

Early Pregnancy Factor (EPF): a Link between Fertilization and Immunomodulation

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Abstract

Early pregnancy factor (EPF) is a pregnancy-dependent, suppressor-releasing hormone, which can be differentiated from other known pregnancy-associated substances by its physiological, biochemical and immunological characteristics. To date studies have shown that the presence of EPF in serum at anytime except during pregnancy is pathological and is associated with tumors of germ cell origin.

EPF can be detected in serum within hours of fertilization, thus providing for the first time a means of monitoring *in vitro* the continuing viability of a fertilized egg *in vivo*. EPF has been shown to be a link between fertilization and immunomodulation. Fertilization of the ovum initiates a chain of events which includes production of EPF. EPF then binds to lymphocytes, releasing soluble suppressor substances, which in turn modulate the mother's immune system. Thus it is proposed that the conceptus ensures its own defence against immunological rejection.

Introduction

Early pregnancy factor (EPF) was discovered in the course of an investigation to find a natural model of immunosuppression for transplantation biology (Morton *et al.* 1974). At that time we were using the rosette inhibition test devised by Bach and Antoine (1968) to evaluate the immunosuppressive properties of antilymphocyte sera (ALS) (Morton *et al.* 1975). The assay was adapted to look for the presence of suppressor activity in pregnant mice and the results showed that the inhibitory activity of immunosuppressive ALS in this test could be enhanced by a factor (EPF) present in pregnant mouse serum (Morton *et al.* 1976).

Further investigations demonstrated the appearance of EPF in serum during pregnancy in all species studied and, although the time course varies, EPF can be found within 24-48 h of a fertile mating until at least the mid-term of pregnancy (Morton *et al.* 1977, 1979a, 1979b, 1983; Nancarrow *et al.* 1981; Smart *et al.* 1982b; Koch *et al.* 1983; Tinneberg *et al.* 1984). During the preimplantation stage of pregnancy EPF is produced by maternal tissue in response to a signal from the conceptus, and its continuing presence is directly related to the presence of a fertilized ovum (Morton *et al.* 1980; Cavanagh *et al.* 1982). Production in this manner continues until shortly after implantation, by which time embryonic tissue has commenced production of EPF (Morton *et al.* 1982a). EPF has also been found in serum of patients with tumors of germ cell origin (Rolfe *et al.* 1983a). Maternal, fetal and tumor EPF are of similar molecular size, exhibit immunological cross-reactivity and give the same responses in the assay for EPF, but preliminary studies have suggested that they may differ in some biochemical characteristics (Wilson *et al.* 1983; Cavanagh 1984; B. E. Rolfe, A. C. Cavanagh and H. Morton, unpublished data).

EPF binds to lymphocytes and in so doing indirectly suppresses immune reactivity both *in vivo* and *in vitro* (Noonan *et al.* 1979; Morton *et al.* 1982a; Rolfe *et al.* 1983b). Thus EPF is a link between fertilization and immunomodulation and therefore is thought to have a role in the protection of the fetus from immune rejection.

Further research into the physiological role of EPF will be possible when a rapid assay for its detection is available. Although the present assay for EPF, the rosette inhibition test, is extremely sensitive and reproducible, it is time-consuming and not suitable for testing large groups of samples. Nevertheless, its use has established EPF as a pregnancy-dependent, immunosuppressive protein and made it possible to investigate the sites of production, to determine the most suitable source of EPF for purification and to monitor the steps involved in this process.

Production of EPF

The presence of EPF can be demonstrated in the serum of the mouse as early as 4–6 h after a fertile mating (Morton *et al.* 1976). The earliest time of detection in other species has not been extensively investigated but preliminary studies have shown that in humans, pigs and sheep, EPF is present in serum after 24–48 h (Morton *et al.* 1979a, 1983; Nancarrow *et al.* 1981; Rolfe 1982). Thus the presence of EPF in serum provides the earliest indication that fertilization has taken place. In all cases of established pregnancy, the continuing presence of EPF has been noted for at least the first half of pregnancy, but the length of time EPF remains in the circulation shows considerable variation. Some women had no detectable EPF during the third trimester, whereas in others EPF was present until just before parturition (Morton *et al.* 1977; Smart *et al.* 1982c). Similar variation has been shown in ewes (Morton *et al.* 1979b), while all sows tested had EPF present throughout pregnancy (Morton *et al.* 1983). In no case has EPF been detected after parturition.

The presence of EPF in the fetal circulation has been investigated using the sheep as the experimental model (see Fig. 3 in Morton *et al.* 1982a). EPF was shown to be present in fetal serum and amniotic fluid during the second third of pregnancy (the earliest that sufficient serum could be obtained for the assay), while levels declined during the last third. In both of these periods, however, the values were higher than those found in maternal serum.

EPF has two known sources of production during pregnancy. Maternal ('early') EPF is produced during the pre- and peri-implantation periods while production of fetal ('late') EPF begins in the peri-implantation stage and persists until at least mid-term. Maternal EPF consists of two components, EPF-A and EPF-B (Clarke *et al.* 1980; Morton *et al.* 1980; Cavanagh *et al.* 1982). EPF-A is oestrus-dependent and its presence in precursor form has been demonstrated in serum around the time of ovulation in mice (Morton *et al.* 1980), humans (B. E. Rolfe, personal communication), sheep (H. Morton, unpublished data) and pigs (Morton *et al.* 1983). Its appearance is related to neither fertilization nor pregnancy, but its time course is prolonged in the pregnant mouse compared with the non-pregnant cycling mouse (Figs 1 and 2). In the mouse EPF-A is produced by the oviduct (Morton *et al.* 1980) and the presence of this component in serum parallels other oestrus-dependent changes, e.g. those observed in the morphology of the vaginal mucosa (Rugh 1968). Although EPF-A is the component of EPF that binds to lymphocytes, it will not bind unless it has first been activated by the presence of EPF-B (Morton *et al.* 1980). Once activated, it will bind to lymphocytes either on its own or in combination with EPF-B (Clarke *et al.* 1980); activated EPF-A will bind to the cells in competition with EPF and, if in high enough concentration, will reduce the responsiveness of lymphocytes to EPF (Morton *et al.* 1983).

EPF-B is the pregnancy-dependent component of EPF which is released from the ovary in response to a signal from the fertilized egg. This signal, called 'ovum factor', is first

liberated from the mouse ovum upon penetration by the fertilizing spermatozoon and production continues at least until blastulation. Processes which parthenogenetically activate the ovum are capable also of liberating ovum factor from the unfertilized egg. However, a specific stimulus is needed, e.g. spermatozoa, hyaluronoglucuronidase or Ca^{2+} - and Mg^{2+} -free medium, because ovum factor is not released from the cells either by diffusion or after sonic disruption (Cavanagh *et al.* 1982). Human ova (A. C. Cavanagh,

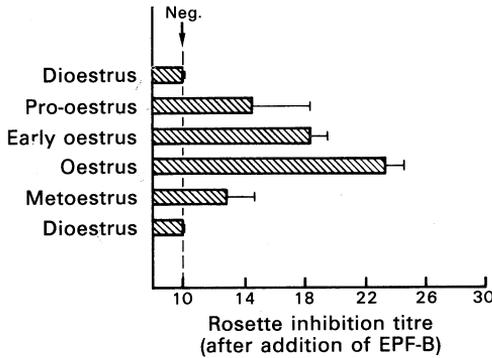


Fig. 1. Mean rosette inhibition titre (RIT) (with s.e.m., $n = 3$), after addition of EPF-B, with mouse serum taken throughout the oestrous cycle. The results demonstrate the presence of EPF-A in serum. EPF-B was prepared by incubation of dioestrous mouse ovaries with ovum factor and prolactin as described by Cavanagh *et al.* (1982). RIT was determined as shown in Fig. 3, and an RIT ≥ 14 is evidence of the presence of EPF-A. The control tests with (i) serum without added EPF-B, (ii) EPF-B alone, and (iii) PBS gave RIT values of 10 (negative test for EPF).

unpublished data), pig and sheep ova (H. Morton, unpublished data), as well as mouse ova (Cavanagh *et al.* 1982), have all been found to produce ovum factor, which is not species-specific in its biological activity; ovum factor produced from cultured human or

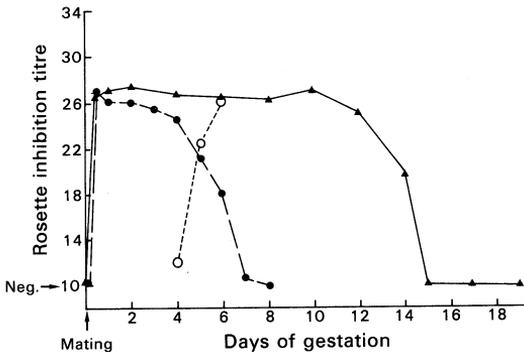


Fig. 2. Rosette inhibition titre (RIT) with mouse serum demonstrating a crossover in production of EPF from maternal to fetal tissue. ●—● At various times after mating, mouse ovaries and oviducts were removed, incubated in RPMI 1640 medium (Flow Laboratories, Irvine, U.K.) at 37°C for 24 h, and the medium tested for EPF. ○.....○ Mice were ovariectomized at various times after mating, killed 3 days later

and the serum then tested for EPF. ▲—▲ Serum was taken from mice at various times after mating and tested for EPF. The results illustrated are the means of triplicate determinations. RIT ≥ 14 is evidence of the presence of EPF. Neg. indicates a negative value for EPF.

pig embryos acts in the mouse similarly to mouse ovum factor. Furthermore, Nancarrow *et al.* (1981) have shown that, after an infusion of mouse ovum factor, EPF can be detected in the serum of oestrous sheep.

Confirmation that the fertilized egg liberates an EPF-B-releasing factor rather than EPF itself has been obtained by several groups. Morton *et al.* (1980) and Cavanagh *et al.* (1982) have shown that medium from fertilized mouse ova cultured *in vitro* does not have any detectable EPF at least until ova develop to the blastocyst stage; from this stage the production of fetal EPF from the embryo commences. Nancarrow *et al.* (1981) similarly showed that infusates prepared from fertilized mouse ova did not contain EPF activity. A. C. Cavanagh (unpublished data) also tested human embryo culture medium, supplied by Dr Alan Trounson, Melbourne, and once again found ovum factor but no EPF. This work has been confirmed recently by Chen and co-workers (1984), who were unable to find any EPF-like activity in their human embryo cultures; in their experiments, the test only became positive when exogenous EPF was added to the culture medium. Preliminary studies on the characteristics of ovum factor by Cavanagh *et al.* (1982) determined that it is liberated from mouse ova within 2 h of mating. Analysis of embryo culture medium by gel filtration detected peaks of activity at M_r of approximately 1500, 2800 and 160 000, but the nature and significance of those forms is yet to be determined.

In the mouse, the release of EPF-B by ovum factor has been shown both *in vivo* and *in vitro* to be dependent on the presence of prolactin. Confirmation of the role of prolactin *in vivo* was obtained from a series of experiments carried out in conjunction with Dr A. Gidley-Baird (see Morton *et al.* 1982a). These experiments showed that hypophysectomy of female mice 6 h after mating resulted in the disappearance of EPF from serum. However, EPF production in these mice was maintained by injection of prolactin. Cavanagh *et al.* (1982) have shown *in vitro* that non-pregnancy mouse ovaries can only be stimulated to produce EPF-B if prolactin is present in the incubation medium along with ovum factor. The role of prolactin in EPF production in other species has yet to be determined.

Whereas EPF-A can be found in serum during oestrus as well as pregnancy, the continuing production of EPF-B, and thus the presence of active EPF, is directly related to the presence of a viable conceptus. Confirmation of this has been obtained *in vivo* from experiments in pigs, sheep and mice. The presence of EPF in serum of pigs was studied before and after surgical removal of ova and related to the development of these ova in culture (Koch *et al.* 1982). The results indicate that: (i) EPF disappears from serum within 24 h of removal of the eggs; (ii) in the presence of unfertilized eggs, EPF is absent from serum; and (iii) for the polytocous pig, more than one fertilized and viable egg seems to be necessary to signal the production of EPF. Similarly Nancarrow and coworkers (1979, 1981) showed that, within 24 h of the conceptus being removed from the oviduct or the uterus of sheep, levels of serum EPF rapidly declined. While these results showed that removal of fertilized ova initiates loss of circulating EPF, further reports (Nancarrow *et al.* 1981; Morton *et al.* 1982a; Chen *et al.* 1984) have described the reverse experiments, showing that EPF appears in serum 1–3 days after embryo transfer.

Nancarrow *et al.* (1981) demonstrated the presence of EPF in the serum of synchronized recipient ewes, 24 h after embryo transfer on day 3 of the cycle. In mice, unfertilized ova and fertilized ova, obtained on days 1, 3 and 4 of pregnancy, were transferred to the uteri of day 3 pseudopregnant recipients; EPF appeared in the serum of only those animals in which healthy, dividing ova or implantation sites were found at autopsy (Morton *et al.* 1982a). More recently, Chen *et al.* (1984) have shown that, after successful embryo transfer in women, EPF was detectable in sera tested on the third day after transfer and was still present after the time of expected implantation and positive hCG assay; this work is discussed further under the section 'EPF in pregnancy diagnosis'. Thus in these controlled experiments, the presence of EPF in serum has been shown to be dependent on the presence of a conceptus. While preimplantation embryos can initiate the formation of maternal EPF through their release of ovum factor (Cavanagh *et al.* 1982), it is only upon reaching the blastocyst stage that the embryo itself has the capacity to produce EPF (Morton *et al.* 1982a). Evidence has been obtained from *in vivo* and *in vitro* studies to illustrate the crossover of EPF production from maternal to fetal tissue (Fig. 2). Oviducts and ovaries,

isolated from mice at various times after mating, cease to produce both EPF-A and EPF-B from day 7 of pregnancy onwards (Morton *et al.* 1982a). Furthermore, when mice were ovariectomized at various times after mating and maintained on progesterone until autopsy 3 days later, EPF could be detected in serum at the time of autopsy only if embryos had developed to at least the blastocyst stage. All animals autopsied at days 5 and 6 gestation and one at day 4 fell into this group. Animals with viable embryos at earlier stages of development, had no detectable EPF still present in their serum. These animals were in the group autopsied at day 4 and, although the embryos were viable, they were retarded in their development, as they were at the 1–2 cell stage and still in the oviduct (Morton *et al.* 1982a). In mice the crossover in production from maternal to fetal EPF, which occurs around the time of implantation, is not evident in EPF levels in maternal serum (Fig. 2). Biphasic production is observed, however, in the pig. There is a marked decline in serum EPF levels at about 3 weeks with a second surge later in pregnancy (Morton *et al.* 1983), suggesting that in the pig also more than one site is involved.

The continuing presence in serum of fetal, like that of maternal EPF, is dependent on the presence of a viable conceptus. Data has been presented from cases of spontaneous or induced abortion of established pregnancies in women, which demonstrate that EPF is lost from the circulation either before, or within 24 h after, loss of the conceptus (Morton *et al.* 1977; Rolfe 1982; Smart *et al.* 1982b).

Purification of EPF

To date, EPF of both maternal and fetal origin has been isolated. Clarke and Wilson (1982) and also Wilson *et al.* (1983) used serum from pregnant sheep as their source of EPF. The period of gestation (1–2 months) during which serum was collected for their study, suggests that the polypeptides described represent fetal ('late') EPF. The isolation procedures involved large scale molecular size fractionation on hollow fibres in combination with conventional ion exchange and high performance gel permeation chromatography. Two homogeneous active fractions were obtained with M_r of 20 000 and 67 000. The 20 000 polypeptide was at least an order of magnitude more active in the assay for EPF than the 67 000 substance and represented the major form of EPF present in pregnant ewes' serum at this time.

For the isolation of maternal ('early') EPF, Cavanagh (1984) used, as starting material, ovaries and oviducts from oestrous mice. These organs were cultured for 5 days in the presence of prolactin and embryo culture medium (Cavanagh *et al.* 1982), then EPF harvested from the culture medium by immunoabsorption, electrofocusing and gel filtration. A fraction was isolated with pI 6.83 and M_r of c. 21 000, which was responsible for 90% of recovered biological activity. This material appeared homogenous when analysed by high performance liquid chromatography.

Maternal (Cavanagh 1984), fetal (Wilson *et al.* 1983) and tumor-derived EPF (Rolfe *et al.* 1983a) appear to have a number of similar properties. The molecules have a monomeric size of 20 000–21 000 and exhibit immunological cross-reactivity, i.e. polyclonal antibodies raised against any one of the purified forms will cross-react with the other two (A. C. Cavanagh, B. E. Rolfe and H. Morton, unpublished data). Furthermore these three forms bind to lymphocytes and in so doing, stimulate suppressor cell activity; this activity can be detected in the rosette inhibition test and by adoptive transfer of contact sensitivity (H. Morton and B. E. Rolfe, unpublished data). However, these three forms do vary in some of their biochemical characteristics. Maternal EPF can be separated by 40% ammonium sulfate into two components (Clarke *et al.* 1980), whereas fetal and tumor EPF are resistant to this form of separation (Wilson *et al.* 1983; A. C. Cavanagh, unpublished data). The components of maternal EPF, separated by ammonium sulfate, have a similar M_r (c. 10 000) and alone exhibit no EPF activity. However, activity can be restored either by recombining these components or by adding the soluble fraction to

EPF-B or the precipitated fraction to EPF-A (Clarke *et al.* 1980). Although neither fetal nor tumor EPF can be separated by 40% ammonium sulfate, there is evidence that these components are produced by both fetal and tumor tissue. Free EPF-A has been found late in pregnancy in fetal serum and amniotic fluid (H. Morton, unpublished data) and free EPF-A and EPF-B in the serum of some patients with germ cell tumors of the testis (Rolfe *et al.* 1983a).

The three forms differ in their ability to bind to a protein carrier. In all species tested within the first few days after fertilization, the major portion of serum EPF activity detected was bound to a protein carrier (Clarke *et al.* 1980; Rolfe *et al.* 1983a; Morton *et al.* 1983). However, when serum obtained later in pregnancy (fetal EPF) or from tumor patients was fractionated, the major portion of EPF activity was not bound to a carrier (Clarke and Wilson 1982; Wilson *et al.* 1983; Rolfe *et al.* 1983a; Morton *et al.* 1983). Thus EPF produced within the first 2–3 days of pregnancy may be conserved in the circulation by its association with a larger carrier protein. EPF produced later in pregnancy by fetal tissue may have a more localized effect at the placental interface, thus eliminating the need to circulate. These proposals are supported by the finding of B. E. Rolfe (unpublished data) which showed that EPF could be detected in serum of pregnant women within 24 h of fertilization, whereas urine EPF was not detectable until 2–3 days later and was most concentrated at 5–10 weeks of gestation.

The sensitivity of the assay for EPF varies when testing EPF from different species. With EPF isolated from sheep serum (fetal EPF), Wilson *et al.* (1983) could detect 1 ng ml^{-1} in the assay with sheep lymphocytes and anti-sheep lymphocyte serum. Cavanagh (1984) found that, with mouse lymphocytes and anti-mouse lymphocyte serum, 100 pg ml^{-1} mouse maternal EPF would give a positive result. With the same assay system, A. C. Cavanagh (unpublished data) compared material purified from mouse and human tissue and found that human EPF was 10^2 – 10^3 times more active in the assay than was mouse EPF. This confirms previous reports (Morton *et al.* 1977; Clarke *et al.* 1978; Nancarrow *et al.* 1981) that EPF can be detected as a contaminant in commercial preparations of hCG, when the hCG preparations are diluted to $<10 \text{ pg ml}^{-1}$; in these experiments, human, mouse and sheep lymphocytes respectively were used to detect EPF derived from human tissue. It may be then, that human EPF has a greater potential for releasing suppressor activity from EPF-bound lymphocytes.

The significance of differences in biochemical characteristics is not yet understood, but should be considered in conjunction with the biological and immunological similarities observed to date. Nevertheless, the true relationship between these forms of EPF, derived from different sources, will eventually be determined only by sequencing of the proteins. In the meantime the different forms will be described as: maternal ('early'), fetal ('late') and tumor EPF. This does not necessarily imply that they are different substances, but simply serves to indicate the tissue of origin.

Assay for EPF

The rosette inhibition test, originally described by Bach and Antoine (1968), has been adapted as the assay for EPF (Morton *et al.* 1976). The modified test depends on the capacity of EPF, in the presence of complement, to enhance indirectly the inhibition by antilymphocyte serum of active rosette formation between lymphocytes and heterologous red blood cells (RBC). The presence of EPF in the rosette inhibition test does not in itself affect the number of active rosettes formed but increases the titre of ALS to which these rosettes are significantly inhibited.

Active rosettes are thought to represent a subpopulation of immunologically active T cells (Felsburg *et al.* 1976); the kinetics of the active rosette-forming lymphocyte response to skin test antigen was shown to parallel closely the delayed-type hypersensitivity (DTH) response, with the levels of these cells varying independently of the total T cell population.

Furthermore surface membrane differences, existing between active rosette forming cells and total T lymphocytes (Yu 1974), support the thesis that the former are a distinct subpopulation of lymphocytes. Only active rosettes, not total T rosettes, are responsive in the rosette inhibition test both with ALS alone and after the addition of EPF (Morton *et al.* 1982*b*, 1983; Smart *et al.* 1982*c*; Tinneberg *et al.* 1984). One important fact is that

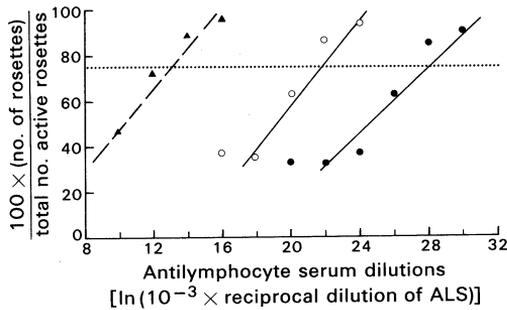


Fig. 3. Regression lines of results from the rosette inhibition test with mouse spleen cells, pre-incubated in non-pregnancy and pregnancy serum. Lines are calculated from values in the zone of greatest sensitivity; in each case $r = 0.94$. The rosette inhibition titre (RIT) is the dilution of anti-mouse lymphocyte serum (ALS) which inhibits rosette formation to 75% of the total active rosettes. ▲ With non-pregnancy serum RIT = 13.2. ○ With pregnancy serum (1) RIT = 21.8. ● With pregnancy serum (2) RIT = 28.0. RIT ≥ 14 is evidence of the presence of EPF.

active rosettes are formed immediately after adding red blood cells to lymphocytes, without incubation at 4°C and without addition of fetal calf serum, bovine serum albumin or any other factor which will enhance rosette formation. Inattention to this fact will lead to inability to carry out the assay successfully with failure to detect EPF, as demonstrated by Cooper and Aitken (1981) and Thomson *et al.* (1980).

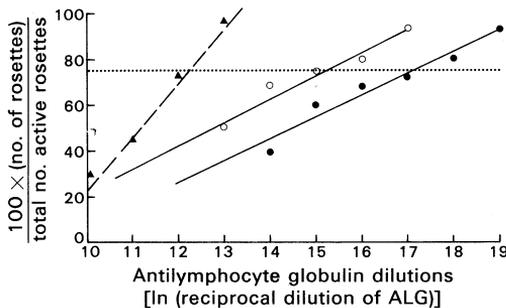


Fig. 4. Regression lines from results of the rosette inhibition test with human lymphocytes, pre-incubated in pregnancy and non-pregnancy serum. Lines are calculated from values in the zone of greatest sensitivity (▲ $r = 0.99$; ○ $r = 0.96$; ● $r = 0.94$). The rosette inhibition titre (RIT) is the dilution of anti-human lymphocyte globulin (ALG) which inhibits rosette formation to 75% of the total active rosettes. ▲ With non-pregnancy serum RIT = 12.2. ○ With pregnancy serum (1) RIT = 15.3. ● With pregnancy serum (2) RIT = 17.2. RIT ≥ 13 evidence of the presence of EPF.

The result of the assay is recorded as the rosette inhibition titre (RIT). This is the highest dilution of ALS [\ln (reciprocal dilution of ALS)] which will inhibit the number of active rosettes formed between lymphocytes and RBC to 75% or less than the number formed without ALS. Alternatively the RIT can be calculated from the regression line of values in the range of greatest sensitivity of the assay as shown in Figs 3 and 4. Only certain

ALS preparations have the ability to inhibit rosette formation. This property is not dependent on the original source of immunizing lymphocytes, as successful antisera have been prepared from thymus, tonsil, spleen and peripheral lymphocytes (Morton *et al.* 1982*b*); nor does it relate to red cell agglutinins, leucocyte agglutinins, or cytotoxins, but rather to an antibody which modifies lymphocyte response at dilutions much higher than used in these reactions (Morton *et al.* 1975).

In an attempt to identify this antibody more precisely, the effect on rosette formation of monoclonal antibodies against murine lymphocyte surface antigens (McKenzie and Potter 1979) has been studied. Monoclonal anti-Ly 1·1 and anti-Ly 2·1 (Australian Monoclonal Development, Sydney) were tested with spleen cells from inbred strains of mice (Morton *et al.* 1982*a*; Rolfe *et al.* 1984). These experiments showed that anti-Ly 1, but not anti-Ly 2, could be substituted for ALS in the detection of EPF; thus anti-Ly 1 inhibited active rosette formation between lymphocytes and RBC, and the reaction was enhanced by the presence of EPF.

Similarly, preparations of monoclonal antibodies directed against human lymphocyte surface antigens have been used with human lymphocytes in the rosette inhibition test for the detection of EPF. Tinneberg and his associates (1984) have used a monoclonal antibody directed against Lyt-3 (New England Nuclear Chemicals, NEN GmbH, Dreieich, F.R.G.), an antigen present on the cell surface of all human T lymphocytes; this antibody can recognize sheep red blood cell receptors, essential in active rosette formation. Rolfe *et al.* (1984) have also used a monoclonal antibody with pan-T cell reactivity in the assay. This antibody, Hu-Ly-m1 (Australian Monoclonal Development, Sydney) does not react with normal B cells, monocytes, granulocytes, platelets or erythrocytes, but recognizes an antigen closely associated with or identical to the E-rosette receptor. These experiments suggest that the activity in some ALS, which enables them to detect the effect of EPF on a population of lymphocytes, may be an antibody to a specific cell surface determinant on T lymphocytes.

Recently the rosette inhibition test has been adapted to assay for EPF in human serum samples using mouse lymphocytes and anti-mouse ALS (Rolfe *et al.* 1984) as the increase in RIT, resulting from the presence of EPF, is greater in the mouse assay than in the human (Figs 3 and 4). Because of the greater precision of this modification as well as the ready availability of defined populations of spleen cells from control-bred mice, we now test serum samples from all species using the mouse system (Rolfe *et al.* 1984). This has been made possible by removing most of the serum proteins with DEAE-cellulose before incubating the test sample with mouse lymphocytes; otherwise the presence of heterologous serum proteins will stimulate enhanced rosette formation, masking inhibition produced by EPF. In this way mouse lymphocytes can be used to test serum samples from any species, including those from which lymphocytes are not available either for the preparation of ALS or for use in the assay.

Binding of EPF to Lymphocytes

Thus EPF is detected by its ability to bind to lymphocytes either *in vivo* or *in vitro* and, in so doing, augment the RIT of an ALS (Morton *et al.* 1975; 1977). Furthermore this reaction is not species-specific as EPF from one species can be detected with lymphocytes from a different species (Rolfe *et al.* 1984). More recent studies have shown that the rosette inhibition test is a two-stage reaction and augmentation of ALS activity in the rosette inhibition test is mediated by EPF-induced soluble suppressor substance(s) (H. Morton, B. E. Rolfe, A. C. Cavanagh and D. J. Morton, unpublished data); EPF first binds to a small population of lymphocytes, inducing the release of suppressor(s) which then reacts with a further population of lymphocytes (Ly 1⁺ cells in the mouse) to give the co-operative effect of rosette inhibition in the presence of ALS.

The lymphocytes binding EPF have been quantitated by tagging them with ^{125}I -EPF, gold-labelled EPF and gold-labelled anti-EPF (D. J. Morton, unpublished data). There was good agreement between all three methods, with *c.* 2% mouse spleen cells binding EPF. In order to determine the optimal conditions for this binding, studies were done with ^{125}I -EPF, varying the number of lymphocytes, time and temperature of the reaction and concentration of EPF.

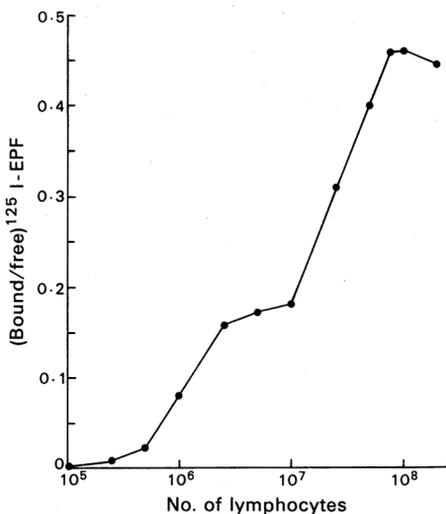


Fig. 5. Binding of ^{125}I -EPF to lymphocytes as a function of cell concentration. EPF was radio-iodinated by the solid-phase lactoperoxidase method. Lymphocyte suspensions in RPMI medium were prepared from Quackenbush mouse spleens. Volumes containing the required number of cells were dispensed into tubes, centrifuged and then 5 ng ^{125}I -EPF in 0.2 ml RPMI medium (25 ng ml^{-1}) was added to each cell pellet. After incubation at 37°C for 0.5 h, the cells were rapidly sedimented, the supernatant removed and the radioactivity of the cells and the supernatant were measured. This experiment was done in duplicate.

With varying numbers of spleen cells, EPF binding is biphasic with a rapid increase occurring at higher cell concentrations (Fig. 5). One possible interpretation of this is that the higher concentrations favour cell-cell interaction and this augments binding; the frequent observation of labelled EPF at the interface between two cells supports this suggestion (D. J. Morton, unpublished data). The uptake of EPF by spleen cells is rapid

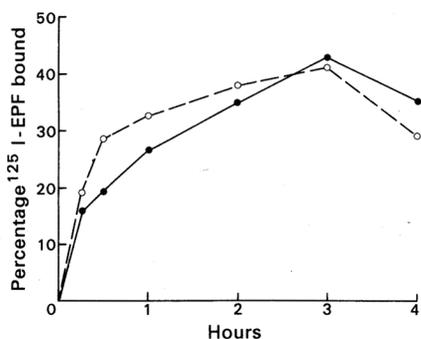


Fig. 6. Binding of ^{125}I -EPF to lymphocytes as a function of time and temperature. Lymphocytes (2×10^7) and ^{125}I -EPF (5 ng in 0.2 ml RPMI) were prepared as described in Fig. 5. These mixtures were incubated for various times at 37°C (\circ --- \circ) and 4°C (\bullet — \bullet), and then the cells were rapidly sedimented, the supernatant was removed and the radioactivity of the cells and the supernatant was measured. These experiments were done in duplicate.

in the first half-hour of incubation, with *c.* 75% of the maximum binding at 37°C being completed within this time (Fig. 6). The binding was marginally less at 4°C in the early stages but, after 3 hours, no temperature difference was observed. In the rosette inhibition test, EPF is bound to lymphocytes by incubating 2×10^7 cells in 0.2 ml test sample at 37°C for 0.5 h. With these conditions and varying EPF concentrations, the results of EPF detection by the rosette inhibition test were compared with those obtained from the binding

of ^{125}I -EPF to lymphocytes. EPF can be detected in the rosette inhibition test in concentrations at least as low as 100 pg ml^{-1} , i.e. 20 pg in 0.2 ml sample (Cavanagh 1984), while significant binding of labelled EPF to spleen cells is not observed below 2 ng in 0.2 ml sample (10 ng ml^{-1} concentration). These results demonstrate that the rosette inhibition test is an extremely sensitive assay for detecting the presence of EPF, and this is considered to result from an augmentation by the release of suppressor(s) from the EPF-bound lymphocytes.

However, high concentrations of EPF ($>50\text{ ng ml}^{-1}$) cause a loss of responsiveness of the lymphocytes in the rosette inhibition test (Wilson *et al.* 1983; Cavanagh 1984) which is manifest by a failure to release suppressor (H. Morton, unpublished data). Loss of responsiveness when excess activated EPF-A is present with EPF has also been demonstrated *in vitro* by adding excess EPF-A to a preparation of EPF and *in vivo* by testing the serum of pigs within the first few weeks of pregnancy (Morton *et al.* 1983). When testing serum samples from sheep, mice and humans, loss of responsiveness is not observed but, with serum from pigs, a preliminary dialysis step is necessary to remove excess activated EPF-A (Morton *et al.* 1983). Since EPF-A is the moiety of the EPF complex which binds to lymphocytes, its inhibitory effect might be explained by competition with EPF for lymphocyte receptors. However, the biological result of the presence of excess EPF-A in serum and subsequent loss of suppressor release will have to await further investigations into the role of EPF in pregnancy.

EPF-dependent Suppressor

The binding of biological concentrations of EPF to lymphocytes causes the release of suppressor(s) and it is this activity rather than EPF as such, which has been shown to inhibit cellular immune responses (H. Morton, unpublished data). This was demonstrated by experiments designed to separate the inducing EPF from any putative suppressor factor(s) liberated after incubation of lymphocytes with EPF. The results of the experiments showed that an increased RIT could still be obtained with lymphocyte supernatant after removal of unbound EPF by an immunoabsorbent. Thus EPF induces the release of suppressor activity which is detected in the rosette inhibition test. While the action of EPF with mouse spleen cells in this test is neither species- nor strain-restricted, the suppressor activity is genetically restricted (H. Morton, B. E. Rolfe, A. C. Cavanagh and D. J. Morton, unpublished data). Suppressor therefore can be differentiated from the inducing EPF by restricted activity; i.e. suppressor produced by EPF binding to C57BL/6 lymphocytes will not give an increased RIT with allogeneic cells (C3H or BALB/c) as it will with syngeneic (C57BL/6) cells. Thus it appears that at least two populations of cells are involved in the rosette inhibition test, the cells that bind EPF, releasing suppressor, and the cells which are affected by suppressor.

EPF has been shown previously to inhibit the adoptive transfer of contact sensitivity (Noonan *et al.* 1979; Rolfe *et al.* 1983b) which, like the rosette inhibition test, is a T-cell dependent assay (Asherson and Zembala 1973; Morton *et al.* 1975; Moorhead 1977). Suppressor has been prepared from C57BL/6 and BALB/c spleen cells and both preparations tested in parallel with EPF in this assay (H. Morton, B. E. Rolfe, A. C. Cavanagh and D. J. Morton, unpublished data). As in the rosette inhibition test, the adoptive transfer of contact sensitivity was inhibited by both EPF and syngeneic suppressor but not by allogeneic suppressor.

Studies were carried out then to determine if suppressor activity could be identified *in vivo* and if so, to investigate its time course in relation to that of the inducing EPF. Male mice were chosen for the experiment to avoid interference from oestrus-related hormones. C3H mice were injected intraperitoneally with 5 ng EPF (Cavanagh 1984) and the serum was tested 3 days later with lymphocytes from C3H and C57BL/6 mice (Fig. 7a). There was no EPF detectable in serum (negative assay with C57BL/6 lymphocytes), but suppressor

activity was still detectable both with the mouse's own cells and after incubation of serum with syngeneic cells. In fact the suppressor activity increased over the 14 days following injection, indicating a recruitment of suppressor cells. With increasing dose, the time course of EPF in serum was prolonged and in all cases, suppressor activity was detectable at 14 days (Figs 7b and c). By 21 days post-injection, neither EPF nor suppressor could be

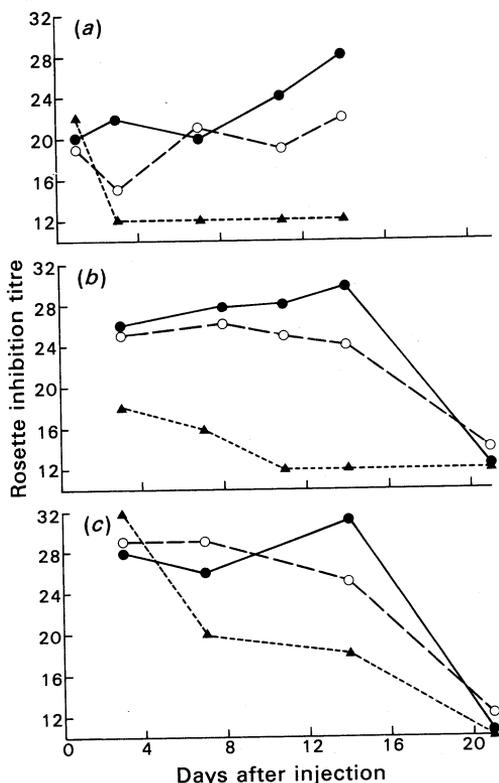


Fig. 7. Time course of EPF and suppressor *in vivo* after i.p. injection of various amounts of EPF into male mice. Purified EPF in 0.9% NaCl was injected into C3H mice (aged 10–12 weeks); (a) 5 ng per mouse, (b) 50 ng per mouse, (c) 500 ng per mouse. The mice from each group were killed at different times after injection and the spleen and serum harvested. Spleen cells from the mice were tested in the rosette inhibition test, as were donor C3H and C57BL/6 spleen cells after incubation for 0.5 h at 37°C in the harvested serum. ●—● C3H spleen cells from injected mice, ○--○ serum from injected mice plus donor C3H spleen cells; both of these tests demonstrate the presence of EPF and suppressor. ▲--▲ Serum from injected mice plus donor C57BL/6 spleen cells; these tests demonstrate only the presence of EPF. RIT ≥ 14 is evidence of the presence of EPF and/or suppressor. Each value is the mean of results from two mice.

demonstrated. These results are in marked contrast to those obtained after loss or removal of the conceptus; under these conditions both EPF and suppressor disappear from the circulation within 24–48 h (H. Morton and B. E. Rolfe, unpublished data). It would appear then that the removal of these substances after abortion is an active process precipitated by the loss of the fetus.

EPF in Pregnancy Diagnosis

The presence of EPF in serum has been used to diagnose pregnancy in sheep (Morton *et al.* 1979a; Nancarrow *et al.* 1979), pigs (Koch *et al.* 1983; Morton *et al.* 1983) and humans (Morton *et al.* 1977; Rolfe 1982; Morton *et al.* 1982b; Smart *et al.* 1982a, 1982b; Tinneberg *et al.* 1984; Chen *et al.* 1984). Evidence has already been presented from animal studies to confirm that the detection of EPF indicates the presence of a fertilized egg(s) and in the studies on humans mentioned above, no variation in the negative control assays has been detected relating to the menstrual cycle, contraception or infertile intercourse. Consequently this marker was used by Rolfe (1982) to assess fetal wastage in the preimplantation period of pregnancy in a random group of 13 nulliparous women who had discontinued oral contraception at least 2 months previously. Of 28 cycles studied, EPF was detected after ovulation and intercourse in 18, giving a fertilization rate of 64%. However, EPF production continued for more than 14 days in only four cases, indicating

an early fetal wastage of 78%. Taking the presence of EPF in serum as a marker, Smart *et al.* (1982b) found a similar fertilization rate (67%) in their group containing 8 nulliparous and 10 multiparous women. However, they demonstrated only a 38% fetal loss by day 14 post-ovulation. Roberts and Lowe (1975) used behavioural data to estimate a total embryonic and fetal loss of 78% by the time of birth, whereas Leridon's results (see Biggers 1984) suggested only 31% of ova exposed to spermatozoa would be expected to develop into live babies (69% fetal wastage). Hertig *et al.* (1959) in their study of ova recovered from women of known fertility, estimated that only 42% of ova exposed to spermatozoa would survive to the end of the second week of ovulation (58% loss). These values appear to vary according to the reproductive history of the women included in the groups. With the development of a rapid convenient assay for EPF, it will be possible to investigate larger, more representative groups of women, to determine the effect of various factors both on fertilization rate and early fetal wastage.

Smart and coworkers (1982a) extended their studies to test the serum of two groups of women for the presence of EPF after intercourse at the time of ovulation. EPF was not detected in the group with tubal ligation, whereas in the group wearing intra-uterine devices, the transitory appearance of EPF was noted 6–7 days after presumed ovulation. A fertilization rate of 26% in this latter group, compared with the values of 67% (Smart *et al.* 1982b) and 64% (Rolfe 1982) in women without contraception, suggests that the intra-uterine device decreases the rate of fertilization as well as preventing implantation.

Chen *et al.* (1984) have used the presence of EPF in serum to monitor patients after transfer of *in vitro* fertilized eggs. As Chen points out, the detection of hCG in the maternal circulation is the only established way of determining whether the embryo has successfully implanted, but this marker will not indicate the viability of the embryo prior to implantation. Edwards (1981) reported an 81% wastage of single embryos transferred to women after *in vitro* fertilization. Chen *et al.* (1984) tested for EPF in the serum of their post-transfer patients, and found that, of the embryos transferred that failed to successfully implant (65%), all were still viable at 7 days after transfer but not at 15 days. In another group, in which transfer was difficult, EPF was not detectable at any time, confirming that there had been no successful transfer. The presence of EPF therefore can differentiate between a successfully implanted embryo leading to an established pregnancy, an embryo that remains viable only to the peri-implantation stage and lastly an unsuccessful transfer; thus the viability of the embryo during the immediate post-transfer period can be studied with the aim of increasing the embryo survival rates in *in vitro* fertilization programs.

EPF as a Tumor Marker

EPF has been found in the serum of some patients with choriocarcinomas (B. E. Rolfe, unpublished data) and with germ cell tumors of the testis (Rolfe *et al.* 1983a). In the latter group, tumor EPF or its free components were associated most frequently with non-seminomatous germ cell tumors, containing embryonic/chorionic elements, but they were present also in some seminoma patients. Nevertheless in this latter group, it is possible that non-seminomatous elements, not observed on histological examination, were present in the tumor (Nørgaard-Pedersen 1978). EPF was not detected in the serum of patients with non-germ cell tumors (Rolfe *et al.* 1983a); however, in the light of findings for other embryonic and placental antigens (Braunstein *et al.* 1973; Rosen *et al.* 1975), it is possible that EPF production may not be restricted to germ cell tumors.

Regardless of the cell type involved in tumor EPF production, EPF and its components provide additional serum markers for germ cell tumors, thus potentially enlarging the group identifiable by such means. Further investigations will be required to determine the exact nature of the cell types involved in EPF production by tumors, the relationship of tumor EPF with other tumor-associated antigens and, finally, the diagnostic and prognostic value of the measurements of EPF in tumors.

Concluding Remarks

EPF is a pregnancy-dependent suppressor-releasing hormone. The ultimate decision of whether it is a novel substance must rest on its full characterization including the determination of a unique amino acid sequence. Nevertheless the research done to date demonstrates that EPF can be differentiated from other known pregnancy-associated substances by its physiological, biochemical and immunological behaviour. The detection of EPF in serum virtually within hours of fertilization has potential for opening up new fields of research. Previously it had not been possible to detect pregnancy in the pre-implantation period other than by surgery, but now, by assaying serum EPF, a means is provided of monitoring *in vitro* the continuing viability of a fertilized egg *in vivo*.

The discovery of EPF and its subsequent characterization has depended entirely on the rosette inhibition test as the assay system. While the development of an immunoassay will permit more rapid progress in clarifying the role of EPF, the RIT still will remain as a reference standard of biological activity, i.e. the ability of EPF to stimulate the release of soluble suppressor substance(s) from lymphocytes. This suppressor activity is capable of affecting cellular immune responses and is detected *in vitro* through its cooperative action with ALS in rosette inhibition and *in vivo* by the suppression of adoptive transfer of contact sensitivity.

The activity of EPF is not species specific; human, sheep and pig EPF will bind to mouse lymphocytes both *in vivo* and *in vitro*, releasing suppressor. It could be proposed then that EPF plays an important role *in vivo* as there appear to be strong evolutionary pressures to conserve its function. In contrast to the unrestricted activity of EPF, that of the EPF-induced suppressor is genetically restricted. In this context it is worth noting that the major histocompatibility complex (MHC) plays a key role in the recognition of foreign antigens by T lymphocytes (Zinkernagel and Doherty 1979), and recent findings have suggested that the MHC is also involved in cell-cell communication and differentiation of both lymphoid and non-lymphoid cells (Parish *et al.* 1981).

EPF has been shown to be a link between fertilization and immunomodulation. Fertilization of the ovum is accompanied by release of ovum factor, which signals the production of EPF-B from the ovary. EPF-B is the pregnancy-dependent component of EPF. It binds to EPF-A, present in serum at ovulation, to form active EPF, which in turn stimulates populations of lymphocytes to produce soluble suppressor substance(s) capable of suppressing cellular immune responses. The reactions involving EPF form a cascade initiated by the conceptus and resulting in modulation of the mother's immune system; thus it is proposed that the conceptus ensures its own defence against immunological rejection.

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