Isolation and Purification of an Early Pregnancy Factor-like Molecule from Culture Supernatants Obtained from Lymphocytes of Pregnant Women

CLARA ARANHA,¹ USHA NATRAJ,¹ K. S. IYER,¹ and SAVITRI SHAHANI^{1,2}

Submitted: August 11, 1997

Accepted: October 31, 1997

Purpose: Our purpose was to determine whether lymphocytes synthesize proteins during pregnancy, to observe whether one of the proteins synthesized has early pregnancy factor (EPF)-like activity and to isolate and purify this molecule from culture supernatants obtained from stimulated lymphocytes of pregnant women.

Methods: Lymphocyte proliferation assay and ³⁵S-methionine labeling were done to study de novo synthesis of proteins followed by autoradiography to confirm synthesis of proteins. The rosette inhibition assay was used for detection of the EPF-like molecule. Gel filtration on Sephadex G-100 and RPHPLC were used for purification of the EPF-like molecule.

Results: The rate of incorporation of ³⁵S-methionine was significantly higher in the lymphocytes of pregnant women compared to those of the control, and autoradiography confirmed the synthesis of proteins during pregnancy. There is a total protein enhancement trend observed during the first trimester that declines toward term. The EPF-like molecule is observed to be synthesized during all the trimesters of pregnancy. This molecule, when purified, showed a single homogeneous biologically active peak.

Conclusions: The results indicated that there is an enhancement of existing protein or synthesis of new proteins during pregnancy. The EPF-like molecule is one of the many proteins synthesized and secreted by lymphocytes during pregnancy that, when purified, is biologically active.

KEY WORDS: early pregnancy factor-like molecule; protein synthesis; lymphocytes; ³⁵S-methionine.

INTRODUCTION

Early pregnancy factor (EPF) is a pregnancy-associated immunosuppressive protein released in the maternal serum within hours after fertilization and detected by an immunologic assay called the rosette inhibition test (1).

The kinetics of this molecule are such that it is present in the serum hours after fertilization, in most of the mammalian species tested so far. In women, it is present 24–48 hr after fertilization, persists throughout the first and second half of pregnancy, and returns to baseline level during the third trimester of pregnancy (2–4).

In no case has EPF been detected after parturition. Studies in humans have shown that the appearance of EPF precedes that of human chorionic gonadotropin (hCG) and it can be detected within 24–48 hr after coitus in the conceptual cycles.

In human beings EPF activity has been found in culture supernatants obtained from fertilized eggs in vitro (5), urine (6), amniotic fluid of pregnant women (7), serum of patients with testicular germ cell tumors (8), and women with choriocarcinoma (9). EPF has also been detected in fetal sera, and its dynamic changes were similar to that in maternal sera (10). The extensive distribution of EPF may therefore be postulated to have a significant role in immunomodulation in pregnancy.

Data published by Rolfe *et al.* (11,12) suggest that EPF bound to spleen cells appears to act through induction of specific suppressor factors (SF₁ and SF₂) that are both active in the rosette inhibition test.

Blalock *et al.* (13) showed that human leukocytes and mouse spleen cells produce hCG, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). It is also well-known that endometrial stromal cells, which are mainly lymphocytes, have the ability to syn-

¹ Institute for Research in Reproduction (ICMR), J. M. Street, Parel, Mumbai 400 012, India.

² To whom correspondence should be addressed.

thesize and secrete specific proteins such as prolactin, pregnancy-associated plasma protein A, progesterone-associated endometrial protein, placental proteins, and epidermal growth factors (14,15). Also, products from concanavalin A (Con A)-stimulated lymphocytes result in a dose-dependent increase in both progesterone and 20 α -hydroxyprogesterone production from cultured rat granulosa cells (16).

Mention has also been made of antigens produced by the trophoblast that are also present on the maternal lymphocyte (17). It is likely that there are a number of proteins common to both the fetal trophoblast and the maternal lymphocyte.

With these observations in mind, we undertook a study to observe whether Con A-stimulated lymphocytes from pregnant women synthesize proteins during pregnancy and whether one of these proteins is the rosette-inhibiting EPF-like molecule and, if present, to purify this molecule from lymphocyte culture supernatants.

MATERIALS AND METHODS

Collection of Samples. Plain and heparinized blood from pregnant women was collected during different trimesters of pregnancy. Blood from confirmed non-pregnant women and males served as a control. Serum was separated from whole blood and stored at -20°C until further use.

Lymphocyte Proliferation Assay. Heparinized blood was centrifuged, and the lymphocyte-rich plasma was layered onto lymphoprep (Nycomed Pharma As, Oslo, Norway) and centrifuged for 30 min at 1500 rpm. The lymphocytes were recovered from the cellular ring formed at the interphase (separating the F/H layer from diluted plasma). The harvested lymphocytes were washed three times with RPMI 1640 medium (with L-glutamine and Hepes buffer without bicarbonate, purchased from Sigma, St. Louis, MO) and resuspended in serum free RPMI medium. Viability of lymphocytes was confirmed by viability test using 0.2% Trypan blue dye. One hundred microliters of cell suspension were added to each well of St. Laxbro microtiter culture plate. The optimum dose of Con A for lymphocyte transformation was determined. Maximum stimulation was observed at a concentration of 0.5 μ g of Con A. Therefore 100 μ l of 0.5 μ g Con A was added to each well. The plate was incubated in an atmosphere of 5% CO₂ at 37°C. Cultures were harvested after 72 hr. After incubation, cells were observed microscopically. Proliferation of

cells was seen and lymphocytes appeared as blastlike cells.

Metabolic Labeling of Lymphocytes and Analysis of Protein Synthesized and Secreted. The lymphocyte proliferation assay was carried out as described earlier. Twenty-four hours before harvesting lymphocytes, 10 μ Ci/10 μ l ³⁵S-methionine was added to each well and incubation continued for another day at 37°C in an atmosphere of 95% O₂ and 5% CO₂. In an Eppendorf tube, 200 μ l of this lymphocyte suspension, 10 μ l of 1% BSA, 20 μ l of 20% Nonidet P-40, 100 μ l of water, and 200 μ l of ice-cold 25% TCA were mixed to precipitate the proteins. The precipitate was vortexed and left on ice for 30 min and then microfuged at 10,000 rpm for 10 min. The supernatant was discarded and the precipitate washed at least twice with 5% TCA. Finally the precipitate was washed with ethanol:ether (1:1) and ether. The pellet was dissolved in 50–100 μ l of SDS sample buffer. A 5- μ l aliquot was dispensed in 5 ml of scintillation cocktail (0.5% PPO in 1:1 methyl cellulose/toluene) and counted in a β -matic Wallace counter.

Analysis of Labeled Protein by SDS. A 12.5% polyacrylamide gel (vertical slab gel) was prepared as described by Laemmli (18) and 50 μ l of medium containing 1,00,000 cpm was loaded. The gel was run at 40 mA until the dye reached the bottom of the gel. The gel was removed, stained with Coomassie blue R (in 2:2:10 of propanol:acetic acid:water) and destained using several changes of destain solution (1:1:10 mixture of propanol:acetic acid:water). The gel was transferred between two sheets of gelatin paper. The dried gel was exposed to X-ray film using intensifying screens and left at -70°C for 2 weeks and the film developed.

Measurement of EPF-like Activity. For EPF estimation cultures were harvested after 72 hr after the lymphocyte proliferation assay was carried out. The supernatant was centrifuged and lyophilized and stored at -20°C.

EPF activity was measured in all the serum samples, culture supernatants, and chromatographic fractions using the rosette inhibition assay as described by Morton *et al.* (3). In this assay the rosette inhibition titer (RIT) was defined as the reciprocal dilution of the antilymphocyte serum (ALS) in which the number of rosettes formed was <75% of the number formed in the absence of ALS and was expressed as the logarithm (base 2) of the reciprocal dilutions of ALS $\times 10^{-3}$. An enhancement of the rosette inhibition titer was taken as an index of EPF activity. The RIT obtained with normal lymphocytes after incubation in

serum from nonpregnant females or in PBS alone was 2–3. Throughout these studies, EPF-positive samples were defined as those that gave a RIT >4.

Gel Filtration. Gel filtration was performed on Sephadex G-100 (Pharmacia Fine Chemicals). Approximately 20 g of the gel was soaked in phosphate-buffered saline (PBS), pH 7.2, and kept overnight at 4°C. The swollen gel was packed into a column (30 × 1.5 cm) and equilibrated with PBS. The column was eluted at a flow rate of 9 ml/hr and the effluents were collected as 1-ml fractions.

Reverse-Phase (RP-) HPLC. RP-HPLC was performed on a preparative column (Bio-Rad; Hipore RP-318, 25 × 2.15 cm) using a gradient system consisting of 0.1% (w/v) TFA in water (solvent A) and 50% acetonitrile in A (solvent B). Analytical RP-HPLC was carried out to check the homogeneity of the purified EPF material using a Lichrosorb RP-18, 5 µm (0.4 × 25 cm).

Protein Estimations. The protein content of the fractions was estimated by the method of Lowry *et al.* (19), using bovine serum albumin as standard.

RESULTS

Our results using ^{35}S -methionine to study *de novo* synthesis of proteins from lymphocyte culture supernatants of pregnant and nonpregnant women indicated that the rate of incorporation of ^{35}S -methionine was significantly higher in the lymphocytes obtained from pregnant women compared to those of the control (Fig. 1).

Fig. 1. *De novo* synthesis of proteins from lymphocytes during different trimesters of pregnancy using ^{35}S -methionine.

Moreover there was an enhancement trend observed in the ^{35}S -methionine incorporation during the first two trimesters of pregnancy, with a decline to nearly control values in the third trimester of pregnancy (Fig. 1). These studies were further confirmed by the autoradiography technique, which showed the synthesis of new proteins or enhancement of existing proteins during pregnancy (Fig. 2).

Twenty-two samples from pregnant women during the different trimesters of pregnancy were tested for the presence of EPF-like activity. Six samples from confirmed nonpregnant females and four samples from males served as a control. The lymphocyte culture supernatant and the serum were both tested for the presence of EPF-like activity.

Our results using the rosette inhibition test confirmed the earlier observation (4) that EPF levels in serum were high during the first two trimesters of pregnancy and returned to baseline levels in the third trimester of pregnancy (Fig. 3b). However EPF was observed to be present in the culture supernatants of Con A-stimulated lymphocytes of pregnant women during all three trimesters of pregnancy (Fig. 3a).

Fig. 2. Autoradiograph of gel showing *de novo* synthesis of proteins during pregnancy.

Fig. 3. Mean rosette inhibiting titer (RIT) of (a) lymphocyte culture supernatant and (b) pregnancy serum from women at different trimesters of pregnancy. Numbers marked with an asterisk indicate the total number of samples.

The rosette inhibiting activity in the culture supernatants was highest quantitatively in the first two trimesters of pregnancy and less, although also present, in the third trimester of pregnancy. EPF was

absent at all dilutions of culture supernatant obtained from nonpregnant women or from males.

When the presence of EPF in serum and lymphocyte culture supernatant was correlated with the outcome of pregnancy, it was observed that there was one patient negative for EPF activity at all the dilutions of lymphocyte cultures supernatant tested and this patient subsequently aborted (Table I).

The lymphocyte culture supernatants obtained by proliferation of lymphocytes from first trimester pregnancies were processed on G-100 gel filtration chromatography and four fractions, designated GI, GII, GIII, and GIV (Fig. 4), were obtained. GI fraction, although positive by the rosette inhibition assay, was found to be associated with the IgG molecule as checked by the Ouchterlony immunodiffusion technique. The bulk of the EPF activity was contained in GIV as tested by RIT. This potent GIV fraction was loaded on preparative RP-HPLC (Fig. 5). The peaks were checked for EPF activity and the positive RP-HPLC peak loaded on analytical RP-HPLC, which showed a single homogeneous peak (Fig. 6). This homogeneous peak observed on analytical RP-HPLC was, however, observed to be heterogeneous by SDS-PAGE electrophoresis showing bands of approximately 70 and 68 kDa (Fig. 7).

DISCUSSION

The establishment of pregnancy and the maintenance of the corpus luteum require the mother and embryo to produce various molecules unique to pregnancy.

These molecules have now been identified as soluble serum factors (20) gestational hormones (21), placental proteins (22), and factors released from immunocompetent cells.

Table I. Detection of EPF-like Activity in Serum and Culture Supernatant from Con- A-Stimulated Lymphocytes of Pregnant Women

Trimester of pregnancy	No. of samples tested	No. of samples positive for EPF ^a		
		Serum	Culture supernatant	Outcome
1	12	12	11	1 aborted 11 FTND ^b
II	5	5	5	5 FTND
III	5	None	5	5 FTND
Control	10	None	None	—

^aA RIT >4 indicated the presence of EPF activity.

^bFull-term normal delivery.

Fig. 4. Lymphocyte culture supernatant (first trimester) processed on G100.

It is now believed that immunocompetent cells, i.e., lymphocytes, during pregnancy develop specific progesterone receptors (23–25).

The binding of progesterone to the receptor induces a structural alternative or transformation of the recep-

tor, which in turn enables the complex to bind specific DNA sequences. This is believed to result in selective transcription of target genes with ultimate synthesis of mediator proteins (26).

The mediator proteins are named progesterone

Fig. 5. Elution profile after application of the EPF fraction on a preparative RP-HPLC Bio-Rad Hipore RP-318, 25 × 2.15-cm column using solvent A (0.1% TFA in water) or solvent B (50% CH₃CN in solvent A).

Fig. 6. Elution profile of purified EPF after application on an analytical RP-HPLC Lichrosorb RP-18 column (0.4 × 25 cm).

Fig. 7. SDS-PAGE electrophoresis of the protein purified on RP-HPLC. Lane 1: Sample. Lanes 2 and 3: Molecular weight markers.

blocking factor (PIBF), as they inhibit the release of arachidonic acid (27) and are endowed with immunomodulatory properties (28).

Szekeres-Bartho *et al.* (29) further observed a lower than normal percentage of PIBF-positive cells in women sampled during pregnancy termination and have postulated the following hypothesis. Due to expression of progesterone receptors pregnancy lymphocytes produce an immunomodulatory protein (PIBF) that can block NK cell activity both in vitro and in vivo. Preceding pregnancy termination progesterone receptors disappear from the peripheral pregnancy lymphocytes. Thus even if progesterone is present at a biologically sufficient concentration, they are unable to produce PIBF.

They also observed that during labor, spontaneous abortion, or premature labor, there is a drop in progesterone receptor expression of pregnancy lymphocytes (30).

Rubesa *et al.* (31) for the first time (using western blotting techniques) showed that TJ6 is present on the surface of human decidual lymphocytes during the first trimester of pregnancy. It is a newly described protein produced locally at the time of implantation.

Recent studies have shown that the membrane form of TJ6 is also expressed on peripheral blood B lymphocytes during pregnancy (32).

The mature protein is expressed as a 70-kDa protein, which is most likely posttranslationally modified by proteolytic cleavage to form two smaller proteins. The larger protein (50 kDa) contains the transmembrane portion of the molecule (TJ6m), while the smaller protein (20 kDa) contains the original N terminus and is a soluble protein (TJ6s).

Our results have also indicated that immunocompetent cells, i.e., lymphocytes, do synthesize and secrete proteins and that there is enhancement of existing proteins or synthesis of new proteins during pregnancy.

Further, the ^{35}S -methionine incorporation studies with lymphocytes of pregnant women during the different trimesters of pregnancy indicated a pattern of increased proteins similar to that of placental proteins (33) in the maternal sera.

Matthiesen *et al.* (34) observed that during normal pregnancy from first trimester to early puerperium, a state of suppression or nonactivity of the maternal cellular immune system was seen as judged by deviations of a T-helper; T-cytotoxic/suppressor subpopulation as well as decreased proportions of activated T cells and natural killer cells and they suggested that this suppressed and inactivated maternal cellular immune response in pregnancy may be one of the mechanism that protects the fetus from rejection. Their data suggest that there may be some relationship between systemic and local immunologic findings during pregnancy.

For the first time (as indicated by our results) it has been observed that one of the proteins synthesized and secreted by lymphocytes during pregnancy is a molecule with EPF-like activity.

EPF is immunosuppressive in nature and its immunosuppressive activity is mediated through induction of at least two lymphokines (11,12). Recent studies indicate the importance of EPF also as a growth regulator of transformed, neoplastic, and normal cells, while its presence in platelets implies a role in inflammation and wound healing (35).

A molecule possessing this exceptional combination of properties must have great biological significance. Hence it is imperative that EPF be characterized in biochemical terms. But despite a number of attempts in different laboratories using a variety of source material, the EPF structure has remained elusive.

In our attempts to characterize biochemically the EPF-like molecule from lymphocyte culture supernatants, it was observed that the G-100 IV fraction on RP-HPLC gave a single peak positive for EPF activity. This single peak, isolated on RP-HPLC and EPF active, represents the basic molecular form of human EPF isolated from lymphocytes of pregnant women.

This homogeneous peak observed on analytic RP-HPLC was, however, observed to be heterogeneous by SDS-PAGE electrophoresis, showing bands of approximately 70 and 68 kDa (Fig. 7).

At this stage it is difficult to say whether PIBF, TJ6, and protein with EPF-like activity are the same or different, with similar functions of modulating immunosuppression during pregnancy.

Attempts are being made to identify the molecule with EPF-like activity that may ultimately provide a satisfying understanding of the molecular nature of the EPF-like molecule secreted and synthesized by lymphocytes of pregnant women.

ACKNOWLEDGMENTS

We are grateful to our director, Dr. H. S. Juneja, for his continued encouragement and guidance in carrying out this work. We also thank Mrs. S. V. Nair for typing the manuscript.

REFERENCES

1. Morton H, Hegh V, Clunnie GJA: Immunosuppression detected in pregnant mice by the rosette inhibition test. *Nature (London)* 1974;247:459
2. Morton H, Nancarrow CD, Scaramuzzi RJ, Everson BM, Clunnie GJA: Detection of early pregnancy in sheep by the rosette inhibition test. *J Reprod Fertil* 1979;56:75-80
3. Morton H, Tinneberg R, Rolfe B, Wolfe M, Mettler L: Rosette inhibition test: A multicentre investigation of early pregnancy factor in humans. *J Reprod Immunol* 1982;4:251-261
4. Mehta AR, Menezes J, Peter J, Hinduja IN, Shahani SK: Detection of immunosuppressive early pregnancy factor in humans. *Ind J Med Res* 1987;85:37-45
5. Smart YC, Roberts TK, Clancy RL, Cripps AW: Early pregnancy factor. Its role in mammalian reproduction research overview. *Fertil Steril* 1981;35:397-402
6. Negami A, Kasuga K: Studies of early pregnancy factor from humans pregnant urine using anti-human T-cell monoclonal antibody. *Jpn J Fertil Steril* 1981;31:502-505
7. Zheng ZQ, Qin ZH: Detection of early pregnancy factor-like activity in human amniotic fluid. *Am J Reprod Immunol Microbiol* 1990;22:9-11
8. Rolfe BE, Morton H, Cavanagh AC, Gardiner RA: Detection of an early pregnancy like substance in sera of patients with testicular germ cell tumors. *Am J Reprod Immunol* 1983;3:97-100
9. Mehta AR, Shahani SK: Detection of early pregnancy factor like activity in women with gestational trophoblastic tumours. *Am J Reprod Immunol* 1987;14:67-69
10. Wang-Houng, Zheng Zheng-Qun: Detection of early pregnancy factor in fetal sera. *Am J Reprod Immunol* 1990;23:69-72
11. Rolfe BE, Cavanagh AC, Quinn KA, Morton H: Identification of two suppressor factors induced by early pregnancy factor. *Clin Exp Immunol* 1988;73:219-225
12. Rolfe BE, Quinn K, Athanasas S, Cavanagh AC, Morton H: Genetically restricted effector molecules released by human lymphocytes in response to early pregnancy factor. *Immunol. Cell Biol* 1989;67:205-208
13. Blalock JE, Harbour MC, Menamin D, Smith EM: Peptide hormones shared by the neuroendocrine and immunologic systems. *J Immunol* 1985;135:858s
14. Healy DL, Hodgen GD: The endocrinology of human endometrium. *Obstet Gynecol Surv* 1983;38:509-530
15. Stabile I, Grudzinskas JG, Chard T: Clinical application of pregnancy protein estimations with particular reference to pregnancy associated plasma protein A (PAPP-A). *Obstet Gynecol Surv* 1985;43:73-82
16. Gorospe WC, Kasson BG: Lymphokines from concanavalin A-stimulated lymphocytes regulate rat granulosa cell steroidogenesis in vitro. *Endocrinology* 1988;123:2462-2471
17. McIntyre JA, Faulk WP: Trophoblast antigens in normal and abnormal human pregnancy. *Clin Obstet Gynecol* 1986;29:976-998
18. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 1970;227:680
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with folinphenol reagent. *J Biol Chem* 1951;193:265-275
20. Hellstrom KE, Hellstrom I, Brown J: Abrogation of cellular immunity to antigenically foreign mouse embryonic cells by a serum factor. *Nature* 1969;224:914-915
21. Adcock EW III, Teasdale F, August CS, Cox S, Maschia G, Battaglia FC, Naughton MA: Human chorionic gonadotropin: Its possible role in maternal lymphocyte suppression. *Science* 1973;181:845-847
22. Grudzinskas JG, Teisner B, Seppala M: *Pregnancy Proteins: Biology, Chemistry and Clinical Applications*. Sydney, Academic Press, 1982
23. Szekeres-Bartho J, Szekeres GY, Debre P, Autran B, Chaouat G: Reactivity of lymphocytes to a progesterone receptor-specific monoclonal antibody. *Cell Immunol* 1990;125:273-283
24. Roussev RG, Higgins NG, McIntyre JA: Phenotypic characterization of normal human placental mononuclear cells. *J Reprod Immunol* 1993;25:15-30
25. Paldi A, d'Auriol L, Misrahi M, Bakos AM, Chaouat G, Szekeres-Bartho J: Expression of the gene coding for the progesterone receptor in activated human lymphocytes. *Endo J* 1994;2:317-319
26. Yamamoto K: Steroid receptor regulated transcription of specific genes and gene networks. *Annu Rev Genet* 1985;19:209-252

27. Szekeres-Bartho J, Kilar F, Falkay G, Csernu V, Torok A, Pacsa AS: Progesterone treated lymphocytes release a substance inhibiting cytotoxicity and prostaglandin synthesis. *Am J Reprod Immunol* 1985;9:15-19
28. Szekeres-Bartho J, Autran B, Debre P, Andreu G, Denver L, Chaouat G: Immunoregulatory effects of a suppressor factor from healthy pregnant women's lymphocytes after progesterone induction. *Cell Immunol* 1989;122:281-290
29. Szekeres-Bartho J, Faust ZS, Varga P: The expression of a progesterone induced immunomodulatory protein in pregnancy lymphocytes. *Am J Reprod Immunol* 1995;34:342-348
30. Szekeres-Bartho J: Immunosuppression by Progesterone in Pregnancy; Monograph. Boca Raton, FL, CRC Press, 1992
31. Rubesa R, Beaman KD, Lucin P, Beer AE, Rukavina D: Expression of TJ6 protein in the human first trimester decidual lymphocytes. *Region Immunol* 1994;6:331-333
32. Rubesa R, Beaman KD, Beer AE, Haller H, Rukavina D: Expression of membrane form of the pregnancy associated protein TJ6 on decidual lymphocytes in the first trimester of pregnancy. *J Reprod Immunol* 1996;30:17-27
33. Chard T, Grudzinskas JG: Pregnancy protein secretion. *Seminars in Reprod. Endocrinology* 1992;10:61-71
34. Matthiesen L, Berg G, Ernerudh J, Hakansson L: Lymphocyte subsets and mitogen stimulation of blood lymphocytes in normal pregnancy. *Am J Reprod Immunol* 1996;35:70-79
35. Cavanagh AC, Rolfe BE, Athanasas Platsis S, Quinn KA, Morton H: Relationship between early pregnancy factor, mouse embryo-conditioned medium and platelet activating factor. *J Reprod Fert* 1991;93:355-365