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Rosette Inhibition Levels
During Early Pregnancy
in Sheep and Swine

by

© Fern Donna Levine, BSc. (Agr.)

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

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ABSTRACT

The rosette inhibition test, an immunologically-based assay, was used to detect an in vitro immunosuppressive early pregnancy factor (EPF) in sheep within 7 days of fertilization. Lack of success with the sheep system led to a re-examination of the factors influencing rosette formation. Many physical and physiological factors were found to affect the test such as working temperature, source of lymphocytes and quality of rosette inhibiting ability of antilymphocyte serum. When a good working system was developed for the pig, further attempts were made to detect EPF. In no case was it possible to distinguish between samples from pregnant and non-pregnant animals. It was concluded that although the rosette inhibition test was unpredictable, nevertheless at present it is the only test which can detect EPF. Therefore, until other techniques are developed, further knowledge about this early pregnancy factor must be learned through the use of an improved rosette inhibition test.

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LIST OF ABBREVIATIONS

AET	aminoethylisothiuronium bromide
ALS	antilymphocyte serum
CG	chorionic gonadotropin
EDTA	ethylenediamine-tetraacetic acid
EPF	early pregnancy factor
E-rosette	erythrocyte rosette
Ficoll	Ficoll-Hypaque
x g	times gravity
g	gram
GPC	guinea pig complement
h	hour
HBSS	Hanks' balanced salt solution
hCG	human chorionic gonadotropin
HRBC	human red blood cell
LH	luteinizing hormone
mg	milligram
min	minute
ml	millilitre
oCS	ovine chorionic somatomammotropin
OF	ovum factor
PBL	peripheral blood lymphocytes
p.c.	<u>post coitum</u>
PGE ₂	prostaglandin E ₂
PMSG	pregnant mare's serum gonadotropin
PS _β G	pregnancy-specific β_1 -glycoprotein

RALS-7 rabbit #7 ALS
RBC red blood cell
RIA radioimmunoassay
RIT rosette inhibition titre
SRBC sheep red blood cell
T-cell thymus derived lymphocyte (or T-lymphocyte)
ul microlitre
UV ultra-violet (short wave)
w/v weight/volume

INTRODUCTION

In adapting to pregnancy mammals must provide for a continuous elevated blood progesterone concentration. The means by which this is done is by the sustained progesterone production by the corpus luteum (Heap, 1972). Since the corpus luteum normally undergoes luteolysis at the end of the estrous or menstrual cycle due to the production of uterine luteolysins, chiefly prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (McCracken et al., 1972), the maternal organism must receive a signal to inform her that a developing embryo is present and that therefore luteal function must be maintained. This interaction has been termed the "maternal recognition of pregnancy" (Short, 1969). Many pregnancy-specific substances, produced during pregnancy, have been reported e.g. human chorionic gonadotropin (hCG) and various serum proteins, but their biological functions have not been determined, although antiluteolytic or luteotropic activities have been proposed for some.

A second aspect of the establishment of pregnancy is that the maternal immune system must make adaptations to the presence of paternal antigens, present on the developing embryo, in order to prevent immunological rejection. Several of the pregnancy-associated factors have been suggested to have an immunosuppressive function either masking the developing conceptus to prevent

immunologic recognition or interrupting the immunologic responses to recognition.

Maternal Recognition of Pregnancy

The evolutionary development of viviparity has demanded the provision of mechanisms for internal nurture of the developing young. Various regulatory mechanisms have been developed according to individual species' needs. Differences exist even within mammals which are divided into three classes; the monotremes, the marsupials and the eutherian mammals. The monotremes do not give birth to live young but encase their embryos, after a short period of development in utero, in leathery-shelled eggs for further incubation (Griffiths, 1968). All other mammals give birth to live young.

Gestation in eutherian mammals is generally characterized by an extension of the lifespan of the corpus luteum beyond the period of the normal breeding cycle. This extension of luteal function may be interpreted as a first step in the evolution of prolonged gestation from a condition such as exists generally in the marsupials, where the lifespan of the corpus luteum in pregnant and non-pregnant females is similar and the gestation period is the same length as the estrous cycle.

This prolonged gestation demanded that the maternal organism recognize the developing conceptus so that

progesterone production by the corpus luteum would be maintained. The pituitary gland of most mammals is supportive (luteotropic) during early pregnancy. The duration of this support varies, depending upon the endocrine capabilities of the placenta. In sheep the pituitary is required for only the first third of pregnancy, while in other species, such as the pig, it is essential throughout gestation (Heap, 1972).

In order for the maternal organism to prolong the function of the corpus luteum, it must be influenced by the embryo. In the dog and ferret this "maternal recognition of pregnancy", as defined by Short (1969), is not necessary for luteal maintenance, since the corpus luteum persists for the same time in both pregnancy and pseudopregnancy (Lawson and Findlay, 1976).

Eutherian mammals have evolved a variety of mechanisms for control of corpus luteum function and maintenance of gestation while retaining the reproductive advantages of being polyestrous (i.e. greater reproductive output), but there is not necessarily a common mechanism for recognizing the conceptus. Three primary mechanisms of pregnancy maintenance have been proposed (Lawson and Findlay, 1976):

a) Recognition is not required, as in the "fail safe" system of luteal maintenance of the dog and ferret.

b) The conceptus may overcome luteolytic actions of

the uterus by:

1) suppressing the production, release or function of uterine luteolysins, as in ungulates and the guinea pig;

2) exerting an overriding luteotropic influence, as in the rat.

c) The pituitary-ovarian axis initiates corpus luteum function and the conceptus provides luteotropic support, which appears to be the situation in the primates.

In the sheep, pig and cow, the corpus luteum normally regresses at the end of a 16-21 day estrous cycle in response to a luteolysin, most likely $\text{PGF}_{2\alpha}$ (McCracken et al., 1972), produced by the uterus and transported locally to the ovaries. When an embryo is present in the uterus on Day 12 in sheep and pigs or Day 16 in the cow (Moor and Rowson, 1967; Dhindsa and Dziuk, 1968; Betteridge et al., 1976), this regression does not occur. This is believed to be due to substances passing from the embryo to its mother, a maternal response being elicited prior to attachment of the embryo to the endometrium (see Table 1). Therefore some embryonic factor acts directly or indirectly on the ovary to maintain luteal function (Heap et al., 1979).

Considerable effort has been made to identify the embryonic signals. Yoshimi et al. (1969) found evidence that hCG, produced by human trophoblast and placenta,

Table 1

A Comparison of the Times of Maternal Recognition of Pregnancy,
Attachment and Length of Gestation in the Pig, Cow and Sheep

Species	Day <u>post coitum</u>		
	Maternal Recognition of Pregnancy	Attachment	Gestation length
sow	12	18	114
cow	16-17	40-45	282
ewe	12-13	16	145

Taken from Heap et al. (1979)

influences the corpus luteum of the cycle so that it persists and continues to produce progesterone. The hCG is first produced about the time of implantation (Day 5.5 to 6).

Several workers have reported evidence of an hCG-like material produced near the time of implantation by the rabbit blastocyst (Haour and Saxena, 1974; Fujimoto, et al., 1975; Holt et al., 1976; Ellinwood et al., 1977). Varma et al. (1979) found a gonadotropin-like substance resembling CG and LH in rabbit blastocysts. The earliest embryonic signal thus far characterized is a contraceptive tetrapeptide, isolated from the two-cell hamster embryo (Kent, 1973, 1975), which, when injected into diestrous hamsters, prevents both ovulation and estrus. Thus in the hamster recognition of pregnancy occurs by Day 4, when the placenta has not yet formed.

In the mare, only fertilized eggs pass into the uterus while unfertilized eggs are retained in the oviducts (van Niekirk and Gerneke, 1966; Betteridge and Mitchell, 1974). No explanation for this phenomenon has yet been provided. Godkin et al. (1978) claimed that Day 13-15 ovine embryos stimulate progesterone production by both ovine and bovine luteal tissue in vitro and postulated that the pre-implantation ovine conceptus produces a non-species-specific luteotropic substance.

Domestic species, however, Godkin's report

notwithstanding, do not appear to produce a luteotropin at the time that the presence of an embryo prevents luteal regression. Pituitary LH is responsible for corpus luteum function at this time. Although ovine chorionic somatomammotropin (oCS) has been detected in ovine pre-attachment chorion as early as Day 14 (Carnegie et al., 1982), this and other pregnancy-associated proteins, which have recently been reported, do not appear to play a primary role in maintenance of the corpus luteum in pregnancy (Schramm, et al., in press).

In the pig, estrogen, which has a luteotropic effect, is synthesized by the blastocyst in the form of the sulfate (Perry et al., 1973) by Day 12 of pregnancy. The luteotropic action is believed to be mediated either by primary action on the uterus, redirecting prostaglandin release to the lumen rather than the venous system or by increasing uterine blood flow and effectively reducing the concentration of $\text{PGF}_2\alpha$ in the uterine vein (Ford et al., 1982). Carnegie and Robertson (1978), also have shown that estrogens are synthesized by the ovine embryo by than Day 30 and that the principal estrogen is also estrone sulfate.

Trophoblastin, a protein claimed to be antiluteolytic, is produced in early pregnancy in the sheep (Martal et al., 1979). Trophoblastin inhibits luteolysis only in the corpus luteum ipsilateral to the

embryo but disappears after Day 21-23 of gestation. Trophoblastin alone does not completely prevent luteal regression. Therefore it has been suggested that there is a complementary action of placental luteotropins in the pregnant ewe (Martal et al., 1979).

Thus interactions between embryo and mother during the establishment of pregnancy appear to be of two types. In some cases the embryo exerts an antiluteolytic action, in others a luteotropic action or both. The result is continuous progesterone production. Eventually in species such as the human and the sheep the placenta becomes a major source of progesterone.

Immunological Aspects of Reproduction

The fact that mammalian embryos and fetuses, although representing an allograft, are not rejected by the maternal organism is an immunological enigma. Several hypotheses have been advanced to explain the tolerance of the fetus by the uterus (reviewed by Beer and Billingham, 1976, 1978; Kiefer, 1977; Stites et al. 1979; Billingham and Head 1980; and Stimson, 1981).

The four principal theories, outlined below, have experimental support but none alone accounts completely for tolerance of the fetus.

1. Although under some circumstances the uterus behaves as an immunologically privileged site, allografts

from the non-pregnant uterine horns of pregnant animals with bicornuate uteri ~~are~~ rejected at approximately normal rates (Stites et al., 1979).

2. The fetus and trophoblast appear, in some cases, not to express transplantation antigens. Billington et al. (1977) found that minor and eventually major paternal transplantation antigens appear sequentially on the plasma membranes of embryonic cells from at least the two-cell stage onwards. Heyner et al. (1980) showed expression of transplantation antigens on the mouse zygote freed mechanically of the zona pellucida but not at the two-cell stage. While no antigens were detected at the four-cell stage either, it was thought that the reactivity seen at the eight-cell stage represented de novo synthesis of these antigens (Heyner et al., 1980).

An antigen masking theory recently proposed is that of low dose antigen tolerance (immunological enhancement), whereby trophoblast surface antigens may be masked by maternal antibodies. Maternal antihistocompatibility antigen antibodies may thus protect fetal tissues and placenta from cellular immune attack (Stites et al., 1979). Knobloch et al. (1979) have shown in rats that both mother and embryo contribute to the syncytiotrophoblast so that a cell hybridization occurs which is postulated to result in a living cytoplasm composed of DNA, RNA and cellular organelles of both

maternal and fetal lineages. The gradual and slowly increasing supply of antigenic material is thought to induce a low dose tolerance which is mediated by thymus-derived lymphocytes. Makino et al. (1978) found that human trophoblast cells express rather weak antigenicity and suggested that this plays a quarantining role in the maintenance of pregnancy by blocking access to strong fetal antigens, while not attracting an immunologic attack on itself.

3. There is a weakening of maternal cellular immunity. Finn et al. (1972) found that lymphocytes from pregnant women showed significantly reduced immunological reactivity when cultured with normal lymphocytes (mixed lymphocyte culture). It was suggested that T-cell activity is reduced during pregnancy and that this reduction may help protect the fetus from rejection. However, many conflicting reports have challenged this view. Kovithavongs and Dossetor (1978) and Stahn et al. (1978) showed the presence of suppressor cells in human pregnancy as early as 6 to 10 weeks after conception. Tofoski and Gill (1977), however, have shown that "migration inhibitory factor" is produced during allogeneic pregnancies but not during syngeneic ones. Billingham and Head (1980) found that although the lymph nodes draining the uterus hypertrophy during allogeneic pregnancies, there is a humoral response which involves

blocking antibodies rather than cytotoxic antibodies. These provide protective advantages to the offspring.

The possibility that the maternal immune system undergoes no changes during pregnancy was suggested recently by Miyasaka and McCullagh (1981). They found no differences in the reactions of peripheral blood lymphocytes (PBL) from ewes before and during pregnancy to the plant mitogen, concanavalin A, and to paternal and third party PBLs.

4. There is a complete separation between fetal and maternal circulations, but placenta is not an absolute barrier between maternal and fetal circulations, since antibodies and blood cells, both erythrocytes and leukocytes, may pass in either direction; antibodies against paternal antigens are detectable in maternal serum. Maternal Rh-antibodies may cross the placenta to the fetus resulting in the Rh-hemolytic condition (Amino et al., 1978). It is, therefore, evident that the placenta does not present a complete immunological barrier and immunological adjustments between mother and fetus must occur in order to ensure survival of the offspring.

It is evident that whatever the degree of immunodeficiency achieved during pregnancy the maternal organism is not rendered vulnerable to lethal infection. The intrinsic reactivity of pregnant maternal lymphocytes may or may not be normal, but circulating suppressive

factors seem to modulate antigen recognition and effector cell function during pregnancy. Consequently, pregnancy serum has been examined for both specific and non-specific effects on immunocompetence. Thus far twenty pregnancy-associated serum proteins have been found, but their biological roles have not been established. Stimson (1981) has reviewed evidence concerning six allegedly immunosuppressive proteins

[pregnancy-associated- α_2 -glycoprotein (called α_2 -PAG, PAG, PAM, SP3, or PZP), pregnancy-specific β_1 -glycoprotein (called PS β_1 G, SP1, PAPP-C or TBG), human placental lactogen (called hPL or hCS), α -fetoprotein (called AFP or α FP), human chorionic gonadotropin (hCG), and early pregnancy factor (EPF)] but it is clear from the conflicting reports of the immunosuppressive actions of these substances that none has a major role in pregnancy-associated immunotolerance.

Although physiological concentrations of gestational hormones, such as estrogen, progesterone and their derivatives do not suppress lymphocyte responses in vitro (Schiff et al., 1975), supraphysiological concentrations are effective. Thus, it has been proposed that the prolonged elevations in the concentrations of those hormones during pregnancy may affect maternal lymphocytes. Moreover the locally elevated concentrations at the maternal-placental interface, may provide

immunosuppression.

As an added note, there is one more hypothesis as to the control of the immunological response to pregnancy, namely that since pregnancy is a normal physiological state, perhaps it is under a different type of genetic control than the immune responses to exogenous foreign antigens (Gill and Kunz, 1978).

Older Methods of Pregnancy Diagnosis

Elucidation of the roles of the pregnancy-specific factors is of vital importance to the understanding of reproductive physiology. Moreover, detection of these factors could be used to monitor pregnancy status. In domestic animals ideally pregnancy failure should be detectable before the first estrus after breeding so that rebreeding may be planned immediately. Throughout this century a multitude of tests for pregnancy has been developed. Some involve physical examination, while others involve various laboratory procedures, but none is useful before the first missed menses or return to estrus.

Cowie (1946) has reviewed the older pregnancy diagnostic tests and classified them into: 1) tests based on clinical methods; 2) tests based on hormonal investigations of body fluids; 3) tests based on biochemical investigations of body fluids; 4) tests based on physiological phenomena; 5) tests based on

immunological phenomena; and 6) tests based on physical changes occurring in body fluids and tissues.

Clinical methods such as rectal palpation are accurate, rapid and immediate when performed by an experienced handler. When performed between 35 and 40 days following service rectal palpation can give a definite diagnosis (Hafez, 1975). Rectal palpation is still the simplest and most widespread method of pregnancy diagnosis in cattle and horses. It cannot be used in the pig or sheep due to physical limitations (Hafez, 1975; McDonald, 1980).

The Benesch-Kurosawa test involving examination of changes in the vaginal walls and mucus was used in the mare and was 95% accurate on days 21-30 post ovulation. (see Cowie, 1946).

The first hormonally-based pregnancy test useable with confidence (98% accuracy) was the Aschheim-Zondek test (1927) based on the observation that pregnancy urine, when injected into immature mice or rats, caused ovulation and formation of corpora lutea. The test could be performed as early as the 35th day after the last menstruation (21 days after ovulation).

The Friedman test (1929), similarly exploited the induction of ovulation in estrous rabbits by pregnancy urine, with an accuracy similar to that of the Aschheim-Zondek test.

Another popular urine test developed in 1934 by Shapiro and Zwarenstein (see Cowie, 1946), used the anuran, Xenopus laevis, which ovulates and oviposits within 18 h after injection of pregnancy urine or plasma. The accuracy and time of use was also similar to that of the Aschheim-Zondek test.

Zondek (1930) used immature female rats or mice as test animals to detect the pregnant mare's serum gonadotropin (PMSG), first discovered by Cole and Hart (1930) (see Cowie, 1946). PMSG is first detectable at 37 to 42 days of pregnancy. The accuracy of this test between 45 and 110 days following service was reported to be 95-98%. Aside from PMSG, early investigators found no gonadotropic activity in the urine or blood of pregnant animals other than primates.

Following the gonadotropin-based tests came the discovery of the role of estrogens during pregnancy. Although steroid hormones are not pregnancy-specific, they may be present in sufficient quantities at some stages of pregnancy to permit diagnosis. The Sulfuric Acid test of Kober (1931) was used to detect estrogens in pregnancy urine of mares (Cuboni, 1934) with 98% accuracy in the diagnosis of pregnancy after the 90th day.

The earliest test for progesterone (Corner and Allen, 1931) made use of its proliferation-inducing activity on ovariectomized rabbit endometrium, but was relatively

insensitive, requiring 1 mg of progesterone.

The only truly valuable techniques of pregnancy diagnosis during the first half of this century were the Aschheim-Zondek test and its modifications (i.e. the Friedman test) which tested for hCG, and the tests of Cole and Hart for PMSG. These methods detected gonadotropin activity. The major problem with these techniques is that none was capable of detecting pregnancy or non-pregnancy before the first missed estrus in domestic animals.

Current Methods of Pregnancy Diagnosis

Currently, the most widely used test for pregnancy in humans is the agglutination-inhibition test, which detects hCG and may even be self-applied through use of readily available kits.

Hormones which can be measured in testing for pregnancy by radioimmunoassay include estrogens, estrone sulfate, progesterone, hCG, PMSG and oCS. Some pregnancy-specific proteins which may be detected by other means, such as gel immunodiffusion, include trophoblastin, PS β , G, and the proposed contraceptive polypeptide from hamster embryos (Kent, 1974).

One of the most popular tests for pregnancy in animals used today is assay of progesterone in peripheral blood or milk at a fixed time after breeding (Robertson and Sarda, 1971; Laing et al., 1979), by radio- or

enzyme-linked immunoassay. The test is based on the fact that the progesterone concentrations, which are zero at estrus, remain elevated during pregnancy past the time of the next expected estrus. The accuracy is reported to be about 87% for cows, pigs and sheep (Robertson and Sarda, 1971), and a single skilled operator is now able to test 200 samples per day.

In sows, the most popular clinical method of pregnancy diagnosis is the Doppler ultrasound technique (Fraser and Robertson, 1968), which gives 95% accuracy in animals at least 30 days pregnant. The cost of equipment is low and diagnosis is done in situ.

Robertson and Sarda (1971) have provided criteria for judging the practicality of a pregnancy test: It must be a) accurate (at least 90%), b) simple and economical to perform and c) give results very early in pregnancy. Another consideration is the number of species on which the test can be performed. The major problem with all hormonally based tests is that none is applicable until the first missed estrus (except, perhaps the estrogen assay in the sow).

"Early Pregnancy Factor"

A claim of the discovery of an "early pregnancy factor" in the serum of pregnant mice within one day after conception (Morton et al., 1974, 1976b) was followed by

claims of similar findings in women (Morton et al., 1977) and in sheep (Morton et al., 1979a). Morton and her colleagues used the rosette inhibition test to evaluate the influence of sera from pregnant mice, women and sheep on the ability of an antilymphocyte serum to decrease the number of spontaneous rosettes formed between lymphocytes and heterologous erythrocytes. The EPF was found in mice from 6 h post coitum (p.c.) until 4 to 6 days before parturition (Morton et al., 1976b), and in sheep and women EPF was detected within 24 h after mating (Morton et al., 1979a, 1977). In the sheep, EPF was shown to persist past one month and in some cases past the fourth month of gestation (Morton et al., 1979b), while in humans it appears only during the first two trimesters of pregnancy (Morton et al., 1977).

The possibility that a test for EPF could be used widely to detect early pregnancy and embryonic mortality has wide implications for both the practical aspect of animal husbandry as well as the scientific interest in the study of reproduction and fertility. The fact that the rosette inhibition test is immunologically-based has been taken to suggest that EPF may play a role in preventing rejection of the embryo by the maternal organism.

Since it has been shown that transplantation antigens are expressed by pre-implantation embryos (Webb et al., 1977), and even by the zygote (Heyner, 1980), it would be

advantageous for survival of the embryo if the maternal organism recognized her pregnant status as soon as possible in order to make various adaptations which may be necessary.

Basic Characteristics of Rosette Formation

Erythrocyte-rosettes (E-rosettes) are formed when thymus-derived lymphocytes from one species are exposed to erythrocytes (RBC) from a heterologous source. The RBCs form a cluster around the lymphocyte which resembles a flower (see Figure 1).

Rosettes cannot be formed between all species of animals. Some combinations work better than others and in most cases the fact that lymphocytes from a particular species will form rosettes with RBCs from another species does not necessarily indicate that the reverse combination will work (Johannsen et al., 1974).

The ability to form E-rosettes has been shown to be a characteristic of thymus-derived lymphocytes (T-cells, T-lymphocytes, thymocytes) in the human (Jondal et al., 1972; Bentwich et al., 1973) and in the pig (Escajadillo and Binns, 1975). It has also been that 2-4% of bursal-equivalent lymphocytes (B-lymphocytes, B-cells) can form E-rosettes (Bentwich et al., 1973).

Gurtler (1981) has claimed that a lipid-protein interaction involving the lipid of the RBC and a protein

Figure 1: Rosette formation between pig lymphocytes and sheep erythrocytes. Lymphocytes are stained with Acridine Orange and fluoresce green. Note how sheep red blood cells surround lymphocytes in a flower-like configuration.

COLOURS
Images en couleur

structure on the lymphocyte membrane is responsible for the T-lymphocyte rosette formation. There are two subpopulations of T-lymphocytes. One subpopulation will form rosettes spontaneously within 10 min while the other subpopulation does so only after 24 h (Richter, 1980).

In human medicine E-rosette formation is used to monitor the immunocompetence of T-cells, since rosette formation with sheep erythrocytes (SRBC) is virtually 100% after 24 h (Richter, 1980).

Inhibition of rosette formation, known as the rosette inhibition test, was first developed to assess the ability of antilymphocyte sera (ALS) to suppress the cellular immune systems of patients receiving organ transplants (Bach et al., 1969; Bewick et al., 1972; Wood and Gray, 1973). Briefly, lymphocytes from thymus, spleen, peripheral blood, tonsils or lymph nodes are washed and incubated with ALS and guinea pig complement. SRBCs are then added, and the rosettes are counted and compared with controls in which no ALS was given. The dilution of a specific ALS which produces a certain amount of rosette formation, such as 75% of control value, is the rosette inhibition titre (RIT) of that ALS.

Statement of Objectives

This study was undertaken with the purpose of validating the rosette inhibition test for the detection of early pregnancy in the sheep and then to test the porcine species for the presence of EPF, since it had not yet been reported. Because EPF appears so early in pregnancy but disappears before parturition, the progress of EPF and its effect on both the maternal organism as well as the embryo were to be studied. In addition, various aspects of the test procedure will be evaluated.

Experimental

The basis of the method for the detection of the early pregnancy factor is the inhibition of rosette formation by EPF as described by Morton et al. (1974).

As previously mentioned, Morton et al. (1979) described the detection of EPF in the maternal serum of sheep 24 h after mating (i.e. fertilization). Since the sheep is one of the more frequently used models for studies in reproductive physiology, it was decided to confirm this finding prior to investigating the possible biological role of this factor. It is important to note that many of the details essential for success in performing this test for EPF were unknown. Many requirements were recognized by Morton, herself, only following the failure of this test in the hands of a

majority of the workers who have attempted to use it (Walton, personal communication, 1980; Thomson et al., 1980; Cooper and Aitken, 1981; Gimenez, personal communication, 1982). In the present study, such fine details of methodology were gleaned only towards its termination from personal discussions and correspondence with Morton or from the experimental work to be described herein.

The methodology followed initially was that reported for the detection of sheep EPF (Morton et al., 1979a); after the failure to detect ovine EPF and to obtain satisfactory spontaneous rosette formation between ovine spleen lymphocytes and human erythrocytes, the investigations were directed towards studying rosette formation using combinations from different sources of lymphocytes and erythrocytes. In the belief that a high percentage of rosette formation between porcine thymocytes and sheep erythrocytes constituted a good working system, the study was finally concentrated on efforts to detect porcine EPF.

MATERIALS AND METHODS

Animals: Breeding Schedule

A) Sheep

Crossbred ewes were fitted with progestogen sponges which were removed after 13 days. At the time of sponge-withdrawal the animals were injected intramuscularly with 500 IU pregnant mare serum gonadotropin (PMSG). Estrus followed 33-36 h later (Day 0) with ovulation occurring approximately 27 h after the onset of estrus.

The ewes were artificially inseminated on the morning and on the afternoon of the day of onset of estrus. Blood samples for the rosette inhibition test were collected from the jugular vein into non-heparinized Vacutainer tubes (Becton-Dickenson) 24 h and 96 h after insemination. Prebreeding samples were also obtained. After allowing clotting at 4°C overnight, the serum was separated by centrifugation at 1300 x g for 20 min at 4°C, complement was inactivated at 56°C for 30 min and the serum was stored in small aliquots at -20°C. On day 18, serum progesterone was assayed by RIA as a test for pregnancy (Robertson and Sarda, 1971).

B) Pigs

Crossbred gilts were monitored for the onset of

estrus (Day 0) and were bred by natural service twice, 24 h apart on Day 0 and Day 1. Ultrasonic scanning (Fraser and Robertson, 1968) was used to confirm pregnancy on Day 30. Farrowing records were also consulted. Blood samples (days -4, 0, 3, and 7) were collected from the orbital sinus into a syringe and placed immediately into non-heparinized glass tubes. Samples were obtained on day 3 and day 7 from animals which were not bred. The blood was processed and stored as described above. Prior to use in the rosette inhibition test, porcine serum samples were dialyzed overnight at 4°C against 500 ml Hanks' Balanced Salt Solution (HBSS) without calcium or magnesium.

Immunization Procedures

Lymphocytes for immunization were prepared from spleen, thymus or peripheral blood using tissue pooled from at least 3 non-pregnant animals. In each case a sample was prepared following the procedures described below (see Preparation of Lymphocytes). A modification of the lymphocyte suspension used for immunization described by Gozzo et al. (1979) was employed as adapted by Morton et al. (1979). A final suspension of 1×10^8 lymphocytes/ml in 0.9% (w/v) NaCl (Morton et al., 1979a) was used to prepare the immunization mixture.

Antilymphocyte sera were raised in rabbits, sheep and goats. Twelve albino male rabbits were immunized against

pig lymphocytes (6 against spleen cell and 6 against peripheral blood lymphocytes). Another 4 rabbits were immunized against sheep spleen cell lymphocytes. Two male goats and two ovariectomized sheep were immunized against pig thymocytes.

The immunization schedule used by Morton et al. (1979a) was adopted from Bach et al. (1969).

The rabbits' backs were shaved and intradermal injections were given at a minimum of ten sites with 0.5 ml of the lymphocyte suspension in 0.5 ml Freund's complete adjuvant (DIFCO Laboratories, Detroit, Michigan). This cell suspension was prepared using a Pasteur pipette. This injection was followed 4 weeks later by an intravenous injection of 0.5 ml lymphocyte suspension on each of 3 successive days. The rabbits were bled either from the ear vein (Williams and Chase, 1967) or by cardiac puncture on the 9th, 11th and 13th days after the last injection. The blood was allowed to clot overnight at 4⁰ C. The serum was separated at 1300 x g for 20 min at 4⁰ C and inactivated at 56⁰ C, for 30 min. Small aliquots were stored at -20⁰ C.

The sheep and goats received intradermal injections once a week for 5 or 6 weeks followed by a blood collection one week later. Thereafter, periodic boosters, either intradermal (but without adjuvant) or intramuscular, were given and blood samples were collected

and processed as above for another 6 months (sheep) or 9 months (goats). The antilymphocyte serum titre was assessed as that dilution which reduced rosette formation by 25% (Morton et al., 1974).

A rabbit anti-porcine lymphocyte serum prepared by Morton was received in the lyophilized form. It was reconstituted to the original volume in distilled water and small aliquots were stored at -20°C . The reported RIT of this serum was at a dilution of 1:4000.

Hanks' Balanced Salt Solution

Hanks' Balanced Salt Solution (HBSS) (Hanks and Wallace, 1949) was the medium used for rosette formation (Morton et al., 1974). HBSS was initially prepared as shown in Appendix 1 from stock solutions or from powdered HBSS obtained commercially (GIBCO). The commercial medium contained phenol red as a pH indicator. Calcium and magnesium were later excluded from the buffer system (Morton, personal communication, Morton et al., 1982). The buffer solution was adjusted to pH 7.2 and brought to room temperature ($18-25^{\circ}\text{C}$) before use.

Glassware

Disposable plastic or glassware was used whenever possible. It was not washed before use. Other glassware was washed in hot water with 7X detergent (Flow

Laboratories) then rinsed with distilled water and dried at 121°C overnight.

Heparin

Pure sodium heparin from porcine intestinal mucosa (without added antiseptic or preservative) (Sigma Chemical Co., St. Louis, Mo.) was used for the collection of peripheral blood from animals at the abattoir. One ml of heparin solution was prepared by adding 1.0 ml sterile saline (0.9% NaCl w/v) to 159 mg sodium heparin (10,000 IU). The heparin solution was stored unrefrigerated and away from light. One hundred μ l heparin solution was used per 50 ml blood collected.

Lymphocyte Separation Medium

A solution of 5.7% Ficoll:8.8% sodium diatrizoate (Hypaque) with a final density of 1.077 was obtained as Histopaque 1077 (Sigma Chemical Co.) or as Lymphodex (Fresenius, Bad Hamburg, FDR), or it was prepared in the laboratory from 57 ml 10% Ficoll:9% Hypaque, 43 ml 9% Hypaque (Sigma Chemical Co.) and 3 ml distilled water.

Preparation of Lymphocytes

A) Spleen

Spleens (>100 g) were obtained from a non-pregnant animal of desired species (pig or sheep) at the abattoir.

The material was cut into small pieces in HBSS and transported to the laboratory within 30 minutes. Connective tissue was removed, the spleen ground in a mincer and suspended in approximately 150 ml HBSS. This suspension was stirred slowly for 15 min to release lymphocytes and then filtered through surgical gauze. The filtrate was centrifuged in 40 ml glass conical centrifuge tubes at $825 \times g$ for 20 min at room temperature (20°C). The supernatant was decanted and the pellets hemolyzed with 5 ml distilled water and resuspended with a Pasteur pipette for 1.5 min. Then 20 ml HBSS were added and the suspension was filtered through a surgical gauze pad and funnelled into a 125 ml Erlenmeyer flask. The mixture was divided between three to six 15 ml disposable plastic conical centrifuge tubes (Corning) and centrifuged at $360 \times g$ at room temperature for 5 min on a clinical centrifuge. If a second hemolysis was required the operation was repeated. If the pellets appeared relatively free of erythrocytes, they were washed once more in HBSS and suspended in HBSS for counting. Cells were stained by adding 25 μl of a 0.05% solution of acridine orange (British Drug Houses, Ltd.) in HBSS per ml suspension of cells. Cells were spread on a hemocytometer with Neubauer mirror finish (CanLab) and counted. Lymphocytes were examined using UV fluorescence microscopy (Zeiss Photomicroscope III) to facilitate recognition in

rosettes as RBCs are not stained by acridine orange.

Following this cell count a final concentration of 1.5×10^4 lymphocytes/ul was prepared in HBSS and the cell suspension placed in a 25 ml Erlenmeyer flask, covered with Parafilm and left at room temperature.

B) Thymus

Thymuses (~ 70 g) were obtained from a young non-pregnant individual of the test species (pig or sheep) and transported whole and unrefrigerated in HBSS. Excess fat and connective tissue were removed. The organ was cut into small pieces, ground and suspended in 50 ml HBSS. This suspension was stirred for 15 min then filtered through several layers of surgical gauze and centrifuged in 15 ml conical plastic tubes at $360 \times g$ for 5 min at room temperature. The supernatant was discarded, the cells washed twice in HBSS and counted as detailed for spleen lymphocytes. A final suspension of 1.5×10^4 lymphocytes/ul HBSS was prepared for the rosette test.

C) Peripheral Blood (Lymphocytes)

The method of Boyum (1968) was used to isolate peripheral blood lymphocytes. Blood was collected from non-pregnant sheep or pigs. Ovine blood was collected by jugular venepuncture into 20 ml heparin-coated Vacutainer tubes. The tubes were inverted several times to ensure

thorough mixing and were transported unrefrigerated to the laboratory for separation of the lymphocytes.

Porcine blood was collected in one of two ways. At the abattoir it was collected into an Erlenmeyer flask coated with 0.1 ml (1000 IU) preservative-free heparin solution for each 50 ml blood that was required. Only 50 ml of blood were required to perform a series of rosette inhibition tests to detect EPF. Blood was collected from the jugular vein immediately following slaughter. The blood was thoroughly mixed, and transported unrefrigerated to the laboratory and diluted 1:1 with 0.9% NaCl, at room temperature, upon arrival at the laboratory. Twelve ml of Ficoll-Hypaque at room temperature were placed carefully into a 30 ml glass round-bottomed centrifuge tube (105 mm x 24 mm, Corning) without touching the sides of the tube. Sixteen ml diluted blood were pipetted carefully over the Ficoll layer in order to establish a clear interface between the blood and the Ficoll. The pipette tip was placed against the inside wall of the tube and the blood was allowed to run slowly down and form a layer over the Ficoll.

Four such tubes supplied sufficient lymphocytes ($>10^6$) for a day's work with rosette tests. The tubes were centrifuged at $1000 \times g$ for 20 min at 20°C . The white lymphocyte layer (at the interface) was removed with a Pasteur pipette and diluted approximately 3:1 with HBSS

in 15 ml disposable plastic centrifuge tubes. The tubes were centrifuged at $360 \times g$ for 5 min at room temperature and the supernatant discarded. (Hemolysis, when necessary, was performed by suspending each pellet in 1 ml of distilled water for 1 min then adding 14 ml HBSS. After Morton (personal communication, 1981) suggested that hemolysis may damage the lymphocytes, this procedure was subsequently omitted.) The cells were resuspended, centrifuged and washed twice in HBSS and combined in a total volume of 10 ml of suspension. A sample of the suspension was diluted and stained with acridine orange for counting, and a final concentration of 1.5×10^4 lymphocytes/ μ l HBSS prepared for the rosette test.

Figure 2 contains a flow-chart of the above procedures.

ROSETTE INHIBITION TEST

Treatment of Lymphocytes

Disposable 5 ml plastic test tubes with plastic stoppers (Sarstedt, FDR) were set up in the following manner: One ml of the lymphocyte suspension (1.5×10^4 lymphocytes/ μ l HBSS) was placed in each of three tubes and centrifuged at $360 \times g$ for 5 min at room temperature. The supernatants were decanted and the tubes were inverted. The pellets were resuspended by gentle rocking in:

Figure 2Preparation of Lymphocyte Suspension

tube 1: 0.2 ml HBSS + 0.2 ml prebreeding serum
tube 2: 0.2 ml HBSS + 0.2 ml postbreeding serum
tube 3: 0.4 ml HBSS

If more than one test was performed on the same day, a pre- and a postbreeding sample was included for each animal.

These tubes were then incubated at 37°C for 0.5 h with occasional resuspension. After incubation the tubes were centrifuged at 360 x g for 5 min at room temperature and the supernatants were removed. The pellets were gently suspended in 1 ml HBSS and washed twice then taken up in 1 ml. This suspension of lymphocytes served for the test series.

Serial Dilutions of Antilymphocyte Serum

Sets of 5 ml (75 mm x 12 mm) disposable plastic test tubes with push-in stoppers (Sarstedt, FDR) were lined up in a rack. Antiserum was thawed and an initial dilution of 1:1000 was made in HBSS. This was followed by a series of doubling dilutions up to $1:1.6 \times 10^9$ with alternate dilutions discarded. The dilution range of ALS varied depending on the rosette inhibition titre of a particular ALS. The RIT was determined as the highest dilution of antilymphocyte serum in which the number of rosettes was less than 75% of the number formed without ALS (Morton et al., 1974).

All work was performed at room temperature following advice from Morton (personal communication, 1981). In earlier experiments, the preparation of lymphocytes was performed on ice with centrifugations at 4°C.

Complement

Commercially prepared lyophilized guinea pig complement (GPC) (GIBCO) was initially used. It was reconstituted to its original volume with "Restoring Solution" (GIBCO) containing 6% sodium acetate, 2% boric acid and 0.25% sodium azide. This preparation was then absorbed with equal volumes of packed, washed donor erythrocytes from all the donor species to be used. For example, if red blood cells (RBCs) from both sheep and human were to be used with a specific preparation of GPC, then the complement would be absorbed as follows: 1 volume GPC: 1 volume packed SRBC: 1 volume packed HRBC. Absorption was performed at 4°C for 2 h. The mixture was then centrifuged at 1300 x g for 20 min at 4°C. The serum was removed and 0.5 aliquots were placed in disposable plastic microcentrifuge tubes (Sarstedt). These were stored at -20°C until needed.

Following advice from Morton (personal communication, 1981), fresh serum was obtained from male guinea pigs. Blood was obtained from at least six animals via cardiac puncture (Health and Welfare Canada or Animal Disease

Research Institute, Agriculture Canada) and pooled. It was absorbed and stored as previously mentioned.

For the rosette test complement was prepared at the last minute after the ALS and lymphocytes were added to the series of tubes. Complement was thawed and diluted 1 part serum to 4 parts HBSS, and 0.05 ml was immediately added to each tube. The tubes were mixed by gentle hand rocking and placed in the incubator at 37°C for 1.5 h. Control tubes received GPC with HBSS instead of ALS.

Erythrocytes

Human blood was collected in 5 ml heparinized Vacutainer tubes from four donors (GM and HR = male; FL and SM = female, non-pregnant).

Sheep blood was drawn from the jugular vein into 20 ml heparinized Vacutainer tubes. The sheep donors were either male or ovariectomized females. When possible the same sheep was used as a constant source of RBCs.

The blood was initially transported on ice to the laboratory and prepared in the cold; however, after receiving advice from Morton (personal communication) the blood was used only at room temperature. It could be stored for up to one week at 4°C and brought to room temperature before use.

The erythrocytes (RBCs) were prepared in one of two ways. 1) Whole blood was centrifuged at 400 x g for

10 min at 20°C and the buffy coat and plasma were removed followed by dilution of 1 ml of packed RBCs into 9 ml HBSS. 2) Two ml of whole blood were diluted to 10 ml in HBSS in 10 ml disposable plastic test tubes (Sarstedt) and centrifuged at 360 x g at room temperature for 10 min. In both cases the pellets were washed 3 times in HBSS and a final concentration of 1×10^5 RBC/ul HBSS was prepared and left at room temperature.

Rosette Formation

Following the 1.5 h incubation of the lymphocytes with the ALS and GPC, 100 µl of the RBC suspension was added to each tube. The tubes were immediately centrifuged at 400 x g for 5 min at room temperature. The pellets were resuspended on a Hematology/Chemistry Mixer Model 346 (Fisher Scientific Co.) for 5 min at room temperature at 12 revolutions per min.

Counting Rosettes

The first method used to count rosettes was that of differential counts, where the number of rosettes per total number on lymphocytes in a microlitre of the hemocytometer was counted. When many samples had to be counted, this method was modified to counting the number of rosettes per 200 lymphocytes. These counts were made using hematology counters. When these differential counts

were performed, it was necessary to stain the lymphocytes so that a lymphocyte in the centre of a rosette was distinguished from clumps of RBCs or lymphocytes. The rosettes were examined by simultaneous light and fluorescence microscopy if they were stained with acridine orange. When only a light microscope was available, the lymphocytes were stained with a 0.33% solution of methylene blue prepared in HBSS.

Morton (personal communication, 1981) recommended another method for counting rosettes, which involved counting all rosettes on the entire field of both sides of the hemocytometer. A differential count was not performed, since it was assumed that the concentration of lymphocytes was approximately equal in all samples. The samples from an entire series of ALS dilutions were spread on hemocytometers immediately following suspension of rosettes. These slides were placed in a moist chamber to prevent dessication of the samples. The counts from the series were performed within a half-hour. This method was used only for the pig lymphocyte-sheep RBC system, since it was unknown earlier.

As various caveats became known from communication with Morton (as noted above) they were incorporated into the testing protocol. These changes were: removal of Ca and Mg from HBSS; performing all work at room temperature (except for incubations at 37°C); using Morton's Ficoll;

using fresh male guinea pig complement (pooled from at least 6 animals); using PBLs for the rosette test; not hemolyzing the lymphocyte preparation; placing the hemocytometers in a moist chamber to prevent dessication of the samples; and counting the rosettes within one hour and counting rosettes only, instead of performing a differential count.

Statistical Analysis

All comparisons, except where noted, were made using Student's t test.

RESULTS AND DISCUSSION

Section A) Physical and Physiological Factors Affecting Rosette Formation

The results presented below represent data collected both before and after learning Morton's recommendations. No differences in results were obtained following these changes, therefore, the results were compiled and considered together. All of the results in Section A were obtained before Morton's modifications were known, while Sections B and C include data obtained using all of Morton's precautions (see Materials and Methods).

Species and Tissue Source of Lymphocytes

During the first few months of this project the sheep lymphocyte-human RBC rosette system was explored in an effort to validate the findings of Morton et al. (1979). It soon became apparent that this system did not produce an "adequate" number of rosettes and rosette formation was extremely variable from day to day. Therefore, it was decided to examine different combinations of rosetting systems in the hopes of finding a good working system. The results of this effort are presented in Table 2. Sheep lymphocytes did not produce more than 5-10% rosettes with RBC from human, pig or cow, however, pig spleen and thymus lymphocytes produced good rosettes (50%) with sheep RBC. Porcine peripheral blood lymphocytes produced only

Table 2

Rosette Formation with Different Species and Different Lymphocyte
Tissue Sources

<u>Source of T-Lymphocytes</u>	<u>Source of RBC</u>	<u>Rosette Formation(8)</u>
sheep spleen (1)	human	low ~ 5%
sheep spleen	pig	very low $\leq 1\%$ (*)
sheep spleen	cow	very low $\leq 1\%$ (*)
sheep thymus (2)	human	low $\leq 5\%$
sheep blood (3)	human	low $\leq 5\%$
sheep blood	pig	low $\leq 5\%$
pig spleen (4)	sheep	very high 50%
pig thymus (5)	sheep	very high 50%
pig thymus	goat	high $\leq 10\%$ (*)
pig thymus	cow	no rosettes (*)
pig blood (6)	sheep	high $\leq 10\%$
cow blood (7)	sheep	low $\leq 5\%$ (*)
cow blood	human	low $\leq 5\%$ (*)

(1) 1×10^8 WBC/g (2) 1×10^9 T-cells/g

(3) 7×10^5 WBC/ml whole blood (4) 1.75×10^7 WBC/g

(5) 1×10^9 T-cells/g

(6)(7) 9×10^5 WBC/ml whole blood

(8) Replicated measurements except where indicated by (*)

Morton et al. 1979: Range = 3-11% (sheep spleen lymphocyte
 with human RBC)

10% rosettes with sheep RBC. The goat and cow did not prove to be of any value in rosette formation with the combinations attempted.

Further testing in the sheep often produced higher numbers of rosettes as seen in the data in Table 3. In comparing rosette formation produced by sheep spleen versus sheep thymus, the thymus cell preparation had twice as many rosette forming cells ($65.4 \pm 3.7\%$) as the spleen cell preparation ($29.7 \pm 2.1\%$), and this difference was significant ($p < 0.05$).

Most consistent results, approximately 50% rosettes, were obtained using porcine lymphocytes with sheep RBCs. As it was thought best to have a high rosette formation in order to detect differences more readily this system was adopted, as well as because of lack of success with the sheep lymphocyte/human RBC system.

Rosette formation by thymocytes was greater than that by spleen or peripheral blood lymphocytes, as expected, because the thymus contains T-cells only, with 65-90% capable of forming rosettes (Escajadillo and Binns, 1975). The spleen contains only 30-50% T-cells (Loor and Roelants, 1977) and about 60% of the white cells recovered from peripheral blood are T-cells. Because E-rosette formation involves only T-cells, it was decided to use the thymus as a source of lymphocytes in further work with the sheep as well as in the porcine studies. Morton later

Table 3Comparison of Rosette Formation Between Spleen and ThymusLymphocytes

Lymphocyte Source		
Thymus <u>% rosettes</u>	Spleen <u>% rosettes</u>	
62.1	31.2	
61.0	29.8	
64.8	36.7	
59.3	24.5	
<u>79.7</u>	<u>26.2</u>	
65.4 <u>+3.7</u>	29.7 <u>+2.1</u>	Mean <u>+S.E.</u>

Source of spleen and thymus lymphocytes - ewe lamb #80-0686

Source of RBC - human

advised, however, that it was preferable to use spleen or peripheral blood as a lymphocyte source (personal communication, 1981).

Erythrocyte Donors

Erythrocytes from three human donors forming rosettes with sheep thymocytes was examined (Table 4a). There were no differences ($p > 0.05$); however, it appeared that HR produced the best rosettes with an unusually high $81.3 \pm 2\%$ rosettes. Each day the rosette formation was apt to change whether using sheep thymus, spleen or peripheral blood lymphocytes. The lowest rosette formation with this combination was about 5%. As the results were so erratic and because of insurmountable obstacles using the sheep lymphocyte-HRBC rosetting system, the test species was eventually changed.

Using porcine thymocytes and SRBCs no significant difference was observed ($p > 0.05$) (Table 4b), although ewe #203 appeared to produce more rosettes than did ewe #202.

Data in Tables 4a and 4b show that rosette formation varies with the donor. As erythrocytes from HR and FL produced higher numbers of rosettes than did RBCs from SM, HR and FL served as RBC donors for further sheep studies. Morton (personal communication, 1981; Morton *et al.*, 1982) has also found that some human subjects produce RBCs that are more apt to form rosettes than those of other individuals.

Table 4Comparison of Rosette Formation Between Erythrocyte Donors

4a) Human erythrocyte donor (% rosettes)

<u>FL</u>	<u>SM</u>	<u>HR</u>	
74.1	66.8	85.5	
66.3	49.4	75.7	
67.6	60.9	77.6	
	68.1	84.6	
—	<u>60.0</u>	<u>82.9</u>	
69.3 $\pm$ 2.4	61.0 $\pm$ 3.3	81.3 $\pm$ 2.0	Mean $\pm$ S.E.

Source of lymphocytes: sheep thymus

4b) Sheep erythrocyte donor (% rosettes)

<u>Ewe #203</u>	<u>Ewe #202</u>	
44.6	46.8	
60.4	32.5	
47.1	34.5	
—	<u>38.1</u>	
50.7 $\pm$ 4.9	38.0 $\pm$ 3.2	Mean $\pm$ S.E.

Source of lymphocytes: pig thymus

Only two sheep were used in the comparison for the pig study and although no significant difference ($p > 0.05$) was detected between these donors, it appeared that RBCs from ewe #203 tended to form more rosettes than RBCs from ewe #202. Thus, in subsequent studies, blood was collected from ewe #203 whenever possible. This confirms work by Steel et al. (1974) who found rosette formation varied with the origin of sheep RBC.

In addition to differences among RBC donors in rosette formation it was also evident that the numbers of rosettes formed under similar conditions changed from day to day. This was confirmed by Morton (personal communication, 1981).

Protein

As it appeared that during processing some lymphocytes were being lost due to adherence to the sides of the plastic test tubes, the effect on rosette formation of protein (gelatin or serum) or the use of siliconized glass test tubes was examined (Tables 5a and 5b). The average rosette formations in rabbit, goat, cow and pig serum show that there is no difference ($p \gg 0.05$) among the results of any of these sera; however, compared with the control, the serum from all species seems to have caused a trend to higher numbers of rosettes ($p < 0.05$).

Using porcine thymocytes and SRBC, rosette formation was examined in the presence and absence of gelatin (Table

5b). Even compared with the blank, there were no significant differences ($p > 0.05$) in rosettes formed under these conditions. Likewise, when incubation was performed using non-pregnant gilt serum (control) or no protein in siliconized glass tubes, rosetting was not significantly increased ($p > 0.05$). Although rosette formation appeared to be greater in the siliconized glass tubes and in the samples containing protein, these differences were due to larger numbers of lymphocytes recovered, and in the final analysis, did not influence the test since the number of rosettes formed was proportional to lymphocyte concentration.

No species-dependent difference in the effect of serum on sheep/human rosetting was noted. Since the diluted guinea pig complement is actually serum, which provides 2.5% of the total sample volume, this amount of protein may not suffice to influence the test. The sera from pig, cow, goat and rabbit comprised 15.6% of the total sample volume and thus may have influenced rosetting. Steel et al. (1974), found that the rosette inhibition test is dependent on the concentration of serum used. They reported that rosettes formed in the absence of serum were disrupted easily, whereas those formed in the presence of serum were more robust, although actual serum concentration (2.5%-25%) was unimportant.

Table 5aEffect of Different Species' Sera on Rosette Formation

<u>Species</u>	<u>% Rosettes</u>
blank + GPC	2.8 $\pm$ 0.6
rabbit + GPC	6.3 $\pm$ 0.6
goat + GPC	7.0 $\pm$ 1.3
cow + GPC	6.0 $\pm$ 1.5
pig + GPC	8.2 $\pm$ 0.9

% rosettes represents mean $\pm$ S.E. of 5 replicates

thymus from ewe lamb plus HRBC

serum from animals diluted 1:400

Table 5bEffect of Serum Protein Concentration on Rosette Formation

<u>Concentration</u>	<u>% Rosettes</u>
blank	12.5 $\pm$ 3.3
1% gelatin	13.6 $\pm$ 1.6
3% gelatin	13.6 $\pm$ 1.1
control serum	14.3 $\pm$ 3.2
siliconized tube - blank	16.9 $\pm$ 2.0

% rosettes represents mean $\pm$ S.E. of 5 replicates

Source of lymphocytes: pig thymus

Source of RBC: sheep

Buffer, Ficoll and Detergent

Initially, the buffer contained calcium and magnesium but following advice from Morton (personal communication, 1981), these cations were omitted in subsequent assays.

It was important to maintain the pH of the Hanks' Balanced Salt Solution below 8.0 and preferably between pH 7.2-7.4, since rosette formation is disrupted at a more basic pH (Morton, personal communication, 1981). Since HBSS has poor buffering capacity, an indicator (phenol red) was necessary to monitor the pH. Acidity was not a problem, since the pH was adjusted to 7.2 during preparation and, when left standing, the buffer tended to become basic.

The Ficoll used to isolate PBLs alternated during the project (see Materials and Methods). Dextran was not listed as an ingredient in any of the products used although it is present in a number of commercial Ficoll preparations (but not in the preparations used during this study) and has been shown to increase rosette formation (Binns, 1978; Morton, personal communication, 1981). The Fresenius Ficoll was recommended by Morton (1981).

The detergent initially used was Decon (British Drug Houses, Ltd), but this was changed following Morton's recommendation (personal communication, 1981) to use "7X" (Flow Laboratories), who found that detergents may affect the lymphocyte cell membranes. More recently, Morton

(personal communication, 1982) has advised against using any detergents, even "7X". Glassware should be rinsed in acetic acid followed by water. Disposableware was recommended when possible.

There were no apparent differences in rosette formation or in inhibition of rosette formation using ALS and GPC alone or in a pregnancy test, following the modifications of buffer, Ficoll and detergent as recommended by Morton.

Complement

Data from Table 6 indicate that the presence of GPC significantly decreased the number of rosettes formed at most dilutions of ALS ($p < 0.05$). At the start of this project, commercial, lyophilized GPC was used. The restoring solution contained sodium azide, which is supposed to inhibit the effect of EPF, however since EPF was not detected in subsequent work with fresh guinea pig serum as the source of complement, it is unlikely that sodium azide has played a major role in causing negative results.

Temperature

When rosetting was allowed to occur at various temperatures, it was found that low temperatures increased the numbers of rosettes ($p < 0.05$) (Table 7). This prompted the decision to chill the cells during rosette

Table 6

Effect of Guinea Pig Complement on Rosette Formation

<u>Dilution of ALS Rabbit anti-pig</u>	<u>Complement</u>	<u># Rosettes/ Total # Lymphocytes</u>	<u>% Rosettes</u>
control	no	143/257	55.6%
1:100	no	13/200	6.5
	yes	1/384	0.3
1:200	no	68/216	31.5
	yes	58/440	13.2
1:400	no	164/420	39.0
	yes	111/480	23.1
1:600	no	272/477	57.0
	yes	168/386	43.5
1:800	no	186/437	42.6
	yes	175/554	31.6
1:1000	no	156/440	35.5
	yes	126/359	35.1
1:1600	no	111/232	47.8
	yes	217/503	43.1
1:3200	no	113/232	48.7
	yes	218/348	62.6

Source of lymphocytes - pig thymus

Source of RBC - sheep

Table 7Effect of Temperature on Rosette Formation

<u>RBC Species</u>	<u>Temperature</u>	<u>% Rosettes</u>	<u>Mean +S.E.</u>
sheep (1)	37 ⁰ C	a) 30	
		b) 22	25.5 <u>+1.8</u>
		c) 23	
		d) 27	
		†	
sheep (1)	20 ⁰ C	a) 70	
		b) 60	59.5 <u>+4.5</u>
		c) 60	
		d) 48	
sheep (1)	0 ⁰ C	a) 63	
		b) 76	68.0 <u>+2.8</u>
		c) 66	
		d) 67	

(1) ovariectomized female

Source of T-cells: pig (gilt) thymus

no ALS or complement used

formation in order to maximize the numbers of rosettes formed. This decision was based on two factors. The first consideration was that the numbers of rosettes formed at 0°C ($68.0 \pm 2.8\%$) appeared to be greater than at 20°C ($59.5 \pm 4.5\%$) although this difference was not significant ($p > 0.05$). The second factor was that degradation of buffers and test material would be less likely to occur at 0°C . However, it was later recommended by Morton (personal communication, 1981) that all work should be performed at room temperature ($20\text{--}25^{\circ}\text{C}$) unless otherwise specified. Since the lymphocytes involved in the cold-increased rosetting might not be the spontaneously active ones, cold might negate the effect of EPF (Morton, personal communication, 1981).

Delay of Counting Rosettes after Addition of RBC

Data in Table 8 show that there is no significant difference ($p > 0.05$) in rosette formation whether SRBCs are added to all tubes and left to stand on ice for up to 2 h ($39.9 \pm 2.7\%$) or the SRBCs are added just prior to centrifugation of the sample tubes ($37.8 \pm 3.1\%$). In subsequent work the RBCs were added just before centrifuging as this was thought to improve the chances of only the spontaneously active T-cells rosetting (Steel et al., 1974; Morton, personal communication, 1981). Morton (personal communication, 1981) recommended that the RBC be added to all the tubes of each series at one time and that

Table 8Influence of Delay after Addition of SRBC on Numbers of Rosettes

Time (min)	Up to 2 h Delay % Rosettes	No Delay % Rosettes
0	45.5 (1)	36.5 (1)
30	47.0	39.7
60	35.5	41.0
90	34.3	38.5
<u>120</u>	<u>37.0</u>	<u>24.0</u>
overall mean <u>+S.E.</u>	39.9 <u>+2.7</u>	37.8 <u>+3.1</u>

(1) Dilution of ALS - 1:16,000

Source of lymphocytes - pig thymus

Source of RBC - sheep

they all be centrifuged, resuspended and counted within within one half-hour after the samples had been spread on the hemocytometers.

Human Factors

Because of the large numbers of samples and replicates which were used at the start of the project it was necessary for more than one person to count rosettes. Although a statistical analysis is not possible due to the limited number of replicates for each dilution, nevertheless data presented in Table 9a indicate that there was no real difference between these observations since the standard error is quite low.

When Morton's counting method was adopted, only two people were involved. With practice (Table 9b) the results from these two observers became similar enough that there was no significant difference ($p \gg 0.05$) between their observations when the same samples were counted.

Wood and Gray (1973) tested for observer error in counting rosettes. They found the error in lymphocyte counting to be 20% while that of rosette counting was only 8%. During this work both lymphocytes and rosettes were counted until Morton (personal communication, 1981) suggested counting rosettes only. As rosettes are much more obvious to the eye there is less chance of observer error. The assumption is that the number of lymphocytes is the same in all samples.

Table 9Human Factors Affecting Rosette Formation and Counts

9a) Five people counting sample replicates

<u>Individual</u>	<u>% Rosettes</u>
SM	58.2
HR	68.8
HR	66.4
FL	61.1
GM	<u>61.3</u>
	63.2 <u>+1.9</u>

Source of lymphocytes: pig thymus

Source of RBC: sheep

9b) Two people counting the same samples

<u>Dilution of ALS</u>	<u>% Rosettes (FL)</u>	<u>% Rosettes (SM)</u>
1:250	38.1	33.3
1:1000	95.2	104.8
1:4000	109.5	100.0

Source of lymphocytes: pig blood

Source of RBC: sheep

Section B) Titration of Antisera

None of the preimmunization sera collected from rabbits, sheep or goats produced any inhibition of rosette formation. For an example of the results obtained, see Appendix II.

Titration of Antisera

Four rabbit anti-sheep lymphocyte sera were produced but none showed adequate or consistent rosette inhibition. It was therefore decided to investigate the pig lymphocyte-sheep RBC rosetting system.

Figures 3 and 4 are typical examples of rabbit ALS titration curves obtained with the best of the twelve antisera prepared (8 anti-spleen cell and 4 anti-peripheral blood lymphocyte sera). Comparing sera from Rabbits #2 (anti-pig spleen lymphocyte) and #7 (anti-pig PBL) (Figure 3) it was clear that Rabbit #7 ALS was more potent, thus it was chosen for further study. The average RIT was between 10 and 12. Figure 4 indicates that the results obtained with Rabbit #7 ALS were consistent from one day to another. This ALS was then used for pregnancy testing. Figure 5 is a titration curve obtained with a rabbit anti-pig lymphocyte serum (Kal:01218) prepared by Morton (1981). The average RIT was between 10 and 12. An RIT above 14 for a test serum was chosen as indicative of enhanced inhibition, i.e. of the presence of EPF.

Figure 3: Titration of rabbit #2 & #7 anti-porcine
ALS (RALS) by inhibition of rosette
formation.

Source of lymphocytes: pig blood

Source of RBC: sheep

Figure 4: Titration of rabbit #7 anti-porcine ALS (RALS)
on two different days by inhibition of
rosette formation

Source of lymphocytes: pig blood

Source of RBC: sheep

Figure 5: Titration of inhibition of rosette formation
by KAL:01218 anti-porcine ALS (Mariensee) on
two different days.

Source of lymphocytes: pig blood

Source of RBC: sheep

At the start of the pig studies four rabbit anti-pig spleen cell sera were prepared, titrated and the best one was used for pregnancy testing. Following failure with the first set of anti-pig ALS, the goat and sheep ALS were produced and tested and still negative results were obtained. The final series of anti-pig spleen and PBL immunizations were done in rabbits and the best ALS was used, but the results indicate great discrepancies between the rosette inhibitory abilities of the different antisera produced.

The major aspect to examine is the range of dilutions over which the serum is functional. Morton et al. (1974, 1976b), using antisera produced in rabbits and goats, reported titres of 1×10^9 in studies on anti-mouse ALS where the RIT for the non-pregnant control ranged between 1×10^6 and 16×10^6 . Similar titres of anti-sheep ALS were reported (Morton et al., 1979a,b) but not for anti-human ALS where dilutions of 1×10^3 to 128×10^3 were used (Morton et al., 1977). In the present study, antisera were prepared in rabbits, goats and sheep, but in no case were titres as high as those reported by Morton obtained. A satisfactory dilution range for antisera prepared against pig spleen and PBL was from 10^3 to 1.6×10^7 , giving RITs of 1×10^3 to 16×10^3 (corresponding in $\log_2$ to 10 to 14).

The major conclusion from the preparation and testing

of antiserum from different animals is that, contrary to Morton's findings (Morton et al., 1982) the ALS titre varied more than twofold from day to day. so that the RIT varied by more than twofold.

The antiserum that produced the smoothest curve and had the highest titre was Rabbit #7 ALS(RALS-7) and it was selected for use in all subsequent work. The rabbit ALS received from Morton (Kal:01218) was also tested since it was claimed to give successful results.

Section C) Pregnancy Tests

a) Sheep

On the assumption that EPF is not species-specific (Noonan et al., 1979), detection of sheep EPF was attempted using the pig lymphocyte-sheep RBC system. The results were inconclusive as no consistent effect of pregnancy serum was noted. Occasionally at lower dilutions of ALS, test serum suppressed rosette formation, while at higher dilutions enhancement occurred.

Serum from one ewe strongly suppressed rosette formation at ALS dilutions from 2.5×10^5 to 10^{12} . It became obvious that the inhibition of rosetting occurred even in the absence of ALS and the serum was effective alone in dilutions of 1:6. Unfortunately prebreeding serum from this animal was not available and the animal was slaughtered before further study could be undertaken.

There were no interpretable or consistent differences between the pregnant and non-pregnant sheep sera using either the sheep-human rosette system or the interspecific pig-sheep system. This was true in over a dozen tests performed. Lack of further information precludes speculation as to the basis of the strong rosette suppressing activity in a single case - except to say that some substance in the serum of one unusual ewe probably bound to the lymphocytes and interfered with other binding interactions.

Following a series of unsuccessful tests using the interspecific system attention was turned to porcine EPF as it was considered possible that proteins present in sheep serum may have interfered with the detection of the sheep EPF.

b) Pig

Figure 6 contains the results of an examination of effects of prebreeding serum on the rosette inhibition test. The RITs were between 11.5 and 12.8 indicating that there was no effect of prebreeding serum on rosette formation. However, increased rosetting was seen in some sera when compared to that obtained with lymphocytes initially incubated in HBSS instead of serum.

Figure 7 shows the results obtained using RALS-7 in a pig pregnancy test. The test included a prebreeding and

Figure 6: Comparison of rosette inhibition titration curves of pig prebreeding sera.

Key: S - sow

Source of lymphocytes: pig blood

Source of RBC: sheep

Figure 7: Comparison of a sample of pregnancy serum with its prebreeding sample and two other samples from nonpregnant animals.

Key: S-137 NP - sow 137 prebreeding
S-137 P - sow 137 pregnant day 7
S-1973 NP - sow 1973 post-breeding
nonpregnant
S-1490 - sow 1490 day 7
post estrus

Source of lymphocytes: pig blood

Source of RBC: sheep

Day 7 pregnancy sample from Sow 137 as well as samples from Sow 1973 Day 7 after an unsuccessful breeding and from Sow 1490 Day 7 post estrus.

As with the previous samples, these samples produced a curve initially fairly steep with wide fluctuations and rosette formations of over 100% of control values and which then dips down to or below the 75% level. All RITs were between 11 and 14 therefore the pregnancy sample was indistinguishable from the prebreeding or other non-pregnant samples.

These results were typical of those obtained in testing other pig pregnancy sera.

Figures 8 and 9 contain the results obtained in two pig pregnancy tests using Morton's ALS, Kal:01218, testing Gilt 26 and Sow 1966, on the same day. The titration curves show great fluctuations, which preclude distinguishing pregnancy serum from non-pregnancy or prebreeding serum.

In ten tests of pig sera, 3 pregnant and 7 non-pregnant, the RIT was between 10 and 14, and therefore no differences were seen. Even the ALS provided by Morton did not allow identification of pregnancy serum.

Figure 8: Pregnancy test of gilt 26 - Comparison of prebreeding and post-breeding titrations.

Key: NP - nonpregnant

P - pregnant

Source of lymphocytes: pig blood

Source of RBC: sheep

Figure 9: Pregnancy test of sow 1966 - Comparison of prebreeding and post-breeding titrations.

Key: NP - nonpregnant

P - pregnant

Source of lymphocytes: pig blood

Source of RBC: sheep

GENERAL DISCUSSION

This study was undertaken to examine the events occurring immediately following fertilization of the ovum and prior to the implantation of the embryo in the uterus in two species of domesticated ungulates, the sheep and the pig. The presence of an early pregnancy factor described by Morton and her colleagues (1974) was to be confirmed and if identified, its presence throughout the preimplantation stages, early pregnancy and subsequent disappearance, monitored.

In order to interpret the results of this research it is necessary to consider the work of Morton and her co-workers. These will then be compared with the findings of this study as well as with results obtained in other laboratories.

Early pregnancy factor (EPF) was reported to be present in the peripheral blood of mice 6 h after fertilization (Appendix II). This is coincident with pronuclei formation but before the first cleavage. The factor disappears 4 to 6 days before parturition (Morton et al., 1974, 1976b). Studies in sheep (Morton et al., 1979a,b; Nancarrow et al., 1978, 1979, 1980, 1981; Evison et al., 1977), cattle (Nancarrow et al., 1978, 1980, 1981) and humans (Morton et al., 1977, 1982) revealed elevated RITs indicative of the presence of EPF within 24 to 48 h p.c. if fertilization had occurred. In sheep, the amount

of EPF may decline some time after the first month of gestation but in some cases it has been detected up to the fourth month (Morton et al., 1979a). In the human, elevated RITs indicate that EPF is present for the first two trimesters (Morton et al., 1977). EPF activity disappears within 24 h after death or surgical removal of embryos or induced abortion, however in the case of spontaneous abortions, RITs approach normal levels one to several days in advance (Morton et al., 1977; Nancarrow et al., 1978, 1979; Rolfe, 1982).

Reported Physical Characteristics

EPF is a non-dialyzable, heat labile serum factor (Morton et al., 1976b) and it has been detected in tissues throughout the body (Nancarrow et al., 1981).

Clarke et al. (1980) reported that EPF exists in three different forms in sheep, depending on the stage of gestation, and two in the mouse (Clarke et al., 1978). In both species, a high molecular weight (250,000 daltons) form appears in the serum soon after fertilization, then gradually disappears and is replaced by a 50,000 dalton form, which is the major form during mid-gestation. Sheep serum contained a third, 20,000 dalton form observed only during the first 24 h. Fetal sheep serum at 3 months of gestation reflects the maternal blood content of EPF, containing both the 250,000 and 50,000 dalton forms. The different forms of EPF were shown to be related and it is

proposed that they arise by different combinations of the two components, EPF-A and EPF-B, with or without protein carrier. These two components, EPF-A and EPF-B, were reported to be distinguishable from each other by ammonium sulfate fractionation, gel filtration and interaction with lymphocytes (Clarke et al., 1978, 1980; Rolfe et al., 1980; Morton et al., 1980). Morton et al. (1980) reported that in the mouse EPF-A is synthesized by the oviduct in the estrous and pregnant animal, while EPF-B is pregnancy-dependent and is formed by the ovary only after ovulation (Nancarrow et al., 1981). EPF-A binds to lymphocytes only when EPF-B is present (Morton et al., 1980; Clarke et al., 1980). Thus an increase in RIT requires the interaction of EPF-A and EPF-B to form a complex which then shows rosette suppression. EPF-B production by non-pregnant mouse ovaries has been stimulated in vitro during estrous or diestrous in the presence of fertilized ova and non-pregnant mouse pituitary or only the pituitary from a pregnant mouse. It has been postulated that the fertilized ovum releases an ovum factor (OF) that stimulates production of EPF-B from the ovary, either by induced formation of a second pituitary factor or by synergism with pituitary hormones (Morton et al., 1980). OF may represent the first communication between the conceptus and its mother (Cavanagh et al., 1982) and it is released from the ovum

upon penetration by the fertilizing spermatozoon. It exists in multiple molecular weight forms and interacts with the pituitary-ovarian axis by cooperating with prolactin to stimulate production of EPF-B by the ovary.

The role of EPF is unknown but its action does not appear to be species-dependent. An immunosuppressive function has been attributed to EPF on the basis of its ability to reduce adoptively transferred contact sensitivity to trinitrochlorobenzene when sensitized donor lymphocytes are exposed to EPF in vitro prior to transfer into unsensitized recipient mice (Noonan et al., 1979). This, however, is not evidence of an immunosuppressive activity in vivo in pregnant animals.

In this connection, it may be noted that in pregnant ewes, ovariectomized 4 to 7 days after breeding, pregnancy is maintained by progesterone treatment alone even though ovary-derived EPF-B would be lacking and thus no binding of EPF-A to lymphocytes could occur (Sarda et al., 1973).

Still, as the ovariectomy was performed only after 4 to 7 days, EPF produced until then may have had a sufficient period of action to play a role within the first few days after fertilization. Morton (personal communication, 1981) has suggested that very little EPF-B is required to transform a large amount of EPF-A, therefore, sufficient residual EPF-B may be available for some time after the ovariectomy. Whatever the function of EPF, it is unlikely

that it is required for maintenance of the corpus luteum.

Many pregnancy-associated serum proteins such as α -fetoprotein, pregnancy zone protein, human and ovine placental lactogen and hCG have been suggested to be involved in suppression of the maternal immune response to the embryo; however, except for hCG, ovine placental lactogen and α -fetoprotein, these substances appear only after 6 to 9 weeks of pregnancy and increase in concentration as gestation progresses. EPF by contrast appears immediately (6-24 h) after fertilization and disappears later in pregnancy.

The 50,000 dalton form of sheep EPF crossreacts with anti-hCG and increases the RIT of human and mouse serum (Clarke et al., 1978; Morton et al., 1976, 1977; Nancarrow et al., 1981). Ovine α -fetoprotein behaves similarly (Nancarrow et al., 1981). It is probable that some common structure or contaminant is present in preparations of these proteins (Nancarrow et al., 1981; Morton, personal communication, 1981). EPF-A has a molecular weight similar to that of uteroglobin (Beier, 1968), a glycoprotein of M.W. 25,000 produced by the rabbit uterus. However EPF-A is not produced by post-breeding mouse uterine tissue (Morton et al., 1980), while uteroglobin is produced by rabbit uterine tissue under the influence of progesterone alone (i.e. even in the non-pregnant rabbit) (Beier and Kuhnel, 1973), thus it is unlikely that the two

substances are related.

All of the work described in this discussion was done by a few individuals working in collaboration, albeit at several institutions. However, efforts to independently confirm the findings described above, particularly with respect to detection of EPF by means of rosette inhibition, have met with conflicting results.

Among the successful efforts are those of Smart et al. (1981a, 1982a,b) in the human, Paisley et al. (1982) in the pig and Heywood et al. (1979) in the rat. Koch et al. (1982) also claimed success in the pig but worked under the direct supervision of Morton. EPF was not detected in the present study nor in innumerable other laboratories from which only personal communications have indicated lack of success (Walton, J., University of Guelph, personal communication, 1980; Gimenez, T., Clemson University, South Carolina, personal communication, 1981, 1982; Nancarrow, C.D., Commonwealth Scientific and Industrial Research Organization, Australia, personal communication, 1980, 1981; Rigby, N.W., A.S. Grewel, A.L.C. Wallace and C.D. Nancarrow, C.S.I.R.O., Australia, personal communication, 1982). Rigby et al., who formerly worked with Morton, could no longer detect EPF independently, after their stock of ALS obtained from Morton, was exhausted. Other reports of failure have been published by Thomson et al. (1980) and Cooper and Aitken

(1981), and it is likely that many other failures have not been reported.

In view of repeated reports by Morton and some others, it is likely that EPF can be detected if all necessary conditions are met. Among the many criticisms of the rosette inhibition test is the fact that the mechanism of the test is unknown. The most likely explanation is that EPF increases steric inhibition by binding to receptors on the lymphocytes other than those to which the ALS binds. EPF may also resemble the surface features of some erythrocytes. This decreases the surface area over which an erythrocyte could attach thereby further reducing the number of rosettes formed at a given dilution of ALS.

Throughout the course of this project (1979-1982) many modifications to the original method of Morton et al. (1974, 1979) were introduced. Many of these were developed in this laboratory, while others were indicated by Morton through personal communications and subsequent publications. Morton has suggested about half of the ALS produced should be useful, but none of the twenty antisera produced and tested in this project gave useful results, nor did Morton's own antiserum.

In Morton's reports RITs without EPF did not vary more than twofold and there was never an overlap between inhibition curves for pregnant and non-pregnant sera.

False positives were never reported. Morton steadfastly claims that negative results are due to technical problems such as the presence of factors interfering with the binding of EPF to lymphocytes. One source could be interfering serum components which Morton claims hamper interspecific tests (Morton, personal communication, 1981; Morton et al., 1982). Such substances are removed, it is claimed, by dialyzing the test serum.

A list of sources of difficulty in applying the rosette inhibition test has only recently been provided by Morton (Morton et al., 1982), presumably in response to repeated criticism of her claims. This list includes:

- a) Presence of sodium azide, anticomplementary substances, added calcium or magnesium ions in test samples or medium.
- b) Attempts to increase or stabilize rosette formation by using sensitized lymphocytes, prolonged incubation of red blood cells with lymphocytes or treating the rosettes with glutaraldehyde.
- c) Temperatures above 25°C during counting. This causes the rosettes to become unstable and disintegrate.
- d) The use of a lymphocyte donor with hyperglycemia or viral infection, after anesthesia or aspirin ingestion.
- e) The use of an ALS which is incapable of differentiating between lymphocytes with or without EPF.

In the present study, Morton's recommendations of

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temperature, $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer, male GPC, rosette counting methods, Ficoll and use of spleen or PBL in diagnosing pregnancy by the rosette inhibition test did not produce useful results.

The findings of others also conflict with those of Morton. Jondal et al. (1972) reported that the number of rosettes decreased in $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer and that EDTA completely suppressed rosette formation. In this laboratory, rosette formation was not affected by the presence of calcium or magnesium in the buffer. There was difficulty, however, in discerning differences because of day to day unpredictability in rosetting, an observation made in other laboratories as well (Paisley et al., 1982; Rigby et al., personal communication, 1982).

Thomson et al. (1981) failed to detect alteration of RIT by human pregnancy serum but did not indicate whether the HBSS used was calcium- and magnesium-free. They did, however, treat the sheep RBCs with fetal calf serum and with AET which supposedly modifies the membrane surface and causes stabilization and enhancement of rosettes. However, Paisley et al. (1982) even using AET-treated erythrocytes were able to detect EPF. Morton et al. (1981) claim that enhanced rosetting prevents EPF detection. Cooper and Aitken (1981) failed to detect EPF, possibly because they also enhanced rosette formation by use of AET-treated SRBC and incubation at 4 C overnight.

Morton et al., (1982) tested one of the "unsuccessful" antisera of Cooper and Aitken (1981) and found that it could be used to distinguish between pregnancy and non-pregnancy sera. Another possible reason for the failure of Cooper and Aitken (1981) and Thomson et al. (1980) is that they did not heat-inactivate either test sera or ALS; this may have contributed to the lysis noted in the former report. Both groups also enhanced rosetting by centrifuging the rosettes at 1000 x g for 2 to 3 min. This would select for firmly bound "total" rosettes. Morton claims that by centrifuging at only 200 x g for 5 min only the "active" or "early" rosettes are sedimented. Cooper and Aitken (1981) also found that in the presence of small amounts of ALS 2 to 3 times, the number of rosettes formed than in the ALS-free controls was obtained. This situation occurred repeatedly during the present work, when increased rosetting occurred at high dilutions of ALS. Nancarrow (personal communication, 1980, 1981) also observed enhanced rosette formation in the presence of higher dilutions of ALS with either sheep or human RBCs.

Walton (personal communication, 1980) and Gimenez (personal communication, 1982) abandoned the rosette inhibition test, because it was too unpredictable. They also tried to detect EPF by exploiting its alleged immunosuppressive activity in phytohemagglutinin

stimulation of lymphocytes, but these tests also failed to detect it.

The function of complement in the rosette inhibition test is unknown. Nancarrow et al. (1981) reported that rosettes would not form in the absence of complement and that the numbers of rosettes decreased either as the complement aged or when lymphocytes incubated with complement stand for prolonged periods. It is postulated that as complement receptors are found on both RBCs and lymphocytes, synergism between EPF and ALS is mediated through increased steric hindrance, preventing approach of red cells. Nancarrow et al. (1981) found that all lymphocytes forming rosettes have complement bound to them. Morton (personal communication, 1981) stressed that complement should be obtained from male guinea pigs to exclude the possibility of contamination by EPF and that complement should not be stored below -20°C or kept for more than one month. In the current investigation complement was not found necessary for rosette formation. Morton et al. (1982) claimed that complement is not necessary for E-rosette formation with human cells but is essential to the inhibition of rosetting by EPF.

Neither Smart et al. (1982) nor Paisley et al. (1982) specified using calcium- and magnesium-free buffers. Smart et al. (1982) used fresh male guinea pig serum absorbed against sheep and human RBCs as a source of

complement, while Paisley et al. (1982) used a commercial lyophilized preparation, absorbed against SRBC only.

Smart et al. (1982) reported that the incubation time of treated lymphocytes, ALS and GPC at 37°C could be shortened from 90 min to 30 min. Treatment of SRBC with papain resulted in a complete loss of sensitivity to inhibition of rosette formation by EPF and no rosette inhibition occurred in the absence of complement. The reported optimum lymphocyte-SRBC ratio was 1:10 which produced a 20% to 30% spontaneous rosette formation between human lymphocytes and SRBCs in the controls. Smart et al. (1982) also found that a source of good distilled water was important.

Many of the recommendations of Morton et al. (1982) had been adopted early in the work in this laboratory. Every effort to meticulously follow Morton's procedures was made as new details became known. The results, however, remained negative.

CONCLUSIONS

The occurrence of the early pregnancy factor described by Morton et al. (1974) could not be detected during this investigation. Without casting doubt on the validity of the claims made, the failure to detect EPF can be attributed only to some elusive factor in the methodology, which still escapes identification by anyone.

Although there are many potential applications of a test for EPF, unfortunately the rosette inhibition test for EPF remains, at best, a cumbersome, slow procedure, incapable of application on the scale needed to be useful in the applications cited above. Even if the test can be rationalized and made reliably reproducible it is likely to remain more of a laboratory tool in reproductive physiology research, than a practical field or clinical test. The latter awaits development of a simple, rapid test, which may depend on isolation of EPF in quantities sufficient to permit raising antisera against it and to provide reference standards.

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APPENDIX IHanks' Balanced Salt Solution

NaCl	140 mM
KCl	5 mM
KH_2PO_4	0.4 mM
CaCl_2	1 mM
MgSO_4	0.8 mM
Na_2HPO_4	0.28 mM
NaHCO_3	4 mM
Glucose	6 mM
Phenol red	2 mg/l

APPENDIX IITable 10Effect of Preimmunization Serum on Rosette Formation

<u>Dilution of preimmunization</u>	<u>% Rosettes</u>	<u>Mean +S.E.</u>	<u>% Control</u>
black goat			
1:8,000	24.0	22.5 <u>+2.2</u>	90.7
	28.5		
	21.0		
	16.5		
1:32,000	19.1	21.8 <u>+1.3</u>	87.9
	21.1		
	26.0		
	21.0		
1:128,000	21.0	21.6 <u>+0.2</u>	87.7
	22.0		
	22.0		
	21.5		
control	26.0	24.8	
	27.5		
	25.5		
	20.3		

7
Table 10 (continued)

<u>Dilution of preimmunization</u>	<u>% Rosettes</u>	<u>Mean +S.E.</u>	<u>% Control</u>
grey goat			
1:8,000	28.5	25.6 <u>+0.8</u>	103.2
	24.0		
	25.5		
	24.5		
1:32,000	19.0	25.8 <u>+2.3</u>	104.0
	31.5		
	28.0		
	24.5		
1:128,000	28.0	25.1 <u>+1.4</u>	101.2
	27.5		
	24.0		
	20.8		

Source of lymphocytes: pig

Source of RBC: sheep

APPENDIX III

Figure 10: Rosette formation with lymphocytes from a pregnant and a nonpregnant mouse plotted against ALS dilution, illustrating the high RIT during pregnancy (taken from Morton et al., 1974).

Key: O+ - nonpregnant

●+ - pregnant

Source of lymphocytes: mouse spleen

Source of RBC: human

Conversion to Log base 2:

<u>Dilution</u>	<u>Log base 2</u>
2×10^6	21
32×10^6	25
512×10^6	29
8×10^9	33

END

1 2 10 9 18 4

FIN