Passive immunization of pregnant mice against early pregnancy factor causes loss of embryonic viability

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Summary. Early pregnancy factor (EPF) is a monitor of the incidence of fertilization and the progress of the early embryo. To determine whether, as well as being a marker of embryonic viability, EPF is also necessary for embryonic survival, passive immunization studies with monoclonal and polyclonal antibodies to EPF were carried out on pregnant mice. In the preparation of monoclonal antibodies, it was noted that most anti-EPF producing hybridomas failed to grow in vitro, while those that did grow produced only low yields of specific IgM antibodies.

Two stable hybridoma cell lines were established both producing low affinity anti-EPF IgM; polyclonal anti-EPF IgG was prepared in rabbits. Mice were passively immunized with 500 µg monoclonal anti-EPF IgM at 32 and 56 h post coitum (total dose 1 mg) or with 500 µg polyclonal anti-EPF IgG at 8, 16, 32 and 40 h post coitum (total dose 2 mg). At 10 days, only 6/18 and 3/6 mice receiving monoclonal antibodies and 2/7 and 1/6 mice receiving polyclonal antibodies had maintained their pregnancies. In contrast, all mice receiving control IgM (N = 14) or control IgG (N = 4) and 22/23 receiving saline were still pregnant at Day 10.

Keywords: pregnancy; early pregnancy factor; monoclonal antibodies; passive immunization; embryonic viability; mice

Introduction

Interplay between maternal and embryonic tissue in the preimplantation period activates systems in preparation for the successful implantation and maintenance of pregnancy. The system thought to be the first signal of pregnancy was the local endometrial reaction to the presence of the embryo, initiated by alterations in oestrogen/progesterone concentrations, detected before implantation (Beier & Mootz, 1979). However, with the discovery of early pregnancy factor (EPF) (Morton et al., 1974, 1976), it appears that, even before this, the maternal system is being prepared immunologically for implantation of the antigenically alien conceptus (Noonan et al., 1979). EPF is released from the ovary in response to the presence of a conceptus as early as 4 h after fertilization (Cavanagh et al., 1982) and has been shown to be capable of initiating production of suppressor factors from lymphocytes, which, in turn, can suppress certain immunological responses such as the cell-mediated (delayed-type) hypersensitivity reaction (Rolfe et al., 1988).

EPF can be detected in serum and, while its presence has been shown to monitor the viability of the embryo (Morton et al., 1987), its role in maintaining this viability has not been determined. To investigate the need for EPF in the maintenance of embryonic viability, monoclonal and polyclonal antibodies to EPF were prepared for use as agents to neutralize EPF activity in vivo.
Purification of EPF

**Human EPF.** Human EPF was purified from medium conditioned by the human choriocarcinoma cell line BeWo (ATCC CCL98) (Cavanagh, 1985). By the criterion of SDS-PAGE, the isolated material appeared to be homogeneous, running as a single polypeptide of apparent M, 12,000.

**Mouse EPF.** Mouse EPF was prepared following the method described by Cavanagh (1984). Briefly, EPF was produced in vitro by culture of oviducts and ovaries from oestrous mice, in RPMI (Flow Laboratories, Irvine, UK), stimulated to produce EPF by the addition of prolactin (PRL-3; NIH, Bethesda, MD, USA) and medium conditioned by mouse embryos. After incubation, EPF was purified from the culture medium; the final product appeared to be homogeneous when analysed by high-performance gel-permeation chromatography.

**Assay for EPF by rosette inhibition test**

This assay is dependent on the original finding of Bach & Antoine (1968) that an immunosuppressive anti-lymphocyte serum (ALS) can inhibit active rosette formation between lymphocytes and heterologous red blood cells. The highest dilution of an ALS to give significant inhibition (rosette inhibition titre: RIT) does not vary with lymphocytes from different donors. A modification of this assay was introduced to detect EPF after it was demonstrated that lymphocytes, preincubated with EPF, give a significantly higher RIT with an ALS than do lymphocytes from the same donor without EPF (Morton et al., 1974).

The assay was performed with spleen cells from outbred, male Quackenbush mice, rabbit anti-mouse lymphocyte serum and human red blood cells (RBC) (Rolfe et al., 1984; Morton et al., 1987), with one batch of ALS used throughout. For each test, 1 x 10^7 freshly isolated spleen cells were incubated at 37°C for 0.5 h with 0.2 ml test sample. After incubation, the cells were washed twice in Hank's balanced salt solution (HBSS), reconstituted to 1.0 ml in HBSS and used to estimate the RIT of an ALS as described by Rolfe et al. (1984). Positive [purified mouse EPF, 5 ng/ml in HBSS/0.01% (w/v) bovine serum albumin (BSA)] and negative [HBSS/0.01% BSA] controls were included with each set of tests. RIT is expressed as log_2 (reciprocal dilution of ALS x 10^-3); an RIT ≥ 16 was positive for EPF and an RIT < 16 was negative for EPF (see Morton et al., 1987).

**Polyclonal anti-EPF IgG**

Four male, outbred male rabbits were injected s.c. at 14-day intervals with 2 μg human EPF emulsified in Freund's adjuvant (CSL, Melbourne, Victoria, Australia), complete for the first injection and incomplete for subsequent injections. Serum was monitored for rabbit anti-EPF Ig by solid-phase radioimmunoassay (RIA) with human EPF [0.1 ml, 1.0 μg/ml in 0.05 M-sodium carbonate/bicarbonate buffer, pH 9.6 (binding buffer)] bound to the solid phase (overnight, 4°C) and tubes blocked with Biotto (Johnson et al., 1984) before addition of rabbit serum [1/1000 in 0.05 M-sodium phosphate buffer, pH 7.4/0.02% (w/v) gelatin (diluting buffer)]. Bound Ig was detected with donkey anti-rabbit Ig, biotinylated F(ab')_2/[125I]streptavidin (Amersham International, Amersham, UK) following the manufacturer's instructions. Rabbits with serum giving results > 10 times the control value were bled from the ear vein 8, 10 and 12 days after the last injection and serum was recovered.

Polyclonal anti-EPF IgG was also prepared against mouse EPF for use in screening hybridoma supernatants for anti-EPF specificity by a two-site immunoradiometric assay (IRMA). Two rabbits were immunized with 4 x monthly injections of mouse EPF (1 μg in Freund's adjuvant/injection) and the serum was tested for anti-EPF activity by RIA, as described above, with human EPF on the solid phase. Rabbit 802, which gave results > 5 times the control value, was selected.

IgG was isolated from rabbit serum by precipitation with 18% sodium sulphate (Johnstone & Thorpe, 1987a), followed by chromatography on DEAE Affi-Gel Blue (Bio-Rad, Richmond, CA, USA), using 20 mm-Tris–HCl, pH 8.0/0.28 mm-NaCl/0.01% sodium azide as elution buffer, according to the manufacturer's instructions. The final preparation was analysed by SDS-PAGE (Gorg et al., 1981), protein concentration was determined (Lowry et al., 1951) and anti-EPF specificity was confirmed by RIA, as described above, and by the rosette inhibition test.

**Monoclonal antibodies**

**Hybridomas.** Adult, male BALB/c mice aged 5–6 weeks (Central Animal Breeding House (CABH), Brisbane, Australia) were injected i.p. monthly for 3 months with 50–100 ng human EPF in Freund's adjuvant. A fourth booster shot was administered to each mouse i.v. in saline 1 month later, 3 days before fusion (Goding, 1986a). Sensitized spleen cells were fused with mouse myeloma cells X63-Ag8-653 (Flow Laboratories) in the presence of polyethylene glycol (PEG, M, 1500; BDH, Poole, UK) following the method of Galfre & Milstein (1981).

Screening supernatants for mouse Ig by RIA. Initial screening of hybridoma supernatants was for Ig production as limited amounts of purified EPF were available for anti-EPF screening. The RIA was carried out as previously...
described with sheep anti-mouse Ig (Silenus, Melbourne, Victoria, Australia; 0.1 ml, 4 μg/ml in binding buffer) on the solid phase. Hybridoma supernatants (0-2 ml; 37°C, 2 h) were added to blocked tubes and bound mouse Ig was detected with sheep anti-mouse Ig, biotinylated F(ab')₂/¹²⁵Istreptavidin. Supernatants giving counts > 5 times that obtained with culture medium alone were selected for further testing.

**Supernatants treated to remove EPF.** In preliminary studies we have found that myeloma and hybridoma cells, like other rapidly-dividing tumour cells in culture (unpublished data), produce EPF (or an EPF-like substance). Therefore, before testing for anti-EPF specificity, selected hybridoma supernatants found to be positive for Ig were treated to remove EPF, free or bound to antibody, as follows. Supernatant medium (0-2 ml) was acidified to pH 2.5 with 1 ml 0-2 M-glycine–HCl, pH 2.0 (to separate antigen–antibody complexes), mixed at 4°C for 0.5 h and passed through an activated C₁₈ Sep-pak (Waters Assoc., Milford, MA, USA) which binds EPF. The flow-through fraction containing Ig was collected, the pH raised to 8/0 with solid Tris, dialysed against 0.05 M-sodium phosphate buffer, pH 7.4/0.15 M-NaCl (PBS)/0.01% (v/w) sodium azide overnight at 4°C and concentrated to 0.2 ml using a Centricon Micro concentrator (Amicon, Danvers, MA, USA).

**Screening supernatants for anti-EPF specificity.** Treated supernatants were tested for anti-EPF specificity by (i) the rosette inhibition test and (ii) IRMA.

In (i) after treatment as above, a sample of supernatant was dialysed against PBS, then incubated (0-1 ml, 1/10 in PBS, 37°C, 0.5 h) with mouse EPF (0-1 ml, 50 ng/ml in HBSS/0.01% BSA). Control tests were mouse EPF incubated with (a) PBS and (b) with rabbit (No. 802) anti-EPF IgG (0-1 ml, 0.5 μg/ml in PBS). After incubation, the mixtures were tested for activity in the rosette inhibition test; a negative result (i.e. RIT < 16) was considered evidence of the presence of anti-EPF Ig.

(ii) As more purified human EPF became available, a modified IRMA (Johnstone & Thorpe, 1987b) was used to confirm specificity of selected positive wells; this assay was used due to the low affinity binding of the anti-EPF monoclonals. Ig in treated supernatants was bound to sheep anti-mouse IgG on a solid phase, as described above. Control tests consisted of anti-EPF mouse serum from immune spleen donors (1/1000 in diluting buffer; positive) and culture medium (negative). The tests were incubated overnight at 4°C with human EPF (0-1 ml, 10 μg/ml in diluting buffer), then rabbit (No. 802) anti-mEPF IgG (0-1 ml, 10 μg/ml in diluting buffer) was added to the washed tubes (3 h, 37°C). Bound rabbit IgG was detected as previously described. A result > 2.5 times the result with culture medium alone was considered positive for anti-EPF (Goding, 1986b).

**Screening supernatants for Ig class.** Monoclonal Ig with anti-EPF specificity was tested for Ig class by RIA as previously described, with sheep anti-mouse IgG and IgM antisera (Nordic, Tilburg, The Netherlands) on the solid phase.

**Monoclonal anti-EPF IgM.** After identification of positive wells, hybrid cells were recloned twice by limiting dilution procedures (Galfré & Milstein, 1981), to ensure monoclonality, and established as stable cell lines. Selected cell lines were grown in vivo as ascitic tumours in BALB/c mice for production of antibodies (Goding, 1986c). Ascitic fluid was treated at pH 2.5 with glycine–HCl followed by passage through a Sep-pak cartridge to remove EPF (see above). IgM was precipitated during dialysis against 5 mM-Tris–HCl buffer, pH 7.5 at 4°C overnight (Goding, 1986d), reconstituted in PBS/0.01% sodium azide, protein concentration measured (Lowry et al., 1951) and the purity of the preparation analysed by SDS-PAGE (Gorg et al., 1981). Anti-EPF specificity was confirmed by the rosette inhibition test and by RIA, as described previously, with human EPF on the solid phase and bound anti-EPF IgM detected with sheep anti-mouse Ig, biotinylated F(ab')₂/¹²⁵Istreptavidin.

**In-vivo experiments.**

**Mating protocol.** Virgin female outbred Quackenbush mice aged 10–12 weeks (CABH) were housed in a light (12 h light, 12 h dark) and temperature-controlled (22–26°C) room, with free access to water and mouse pellets. Female mice were caged overnight with males (1:1) and mating was confirmed the next morning by the appearance of a vaginal plug; 0 h was taken as 02:00 h on the day of mating (Day 1). Blood was collected 32 h after mating, from the retrobulbar sinus (Herbert & Kristensen, 1986). The serum was separated, inactivated at 56°C for 0.5 h, diluted 1/8 in HBSS and tested for EPF in the rosette inhibition test. Mice were autopsied on Day 10 and examined for the presence of embryos.

**Control groups: correlation between positive assay for EPF at 32 h post coitum (p.c.) and an established pregnancy at 10 days.** One group of 26 virgin female Quackenbush mice was mated, and serum was collected and tested as described above; a second group of 20 mice was unmated but blood was collected and serum tested as described (negative control). Correlation between the presence of EPF in serum at 32 h, as denoted by a positive value in the rosette inhibition test, and established pregnancy at 10 days was determined.

**Passive immunization.** Having established the above, a third group of female Quackenbush mice was mated as described above and tested for serum EPF at 32 h p.c.; only those mice with a positive test for EPF were included in the study. Before injection into mice, antibody preparations were exchanged into 0.15 M-NaCl on a PD-10 column (Pharmacia, Uppsala, Sweden). Mice received 500 μg anti-EPF monoclonal IgM, control monoclonal IgM or saline i.p. at 32 h and 56 h p.c. (total dose 1 mg). Autopsies were performed on Day 10 and viable embryos counted. The same protocol was used for the polyclonal antibodies, but mice were bled at 8 h p.c. before receiving 500 μg anti-EPF IgG, control rabbit IgG (Silenus) or saline i.p. at 8, 16, 32 and 40 h p.c. (total dose 2 mg).
Statistical analysis

Significant differences between number of mice, pregnant at 10 days, in the treatment groups and the control groups were determined by $\chi^2$ test. Differences were considered significant when a $P$ value of $\leq 0.05$ was obtained.

Results

Initially 9 fusions were carried out and approximately 18% of the wells were found to be positive for anti-EPF Ig by the criteria already described. Only 2 clones, 7/342 and 5/341, positive for anti-EPF IgM from different fusions, were eventually established as stable cell lines. A third clone, 7/331, producing an irrelevant IgM not of anti-EPF specificity (control IgM), was also enlarged to establish a stable cell line. These were grown as ascitic tumours in mice and IgM purified from the ascitic fluid. In the RIA, with human EPF (1-0 µg/ml) on the solid phase, 7/342 and 5/341 (5 µg/ml) gave mean values $>2$ to 2.5 times background (Goding, 1986b); binding with 7/331 did not differ from background.

As more purified EPF became available, mice were immunized with more realistic amounts of antigen (5–20 µg/injection). Supernatants containing anti-EPF IgG were identified by RIA, as described, with positive wells giving counts $>5$ times background. Positive hybridomas were cloned but none was established as a stable cell line. As a result, monoclonal IgG was not available, and so experiments were carried out with polyclonal IgG.

Polyclonal IgG

Purified IgG from Rabbits 810 and 816 (5 µg/ml), tested in the RIA with human EPF (1-0 µg/ml) on the solid phase, gave values $>5$ times background; binding with rabbit IgG (Silenus) did not differ from background.

Neutralization of EPF activity with anti-EPF IgM and IgG

Purified preparations of 7/342, 5/341 and 7/331 were tested by the rosette inhibition test for their ability to neutralize the activity of mouse EPF. The results of duplicate experiments with mouse EPF showed that 500 ng and 50 ng, but not 5 ng, of both 7/342 and 5/341 neutralized the activity of 0-5 ng mouse EPF; 500 ng of the control 7/331 did not affect EPF activity (Fig. 1). Similar results were obtained with the polyclonal IgG (Fig. 1).

Correlation between the presence of EPF in serum at 32 h after mating and the outcome of pregnancy

Serum from all animals in the group of 26 mated mice was positive for EPF at 32 h p.c. and, at autopsy on Day 10, 25 (96%) of the mice were pregnant. In the group of 20 unmated mice none of the serum samples was positive for EPF ($P < 0.001$; $\chi^2$ test). These results demonstrate that the presence of EPF in serum at 32 h after mating is an indication of pregnancy.

Effect of passive immunization of mated mice with anti-EPF immunoglobulin

Monoclonal anti-EPF IgM. Administration of anti-EPF monoclonal IgM 7/342 and 5/341 resulted in only 6/18 (33%) and 3/6 (50%), respectively, of the mice maintaining their pregnancies. In contrast, after treatment with monoclonal IgM 7/331, not of anti-EPF specificity, all 14 (100%) of the pregnancies continued and, with saline, 17/18 (94%) of the pregnancies continued. The number of mice maintaining their pregnancies in the test groups was significantly different from that of the control group (Fig. 2a). The average number of embryos in the test groups of mice that remained pregnant did not vary significantly from that in the control groups (Fig. 2a).
Fig. 1. Neutralization of EPF activity in vitro by monoclonal anti-EPF IgM (7/342, 5/341) and polyclonal anti-EPF IgG (No. 816, No. 810). Various concentrations of antibodies and controls (IgM 7/331, rabbit IgG) were incubated with EPF for 0.5 h at 37°C, then the mixture tested for EPF in the rosette inhibition test. Results are expressed as RIT, i.e. log₂ (reciprocal dilution of ALS × 10⁻³); values of ≥ 16 indicate the presence of EPF. Each bar represents the mean of duplicate experiments.

Fig. 2. Effect of passive immunization of mice with monoclonal and polyclonal anti-EPF antibodies on fetal viability. Confirmed mated mice received 500 μg (a) monoclonal anti-EPF IgM, control IgM or 0.9% NaCl at 32 and 56 h p.c., (b) polyclonal anti-EPF IgG, control IgG or 0.9% NaCl at 8, 16, 32 and 40 h p.c. Autopsies were carried out on Day 10. ■ No. of mice pregnant at Day 10; □ no. of mice not pregnant at Day 10. Values in parentheses are mean ± s.e.m. embryos per pregnant mouse. ***P < 0.001, **P < 0.01, *P < 0.02 compared with 0.9% NaCl group (χ² test).
**Polyclonal anti-EPF IgG.** Administration of anti-EPF polyclonal IgG from Rabbits 816 and 810 resulted in only 2/7 (28.5%) and 1/6 (17%) respectively, of the mice maintaining their pregnancies. The number of mice maintaining their pregnancies in the test groups was significantly different from that in the control groups receiving rabbit IgG or saline, in which pregnancy continued in all 4 (100%) and all 5 (100%) respectively of the mice studied (Fig. 2b). In the one mouse remaining pregnant after treatment with IgG from Rabbit 810, only 8 viable embryos were present at autopsy at 10 days gestation, compared with an average of 15 embryos per mouse in the control groups.

**Discussion**

Since EPF was first described as a pregnancy-associated substance, present in serum of mice within 6 h of fertile mating (Morton et al., 1976), many groups have carried out investigations to determine whether EPF can be used as a marker for early pregnancy in humans and animals. EPF has been found in serum within 4-48 h of fertile mating (Morton et al., 1979; Rolfe, 1982; Koch et al., 1983; Sueoka et al., 1988), or within 12-48 h after embryo transfer (Chen et al., 1985; Morton et al., 1987); the continuation of pregnancy can be monitored by following serum EPF values (Morton et al., 1976, 1977, 1983; Tinneberg et al., 1984; Qin & Zheng, 1987; Sueoka et al., 1988). Furthermore, after death or loss of the conceptus, EPF is no longer detectable in serum (Koch et al., 1982; Rolfe, 1982; Qin & Zheng, 1987); indeed, in humans after therapeutic abortion, disappearance of EPF is the earliest marker of embryonic loss (Mettler et al., 1985). Taken together, these studies closely link EPF with the fate of the embryo.

The ability of EPF to monitor embryonic viability raised the possibility that it may have a role in maintaining this viability. Therefore, it was planned to prepare a number of monoclonal antibodies for use in passive immunization studies, to determine whether neutralization of EPF in vivo affected pregnancy. During the preparation of anti-EPF monoclonal antibodies, we encountered a number of very interesting problems. Initially, all the anti-EPF monoclonals produced were IgM, possibly because a low dose of immunogen was administered. Most of the anti-EPF producing hybridomas failed to grow in vitro while those that did grow produced only low yields of specific IgM antibodies. By increasing the immunizing dose of EPF, hybridomas producing IgG monoclonals were prepared but these also failed to survive in culture.

The results may be explained by recent studies in our laboratory which have shown that EPF is produced not only by embryonic cells but also by actively-dividing tumour cells including myeloma and hybridoma cells. As well as producing EPF, these cells appear to require EPF for their continued survival. These findings can be likened to those of Matsuda et al. (1988), who reported that efforts to obtain murine hybridomas, producing monoclonal antibodies specific for human interleukin 6 (IL/6; B cell stimulatory factor 2, BSF-2; hybridoma growth factor, HGF; Sugawara, 1988), were not successful unless recombinant IL-6 was added directly after cell fusion. A partial N-terminal amino-acid sequence of purified human EPF (unpublished data) shows no similarity with the IL-6 sequence, indicating that we have identified a further substance essential for growth of hybridomas.

While investigations were continuing into means of maintaining growth of anti-EPF IgG producing hybridomas in culture, polyclonal anti-EPF IgG was prepared and included in passive immunization experiments with pregnant mice. The affinity of the polyclonal IgG in solid-phase RIA was much greater than that of the monoclonal IgM, although the ability to neutralize the activity of EPF in liquid phase, as demonstrated in the rosette inhibition test, was similar. These characteristics are in keeping with those generally found for IgM, namely a high avidity but low affinity (Roitt, 1980); significant binding with IgM is not readily observed in a solid-phase immunoassay.

Initially, passive immunization experiments were performed with two anti-EPF IgM monoclonals administered at 32 and 56 h p.c. to outbred mice. Pregnancy was interrupted in a significant number of cases but not in all. Investigation of whether this was simply a function of dose was
precluded by the limited amount of antibody available and by the poor solubility of IgM. Therefore, further experiments were conducted with polyclonal anti-EPF IgG and, in the light of the study by Rider et al. (1987), administration of antibody was commenced at 8 h p.c. Rider et al. (1987) found that the establishment of pregnancy in the mouse is susceptible to passive immunization against progesterone at about the 4-cell stage of development, as well as at the time of implantation. However, when using F₁ hybrids rather than inbred mice, they showed that an earlier exposure of pregnancy hormone (in their case progesterone) to antibody was required to disrupt the hormonal sequence and hence implantation.

Passive immunization experiments with higher doses of polyclonal anti-EPF IgG, administered from 8 h p.c., did result in an increased loss of embryonic viability, compared with monoclonal anti-EPF IgM administered from 32 h p.c. With mice receiving polyclonal anti-EPF IgG from Rabbit 810, pregnancy was affected in almost all cases; only 1 of 6 mice was still pregnant at Day 10 and this had a reduced litter size. The greater effect achieved with anti-EPF IgG in these experiments may have been due to timing of injection, higher doses of antibody or to the ability of IgG to circulate more freely in vivo than IgM. Nevertheless, these experiments showed that neutralization of EPF results in embryonic loss.

The function of EPF is still unclear but the effect of anti-EPF on hybridoma growth suggests that EPF may act as a growth factor and be required directly by the embryo for growth and development. To determine whether EPF has a direct influence on embryonic survival, an in-vitro system will be studied, following the protocol of Hill et al. (1987). Monoclonal anti-EPF will be added to mouse embryo cultures and the effect on the development of the mouse embryo from the two-cell stage through to the blastocyst stage will be observed.

On the other hand, EPF may act indirectly by initiating systems in vivo which are important to the survival of the embryo. It has been postulated that suppressor factors, induced by EPF (Rolfe et al., 1988), may play a role in the prevention of maternal anti-fetal reactions at the time of implantation, when the implanting blastocyst comes in close contact with the maternal circulation. Beaman & Hoversland (1988) have shown that passive immunization of mice on Days 2 to 6 with mAb 14–30, a monoclonal antibody which binds T-cell-produced suppressor factors, results in embryonic loss. Whether these suppressor factors are related to those induced by EPF has not yet been determined but the results suggest that loss of suppressor induction following EPF neutralization may be a contributing factor in the failure to maintain pregnancy. Evidence against the possibility that any antibody–antigen reaction within the genital tract/uterus will lead to termination of pregnancy has been provided by Sulila et al. (1988), who showed that passive immunization with a rat monoclonal antibody to the T-cell surface molecule CD8, while depleting the population of CD8-positive lymphocytes, did not affect the outcome of pregnancy.

From the studies presented in this paper, it can be concluded that, since neutralization of EPF results in embryonic loss, EPF, whether directly or indirectly, is a necessary element in the complex interplay of events leading to the successful establishment of pregnancy.

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