Detection of early pregnancy in sheep by the rosette inhibition test


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Summary. The rosette inhibition test, an established test for determining the immunosuppressive potential of antilymphocyte serum, has been applied to the serum of sheep after mating. The rosette inhibition titre was much higher (12–26) in 7 sheep which were fertilized and remained pregnant for up to 21 days than in 5 sterile ewes mated with intact rams (8–10). The difference was apparent by 24 h after mating. One ewe had high titres for 6 days after mating but these then dropped and she returned to oestrus; early embryonic loss was suspected. Another ewe which returned to oestrus had consistently low titres. The results indicate that the rosette inhibition test can be used to detect fertilization, early embryonic death and continued pregnancy in sheep.

Introduction

The early detection of pregnancy in domestic animals is a problem of practical importance in animal husbandry and of scientific interest in the study of production and fertility. In sheep and cattle, pregnancy cannot as yet be detected by non-surgical methods before the expected return to oestrus. Although a fetus-derived antigen has been detected in circulating maternal blood (Cerinii, Findlay & Lawson, 1976), its presence does not enable accurate identification of those animals in which fertilization has occurred.

The discovery of an "early pregnancy factor" in the serum of pregnant mice (Morton, Hég & Clunie, 1974, 1976a) and women (Morton, Rolfe, Clunie, Anderson & Morrison, 1977) has raised the possibility of accurate diagnosis of early pregnancy in domestic animals. Morton et al. (1974, 1976a, 1977) used a modification of the rosette inhibition test of Bach, Dormont, Dardenne & Balner (1969) to assess the influence of sera from pregnant mice and women on the ability of an immunosuppressive antilymphocyte serum to decrease the number of spontaneous rosettes formed between lymphocytes and heterologous red blood cells. The "early pregnancy factor" in mice was detectable from 6 h after mating until 4–6 days before term (Morton et al., 1976a), and it was therefore considered that the presence of a similar factor might prove a means of determining pregnancy in sheep. The present study is an investigation of the use of the rosette inhibition test for early detection of fertilization and continued pregnancy in ewes.

Materials and Methods

Management of animals and collection of serum

Nine (9) Merino ewes were injected with 125 µg of an analogue of prostaglandin F-2α (Cloprostenol: ICI, Australia Ltd) and kept with 2 raddled vasectomized rams. Ewes detected in
oestrus were mated with intact rams twice within 30 min. Blood was collected from the ewes before and at 24 h, 48 h, 6, 11, 16 and 21 days after service. The separated serum was inactivated by heating at 56°C for 30 min and stored at −30°C. Returns to oestrus were recorded by using raddled teaser rams.

Five (5) ewes were bilaterally salpingectomized to prevent fertilization. Ovulation was induced in these control animals by using withdrawal of a progestagen-impregnated intravaginal sponge (Upjohn Pty Ltd) after 12 days and s.c. injection of 700 i.u. PMSG 24 h before sponge withdrawal. Oestrus was recorded and oestrous ewes were mated as described above. Serum samples were obtained before and at 1, 2 and 6 days after mating.

**Preparation of materials**

*Spleen cell suspension.* The method used was based on that previously described (Morton et al., 1975, 1976b). Spleens were obtained daily from wethers immediately after slaughter at the abattoir. The spleen was more convenient than peripheral blood as a source of lymphocytes because of the large number of cells required for the test. A spleen was finely chopped, then suspended in Hank’s Balanced Salt Solution (BSS) at room temperature. After removal of the sediment, the supernatant solution containing the spleen cells was centrifuged at 200 g for 5 min. Distilled water (0.5 ml) was added to the spleen cell pellet for 5 sec to haemolyse any sheep red blood cells present, then the cells were washed twice in BSS and resuspended to give a final preparation of 1.5 × 10⁴ spleen cells/μl BSS.

**Antilymphocyte serum.** A rabbit anti-sheep lymphocyte serum was prepared by a modification of the method described by Gosso, Wood & Monaco (1972). The suspension of sheep spleen cells for injection was prepared as previously described but to give 1 × 10⁵ nucleated cells/μl 0.9% (w/v) NaCl. Rabbits were injected subcutaneously at multiple sites with 0.5 ml spleen cell suspension in 0.5 ml Freund’s complete adjuvant. Intravenous injections of 0.5 ml spleen cell suspension were given 4 weeks later on each of 3 successive days. The rabbits were bled from the ear vein on the 9th, 11th and 13th days after the last injection, the serum was separated, inactivated at 56°C for 30 min and stored at −30°C. The rosette inhibition titre of these samples of antilymphocyte serum was estimated using normal sheep spleen cells and human red blood cells (Morton et al., 1975). One batch of antilymphocyte serum was used throughout, the serum being dispensed into small aliquots before freezing.

On the day of testing, the antilymphocyte serum was diluted to 1 in 256 × 10⁵ in BSS followed by serial dilution to 1 in 1 048 576 × 10⁶. Dilutions below 256 × 10³ were not used because of the presence of non-specific antibodies (Morton et al., 1976b). For each test, 12 different dilutions of antilymphocyte serum and 2 controls (BSS alone) were used (see Text-fig. 1). The dilutions are expressed as the logarithm (base 2) of their reciprocal × 10⁻³; e.g. the dilution of 1 in 256 × 10³ is equivalent to 8.

**Guinea-pig serum.** The serum from 6 animals was pooled then absorbed with equal volumes of washed human RBC and sheep RBC at 4°C for 2 h before storage at −30°C in 0.3 ml aliquots. The serum was then thawed and diluted 1 in 5 in BSS immediately before use. The serum was tested for complete absorption against the human RBC suspension.

**Human RBC suspension.** Fresh human RBCs were obtained daily from a donor, washed 3 times in BSS and made up to a final suspension of 1 × 10⁵ cells/μl.

**Rosette inhibition test**

A lymphocyte pellet was prepared by centrifuging 1.4 ml spleen cell suspension for 5 min at 200 g. The serum from the mated sheep was thawed, diluted 1 in 2 in BSS and 0.4 ml was added to the lymphocyte pellet which was resuspended and incubated for 30 min at 37°C. After incubation, the cells were washed twice, the final volume was made up to 1.4 ml with BSS and
this spleen cell suspension was used for the estimation of the rosette inhibition titre of the antilymphocyte serum.

A known positive and negative test were included with each batch of test sera. The positive test contained serum from a sheep known to be pregnant; the ewe had been bled 24 h after mating, the serum stored in 0-5 ml volumes at —30°C and a fresh sample used each day. The negative test contained spleen cells incubated in 0-4 ml BSS without serum.

Serially diluted antilymphocyte serum (0·25 ml), absorbed diluted guinea-pig serum (0·05 ml) and spleen cell suspension (0·1 ml) were incubated at 37°C for 1½ h. After incubation a 0·1 ml suspension of human RBCs was added and the tubes were immediately centrifuged at 200 g for 5 min. The cells were then resuspended by rotation on a Matburn Wheel (10 rev./min for 5 min) and this suspension was spread on a haemocytometer. The number of rosettes per 2 × 10³ lymphocytes was counted. Each count was done in duplicate. The rosette inhibition titre was recorded as the highest dilution of antilymphocyte serum in which the number of rosettes formed was less than 75% of the number formed in BSS alone (control count).

Results

Representative titration curves of the rosette inhibition test on serum samples from a sheep before and 24 h after mating are illustrated in Text-fig. 1. The highest dilution of the antilymphocyte serum to give less than 75% rosette formation was 10 before mating and 26 after mating. The number of rosettes per 2 × 10³ lymphocytes formed in BSS alone, using spleen cells from different sheep and human RBC from different donors, ranged from 60 to 225, with a mean

Text-fig. 1 Titration curves for rosette formation obtained using spleen cells from a non-pregnant sheep tested after incubation in serum taken from an ewe before (O—O) and 24 h after (●—●) mating. The antilymphocyte serum dilutions are represented as the logarithm (base 2) of the reciprocal of the dilution × 10⁻³. The number of rosettes formed in each dilution of antilymphocyte serum is expressed as a percentage of the number of rosettes formed without antilymphocyte serum (control). The shaded area indicates the range of values obtained with spleen cells from the negative controls and sheep known to be non-pregnant.
of 133 ± 6·9 (s.e.m.) for 49 observations, i.e. rosette formation was 2–11% of the total lymphocyte count.

The negative control group, i.e. spleen cells tested alone without incubation in serum, gave a mean inhibition titre of 9·14 ± 0·22 (s.e.m.) (n = 21). The range of these values was from 8 to 10 and is illustrated in Text-figs. 1 and 2 as the shaded area. The range of values of the known positive test, i.e. the serum from one ewe retested, was 20 to 22.

Of the group of 9 mated sheep, 2 ewes returned to oestrus 19 days later and were assessed as non-pregnant. The results for the serum samples from the remaining 7 ewes, and those for 5 mated but sterile ewes are shown in Text-fig. 2. The mean ± s.e.m. titre for serum sample taken before mating was 8·57 ± 0·37 (n = 9) and was not significantly different from the values for the sterile ewes (9·60 ± 0·40, n = 5) and the negative control group (see above). However, by 24 h after mating, the titre (22·28 ± 1·27) had increased significantly (P < 0·001, Behrens–Fisher test). Although lower than the values on Days 1 and 2 the titres on Days 6, 11, 16 and 21 were all greater (P < 0·001) than those for the same ewes tested before mating.

Of the 2 sheep that returned to oestrus 19 days after mating one showed no increase in titre up to its return to oestrus, all values remaining within the range for the negative controls and non-pregnant ewes (8 to 10). Significantly increased titres of 24, 26 and 18 were found for the other ewe at 1, 2 and 6 days, respectively, after mating, but the titre returned to the non-pregnant range by Day 11.

**Discussion**

The earliest overt indication of pregnancy in sheep is the failure to return to oestrus at the expected time. Confirmation of pregnancy could possibly be obtained from progesterone estimates in blood samples collected 16–18 days after mating. However, the methods employed in the current investigation were able to detect, at 24 h after mating, those sheep which became and remained pregnant. Two sheep were considered to be non-pregnant since they returned to service 19 days after mating. At the time of return to service, the rosette inhibition titres were within the range for the negative controls and those known to be non-pregnant. However, one of these sheep had had high levels of the ‘early pregnancy factor’ present for at least 6 days after
mating, and it is suggested that these observations may represent the serological detection of natural embryonic death before Day 11 of pregnancy. A similar occurrence has been reported for women (Morton et al., 1977), and surgical removal of embryos in sheep is known to result in the disappearance of the early pregnancy factor within 8–24 h (C. D. Nancarrow, B. M. Evison & R. J. Scaramuzzi, unpublished observations).

In this study the rosette inhibition test, described originally as a measure of the potential immunosuppressive properties of antilymphocyte serum, has been used to detect the appearance of a factor in serum which can modify the ability of lymphocytes to form rosettes with human RBCs. This enhanced inhibition of rosette formation has been interpreted as indicating the presence of an 'early pregnancy factor' which may have immunosuppressive qualities, as demonstrated in mice and women (Morton et al., 1974, 1976a, 1977). It is therefore possible that a similar mechanism might operate in all mammals and that this process may be responsible in part for the maternal acceptance of the antigenically alien fetus.

The nature of the factor responsible is not yet clear, although that in the mouse can be heat-inactivated at 72°C but not at 56°C and is not dialysable (Morton et al., 1976a), suggesting that it is a large peptide or protein. The early pregnancy factor is not species specific; it can be detected in sheep serum by mouse spleen cells. However, there is interference in the rosette formation by the sheep serum proteins, causing increased rosetting and masking inhibition (H. Morton, unpublished observations).

In women, other circulating proteins associated with later pregnancy have been shown to be immunosuppressive. Pregnancy-associated α-macroglobulin/α2-glycoprotein (Stimson & Eubank-Scott, 1972; Von Schoultz & Stigbrand, 1973) and α-fetoprotein (Yachin, 1976) reach peak concentrations in mid-pregnancy, while human chorionic gonadotrophin can first be detected around 7 days after fertilization (Saxena, Hasan, Haour & Schmidt-Gollwitzer, 1974). In early gestation in the sheep, it has been suggested that proteins such as ovine chorionic somatomammotrophin may have luteotropic and possibly growth stimulatory effects (Martal & Djiane, 1977), but none of these proteins has been investigated for possible immunosuppressive activity. Maximum suppression of rosette formation is achieved in the mouse and sheep within 48 h of mating, suggesting that the 'early pregnancy factor' is a separate entity from those proteins associated with mid- and late gestation.

The data presented here suggest that, in sheep as in mice and man, a factor is released into the maternal circulation soon after fertilization and is not due to ovulation, to an unspecified reaction of the ewe to mating or to the presence of spermatozoa in the female reproductive tract because the salpingectomized ewes also experienced these events. It is postulated that this factor may act by modifying lymphocyte activity in order to protect the fertilized ovum from rejection by the maternal tissues.

These preliminary studies suggest that the rosette inhibition test may provide a precise indication of both fertilization and continued pregnancy in the sheep.

The technical help of Mr K. E. Turnbull and Mr D. Norton is gratefully acknowledged. The Cloprostenol was a gift from ICI Australia Ltd.

References


Received 5 July 1978