Isolation and Purification of an Early Pregnancy Factor-like Molecule from Culture Supernatants Obtained from Lymphocytes of Pregnant Women: II. Identification of the Molecule as a FC-Receptor-like Molecule: A Preliminary Report

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Purpose: Early pregnancy factor (EPF)-like activity from culture supernatants obtained from stimulated lymphocytes of pregnant women was characterized and identified.

Methods: The enzyme-linked immunosorbent assay depending on the presence of "Fc" receptors on bovine spermatozoa was used to identify the EPF-like molecule purified by gel filtration and reverse-phase high-performance liquid chromatography.

Results: The results indicated that the crude lymphocyte culture supernatant, the EPF-positive G IV fraction obtained on gel filtration, and the EPF-positive reverse-phase high-performance liquid chromatography protein readily bound with the different concentrations of aggregated human γ -globulin in a manner similar to that in which the standard control of aggregated human γ -globulin binds to the bovine spermatozoa.

Conclusions: EPF-like activity synthesized and secreted by lymphocytes during pregnancy may be a Fc-receptor-like molecule.

KEY WORDS: early pregnancy factor-like activity; lymphocytes; Fc-receptor-like molecule; human γ -globulin.

INTRODUCTION

The puzzle of fetal allograft survival has intrigued immunologists ever since Medawar, in 1953, formulated the concepts of histocompatibility and tissue rejection (1). Attempts to explain maternal acceptance

of fetal allograft have focused on both the immunogenicity of placental tissue and the immunosuppressive activities of uterine secretions and lymphoid cells. Immunologic alterations occur during pregnancy to facilitate the process of implantation, development, and, ultimately, delivery of the allogenic fetus.

This is a complex process thought to involve the dampening of some aspects of the immune response and the enhancement of others, depending on the stage of pregnancy. It has been proposed that a pregnancy-related factor(s) may play an important role in altering the maternal immune response. Such factors include hormones, circulating proteins, and other soluble factors.

Among the several hormones and factors known is the early pregnancy factor (EPF), which is detected by the rosette inhibition assay (2). This pregnancy-associated molecule has attracted considerable interest, as it is released into the maternal blood within hours after fertilization (3).

EPF has been found in the blood within hours after mating in all species tested so far (4). It persists for the first and second trimesters of pregnancy and returns to baseline levels during the third trimester of pregnancy. In no case has EPF been detected after parturition (5).

In humans EPF activity is found to be present in the serum of pregnant women (6,7), culture supernatants obtained from fertilized eggs in vitro (8), urine (9), amniotic fluid (10), patients with testicular tumors (11), women with choriocarcinoma (12), and fetal sera (13). Recently it has also been found to be present in culture supernatants obtained from lymphocytes of

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pregnant women (14). This extensive distribution of EPF is postulated to play a significant role in immunomodulation during pregnancy.

EPF is immunosuppressive in nature (15,16) and its immunosuppressive activity is mediated through the induction of at least two lymphokines (17,18).

Recent studies indicate the importance of EPF also as a growth regulator of transformed, neoplastic, and normal cells (19,20), while its presence in platelets implies a role in inflammation and wound healing (21). A molecule possessing this exceptional combination of properties must have great biological significance. Hence it is imperative that EPF be characterized in biochemical terms.

Our attempts to characterize biochemically the EPF-like molecule from culture supernatants of lymphocytes from pregnant women (14) indicated that the homogeneous single peak isolated on reverse-phase high-performance liquid chromatography (RP-HPLC), and EPF active, represents the basic molecular form of human EPF isolated from lymphocytes of pregnant women.

This homogeneous peak observed on analytical RP-HPLC was, however, observed to be heterogeneous by sodium dodecyl sulfate (SDS)/polyzorylamide gel electrophoresis (PAGE) showing bands of approximately 70 and 68 kDa.

The present study shows the progress made in the endeavor to identify the purified protein with EPF-like activity from culture supernatants obtained from lymphocytes of pregnant women.

MATERIALS AND METHODS

For the purification, blood was collected (about 4–5 ml each) from 189 women during the first trimester of pregnancy. Peripheral blood mononuclear cells were obtained from peripheral blood by the standard Ficoll–Hypaque technique and adjusted after estimation of viability by the trypan blue dye exclusion assay.

Purification of EPF-like activity from culture supernatants was achieved by gel chromatography and RP-HPLC as described earlier (14). The homogeneous peak on RP-HPLC and positive for EPF activity was tested for “Fc”-receptor-like activity.

Preparation of Antiserum to the Purified Protein

The protein purified on RP-HPLC was dissolved in normal saline. Approximately 1 mg protein in 0.5 ml saline was used per animal per injection. The injections

were given at weekly intervals at multiple sites. Only the first injection was given along with Freund's complete adjuvant. Four and six weeks after the first injection, the rabbits were bled and the serum was separated and tested for the presence of antibodies. Nonimmune sera (NRS) was obtained prior to immunizing the rabbits. Antibodies to purified protein were tested by enzyme-linked immunosorbent assay (ELISA) by coating the purified protein in carbonate bicarbonate buffer onto the wells of a microtiter plate.

ELISA Depending on the Presence of Fc Receptors on Bovine Spermatozoa

The presence of Fc receptors on bovine spermatozoa is analogous to the presence of Fc receptors on a wide variety of cells. Witkin *et al.* (22) demonstrated that aggregated human γ -globulin (AHG), a complex possessing many of the properties of immune complex (23), binds to spermatozoa that are either free in solution or fixed to wells of a microtiter plate and, thus, developed an ELISA for the estimation of circulating immune complexes (CICs). This principle has been used in our study for the identification of our purified protein as immunoglobulin (Ig)G Fc-binding protein, or Fc-receptor-like molecule.

Bovine Spermatozoa

Bovine semen (approximately 10^9 spermatozoa/ml) were divided into aliquots and stored at -20°C . Prior to use, the spermatozoa were washed three times with phosphate-buffered saline (PBS). No differences were observed using fresh or once-frozen/thawed spermatozoa. Additional freezing and thawing, however, led to a progressive diminution in the binding capacity of AHG (22).

Coating of the Plates with Bovine Spermatozoa

Bovine spermatozoa were attached to wells of a microtiter plate (Dynatech, Alexandria, VA) using glutaraldehyde. Washed spermatozoa ($2 \times 10^7/\text{ml}$) were pipetted into individual wells of microtiter plates. The plates were then incubated at 37°C overnight for the formation of spermatozoa pellet. The next day the entire plate was immersed in cold PBS containing 0.25% glutaraldehyde as described by Stocker and Hensser (24). After 10 min at 4°C the plates were removed, the liquid was shaken out, and the plate was washed three times with PBS Tween 20 to remove excess glutaraldehyde. The plates were either used

immediately or stored at 4°C in PBS Tween 20 with 0.1% NaN₃. Storage for up to 2 weeks under these conditions had no effect on subsequent AHG binding.

Preparation of AHG

Human γ -globulin was obtained as a 0.5 g/10 ml solution from the National Plasma Fractionation Centre, K.E.M. Hospital, Mumbai. γ -Globulin (4 mg/ml) in PBS was freed of aggregates by centrifugation at 40,000g for 90 min, and the supernatant was heated at 63°C for 30 min. Insoluble aggregates were removed by subjecting the heated γ -globulin to centrifugation at 5000g for 30 min and the soluble AHG was assayed for protein concentration by the Lowry method (25), divided into 100- μ l aliquots, and stored at -20°C.

Standardization of the Assay Procedure

In the actual assay, the test protein (1 μ g/100 μ l in carbonate buffer) was coated onto the wells of a microtiter plate in duplicate and incubated overnight at 4°C. The bovine spermatozoa attached and fixed by glutaraldehyde to the wells of microtiter plate served as the control. Simultaneously different concentrations of AHG (0.05 to 40 μ g/0.1 ml) were added to the test and control. The optimum temperature and time of incubation were determined for the binding of AHG to the purified protein or Fc receptors on the bovine spermatozoa.

Results indicated that the optimum time for binding of the AHG to the purified protein was 60 min. Room temperature was the optimum temperature required for binding of AHG to the Fc receptors on spermatozoa or to the purified EPF-positive RP-HPLC protein.

In all subsequent experiments the plate was incubated for 60 min at room temperature. After incubation 0.1 ml of the enzyme conjugate (1:1000) goat anti-human γ -globulin coupled to horseradish peroxidase (Sigma) was added. The plate was then incubated for 60 min at 37°C and washed three times with PBS-Tween 20. The substrate (OPD), 0.1 ml, was then added. The reaction was allowed to continue in the dark for 30 min at room temperature and then stopped with 0.1 ml of 2 N H₂SO₄. Readings were taken with the ELISA reader at 492nm.

RESULTS

The antiserum prepared to purified protein was first tested for its antibody titer by ELISA. Even after four

immunizations, it was observed that the antiserum did not react to give a high titer of antibody. However, the antiserum gave a graded effect with increasing dilutions of the serum. It was also observed that the normal rabbit serum (preimmunized), which was kept as control, gave a high reading with RP-HPLC-purified protein fixed to the microtiter plate. This observation led us to believe that there was some reactant common to nonimmunized and immunized serum which reacted with the RP-HPLC-purified protein (Fig. 1).

In the next experiment, in which the binding of AHG (different concentrations) to bovine spermatozoa and the purified protein was compared, the results indicated that the crude lymphocyte culture supernatant, the EPF-positive GIV fraction obtained on gel filtration, and the EPF-positive RP-HPLC protein readily bound with the different concentrations of AHG, in a manner similar to that of the standard control, i.e., AHG binding to the bovine spermatozoa (Fig. 2). The binding of RP-HPLC fraction to AHG was found to be maximum. Even 1 μ g/well or less was observed to react with AHG, which compared with 48 μ g of lymphocyte culture supernatant and 25 μ g of GIV-positive fraction (Fig. 2). Thus, our results indicated that the protein molecule is an IgG Fc-binding protein or a Fc-receptor-like molecule.

This was further confirmed by carrying out inhibition experiments. In these experiments the AHG aggregate was first absorbed with crude material, and then its binding to bovine spermatozoa was observed. The results indicated that the aggregate did not attach to the bovine spermatozoa, thus indicating that the Fc-binding protein in the culture supernatant had neutralized the aggregate, with the result that no binding was observed on the bovine spermatozoa.

Fig. 1. Determination of antiserum titer.

Fig. 2. Detection of aggregated human IgG binding by ELISA.

From our results we thus infer that the purified protein with EPF-like activity was perhaps one of the Fc-binding proteins reported in the literature. The Fc-binding protein, when treated with SDS for PAGE, gave two bands, corresponding to 70 and 68 kDa (14).

DISCUSSION

For a long time the EPF has defied molecular definition. First described by Morton *et al.* in 1974 (2), this phenomenon is revealed in vitro by the rosette inhibition assay, in which lymphocyte-modifying activity in the maternal serum is detected within hours of fertilization. It is present for at least the first two-thirds of pregnancy, with continued detection dependent on the presence of viable embryo.

Since its discovery almost two decades ago, in 1974, the identity and structure of EPF have remained elusive. The principal reasons for the disappointingly slow progress in molecular characterization are the scarcity of the active material and the heterogeneity in the molecular weight of the EPF molecule among the different species studied, as well as in the same species during different stages of pregnancy.

Many studies including an earlier one from our laboratory (26) have attempted to isolate the molecules responsible for EPF expression. Preparations of varying purity and composition have been obtained (4).

The common usage of the term EPF has implied the existence in pregnancy sera of a unique factor which has often been assumed to be a novel pregnancy-specific protein with the unique capacity to induce increased rosette inhibition titers.

Recent studies render this concept untenable. These studies have begun to identify molecules and mechanisms which cause the expression of increased rosette inhibition titers. The molecules identified to date as related to the EPF phenomenon are

- (1) chaperonin from platelets (21),
- (2) thioredoxin from ovine placenta (27) and human placenta (28), and
- (3) Fc receptors from human trophoblast and tumor cell lines (29).

Receptors for IgG Fc are usually expressed on various lymphoid cells such as natural killer cells, K cells, T cells, macrophages, and neutrophils (30).

Naturally occurring suppressor cells have receptors for the Fc portion of IgG (31–33).

The immune complexes between maternal antibodies and paternal antigens during pregnancy interact with IgFc-receptor-bearing maternal or fetal suppressor T cells and, thereby, implement the release of soluble factors that suppress cytotoxic and rejection reactions.

It has also been observed that human trophoblast and many, if not all, cancer cell lines from various tissue origins release mutually related immunosuppressive factors, which could account for the escape mechanism in both semiallograft (trophoblast) and tumor rejection (29). Interestingly, the factors from both origins show Fc-binding properties.

These factors from both trophoblast and cancer cells induce potent antigen nonspecific suppressor cell activity in vitro within 24–48 hr after addition to normal lymphocyte culture and thus induce suppressor cells which further release another potent suppressor factor distinct from the initially added factor, indicating the existence of mutually interrelated diverse immunosuppressive mechanisms.

In our attempts biochemically to characterize and identify the partially purified protein on RP-HPLC, using bovine spermatozoa ELISA (22), the results indicated that the bovine spermatozoa, crude lymphocyte culture supernatant, G IV fraction, and RP-HPLC-purified protein, when fixed to a microtiter plate, behave identically in their ability to bind AHG (Fig. 2).

In the inhibition experiments we further observed that when AHG was absorbed with crude material (lymphocyte culture supernatant) prior to addition to the microtiter plate, no binding of the aggregate was observed on the bovine spermatozoa, thus indicating that the Fc binding protein in the culture supernatant had neutralized the AHG, with the result that no binding was observed on the bovine spermatozoa.

We thus infer that the purified protein with EPF-like activity was perhaps one of the Fc-binding protein-like molecules reported in the literature.

Recently it has been reported that seminal fluid from intact or vasectomized men contains a protein that specially reacts with the Fc region of IgG, which could be one of the factors regulating the female response to inseminated spermatozoa and/or protecting the spermatozoa from immune destruction (34,35).

There are also reports describing the presence of a soluble form of IgG Fc-binding proteins in human serum and plasma (36,37). It has been proposed that the main role of this group of molecules is to prevent sperm from antibody-mediated damage (38–41). The question that arises is: Because Fc receptors are present as constituents of normal serum, why is there induction of increased rosette inhibiting titers during pregnancy?

Here it can be concluded from literature studies and the above discussion that Fc receptors are present in seminal fluid (34,35). These Fc receptors and trophoblast antigens in the seminal plasma may act as potential stimuli to prime the mother for implantation. After implantation the trophoblast also contributes to the pool of Fc receptors (42–44).

Hence it can be speculated that there is an enhancement of Fc-receptor molecules during pregnancy and increasing expression of these suppressive Fc-receptor molecules during the early phase of pregnancy, i.e., especially during the preimplantation stage, may bear relevance to the establishment of successful pregnancy and success of fetal allograft.

We now believe that from the beginning of the process of reproduction, conditions exist that allow the survival and function of gametes, promote protection and proliferation of the autonomously developing embryo during its transit to the uterus, and ultimately permit nidation.

Thus the research dealing with EPF has clearly shown that the female develops nonspecific immunoregulatory mechanisms from the first hours following pregnancy/fertilization.

The availability of pure material allows the characterization of EPF activity to move into a new molecular phase, which should ultimately provide a complex and satisfying understanding of the biochemistry of early pregnancy factor.

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REFERENCES

1. Medawar PB: Some immunological endocrinological problems raised by the evolution of viviparity in vertebrates. *Symp Soc Exp Biol* 1953;7:320 (Cited in *The immunobiology maternal fetal relationship*. Mount Sinai J Med 1980;45:5)
2. Morton H, Hegh V, Clunnie GJA: Immunosuppression detected in pregnant mice by the rosette inhibition test. *Nature (London)* 1974;249:459–460
3. Morton H, Rolfe B, Clunnie GJA, Anderson MI, Morrison J: An early pregnancy factor detected in human serum by the rosette inhibition test. *Lancet* 1977;1:394–397
4. Morton H, Rolfe BE, Cavanagh AC: Early pregnancy factor. *Semin Reprod Endocrinol* 1992;10:72–82
5. Morton H: EPF as a pregnancy protein. In *Early Pregnancy Factors*, F Ellendroff, E Koch (eds). Perinatology Press, 1984, pp 53–64
6. Mehta AR, Menezes J, Peter S, Hinduja IN, Shahani SK: Detection of immunosuppressive early pregnancy factor in humans. *Ind J Med Res* 1987;85:37–45
7. Quin Zhihai, Zheng Zheng Qun: Detection of early pregnancy factor in human sera. *Am J Reprod Immunol Microbiol* 1987;13:15–18
8. Smart YC, Cripps AW, Clancy RL, Roberts TK, Lopata A, Shutt DA: Detection of an immunosuppressive factor in human preimplantation embryos. *Med J Aust* 1981;1:78–79
9. Negami A, Kasuga K: Studies of early pregnancy factor from human pregnant urine using anti-human T cell monoclonal antibody. *Jpn J Fertil Steril* 1986;31:502–505
10. Zheng ZQ, Qin ZH, Aiying M, Changxin Q, Houg W: Detection of an early pregnancy factor-like activity in human amniotic fluid. *Am J Reprod Immunol Microbiol* 1990;22:9–11
11. 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;3:92–100
12. 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
13. Wang Houg, Zheng Zheng-Qun: Detection of early pregnancy factor in fetal sera. *Am J Reprod Immunol* 1990;23:69–72
14. Aranha C, Natraj U, Iyer KS, Shahani SK: Isolation and purification of early pregnancy factor (EPF)-like molecule from culture supernatants obtained from lymphocytes of pregnant women. *J Assist Reprod Genet* 1998;15:117–124
15. Noonan FP, Halliday WJ, Morton H, Clunnie GJA: Early pregnancy factor is immunosuppressive. *Nature* 1979;278:649–651
16. Bordekar AD, Mehta AR, Moulik SB, Shahani SK: Evaluation of immunosuppressive property of partially purified EPF from human pregnancy serum by an *in vivo* immunobioassay. *Indian J Exp Biol* 1987;25:487–488
17. 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
18. Rolfe BE, Quin 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

19. Quinn KA, Athanasas-Platsis S, Wong FY, Rolfe BE, Cavanagh AC, Morton H: Monoclonal antibodies to early pregnancy factor perturb tumour cell growth. *Clin Exp Immunol* 1990;80:100–108
20. Quinn KA: Early Pregnancy Factor: A Novel Factor Involved in Cellular Proliferation. PhD thesis, University of Queensland, Australia, 1991, pp 128–138
21. Cavanagh AC, Morton H: The purification of early pregnancy factor to homogeneity from human platelets and identification as chaperonin 10. *Eur J Biochem* 1994;222:551–560
22. Witkin SS, Shahani SK, Gupta S, Good RA, Day NK: Demonstration of IgG-Fc receptors on spermatozoa and their utilization for the detection of circulating immune complexes in human serum. *Clin Exp Immunol* 1980;41:441–452
23. Christian CL: Studies of aggregated γ -globulin. Sedimentation electrophoresis and anticomplementary properties. *J Immunol* 1960;84:112
24. Stocker JW, Hensser CH: Methods for binding cells to plastic. Application to a solid phase radioimmunoassay for all surface antigens. *J Immunol Methods* 1979;26:87–96
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with folin phenol reagent. *J Biol Chem* 1951;193:265–275
26. Mehta AR, Moodbidri SB, Raghavan VP, Shahani SK: Isolation and partial characterization of early pregnancy factor (EPF) from human pregnancy serum. *J Reprod Immunol* 1986;9:247–260
27. Clarke F, Wilson S, McCarthy R, Perkins T, Orozco C: Early pregnancy factor: Large scale isolation of rosette inhibition test active polypeptides from ovine placental extracts. *J Reprod Immunol* 1987;10:133–156
28. Di Trapani G, Orozco C, Perkins A, Clarke F: Isolation from human placental extracts of a preparation possessing “early pregnancy factor” activity and identification of the polypeptide components. *Hum Reprod* 1991;6:450–457
29. Hamoaka T, Matsuzaki N, Itoh K, Tsuji Y, Izumi Y, Fujiwara H, Ono S: Human trophoblast and tumor cell derived immunoregulatory factor. In *Reproductive Immunology*, S Isojima Billington (eds). Amsterdam, Elsevier Science, 1983, pp 133–146
30. Anderson RH, Looney RJ: Human leukocyte IgG Fc receptor. *Immunol Today* 1986;7:264–266
31. Moretta L, Webb SR, Grossi CE, Lydyard PM, Copper MD: Functional analysis of two human T-cells subpopulations. Help and suppression of β cell responses by T cells bearing receptors for IgM (Tm) or IgG (TG). *J Exp Med* 1977;146:184
32. Victorino RMM, Hedgson MJF: Relationship between T-cell subpopulation and the mitogen responsiveness and suppressor cell function of peripheral blood mononuclear cells in normal individuals. *Clin Exp Immunol* 1980;42:571–578
33. Fox RI, Thompson LF, Huddleston: T γ cells express T lymphocyte associated antigens. *J Immunol* 1981;126:2062–2063
34. Witkin SS, Richard JM, Bongiovanni AN, Zelikovski G: An IgG-Fc binding protein in seminal plasma. *Am J Reprod Immunol* 1983;3:23–27
35. Thaler CJ, Faulk WP, McIntyre JC: Soluble antigens of IgG receptor FcY RIII in human seminal plasma. *J Immunol* 1989;143:1937–1942
36. Kjayat D, Gefferiero, Yoon S, *et al.*: Soluble circulating Fc receptor in human serum. A new ELISA assay for specific and quantitative detection. *J Immunol Methods* 1987;100:235–241
37. Huizinga TW, deHas M, Kleijer M, Naijens JM, Roos D, Von dem Borne Jr. AEG: Soluble Fc receptor III in human plasma originates from release by neutrophils. *J Clin Invest* 1990;86:416–423
38. Kamada M, Liang Z, Koide SS: Identification of IgG and Fc binding proteins in human seminal plasma and sperm. *Arch Androl* 1991;27:1–7
39. Liang ZG, Mitsudo SM, Koide SS: Prostatic specific antigen and immunoglobulin binding protein in human seminal plasma and prostate. *Arch Androl* 1992;29:225–231
40. Thaler CJ, McConnachie PR, McIntyre JA: Inhibition of immunoglobulin (Ig) G-Fc-mediated cytotoxicity by seminal plasma IgG-Fc receptor III antigens. *Fertil Steril* 1992;57:187–192
41. Maeda N, Kamada M, Daitoh T, Aonot T, Rutaki S, Liang ZG, Koide SS: Immunoglobulin binding factor in human seminal plasma: Immunological function. *Arch Androl* 1993;31:31–36
42. Kristoffersen EK, Ulvestad E, Vedeler CA, Matre R: FcY receptor heterogeneity in the human placenta. *Scand J Immunol* 1990;32:561–564
43. Sedmak DD, Davis DH, Singh V, vande Winkel JG, Anderson CL: Expression of IgG Fc receptor antigens in placenta and on endothelial cells in human. An immunohistochemical study. *Am J Pathol* 1991;138:175–181
44. Nishikiori N, Koyama M, Kikuchi T, Kimura T, Ozaki M, Harada S, Saji F, Tanizawa O: Membrane spanning Fc gamma receptor III isoform expressed on human placenta trophoblast. *Am J Reprod Immunol* 1993;29:17–25