The appearance and characteristics of early pregnancy factor in the pig

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Summary. The occurrence of early pregnancy factor in the pig has been established by the rosette inhibition test and by the criteria that gel filtration of serum resulted in a number of peaks of activity similar to those observed in other species. In the pig EPF is present virtually to the end of pregnancy, with a biphasic production in which the titres of EPF decline markedly in mid-pregnancy. Free EPF-A appears concurrently with EPF in the first 3 weeks of pregnancy in some but not all pigs. The presence of excess EPF-A has an inhibitory effect in the rosette inhibition test and modifications, including an initial serum dialysis step, have been introduced into the test to take account of this inhibitory effect.

Introduction

Early pregnancy factor (EPF) is a pregnancy-dependent protein complex which has been detected in the serum of all species so far tested including mouse, man and sheep (Morton, Hegh & Clunie, 1976; Morton, Rolfe, Clunie, Anderson & Morrison, 1977; Morton, Nancarrow, Scaramuzzi, Evison & Clunie, 1979a). The assay for the detection of EPF is the rosette inhibition test, originally devised to test the immunosuppressive potential of antilymphocyte serum (Bach, Dormont, Dardenne & Balner, 1969). These authors found that some preparations of antilymphocyte serum had the ability to inhibit spontaneous (Bach *et al.*, 1969), sometimes called active (Felsburg, Edelman & Gilman, 1976), rosettes formed between lymphocytes and heterologous red blood cells. As applied to the detection of EPF, less antilymphocyte serum is required to inhibit rosette formation if lymphocytes have previously been treated with EPF. As a consequence of the indirect character of the rosette inhibition test, it is essential, when establishing the presence of EPF in a species not previously investigated, to demonstrate that any increase in the rosette inhibition titre (RIT) is in fact due to a substance with properties the same as or similar to those of EPF in the species already examined.

Studies of mouse, sheep and human EPF have resulted its partial characterization (Morton *et al.*, 1980; Clarke & Wilson, 1982; Rolfe, Morton & Clarke, 1983). One of the principal characteristics of EPF established in other species is its occurrence after gel filtration as a multimolecular weight substance either free or in association with a protein carrier (Clarke, Morton, Rolfe & Clunie, 1980). EPF itself may be dissociated into two separate moieties termed EPF-A and EPF-B which have separate sites of synthesis *in vivo* (Morton *et al.*, 1980). EPF-A is formed in the oviduct

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during oestrus and pregnancy; production of EPF-B by the ovary, however, is associated only with pregnancy and is a result of the combined action of signals from the pituitary and the zygote (Cavanagh, Morton, Rolfe & Gidley-Baird, 1982). EPF-A and EPF-B are both required for EPF activity (Clarke *et al.*, 1980).

The objectives of this investigation were (1) to establish the appropriate conditions for the rosette inhibition test in the pig, (2) to determine whether EPF with its characteristic physical properties is present in the pregnant sow and (3) if detected, to follow the time course of EPF production throughout pregnancy.

Materials and Methods

Animals. German Landrace gilts and sows of the Institute stock were housed under normal daylight conditions at the experimental farm. A standard pelleted pig chow and water were provided twice daily. As a routine procedure, all sows were tested daily for the occurrence of oestrus and were mated with an intact boar on the morning of Day 1 or Day 2 of oestrus. Subsequently, animals were tested daily for the recurrence of oestrus to indicate failure to establish pregnancy. Blood samples were collected from an ear vein of unrestrained animals as described previously (Hultsch & Ellendorff, 1979). Two groups of animals were tested in this investigation. (1) In Group 1, of 20 animals, random blood samples (2–3 ml) were collected into dry tubes in the first 2 weeks after mating; control samples were collected from unmated sows at various stages of oestrus. (2) In Group 2, of 10 animals, blood was collected at 2 h, 1 and 2 days and 1, 2, 3, 8, 12 and 14 weeks after mating. Blood was also collected from 2 animals 1 week after parturition.

Serum. Blood samples from sows for assay of EPF were allowed to clot, the serum removed, incubated at 56°C for 0.5 h, then frozen in 0.5 ml amounts at –30°C. Samples were only thawed once before testing.

Lymphocytes. Blood (40 ml) was obtained from a chronic jugular catheter (Ellendorff, Parvizi, Elsaesser & Smidt, 1977) in castrated male pigs, mixed with 4×10^3 units preservative-free heparin (Serva, Heidelberg, West Germany) and maintained at room temperature. The blood was diluted to 2 parts with 0.9% (w/v) NaCl, mixed and 15 ml layered carefully on top of 12 ml Lymphodex (Fresenius, West Germany) being very careful not to disturb the interface. After centrifugation at 1000 g for 20 min, the lymphocyte layer was removed, the cells washed twice in Hanks' balanced salt solution without calcium or magnesium (BSS; Serva) and made up to a final volume to contain 1.5×10^4 cells/ μ l BSS. Cells were prepared daily for the assay.

Antilymphocyte serum. Lymphocytes were obtained from the spleens of freshly killed male pigs, by the method described previously (Morton *et al.*, 1979a). The lymphocytes were made up to 10^9 cells/ml in 0.9% NaCl, suspended in an equal volume of Freund's complete adjuvant (Difco, Detroit) and injected subcutaneously at multiple sites into outbred rabbits. After 1 month, intravenous injections, each of 10^9 cells in 0.5 ml 0.9% (w/v) NaCl, were given on 3 consecutive days; 10 days later the animals were bled from the ear vein into dry bottles. The serum was harvested, inactivated at 56°C for 0.5 h, dispensed into 2 ml lots and then frozen. One sample was dispensed into 20 μ l lots, frozen and a 20 μ l sample thawed each day for use in the assay.

Sheep red blood cells. Blood (20 ml) was collected into preservative-free heparin (2×10^3 units) from the jugular vein of a non-pregnant sheep and stored at 8°C. The blood was obtained fresh at weekly intervals. A red blood cell suspension (washed 3 times in BSS) containing 10^5 cells/ μ l was prepared daily.

Guinea-pig serum. Cardiac blood (10–15 ml) was taken from each of 6 mature, male guinea-pigs. As soon as the clot had started to form (10–15 min after bleeding) the blood was refrigerated, then the serum removed as soon as possible after centrifugation at 4°C. The serum was absorbed at 4°C for 2 h with equal quantities of 3 times washed, packed pig and sheep red blood cells. The mixture was again centrifuged at 4°C, the serum removed and stored at –30°C in 0.3 ml volumes. The serum was thawed only once before use.

Rosette formation. Rosettes were formed between pig lymphocytes and sheep red blood cells. As with human lymphocytes and sheep red blood cells, complement (i.e. guinea-pig serum) was not necessary for the formation of spontaneous rosettes. However, complement is necessary in the rosette inhibition test for maximum rosette inhibition by antilymphocyte serum (Bach *et al.*, 1969) and for increased rosette inhibition due to the presence of EPF (Morton *et al.*, 1977). So in the preliminary tests to determine the number of rosettes formed between pig lymphocytes and sheep red blood cells, guinea-pig serum was added. A mixture containing 1.5×10^6 lymphocytes in 100 μ l BSS, 10^7 sheep red blood cells in 100 μ l BSS, 50 μ l guinea-pig serum diluted 1 in 5 in BSS and 250 μ l BSS was centrifuged at 200 *g* for 5 min immediately after the addition of the red blood cells to the lymphocytes. After centrifugation the cells were immediately resuspended by rotation on a Matburn wheel (10 rev/min for 5 min), then spread on a haemocytometer and the number of rosettes per 200 lymphocytes was counted in duplicate. A rosette was defined as a lymphocyte with 3 or more adhering red cells.

To determine the accuracy of counting rosettes, lymphocyte suspensions were prepared from 5 pigs. Ten identical mixtures were prepared (as described above) from each lymphocyte suspension and, for each, the rosettes in the ruled area of the haemocytometer (3 mm $\times$ 3 mm $\times$ 0.1 mm) were counted in duplicate, then the mean and s.d. of the 10 counts determined.

Rosette inhibition test. Antilymphocyte serum was diluted 1×10^3 in BSS, followed by serial double dilutions (250 μ l volumes) to 256×10^6 . Every second dilution was discarded, leaving 10 dilutions with 2 tubes containing 250 μ l BSS alone. To each of these 12 tubes was added 50 μ l guinea-pig serum, diluted 1 in 5 with BSS, and 100 μ l lymphocyte suspension. These mixtures were incubated at 37°C for 1.5 h and were then equilibrated at room temperature for 10 min before the addition of 100 μ l sheep red blood cells. Immediately, the tubes were centrifuged at 200 *g* for 5 min, the pellet resuspended by rotation on a Matburn wheel at 10 rev/min for 5 min, and each suspension spread at the same time in duplicate on haemocytometers. In the ruled area of the haemocytometer, i.e. in 0.9 μ l suspension, the number of rosettes was counted in each spread and the average number of rosettes in each antilymphocyte serum dilution was recorded as a percentage of the number in the tubes containing BSS alone, i.e. without antilymphocyte serum. It is important to complete these steps in the minimum time possible without interruptions. The rosette inhibition titre (RIT) is defined as the highest titre of antilymphocyte serum in which the number of rosettes formed is less than 75% of the number of rosettes formed in the absence of antilymphocyte serum. This titre is recorded at $\log_2$ reciprocal dilution of antilymphocyte serum.

For each sample, the results of rosette formation in each antilymphocyte serum dilution were subjected to a linear regression test and the regression coefficient was determined. The antilymphocyte serum dilution to give 75% and 50% rosette formation was then calculated.

Assay for EPF. To test for EPF in serum samples, 0.2 ml serum (diluted 1 in 2) was incubated with the pellet of lymphocytes from 1.2 ml lymphocyte suspension for 0.5 h at 37°C. The cells were then washed twice in BSS, made up to a volume of 1.2 ml and used in the rosette inhibition test as previously described.

Identification of serum inhibiting factor. In testing the serum from the first group of pigs, some samples had shown a marked increase in RIT after dialysis (see 'Results'). To identify the factor involved, duplicate serum samples (10 ml) were dialysed (Visking dialysis membrane, M_r cutoff 15 $\times 10^3$; Union Carbide Corp., Chicago, U.S.A.) against 1 litre phosphate-buffered saline for 24 h. The dialysate was then concentrated to the original serum volume in a concentration-dialysis cell using an Amicon UM01 membrane (Amicon, U.S.A.).

Column chromatography. The apparent molecular weight of pig EPF was obtained by passage of pig serum through a column (60 $\times$ 3 cm) of Sephacryl S-300 (Pharmacia Fine Chemicals, Sweden), calibrated using the molecular weight marker kit of Pharmacia Fine Chemicals. The column was equilibrated with 0.01 M-Tris acetate, 1.0 M-NaCl, 0.2% sodium azide, 0.03 M-EDTA, pH 7.4; the volume of serum applied was 1 ml and 3-ml fractions were collected. Before testing, fractions were dialysed against PBS and concentrated to 1 ml. Alternate fractions were tested for EPF and the results expressed as the RIT.

Ammonium sulphate fractionation. Serum taken from a pig 48 h after mating, and positive for EPF, was separated into components EPF-A and EPF-B with 40% ammonium sulphate as described by Clarke *et al.* (1980).

Preparation of mouse EPF-A and EPF-B. These EPF-components were produced by separate culture of oviducts and ovaries from 24-h pregnant mice at 37°C for 24 h in RPMI. The method was as described by Morton *et al.* (1980).

Results

Under the conditions described in the methods, a mean of 6.1% pig peripheral blood lymphocytes formed spontaneous rosettes with sheep red blood cells (s.e.m. 0.31, $n = 30$, each assay counted $\times 4$). The range of individual assays was 3.0 to 9.3%. After 0.5 h incubation in non-pregnancy serum, the number of rosettes formed per 100 lymphocytes was 6.5% (s.e.m. 0.58, $n = 25$) compared with 6.7% (s.e.m. 0.31, $n = 100$) formed after incubation in pregnancy serum. There was no significant difference between these three means.

The results of the 5 repetitive assays gave mean $\pm$ s.d. rosette counts of 115 ± 10.7 , 152.8 ± 7.8 , 104.4 ± 9.0 , 182.7 ± 6.4 and 67.5 ± 5.3 in the ruled area of the haemocytometer, i.e. in a volume of 0.9 μ l suspension. By obtaining the standard error of 25% of the mean rosette count and performing a *t* test, in each case 75% of this rosette count would be significant inhibition ($P < 0.001$).

There was a wide variation in the response of individual antilymphocyte sera tested using lymphocytes with and without preincubation in pregnancy serum taken from a sow at 48 h gestation (Table 1). For the present studies, antilymphocyte serum No. 247 was used since it gave inhibition as an antilymphocyte serum as well as an enhanced response due to the presence of EPF. The RIT of antilymphocyte serum No. 247, determined with lymphocytes from different male and non-pregnant female pigs, was 10.2 (s.e.m. 0.14, $n = 20$), range 10–12. With lymphocytes pre-incubated in non-pregnancy serum from different sows, the RIT was 10.4 (s.e.m. 0.18, $n = 20$), range 10–12. The reproducibility of the method was such that an RIT of 10 or 12 was found in all assays when testing lymphocytes with or without preincubation in non-pregnancy serum. Consequently, an RIT > 12 was considered evidence of the presence of EPF.

Representative curves of the rosette inhibition test on 3 samples, taken from pigs 48 h after mating, are shown in Text-fig. 1. RIT of these serum samples are 16, 20 and 24. RIT with non-pregnancy serum samples, 10 and 12, are also illustrated. The RIT has been defined as the highest serial dilution of antilymphocyte serum to give less than 75% rosette formation. The antilymphocyte serum dilution to give 75% rosette formation was also calculated from the line of regression. The mean of these values was 12.1 (s.e.m. 0.27, $n = 20$) for the non-pregnancy group and 20.4 (s.e.m. 0.71, $n = 46$) for the pregnancy group (see Text-fig. 4). The difference between these means

Table 1. RIT ($\log_2$ reciprocal dilution) with and without pregnancy serum using different preparations of rabbit anti-pig lymphocyte serum

	Antilymphocyte serum					
	216	218	247	248	250	323
Non-pregnancy serum	12	18	12	10	12	10
Pregnancy serum	30	34	20	28	14	10

Pregnancy serum was from a sow at 48 h gestation and each assay was performed in duplicate. Antilymphocyte serum was considered suitable for detection of EPF if the RIT was 2 or more dilutions greater in the presence of EPF.

Text-fig. 1. Titration curves for rosette formation with lymphocytes, previously incubated in non-pregnancy (○—○) and pregnancy (*—*) serum. Non-pregnancy serum samples were collected from 2 sows before mating and pregnancy serum samples from 3 sows, 48 h after mating. The antilymphocyte serum dilutions are represented as the logarithm (base 2) of the reciprocal of the dilution. The number of rosettes formed in each dilution of antilymphocyte serum is expressed as a percentage of the number of rosettes formed without antilymphocyte serum (control). The RIT of each serum sample is the highest dilution of antilymphocyte serum to give < 75% rosette formation; with the non-pregnancy samples RITs are 10 and 12 and with the pregnancy samples, 16, 20 and 24.

was significant ($P < 0.001$, variation of Behrens-Fisher Test; Cochran, 1967). However, if this value was calculated for 50% rosette formation, the mean of the non-pregnancy group was 9.1 (s.e.m. 0.37) and of the pregnancy group was 11.0 (s.e.m. 1.14). These values are not significantly different.

To determine whether EPF was detectable in a variety of molecular weight forms as in other species, serum from a sow obtained 2 days after mating was examined by gel filtration and fractions tested for their RIT. EPF could be resolved into 5 peaks of activity corresponding to apparent molecular weights of 450 000, 250 000, 90 000, 45 000 and 20 000 with the predominant forms being of the higher molecular weight (Text-fig. 2a).

Pig EPF (from a serum sample taken at 24 h *post coitum*) could be separated into EPF-A (supernatant) and EPF-B (precipitate) by addition of 40% ammonium sulphate. These separated components had no rosette-inhibiting activity but RIT values similar to those of the original sample were obtained when pig EPF-A and EPF-B were combined with mouse EPF-B and EPF-A respectively (Table 2).

Table 2. RIT obtained after combining EPF components prepared from pig and mouse EPF

	BSS	Pig EPF-A	Pig EPF-B	Pig recombinant (EPF-A + EPF-B)	Concentrated dialysate from pig pregnancy serum
BSS	10	10	10	20	10
Mouse EPF-A	10	10	18	—	10
Mouse EPF-B	10	20	10	—	26
Mouse recombinant (EPF-A + EPF-B)	18	—	—	—	—
Concentrated dialysate from pig pregnancy serum	10	10	26	—	—

The RIT of all recombinant preparations was assayed with pig lymphocytes. The values given are the means of duplicate assays and an RIT > 12 is evidence of the presence of EPF.

Text-fig. 2. (a) Elution profile of EPF activity obtained by passage of serum from a 48 h pregnant sow through a Sephacryl 300 gel fractionation column. ▨ Pooled fractions of peak EPF activity in serum from the same sow at (b) 3 weeks and (c) 2 months of pregnancy are also shown. Fractions were tested for EPF and the results expressed as RIT. ■ RIT of serum samples before fractionation. RIT > 12 is evidence of the presence of EPF. The results represent the mean of duplicate assays. The column was calibrated with the following standard proteins, ferritin (M_r 440 000), catalase (M_r 232 000), albumin (M_r 67 000), ovalbumin (M_r 43 000) and chymotrypsinogen (M_r 25 000).

Of an initial 35 post-coital serum samples taken within the first 2 weeks after mating and tested for the presence of EPF, only 43% gave an RIT > 12, corresponding to the presence of EPF. It was suspected that there was some factor present in the serum which was interfering in the assay. This supposition was confirmed when dialysis of the serum gave titres comparable to those obtained after gel filtration (see Text-fig. 2) and a comparison of the serum sample titres before and after dialysis is shown in Text-fig. 3. All samples were thereafter routinely dialysed before testing.

To establish the identity of the inhibitory factor responsible for the low and erratic titres during the early stages of pregnancy, a concentrated dialysate, prepared from pooled samples shown to have an increased titre after dialysis, was tested in the presence and absence of added EPF-B. Neither EPF-B nor the concentrated dialysate gave a titre for EPF when incubated alone with lymphocytes. However, addition of the dialysate and EPF-B together gave an RIT of 26, demonstrating that the inhibitory factor was EPF-A. This result was the same irrespective of whether the EPF-B was obtained by ammonium sulphate fractionation of the same pig serum sample from which the concentrated dialysate was obtained or from culture of mouse ovaries (Morton *et al.*, 1980) (Table 2).

To follow the time course of EPF production, 10 sows were tested before mating and at various intervals after mating, as shown in Text-figs 4 and 5. Before mating there was no increased RIT in any of the sows but peak levels of EPF were detected 24–48 h after mating. While there was a marked decline in the RIT around 3 weeks, EPF persisted virtually throughout the entire pregnancy in the 8 animals which subsequently farrowed successfully. After parturition, RIT

Text-fig. 3. RIT (mean with s.e.m. for the no. pigs indicated) with serum samples tested before $\square$ and after $\blacksquare$ dialysis at various times after mating. RIT > 12 is taken as evidence of the presence of EPF.

Text-fig. 4. RIT (mean with s.e.m. for the no. of animals indicated) with serum samples taken from sows before and at various times after mating; mating was 24–48 h after detection of oestrus and all animals farrowed (P) at the expected time. Broken lines indicate an RIT of 10 or 12 obtained with non-pregnancy serum samples.

values were within normal non-pregnancy levels. Two of the sows showed an early increased RIT but subsequently returned to oestrus; the results in Text-fig. 5 show that by 3 weeks the RIT had also declined to non-pregnancy levels.

To test whether the low titres found at the mid-stages of pregnancy reflected the comparative absence of EPF or the presence of further interference with the assay, a serum sample from a sow with low titres at 3 weeks was fractionated by gel filtration and the pooled fractions corresponding to the peaks of activity (see Text-fig. 2a) were tested for EPF. The results in Text-fig. 2(b) suggest that there were no further inhibitory factors responsible for the low titre. Text-figure 2 compares the molecular weight profile of samples taken from the same pig 48 h, 3 weeks and 8 weeks after mating.

Text-fig. 5. RIT with serum samples of sows which returned to oestrus 26 days ▨ and 21 days ■ after mating. RIT with serum samples of the pregnant sows, illustrated in Text-fig. 4, are shown for comparison □. The broken line indicates an RIT of 12. Any value greater than this is evidence of the presence of EPF.

Discussion

The appropriate conditions for the assay of EPF in the pig using the rosette inhibition test have now been established. The primary requirement for the assay is an effective antilymphocyte serum which gives rosette inhibition further enhanced in the presence of EPF. There is a wide variation in the response of different animals in the production of antilymphocyte serum which presumably reflects the different responses to cell surface antigens present on the lymphocyte. This is consistent with the ability of a limited number of monoclonal antibodies, notably anti-human Lyt 3, to replace antilymphocyte serum in the rosette inhibition test to detect EPF (Morton, Tinneberg, Rolfe, Wolf & Mettler, 1982a).

The precautions previously noted for the assay of human EPF (Morton *et al.*, 1982a) apply with equal force for the pig assay system. The rosettes involved in the present test are active or spontaneous rosettes, also called fast rosettes, which are formed immediately after the addition of sheep red blood cells to lymphocytes (Felsburg *et al.*, 1976). These rosettes in the pig occur to the extent of about 30–100 rosettes per 1000 lymphocytes; the number may be enhanced by the addition of dextran (Binns, 1978), bovine serum albumin or incubation of the lymphocyte/sheep red cell mixture (Escajadillo & Binns, 1975). However, these enhanced rosettes do not respond to the presence of antilymphocyte serum in the assay for EPF (Morton *et al.*, 1982a). Some confusion has arisen when other lymphocyte rosetting methods have been used in the assay (Thomson, Milton, Campbell & Horne, 1980; Cooper & Aitken, 1981) but an explicit description of active rosettes with the appropriate conditions for their formation has been provided (Bach *et al.*, 1969).

The full expression of rosette-inhibiting activity depends on an initial dialysis of the serum to remove the inhibitory effect of the presence of excess activated EPF-A. Previous studies have demonstrated that EPF-A is the moiety of the EPF complex which determines binding to lymphocytes, presumably to a receptor (Clarke *et al.*, 1980; Morton *et al.*, 1980). The inhibitory effect of EPF-A when present in an unassociated form would then be explained by competition between EPF-A and EPF for binding to this receptor. In the mouse, EPF-A has been shown to be produced by the oviduct during oestrus and during subsequent pregnancy (Morton *et al.*, 1980). There are several aspects of the concurrent appearance of free EPF-A with EPF during early pregnancy in pigs that warrant comment. The fact that free EPF-A occurs in about half the samples of pig serum tested indicates differences between individual pigs. Further the production of EPF-B does not

appear to be regulated by the presence of EPF-A. The known factors involved in the production of EPF-B by the ovaries in the mouse are prolactin and ovum factor, the latter coming from the zygote (Cavanagh *et al.*, 1982); under certain conditions, i.e. those known to stimulate parthenogenetic activation, the unfertilized ovum may also be triggered to release ovum factor (Cavanagh *et al.*, 1982).

Apart from the presence of excess activated EPF-A, the other novel aspect of EPF in the pig is its persistence virtually throughout the entire pregnancy. In other species, there is an early production of EPF which is present with steadily declining levels for the first two-thirds of pregnancy and thereafter may be absent (Morton *et al.*, 1977; Morton, Clunie & Shaw, 1979b; Morton, Rolfe & Cavanagh, 1982b). In the pig there appears to be a biphasic production of EPF with a second surge of production later in pregnancy. This may suggest that there is possibly more than one site of EPF production or that there is a shift in the regulation of EPF during the last month of pregnancy. The present study has not sought to focus upon the time of the first appearance of EPF which must await a more detailed study. However, it seems likely that it can be demonstrated within 24 h of mating, as with the other species so far tested (Morton *et al.*, 1979a; Smart, Roberts, Clancy & Cripps, 1981; Rolfe, 1982).

The fact that it was possible to hybridize EPF-A from the pig with EPF-B from another species to give full activity *in vitro* is in keeping with the lack of species specificity of EPF action on lymphocytes. This genetically unrestricted activity allows the use of the lymphocyte/antilymphocyte serum system from an entirely different species to be used for the detection of EPF, providing other interfering serum proteins are first removed.

At present the role which has been proposed for EPF is one of immuno-modulation based upon the ability of EPF to inhibit the expression of an immune response *in vivo* (Noonan, Halliday, Morton & Clunie, 1979). It has been suggested, therefore, that EPF may prevent the maternal recognition of the alien antigenic status of the embryo (Morton *et al.*, 1976). That EPF-A is able to modify the lymphocyte response to EPF clearly deserves further investigation from this aspect.

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