖							
		0·041	898	44614	48·2	0·11	0·03
Steroid resistant subjects							
J.R.	M	4·4	1005	80650	79·2	4·4	0·9
J.T.	F	22·0	1213	125255	102·3	5·7	1·3
K.B.W.	M	13·4	1097	32579	28·7	9·7	1·7
		119·0	1448	31324	20·6	7·9	1·5
Geometric mean‖							
		10·9	1102	69042	61·5	6·2	1·3

* Counts per minute ^{3}H-thymidine per 10^7 cells.

† Stimulation index (SI) = ct/min PHA − ct/min background/ct/min background.

‡ IL-2 activity as units per ml culture media from PHA-stimulated lymphocytes generated in the absence of prednisolone.

§ IL-2 activity as units per ml culture media from PHA-stimulated lymphocytes generated in the presence of 2·8 μmole/l prednisolone.

‖ In subjects in whom multiple studies were carried out, statistics were calculated using only the first study.

NA, Data not available.

¶ IL-2 activity below the limit of detection of the assay.

concentration of 2·8 μmole/l) was found to suppress the synthesis of IL-2 in both steroid sensitive and steroid resistant subjects by about 75% (resistant = 79%; sensitive = 76%) when compared with PHA stimulated synthesis of IL-2 (Table 1). However, the steroid treated cultures of steroid resistant subjects retained an IL-2 activity comparable to that found in the untreated steroid sensitive subjects (Table 1). Repeated studies over time in four of the subjects gave very similar results for IL-2 production tested both in the presence and absence of prednisolone.

The addition of IL-2 containing supernatant (LCM) (giving an initial concentration of 0·1 unit/ml) to the steroid sensitivity assay resulted in a significant increase in the prednisolone ID_{50} of both steroid sensitive and steroid resistant lymphocytes (Table 2), such that prednisolone sensitive lymphocytes behaved as resistant cells, with the prednisolone ID_{50} near or above 1 μmole/l.

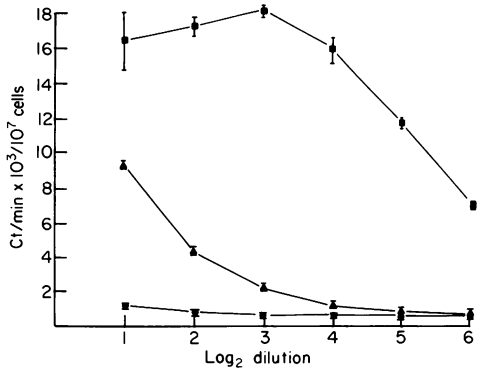

Fig. 2. ^{3}H-thymidine uptake in IL-2 dependent target lymphocytes. This figure represents the mean ± s.e.m. of triplicate cultures from one assay. These responses are representative of this assay system. (■) High activity IL-2 standard medium; (▲) representative subject supernatant; (●) PHA response of target cells. Comparison of the blastogenic response of the target IL-2-dependent lymphocytes, as measured by ^{3}H-thymidine incorporation, is made with that produced by addition of unknown medium. The standard is designated as having an IL-2 activity of 10 units/ml in the dilution giving 50% of maximal blastogenesis of the target cells.

Table 2. Effect of IL-2 on sensitivity to prednisolone *in vitro*

	Prednisolone ID_{50} (μmole/l)	
Subject	Standard assay*	Addition of IL-2†
Steroid sensitive subjects		
M.M.	0·22	0·99
J.P.	0·11	2·10
P.H.	0·07	5·80
Steroid resistant subject		
K.B.W.	16·90	169·50

* ID_{50} of subject in sensitivity assay without the addition of exogenous IL-2.

† ID_{50} of subject in sensitivity assay with the addition of exogenous IL-2 as 100 μl of LCM, giving an initial IL-2 concentration of 0·1 unit/ml.

DISCUSSION

Resistance to steroids *in vitro* has been related to clinical outcome in renal allograft recipients (Walker *et al.*, 1985; House *et al.*, 1986; Dumble *et al.*, 1981). In retrospective studies, using the lymphoproliferative model described in this paper, steroid resistance *in vitro* among patients on maintenance haemodialysis was associated with an increased incidence of previous renal allograft loss due to acute rejection (Walker *et al.*, 1985; House *et al.*, 1986).

The frequency of steroid resistance in the normal population appears somewhat low, of the order of 15% and fewer than in a haemodialysis population (Walker *et al.*, 1985; House *et al.*, 1986); the biological significance and mechanism of this resistance to steroids is unclear. Resistance to steroid therapy in leukaemic patients has been attributed to low steroid receptor numbers (Lippmann *et al.*, 1973), but there is little evidence to suggest such an association in the normal population (Homo-Delarche, 1984). On the contrary some recent observations by our group have suggested the opposite, that is, high receptor numbers in individuals may correlate with steroid resistance *in vitro* (unpublished observations).

A wide variation in IL-2 synthesis among normal subjects has been observed (Bonnard, Yasaki & Maca, 1980) and defective IL-2 production has been implicated in some immunoregulatory dysfunctions (Paganelli *et al.*, 1983); unfortunately, these studies did not attempt quantification of IL-2 synthesis. Steroids, including dexamethasone, methylprednisolone and prednisolone have been shown to suppress mitogen-induced blastogenesis in lymphocytes by specific suppression of IL-2 production (Gillis *et al.* 1979a, b; Larsson, 1980; Kaplan *et al.*, 1983). In this study of normal individuals, IL-2 synthesis in response to PHA challenge is significantly greater in steroid resistant subjects than those classified as steroid sensitive. This was apparently independent of blastogenesis as measured by ^{3}H-thymidine incorporation. Significant IL-2 activity was noted in the culture supernatants of the resistant subjects in the presence of high concentrations of prednisolone (2·8 μmole/l) which markedly suppressed IL-2 synthesis by steroid sensitive lymphocytes.

In this current study it is apparent that the high level of IL-2 synthesis is associated with continued blastogenesis in the presence of steroids. Exogenous IL-2 significantly increased the ID_{50} in an individual assay, lending further support to the hypothesis that steroid resistance *in vitro* and increased IL-2 activity are related and confirming earlier observations on the effect of exogenous IL-2 on steroid-induced suppression of lymphocyte blastogenesis (Palacios & Sugawara, 1982). In the case of renal allograft recipients, continued lymphocyte responsiveness in the presence of pharmacological concentrations of prednisolone may explain the association between steroid resistance *in vitro* and graft loss from acute rejection (Walker *et al.*, 1985; House *et al.*, 1986; Dumble *et al.*, 1981).

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