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Placental protein 14 (PP14) inhibits the synthesis of interleukin-2 and the release of soluble interleukin-2 receptors from phytohaemagglutinin-stimulated lymphocytes

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SUMMARY

Crude human decidual extracts containing up to 15·0 mg/l PP14 were investigated for their effects on the release of interleukin-2 (IL-2) and of IL-2 receptor (IL-2R) from phytohaemagglutinin (PHA) stimulated lymphocytes. The crude decidual extract suppressed IL-2 production over the culture period investigated (0–90 h) with maximal suppression observed at around 66 h of culture. The suppression was dose dependent over the range of PP14 concentrations used (0–7·0 mg/*l*). Decidual extracts also inhibited the release of soluble IL-2R into the culture supernatants over the same period. In both cases, the specific reduction of the PP14 content of the extracts by a monoclonal anti-PP14 immunoadsorbent reduced the observed suppression. These results suggest that PP14 inhibits the production of IL-2 from mitogenically stimulated lymphocytes, and leads to a reduced IL-2R release. The mechanism for such an effect is as yet unknown; however, it may explain the previously reported immunosuppressive activity of PP14.

Keywords placental protein immunosuppression interleukin-2 interleukin-2 receptors

INTRODUCTION

The female reproductive tract is not an immunologically privileged site, and a number of mechanisms have been proposed to explain the continued survival of the fetal allograft. One such mechanism may be the presence of immunomodulatory molecules within the feto-placental unit. PP14 appears in the maternal circulation at peak levels during the first trimester of pregnancy and is also found in decidual tissue (Julkunen *et al.*, 1985), and has previously been investigated for such activity; studies have shown PP14 to exhibit immunosuppressive activity in both the allogeneic mixed lymphocyte reaction (Bolton *et al.*, 1986; 1987) and lymphocyte responsiveness to phytohaemagglutinin (PHA) (Pockley *et al.*, 1988).

Central to the proliferative responses observed in both of these culture systems is the secretion of interleukin-2 (IL-2) and the expression of the IL-2 receptor (IL-2R). The detectable levels of IL-2 in culture supernatants stimulated with PHA have been reported to peak at 18–24 h (Bruserud, Degre & Thorsby, 1986), preceding peak IL-2R expression which occurs after 48–72 h (Depper *et al.*, 1984). The expressed receptors comprise two populations, high- and low-affinity receptors (Robb *et al.*, 1984). The interaction of IL-2 with the high-affinity IL-2R mediates the growth-promoting response of IL-2 on lymphocytes (Robb, Greene & Rusk, 1984).

It has been shown that IL-2R are released by activated lymphocytes and can be detected in culture supernatants of lectin-activated cells. The soluble IL-2R is of lower mol. wt than the IL-2R isolated from the cells (Rubin *et al.*, 1985). Receptor release may represent a method by which an immune response is terminated, or it may serve as an immunoregulatory mechanism by competing for available IL-2 (Rubin *et al.*, 1985).

In this study we have investigated the effects of PP14 on the production of IL-2 by mitogen-stimulated lymphocytes and also on the levels of soluble IL-2R in cell culture supernatants, in an attempt to confirm and further identify the levels of control of the immunosuppressive activity of PP14.

MATERIALS AND METHODS

Decidual tissue extracts

Following aspiration of the uterine contents at legal pregnancy termination at 12–14 weeks gestation, decidual tissue was immediately separated by dissection, and weighed. The nature of the tissue was subsequently confirmed by histological examination. The tissue was rinsed in ice-cold, phosphate-buffered saline (PBS) containing 0·1 mmol/*l* of the protease inhibitor phemylmethylsulphonyl fluoride (PMSF, Sigma Chemical Co.,

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Poole, UK). The tissue samples were homogenized in PBS containing PMSF (10 ml/g wet weight of tissue) and the cell debris was removed by centrifugation (5000 *g* for 30 min at 4°C). The extracts were dialysed against PBS containing PMSF (100 volumes, three changes) at 4°C for 24 h. Extracts were stored at −20°C before use.

PP14 monoclonal antibody immunoadsorbent

Monoclonal antibody was generated by standard procedures as described previously (Bolton *et al.*, 1987), using selected hybridomas derived from splenocytes of mice immunized with extracts of first-trimester human deciduum, to induce ascites tumours. IgG was isolated from ascitic fluid by HPLC ion-exchange chromatography on TSK 5000 PW (Pharmacia, Milton Keynes, UK). This monoclonal IgG antibody showed no detectable binding (<1% cross-reaction) to the following purified human proteins: chorionic gonadotrophin, placental lactogen, pregnancy-associated plasma protein A, placental protein 5, placental protein 12, prolactin, pregnancy-specific beta-1 glycoprotein (SP1) and the placental enzymes malic dehydrogenase, alkaline phosphatase, arylamidase, sphingomyelinase, choline acetyl transferase, and RNAse inhibitor. Such binding was assessed both in a two-site immunoradiometric assay using polyclonal anti-whole human placenta (shown to bind PP14) as capture antibody, and the monoclonal antibody as label (all the proteins tested), and by direct binding of radioiodinated antigen to the monoclonal antibody (all the proteins tested, except the placental enzymes).

The immunoadsorbent was prepared by coupling the monoclonal antibody to cyanogen bromide-activated sepharose 4B (Pharmacia) according to the manufacturers' instructions. Control immunoadsorbent was prepared by blocking cyanogen bromide-activated sepharose with an excess of glycine. Immunoadsorbtion was carried out by treating 1·0 ml of decidual extract with an equal volume of immunoadsorbent or control gel, followed by end-over-end mixing for 3 h at room temperature. The gel was gently centrifuged (300 *g* for 5 min at 4°C), and the supernatant was aspirated and used either as immunoadsorbed (treated with immunoadsorbent gel) or non-adsorbed (treated with control gel) decidual extract in the experiments described.

PP14 radioimmunoassay

The PP14 content of the decidual extracts was measured by radioimmunoassay as described previously (Bolton *et al.*, 1983). Briefly, 100-μl aliquots of standards of purified antigen (PP14 lot 120/135, Behringwerke, Marburg-Lahn, FRG, prepared from term placenta) or decidual extract diluted in assay buffer (0·05 mol/*l* phosphate, pH 7·5, containing 1·0% w/v bovine serum albumin and 0·01% sodium azide) were mixed with 200 μl of radio-iodinated PP14 at a concentration of 1·0 μg/l and containing a 1:133 dilution of normal rabbit serum; 200 μl of polyclonal antiserum (rabbit anti-human PP14, lot 201 ZA, Behringwerke) were added at a dilution to bind about 50% of the added tracer. After incubation overnight at room temperature, antibody-bound was separated from free antigen by the addition of 1·0 ml of a 1:67 dilution of donkey anti-rabbit precipitating antiserum (RD17, Wellcome Diagnostics, Dartford, UK) in 7% w/v polyethylene glycol 6000 in assay buffer, followed by incubation for 30 min at room temperature and sedimentation of the precipitated antibody by centrifugation at 1500 *g* for 30 min at room temperature. The precipitate (antibody bound) fraction of the tracer was measured.

Table 1. The effect of exogenous rIL-2 (2·5 U/ml) on the inhibition of ^{3}H-Tdr uptake into PHA-stimulated lymphocytes by extracts of human decidual tissue

Decidual extract	PP14 content (mg/*l*)	Inhibition of ^{3}H-Thymidine uptake (%) without IL-2	Inhibition of ^{3}H-Thymidine uptake (%) with IL-2
A	5·0	31·0±1·0	15·0±1·8
B	3·5	46·0±1·7	18·0±0·9
C	4·0	48·0±7·3	36·0±5·7
D	1·6	20·0±3·2	0
E	2·2	35·0±9·5	25·0±3·1
F	4·6	58·0±14·5	33·0±3·6
Mean		40·0±14·0	21·0±13·2*

Results are expressed as mean ± s.d.; $n=3$.
* $P<0·002$ (paired Student's *t*-test).

Mitogenic responses

Peripheral blood mononuclear cells were isolated from whole blood by density gradient centrifugation as described by Boyum (1968), using Lymphopaque lymphocyte separation medium (specific gravity 1·084, Nycomed, UK). The isolated lymphocytes were washed and finally resuspended at a concentration of 1×10^6 viable cells/ml (as assessed by Trypan blue dye exclusion) in SF1 serum-free hybridoma growth medium (Northumbria Biologicals, UK) containing 3·7 g/l $NaHCO_3$, 200 IU/ml penicillin, and 200 μg/ml streptomycin.

For the mitogenic stimulation assay, 100 μl-aliquots of the washed cell suspension (1×10^5 cells/well) were incubated in the presence of 50 μl of the PP14 preparation, 50 μl of PHA (2·5 μg/ml final concentration, Sigma), and 50 μl of growth medium, in 96-well microtitre culture plates (Linbro, Flow Laboratories, Inglewood, CA). The plates were incubated at 100% humidity, 37°C and 5% CO_2 for 72 h. Six hours prior to the termination of the cultures, the cells were pulsed with 1 μCi of ^{3}H-thymidine (90 Ci/mmol, Amersham International, Amersham, UK) and on termination were harvested onto glass fibre filters using a cell harvester (Skatron, Norway). The degree of lymphoproliferation was assessed by liquid scintillation counting (LKB Rackbeta). All cultures were performed in triplicate.

Preparation of IL-2-containing supernatants

Peripheral blood mononuclear cells were isolated as described earlier; 10^6 cells were cultured in 24-well culture plates (Linbro, Flow Laboratories) in the presence of 200 μl of the PP14 preparation and 200 μl of PHA (5 μg/ml final concentration) for the times indicated in Results. Control cultures contained PBS in place of the PP14 preparation, and Dulbecco's modification of Eagle's medium (DMEM) in place of the mitogen solution. At the termination of the culture period, the culture supernatants were harvested and stored at −20°C until assay.

Cell culture supernatants were assayed for IL-2 following the manufacturer's recommendations, using a Genzyme Inter-

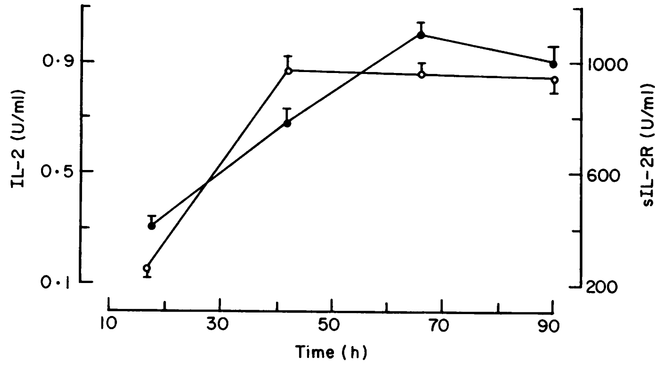

Fig. 1. Production of IL-2 (●) and the release of sIL-2R (○) from PHA-stimulated lymphocytes. Mean ± s.d.; $n=3$.

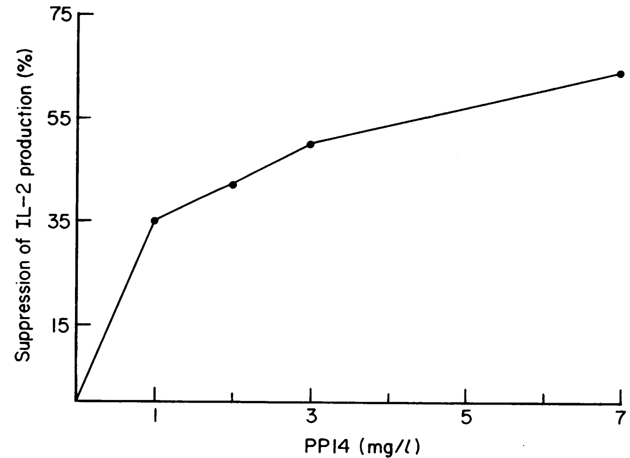

Fig. 3. Effect of crude decidual extracts, related to the PP14 content, on the release of IL-2 from PHA-stimulated lymphocytes. Means of duplicate experiments, concordant in their interpretation.

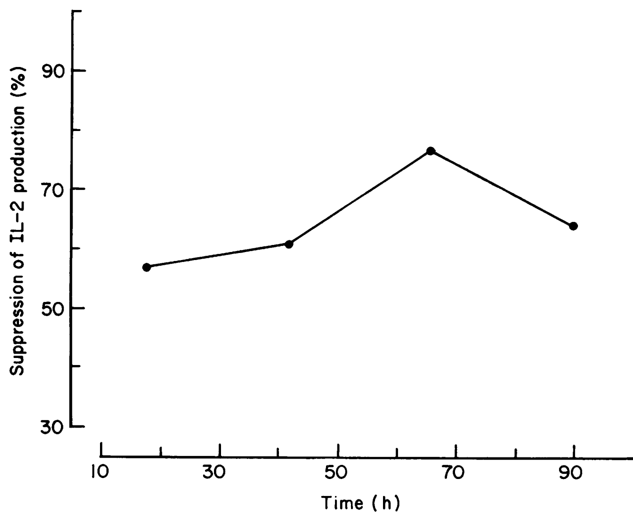

Fig. 2. Time-dependent inhibition of IL-2 release from PHA-stimulated lymphocytes by non-immunoadsorbed decidual extract (PP14 concentration 8·0 mg/*l*), expressed as a percentage of levels in the control (immunoadsorbed decidual extract, PP14 concentration < 1·0 mg/*l*). Means of duplicate experiments, concordant in their interpretation.

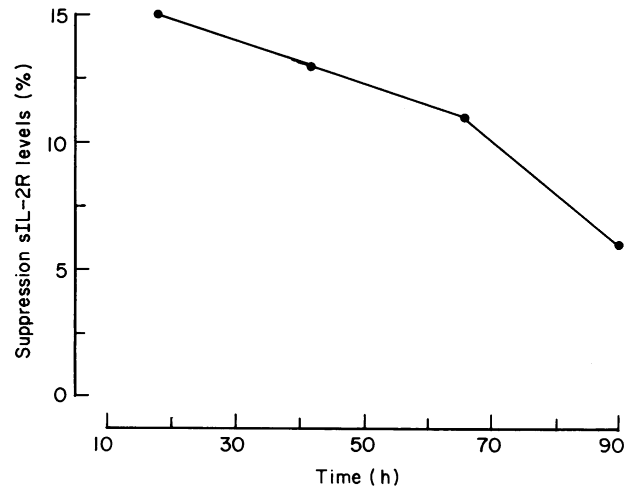

Fig. 4. The suppression of sIL-2R levels by non-immunoadsorbed decidual extract (PP14 concentration 8·0 mg/*l*) expressed as a percentage of levels of the control (immunoadsorbed decidual extract, PP14 concentration < 1·0 mg/*l*). Means of duplicate experiments, concordant in their interpretation.

test 2 human IL-2 ELISA kit (Genzyme Corporation, Boston, MA). The resulting colour was read using a CLS 962 plate reader (Cambridge Life Sciences, Cambridge, UK). Cell culture supernatants were also assayed for the released form of the IL-2R protein from activated T lymphocytes (sIL-2R) using a T cell Sciences Cellfree IL-2R test kit (T Cell Sciences, Cambridge, MA).

RESULTS

The incorporation of six individual decidual extracts with PP14 concentrations of between 1·6 and 5·0 mg/*l* into the mitogenic stimulation assay significantly inhibited the uptake of ^{3}H-Tdr into the stimulated lymphocytes as compared with controls containing no tissue extract (40 ± 14% inhibition, $P<0{\cdot}001$). The observed inhibition was significantly reduced by adding 2·5 U/ml of recombinant IL-2 (rIL-2 ala-125, Amersham International) (21 ± 13% inhibition, $P<0{\cdot}002$; Table 1).

On assaying cell culture supernatants for IL-2, detectable levels of IL-2 (0·25 U/ml) were observed after 18 h of PHA stimulation with a maximum level of IL-2 detectable in the culture supernatants of PHA stimulated lymphocytes, occurring after around 66 h of incubation (Fig. 1). The incorporation of decidual extract (PP14 concentration 8·0 mg/*l*) into the culture system elicited an inhibition of IL-2 production throughout the culture period investigated (0–90 h) as compared with the immunoadsorbed control extract (PP14 concentration < 1·0 mg/l). The maximum inhibition of IL-2 production by decidual extract, expressed as a percentage of the immunoadsorbed control, was observed after around 66 h of culture (Fig. 2); this coincided with maximum IL-2 levels. After 72 h of culture, the

suppression of IL-2 levels by decidual extracts was dose dependent and related to their PP14 content as measured by radioimmunoassay (Fig. 3).

The levels of sIL-2R released from PHA-stimulated cells reached a maximum after around 40 h of culture and remained at this level until the end of the culture period investigated. The highest levels of sIL-2R reached preceded the highest levels of IL-2 by around 24 h (Fig. 1). sIL-2R were not detectable in the culture supernatants of unstimulated cells at any time during the culture period. There was a small, but significant ($P < 0{\cdot}001$, paired Student's *t*-test) inhibition of sIL-2R levels by unadsorbed decidual extract, compared with immunoadsorbed decidual extract over the whole culture period (Fig. 4). The observed inhibitory effect of PP14 decreased towards the end of the culture period investigated.

DISCUSSION

Much previous work on the effect of pregnancy-associated proteins on the lymphoproliferative response has been suspect due to the lack of purity of the protein preparations used with the possibility of impurities exhibiting any observed inhibitory effects. In an attempt to overcome such problems, we employed an appraoch involving the specific removal of PP14 from crude decidual extracts by immunoadsorption with a sepharose 4B-linked monoclonal anti-PP14 immunoadsorbent, using unsubstituted gell as a control absorbent. Using this approach we have previously shown that the suppressive effect of decidual extracts on mitogen-stimulated lymphoproliferation was significantly correlated to the PP14 ($r = 0{\cdot}96$, $P < 0{\cdot}001$), but not the total protein ($r = -0{\cdot}26$, NS) content of the extracts (Pockley *et al.*, 1988).

An involvement of PP14 at the IL-2 level of the immune response is suggested by the ability of exogenous recombinant IL-2 to partially reverse the suppressive activity of decidual tissue extracts in the mitogenic stimulation assay (Table 1); a similar effect was observed using conditioned medium (supernatants from allogeneic mixed lymphocyte cultures) and allogeneically stimulated lymphocytes (Bolton *et al.*, 1988). Furthermore, the time of incubation at which maximal suppression of IL-2 levels by PP14 occurred coincides with the incubation time at which maximal levels of IL-2 were detected (66 h).

We have previously shown that there is no apparent inhibition of Tac antigen expression by PP14 (Pockley & Bolton, 1988) although IL-2 is considered to be essential for IL-2R expression (Diamantstein & Chen, 1982). The suppression of IL-2 levels by PP14 seen here cannot therefore be sufficient to elicit control on IL-2R expression.

Analysis of sIL-2R levels in the culture supernatants revealed a small but significant reduction when cells were incubated in the presence of PP14, probably a result of the observed suppression of IL-2 levels. The soluble form of the IL-2R may serve a regulatory role with low-affinity sIL-2R delivering IL-2 to high-affinity membrane receptors (Baran, Moreau & Theze, 1986). The observed reduction of sIL-2-R would thus tend to reinforce the inhibitory effects of PP14 on lymphocytes.

In several studies, maternal sera have been reported to suppress IL-2 secretion (Domingo *et al.*, 1985; Nicholas, Panayi & Nouri, 1984). In one study, this lowered IL-2 secretion was shown to result from an interference with the capacity of IL-1 to promote the production of IL-2 by lymphocytes (Domingo, Moreno & Palomino, 1988). These studies have generally used sera collected at around 8 weeks of gestation, which would be expected to contain measurable levels of PP14 (typically 2·2 mg/*l* at between 6 and 12 weeks of gestation; Julkunen *et al.*, 1985). It may be that PP14 could explain some of the immunosuppressive activities previously reported.

It is not known whether the *in vitro* activities of PP14 have physiological importance *in vivo*. It would be too simplistic to suggest that a single mechanism can confer protection on the fetus in such a potentially hostile environment as the female reproductive tract. However, PP14 may have a role to play during implantation and the early survival of the developing fetoplacental units. This protein may also prove to be of interest in the wider field of transplantation biology and in investigating and modifying the immune response.

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