Progesterone inhibits in-vitro embryotoxic Th1 cytokine production to trophoblast in women with recurrent pregnancy loss

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A dichotomous T-helper 1 (Th1) versus T-helper 2 (Th2) cytokine response to trophoblast has been proposed to mediate reproductive failure and success, respectively. Progesterone has immunosuppressive properties. In this study, peripheral blood mononuclear cells from women with and without unexplained recurrent pregnancy loss who had and did not have evidence of embryotoxic, Th1 immunity to trophoblast were cultured with progesterone (10^-5 mol/l) or interleukin (IL)-10 (1500 pg/ml) to determine whether these agents were capable of inhibiting embryotoxic, Th1 immunity to trophoblast. The effects of progesterone on Th2 cytokines and transforming growth factor (TGF)-β secretion were also assessed. Progesterone was found to specifically block Th1 immunity to trophoblast, as was IL-10. Progesterone also appeared to upregulate TGF-β secretion in response to trophoblast but had no effect on Th2 cytokine secretion. Our data suggest that assaying Th1 cytokines in supernatants of peripheral blood mononuclear cells cultured with a protein extract from trophoblast may identify individuals more likely to benefit from potentially immunosuppressive doses of progesterone. An appropriately designed clinical trial is needed to determine whether therapies modifying Th1 cytokine secretion in response to trophoblast are useful in the clinical management of recurrent pregnancy loss in women producing these cytokines in response to reproductive antigen stimulation.

Key words: embryotoxicity/progesterone/recurrent abortion/Th1 cytokines

Introduction

Progesterone has been termed ‘nature’s natural immunosuppressant’ (Siiteri et al., 1977) because the concentration attained at the maternal-fetal interface...
Progesterone inhibits Th1 cytokines

[10^{-5} \text{ mol/l}] is capable of inhibiting a variety of in-vitro assays of immune and inflammatory cell function (Siiteri et al., 1977; Grossman, 1985; Hill et al., 1987; Van Voorhis et al., 1998). Progesterone also regulates the migration and proliferation of immune and inflammatory cell populations in the endometrium of animals (Gottshall and Hansen, 1992) and may play a similar role in women (Critchley et al., 1996).

A novel alloimmune hypothesis has been proposed for human reproductive failure termed immunodystrophism (Hill, 1991) involving the production of embryotoxic factor(s), specifically T helper 1 (Th1) embryotoxic cytokines in response to trophoblast (Hill et al., 1992, 1995; Ecker et al., 1993). According to this hypothesis, a dichotomous Th1 versus Th2 cytokine response to trophoblast or potentially other inciting antigens occurs in a subgroup of women with reproductive difficulty (Hill et al., 1995). The Th1 cytokines interferon-gamma (IFN-γ) and tumour necrosis factor (TNF) have been reported to hinder in-vitro embryo development, implantation events, and trophoblast proliferation (Hill et al., 1987a; Berkowitz et al., 1988; Haimovici et al., 1991). These same Th1 cytokines have been associated with a history of reproductive failure in a subgroup of women with recurrent pregnancy loss; while Th2 cytokine production of interleukin (IL)-4 and IL-10 by trophoblast-activated peripheral blood mononuclear cells (PBMC) were associated with a history of reproductive success (Hill et al., 1995).

Transforming growth factor-beta (TGF-β) is an immunoregulating cytokine with both growth enhancing and inhibiting effects depending upon the cellular target and the presence of other cytokines (Sporn and Roberts, 1988). TGF-β in the decidua has been proposed to play an immunosuppressive role in regulating the response of maternal leukocytes to trophoblast (Lea et al., 1992).

The purpose of this study was to determine whether progesterone can block the in-vitro production of embryotoxic factor(s) and affect Th1, Th2 and TGF-β1 cytokine production by trophoblast-activated PBMC from women with and without a history of unexplained recurrent pregnancy loss.

**Materials and methods**

**Experimental design**

Three different series of experiments were performed on different patient samples. In the first study (i), assessment was made of the propensity of PBMC from 39 unselected women with unexplained recurrent spontaneous abortion (URA) and from 10 women with normal reproductive histories to produce factor(s) toxic to mouse embryo development in vitro. For the second study (ii), 32 additional women were recruited with URA whose trophoblast-activated PBMC culture supernatants had been found previously to contain factor(s) toxic to mouse embryo development and from 10 additional unselected women with normal reproductive histories. Supernatants were prepared from these women for cytokine determinations.
In the third study (iii), five additional women were recruited with URA whose trophoblast-activated PBMC culture supernatants had been found to contain factors toxic to embryo development and to contain significant levels of the Th1 cytokine, IFN-γ. Supernatants were prepared from these individuals to determine whether there was a dose response effect of progesterone (Sigma Chemical Co., St Louis, MO, USA) with and without equivalent molar concentrations of the antiprogestin RU486 on Th1 and Th2 cytokine production.

**Study subjects**

PBMC were isolated aseptically by venepuncture into sodium heparin containing green top Vacutainer tubes (Becton, Dickinson, Livingston, NJ, USA) from a total of 96 women following informed consent. A total of 76 women had a history of three or more first-trimester spontaneous abortions (median 4, range 3–10 prior losses) of unexplained aetiology as assessed by the following criteria: normal parental chromosomes, intrauterine structural study, thyroid function, luteal-phase endometrial biopsy, negative cervical cultures and absent lupus anticoagulant, antiphosphatidyl serine and anticardiolipin antibodies. PBMC were also isolated from 20 women with successful reproductive histories and no prior history of reproductive difficulty. All women were between 30 and 40 years of age and on no medications other than multivitamins at the time of blood collection. All women were in excellent health with no history of allergy, atopy or a recent infection. The time of blood collection relative to the menstrual cycle was not constant, although, none of the women had been pregnant within 3 months of blood collection.

**Antigen preparation**

Trophoblast antigen extracts were prepared from the human choriocarcinoma cell line JEG-3 (American Tissue Type Collection, Bethesda, MD, USA) and red blood cell (RBC) membranes as described previously (Yamada et al., 1994; Hill et al., 1995). JEG-3 cells were chosen as the source of trophoblast antigen because of its monocellular nature and because it contains many antigenic factors identical to early normal invasive trophoblast (Berkowitz et al., 1985, 1986, 1988) JEG-3 cells were grown by standard culture techniques until 80% confluent and then washed twice in Hanks’ balanced salt solution without calcium and magnesium (Gibco, Grand Island, NY, USA). Cells were harvested without trypsinization and disrupted by a Dounce homogenizer (100 strokes). Approximately 10^6 cells gave 30 μg/ml protein extract. Following centrifugation at 400 g for 10 min, the supernatants were saved. The protein concentration was determined by BCA reagent kit (Pierce, Rockford, IL, USA) and antigen extracts were adjusted to 333 μg/ml. Heat-inactivated fetal calf serum was added to a final concentration of 10% and 1 ml aliquots containing approximately 300 μg of trophoblast extract each were stored at −70°C until use. A 30 μg/ml protein extract from RBC membranes was prepared similarly as a non-HLA-bearing
Progesterone inhibits Th1 cytokines

antigen control from peripheral blood collected from paid donors as described previously (Yamada et al., 1994).

**Supernatant preparation**

PBMC were isolated by Ficoll–Hypaque (Pharmacia Uppsala, Sweden) centrifugation as described previously (Hill et al., 1992, 1995; Yamada et al., 1994). Briefly, washed cells were resuspended to a concentration of $10^6$ cells/ml in Roswell Park Memorial Institute (RPMI) medium (Gibco) supplemented with 0.3 mmol/l glutamine, 100 IU/ml benzylpenicillin potassium, and 100 ng/ml streptomycin sulphate (Sigma) and 10% heat-inactivated fetal bovine serum.

For experiments involving embryotoxicity (Study A), PBMC from 39 women with URA were equally divided into six polystyrene flasks (Falcon, Becton Dickinson) containing 10 ml of $10^6$ cells/ml of medium and cultured under the following conditions: Flask 1: PBMC in medium only (control); Flask 2: PBMC plus RBC membrane antigen extract (30 µg/ml) plus ethanol less than 1% (vehicle control); Flask 3: PBMC plus trophoblast antigen extract (30 µg/ml); Flask 4: PBMC plus trophoblast antigen extract (30 µg/ml) plus a final concentration of progesterone [$10^{-5}$ mol/l] dissolved in less than 1% ethanol; Flask 5: PBMC plus trophoblast antigen extract (30 µg/ml) and less than 1% ethanol (progesterone vehicle control); Flask 6: PBMC plus trophoblast antigen extract (30 µg/ml) plus IL-10 (1500 pg/ml).

The dose chosen for IL-10 (1500 pg/ml) had been found previously to inhibit IFN-γ production by trophoblast-stimulated PBMC cultures. All cultures were incubated for 96 h at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The final 12 h supernatants were harvested after the medium was changed to Whitten’s medium supplemented with 0.3% bovine serum albumin, which is more physiological for embryo development (Biggers et al., 1971), in a final concentration of $10^6$ cells/ml. Supernatants were each filter sterilized through a 0.22 µm Miller GS filter unit (Millipore, Bedford, MA, USA) and stored separately at −70°C until use.

**Embryo preparation**

Virgin female mice (CF1 strain, Charles River Laboratories) 35–42 days old had ovulation induced by intraperitoneal gonadotrophin injection (pregnant mare’s serum, 5 IU/ml) followed 48 h later by a 5 IU/ml injection of human chorionic gonadotrophin (HCG, from human pregnancy urine). Mating was accomplished with BGSJLF/j males immediately following injection of HCG.

Mice with vaginal plugs were sacrificed by cervical dislocation and their oviducts flushed with Whitten’s medium to remove two-cell embryos 44 h after HCG administration. Embryos were cultured in microdroplets (20 µl) of Whitten’s medium supplemented with 0.3% heat-inactivated bovine serum albumin under CO₂-equilibrated paraffin oil in tissue culture dishes. The media and supernatant (1:1) were equilibrated in 5% CO₂ before adding embryos. Embryos cultured in
medium alone served as additional controls. A minimum of 11 embryos was cultured in each microdroplet as described previously (Hill et al., 1992, 1995). Experiments were performed in triplicate on three separate occasions.

Embryo development was assessed after 4 days of culture by established criteria (Ducibella, 1980). Embryotoxicity was assumed when the median percentage of two-cell embryos developing to blastocysts was less than or equal to 45% (Hill et al., 1987a, 1992, 1995).

Cytokine analysis

PBMC culture supernatants were prepared from an additional 32 women with unexplained recurrent abortion whose PBMC had been previously found to secrete embryotoxic factor(s) (study ii) following coculture with trophoblast (30 μg/ml) as described above except that coculture of PBMC plus trophoblast with IL-10 was not performed.

Cytokines were measured in supernatants by enzyme-linked immunoabsorbent assay (ELISA) kits according to manufacturer’s instructions. IFN-γ, TNF-α, IL-4 and IL-10 were obtained from Endogen (Boston, MA, USA). The lower limit of sensitivity of the ELISA assay was for IFN-γ, 25.6 pg/ml; 10 pg/ml for TNF-α; 4 pg/ml for IL-4, and 3.3 pg/ml for IL-10. TGF-β1 kits were obtained from Genzyme (Cambridge, MA, USA; lower limit of sensitivity, 01 ng/ml). All ELISAs were solid phase sandwich kits in which specific monoclonal antibodies were attached to wells in 96-well plates and a secondary enzyme-conjugated coloured product was measured in each well with a Du Pont microplate reader (Du Pont Pharmaceuticals, Dover, DE, USA) using a wavelength appropriate for the substrate used. Cytokine concentrations were calculated from a standard curve generated with specific cytokine standards provided with each kit as described previously (Hill et al., 1995). All values below the lowest limit of sensitivity as determined by test kit standards were considered negative or zero.

Dose response analysis

PBMC from an additional five women with unexplained recurrent pregnancy loss known to secrete Th1 embryotoxic cytokines (study C) were isolated and placed in coculture with trophoblast extract (30 μg/ml) and different concentrations [10⁻⁵, 10⁻⁷, 10⁻⁹ mol/l] of progesterone with and without equivalent concentrations [10⁻⁵, 10⁻⁷, 10⁻⁹ mol/l] of the antiprogestrone, RU486 (mifepristone; 17 beta-hydroxy-11-beta [4-(dimethylamino)-phenyl]-17-alpha-propynl-estra-4,9 dien-3 one; Sigma), kindly supplied by Dr Samuel Yen, UCSD Medical Center, USA. Supernatants were prepared and processed as described above.

Statistical analysis

ANOVA and the Mann–Whitney U test were used where appropriate to analyse the data, with P < 0.05 considered statistically significant.
Progesterone inhibits Th1 cytokines

Table I. Study A. Median percent blastocyst development (range) in supernatants of PBMC cultures from all of the study participants

<table>
<thead>
<tr>
<th>Activation status</th>
<th>Median percent development (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media alone</td>
<td>URA (n = 39) 80% (70–90%)</td>
</tr>
<tr>
<td>RBC Membrane. (30 µg/ml) plus ethanol (&lt; 1%)</td>
<td>Normal control (n = 10) 82% (73–91%)</td>
</tr>
<tr>
<td>Trophoblast (30 µg/ml) plus ethanol (&lt; 1%)</td>
<td>70% (60–96%) 64% (55–82%)</td>
</tr>
<tr>
<td>Trophoblast (30 µg/ml) plus ethanol (&lt; 1%)</td>
<td>30% (24–36%)* 73% (64–82%)</td>
</tr>
<tr>
<td>Trophoblast (30 µg/ml) plus progesterone [10–5 mol/l]</td>
<td>37% (30–44%)* 64% (55–82%)</td>
</tr>
<tr>
<td>Trophoblast (30 µg/ml) plus IL-10 (1500 pg/ml)</td>
<td>57% (51–65%) 73% (64–91%)</td>
</tr>
<tr>
<td></td>
<td>60% (50–70%) 64% (55–82%)</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with all of the other cultures.

Results

Study A: embryo development

Supernatants prepared from trophoblast-activated PBMC cultures significantly inhibited (30%) embryo development (P < 0.05) in 26 of 39 women with URA. In contrast, embryo development was unaffected by similarly prepared supernatants from the 10 women with normal reproductive histories. As shown in Table I, the embryotoxicity contained in supernatants prepared from PBMC cultures of women with URA stimulated by trophoblast was inhibited by coculture with [10^-5 mol/l] of progesterone. Similarly the addition of IL-10 (1500 pg/ml) at initiation of PBMC and trophoblast extract culture also abrogated the embryotoxicity otherwise contained in the supernatants from women whose PBMC produced embryotoxic factor(s) in response to trophoblast. Embryo development was not affected adversely by either the 30 µg/ml protein extract derived from RBC membranes (70 versus 80% from medium alone from women with URA) or from the less than 1% ethanol vehicle (37 versus 30% from trophoblast alone from women with URA) used to dissolve progesterone (Table I).

Study B: cytokines

Cytokine results from the 32 women with URA whose supernatants were known to contain embryotoxic factor(s) and from 10 additional women with normal reproductive histories are shown in Table II. Detectable levels of IFN-γ were found in all trophoblast-stimulated PBMC culture supernatants from the 32 women whose trophoblast-stimulated supernatants were known to contain embryotoxic factor(s). The levels of IFN-γ in trophoblast-stimulated cultures in women with URA were significantly higher (425.42 ± 67.25, mean ± SEM) than in unstimulated samples from these individuals as a whole (n = 32, P < 0.05),
Table II. Study B. Cytokine levels (mean ± SEM) in culture supernatants from 32 women with URA and known embryotoxic factor(s) and 10 women with normal reproductive histories

<table>
<thead>
<tr>
<th>Group</th>
<th>IFN-γ pg/ml</th>
<th>IL-10 pg/ml</th>
<th>TGF-β1 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Women with URA (n = 32)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>320.34 ± 54.49</td>
<td>45.75 ± 0.68</td>
<td>0.87 ± 0.08</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(n = 21)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>Trophoblast-stimulated</td>
<td>425.42 ± 67.25**</td>
<td>58.23 ± 6.75</td>
<td>0.98 ± 0.12</td>
</tr>
<tr>
<td>(n = 32)</td>
<td>(n = 21)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>Trophoblast-stimulated plus progesterone [10⁻⁵ mol/l]</td>
<td>362.7 ± 47.98*</td>
<td>53.65 ± 2.41</td>
<td>0.95 ± 0.09</td>
</tr>
<tr>
<td>(n = 32)</td>
<td>(n = 21)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>Trophoblast-stimulated plus 1500 pg/ml IL-10</td>
<td>None detected§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 32)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Women with normal reproduction (n = 10)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>29.15 ± 7.2 + 78.4 ± 6.31</td>
<td>1.47 ± 0.22***</td>
<td></td>
</tr>
<tr>
<td>(n = 4)</td>
<td>(n = 10)</td>
<td>(n = 7)</td>
<td></td>
</tr>
<tr>
<td>Trophoblast-stimulated</td>
<td>172.98 ± 61.42a</td>
<td>65.74 ± 4.81</td>
<td>0.92 ± 0.21</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 7)</td>
<td></td>
</tr>
<tr>
<td>Trophoblast-stimulated plus progesterone [10⁻⁵ mol/l]</td>
<td>None detected§</td>
<td>60.31 ± 3.24</td>
<td>1.09 ± 0.19</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 7)</td>
<td></td>
</tr>
<tr>
<td>Trophoblast-stimulated plus 1500 pg/ml IL-10</td>
<td>None detected§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05 compared with unstimulated cultures from normal women (line above).
**P < 0.05 compared with trophoblast-stimulated group (line above).
***P < 0.05 compared with unstimulated cultures from all women with URA.
+P < 0.01 compared with unstimulated cultures from all women with URA.
§P < 0.001 compared with trophoblast-stimulated cultures.
aP < 0.01 compared with trophoblast-stimulated cultures from women with URA.
bP < 0.05 compared with trophoblast-stimulated cultures from normal women (line above).

and in the nine of 32 unstimulated culture supernatants that contained IFN-γ (320.34 ± 54.49, P < 0.05). Supernatants from trophoblast-stimulated PBMC cultures from women with URA where progesterone [10⁻⁵ mol/l] had been added concomitantly contained significantly lower levels of IFN-γ (362.7 ± 47.98) than supernatants where progesterone had not been part of the original culture (425.42 ± 67.25, P < 0.05). The inhibitory effects of IL-10 on IFN-γ production by trophoblast-stimulated PBMC cultures from women with URA were more dramatic than the effects of progesterone (P < 0.001, Table II).

Low levels of IFN-γ were detected in four of 10 unstimulated PBMC culture supernatants from women with normal reproductive histories; however, these levels were significantly lower (29.15 ± 7.2 pg/ml) than corresponding supernatants from women with URA (320.34 ± 54.49 pg/ml, P < 0.01). Trophoblast-activated PBMC culture supernatants from seven of 10 women with normal reproductive histories also contained measurable levels of IFN-γ; however, these levels were significantly lower than the levels contained in supernatants from women with URA known to produce embryotoxic factor(s) (172.98 ± 61.42 pg/ml versus 425.42 ± 67.25 pg/ml, P < 0.01). The inhibitory effect of progesterone on IFN-γ production by trophoblast-stimulated PBMC cultures was most pronounced in women with normal reproductive histories (Table II).
Progesterone inhibits Th1 cytokines

Low levels of IL-10 were detected in 21 of 32 supernatants from women with URA (45.75 ± 0.68). Higher levels of IL-10 were detected in supernatants from the 10 women with successful reproductive histories (78.4 ± 6.31), although these levels were not statistically significant (Table II). Neither trophoblast nor progesterone significantly affected the levels of IL-10 contained in PBMC culture supernatants (Table II).

TGF-β1 was detected in 11 of 32 women with URA. No differences were noted in the levels of TGF-β1 contained in the supernatants from these women with respect to either unstimulated or trophoblast-stimulated PBMC cultured with or without progesterone. In contrast, seven of 10 supernatants from women with normal reproductive histories contained significantly higher levels of TGF-β1 (1.47 ± 0.22) compared to corresponding culture supernatants from women with URA (0.87 ± 0.08, P < 0.05). Also in contrast to women with URA, supernatants from cultures of women with normal reproductive histories that had been stimulated by trophoblast contained significantly lower levels of TGF-β1 than supernatants from unstimulated PBMC cultures (0.92 ± 0.21 ng/ml versus 1.47 ± 0.22 ng/ml, P < 0.05). Progesterone did not significantly affect TGF-β1 secretion in trophoblast-stimulated PBMC culture supernatants (1.09 ± 0.19 ng/ml, Table II).

Study C: dose response effects of progesterone with and without RU486

The effects of different concentrations [10⁻⁵, 10⁻⁷, 10⁻⁹ mol/l] of progesterone with and without equivalent concentrations of RU486 on Th1 and Th2 cytokine production in response to trophoblast-activated PBMC cultures from the five women whose trophoblast-activated PBMC cultures were known to contain embryotoxic factor(s) and significant levels of IFN-γ are shown in Table III. Progesterone significantly (P < 0.05) suppressed Th1 cytokine production in a dose-dependent manner in trophoblast-activated PBMC cultures from women known to produce embryotoxic Th1 cytokines upon trophoblast stimulation. A dose response effect of progesterone was observed with [10⁻⁵ mol/l] having the most dramatic immunosuppressive effect (five of five samples completely suppressed, P < 0.001). The effect of progesterone was specific as immunosuppression caused by [10⁻⁵ mol/l] progesterone was abrogated by an equivalent dose of RU486 [10⁻⁵ mol/l]. Trophoblast-activated PBMC culture supernatants from these women did not contain measurable levels of either IL-4 or IL-10. Similarly, neither IL-4 nor IL-10 were detected in supernatants from women where progesterone had been added to trophoblast-activated PBMC cultures. RU486 also did not appear to effect Th2 cytokine secretion except in one supernatant from a trophoblast-activated PBMC culture containing equivalent molar concentrations [10⁻⁷ mol/l] of both progesterone and RU486. In this supernatant only a very low concentration of IL-10 was detected (48.14 pg/ml, lower concentration of sensitivity, 33.0 pg/ml).
Table III. Study C. The effects of different concentrations of progesterone with and without equivalent concentrations of RU486 on Th1 and Th2 cytokines (mean ± SEM)

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>PBMC + trophoblast</th>
<th>PBMC + trophoblast + progesterone</th>
<th>PBMC + trophoblast + progesterone + RU486</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻⁵ mol/l</td>
<td>10⁻⁷ mol/l</td>
<td>10⁻⁹ mol/l</td>
</tr>
<tr>
<td><strong>Th1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>284.88 ± 11.53</td>
<td>ND</td>
<td>131.14 ± 80.33</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>(n = 5)</td>
<td>(n = 4)³</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1429.6 ± 74.29</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>(n = 5)</td>
<td>(n = 2)²</td>
</tr>
<tr>
<td><strong>Th2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>IL-10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
</tbody>
</table>

ND = none detected; a one did not suppress; b two did not suppress; c three did not suppress; d none detected in four.
Discussion

Human pregnancy is largely an inefficient process as the majority of conceptions fail to attain viability. Clinically detectable pregnancy loss occurs in ~ 15% of couples desiring children, and 1 in 300 pregnancies has been estimated to belong to a woman who has experienced three or more clinical losses (Stirrat, 1990). The majority of individual pregnancy losses are due to structural chromosomal abnormalities in the abortus (Boue et al., 1975). Many other potential aetiologies have been proposed to explain loss, with recent historical attention focused on potential immunological factors (reviewed in Hill, 1998). A dichotomous Th1 versus Th2 cytokine response to trophoblast or other inciting antigens has been the most recently proposed alloimmune hypothesis concerning human reproductive failure (Hill et al., 1995). The response of PBMC to trophoblast may not necessarily be representative of a local (decidual) response; however, the fact that CD4 and CD56 cells are contained within the decidua and the decidual cells themselves are capable of cytokine secretion (reviewed in Tabibzadeh, 1991; Hill, 1998) under gonadal steroid regulation (Critchley et al., 1996) suggests that similar mechanisms may be involved locally as are found peripherally. The association of recurrent pregnancy loss in women with the preferential secretion of embryotoxic factors in response to trophoblast stimulation and their correlation with Th1 cytokine secretion in response to trophoblast-stimulation (Hill et al., 1992, 1995; Yamada et al., 1994) supports similar observations made in animal models (reviewed in Wegmann et al., 1993; Guilbert, 1996; Raghupathy, 1997; Takabatake et al., 1997a,b; Fujita et al., 1998; Rinkerich, 1998). The Th1 cytokine, IFN-γ, has been shown to interfere with many reproductive processes as measured in vitro (Hill et al., 1987a; Berkowitz et al., 1988; Haimovici et al., 1991) and has been reported to mediate abortion in animals (reviewed in Wegmann et al., 1993; Guilbert, 1996; Raghupathy, 1997). Th1 and Th2 cytokines have been demonstrated in human reproductive tissues (reviewed in Cohen and Pollard, 1996). IFN-γ is implicated in endometrial decidual cell remodelling (Tabibzadeh et al., 1989). A delicate cytokine balance between growth-enabling Th2 cytokines and growth-inhibiting Th1 cytokines has been proposed to exist within the decidua. This has been speculated to regulate the depth of trophoblast invasion, with excess Th2 cytokine secretion leading potentially to over-vigorous invasion such as occurs in placenta accreta, while excess Th1 cytokine production within the decidua is speculated to result in limited trophoblast invasion culminating in adverse pregnancy outcome such as pregnancy loss, pre-eclampsia or intrauterine growth restriction (Hill, 1998).

TGF-β has also been implicated in the establishment and maintenance of pregnancy. TGF-β has both growth-enabling and inhibiting properties depending upon the cellular target and the presence of other growth factor/cytokines (Sporn and Roberts, 1988). TGF-β is secreted by trophoblast and decidual natural killer (NK) cells, T cells and stromal cells where it may modulate the response of maternal immune and inflammatory cells to trophoblast (Lea et al., 1992).

Progesterone has been termed, ’nature’s natural immunosuppressant’ because
levels attained at the maternal-fetal interface \([10^{-5} \text{ mol/l}]\) are capable of inhibiting a myriad of immunological processes including macrophage phagocytosis, lymphocyte proliferation, and NK cell activity \(\textit{in vitro}\) (Siiteri et al., 1977; Grossman, 1985; Hill et al., 1987; Van Voorhis et al., 1998). Progesterone has been reported to mediate Th2 (IL-4, IL-10) cytokine secretion by Th1 producing T-cell clones (Piccinni et al., 1995). The precise mechanism of how progesterone may mediate these effects remains unknown, as classical progesterone receptors are not present on immune and inflammatory cells (Schust et al., 1996).

The results of our study confirm and further our previous findings (Hill, 1991; Hill et al., 1992, 1995; Ecker et al., 1993; Yamada et al., 1994) that PBMC from a subgroup of women with otherwise unexplained recurrent pregnancy loss produce embryotoxic Th1 cytokines upon trophoblast-stimulation. Our current data indicate that this effect can be inhibited specifically \(\textit{in vitro}\) by progesterone, especially by concentrations attained locally at the maternal–fetal interface.

We also found that IL-10 inhibited embryotoxicity and downregulated Th1 cytokine production to trophoblast, which was expected since bi-directional feedback is known to occur between IFN-\(\gamma\) and IL-10, with the preferential secretion of one known to downregulate production of the other (Mosmann and Moore, 1991).

Unlike our previous study (Hill et al., 1995), PBMC from a subgroup of women with normal reproductive histories were also found to produce IFN-\(\gamma\) both in their unstimulated and trophoblast-stimulated cell culture supernatants, albeit at significantly lower concentrations than women with URA. All of the individuals included in our study had no evidence of a recent infection and were asymptomatic for any illness, yet IFN-\(\gamma\) was detected in nine of 32 unstimulated (background) supernatants from women with URA. Much lower concentrations of IFN-\(\gamma\) were also detected in unstimulated supernatants from seven of 10 women with normal reproductive histories. These data suggest immune activation in these individuals. Perhaps their set-point to secrete Th1 cytokines is higher due to genetic polymorphisms in the ability to secrete these factors (Fanning et al., 1997). This possibility is being investigated currently in our laboratory. Our data also indicate the need to subtract baseline (unstimulated) IFN-\(\gamma\) levels from trophoblast-stimulated PBMC culture supernatants used in commercially available assays testing for this potentially new alloimmune disorder (embryotoxic factor lymphocyte, ETF-L assay; Sage BioPharm, Bedminster, NJ, USA) before assuming that a true positive value exists in trophoblast-stimulated PBMC culture supernatants.

Our finding that progesterone had no effect on PBMC production of Th2 cytokines does not support the previously reported finding that progesterone mediates Th2 cytokine secretion by Th1 producing T cells clones (Piccinni et al., 1995). This should not be surprising since \([10^{-5} \text{ mol/l}]\) of progesterone is immunosuppressive, as evidenced by diminished lymphocyte proliferation in response to mitogens and suppression of NK cell activity (Siiteri et al., 1977; Grossman, 1985; Hill et al., 1987b; Van Voorhis et al., 1998). Thus, progesterone
should not be expected to facilitate secretion of any cytokine that would occur in response to lymphocyte proliferation or NK cell activation.

The results of our study also indicated that unstimulated PBMC from women with normal reproductive histories secrete significantly higher levels of TGF-β than unstimulated PBMC from women with URA. Levels of TGF-β secreted by PBMC from fertile women were significantly decreased in response to trophoblast but were not significantly different from baseline levels when PBMC were cultured with trophoblast and progesterone. These data suggest that maternal cell secretion of TGF-β in response to trophoblast may be upregulated by local progesterone levels. In contrast, women with URA did not secrete as high a level of TGF-β in response to trophoblast and progesterone as women with normal reproductive histories, suggesting that inadequate secretion of TGF-β by decidual cells in response to trophoblast and progesterone may be associated with recurrent pregnancy loss.

Our data further imply that therapies modifying Th1 cytokine secretion in response to trophoblast may be useful in the clinical management of recurrent pregnancy loss in women who produce IFN-γ in response to reproductive antigen stimulation. Progesterone has been used anecdotally and empirically for decades to treat women with recurrent pregnancy loss. Meta-analyses of studies to date (Karamardian and Grimes, 1992) using inconsistent and often unspecified dosages of progesterone indicate that progesterone is not useful in the routine clinical management of recurrent pregnancy loss. Perhaps assaying IFN-γ production in supernatants from PBMC activated by trophoblast (30 μg/ml) such as with the ETF-L assay (Sage BioPharm) may identify a cohort of individuals more likely to benefit from potentially immunosuppressive doses of progesterone. This hypothesis needs to be tested in a properly designed placebo-controlled trial where patients are pre-stratified by age and number of prior losses before randomization, and chromosome analysis is obtained on all subsequent losses (Hill, 1997). In this way, level one evidence could be obtained to determine whether progesterone is effective in preventing Th1 embryotoxic cytokine-mediated pregnancy loss.

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Bum Chae Choi et al.


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Progesterone inhibits Th1 cytokines


