Effect of administration of antibody against GnRH on preovulatory LH and FSH surges in the ewe

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Summary. An antibody, raised in ewes against synthetic GnRH, was injected intravenously (a) before the expected synchronous surges of LH and FSH or (b) before the second FSH surge, i.e. at 0 or 20 h after the onset of behavioural oestrus. Control ewes were given normal sheep plasma at the same times.

One of the 4 ewes given antibody at 0 h showed blockade of the LH surge, whereas in the other 3 ewes there was an average delay of 16 h in the appearance of the LH peak and in 1 of the 3 there was a surge of FSH coincident with the delayed LH surge; no other surges of FSH were detected in any of the ewes between 0 and 48 h after the onset of oestrus. Endoscopic examination at 48–52 h showed follicles of 6–8 mm diameter with a papilla in all the 4 ewes but 2 ewes showed an abnormal luteal phase (32 and 34 days), indicated by plasma progesterone values.

The magnitude and the time of appearance of the second FSH surge was unaffected in all the 4 ewes treated with antibody 20 h after the onset of oestrus: 2 of the ewes had 6–8 mm follicles at the time of endoscopy, while the other 2 ewes and the controls had fresh corpora lutea haemorrhagica. Apart from 1 ewe with a long luteal phase all the other 5 treated and control ewes had a fertile oestrus 19.0 ± 0.4 days after the experiment.

Administration of antibody 2 h after a 4-h infusion of oestradiol (3 µg/h) to 3 anoestrous ewes completely blocked the LH surge in one and produced a 6 and 3 h delay of an LH surge of decreased magnitude in the other 2 ewes. The FSH surge was blocked in 2 ewes and in the other was asynchronous with the LH surge.

The results suggest that GnRH controls the preovulatory synchronous LH and FSH surges, but not the second FSH surge.

Introduction

The evidence that gonadotrophin-releasing hormone (GnRH) is the sole releasing hormone for both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is based on the fact that pharmacological doses of GnRH cause the release of LH and FSH (Schally *et al.*, 1976). Also, administration of GnRH antibody has been shown to block LH and FSH surges in the rat (Arimura, Debeljuk & Schally, 1974; Kerdelhue, Catin, Kordon & Jutisz, 1976) and the hamster (De La Cruz, Arimura, De La Cruz & Schally, 1976). There is a report (Kerdelhue, Jutisz, Gillesen & Studer, 1973) of a rise in jugular plasma GnRH concentration synchronous with LH and FSH surges in the ewe. Later studies, however, have indicated no relationship between LH and GnRH in the ovine jugular plasma (Nett, Akbar & Niswender, 1974; Jonas, Burger, Cumming, Findlay & de Krester, 1975; Foster, Jeffcoate, Crighton & Holland, 1976), and it is considered that the concentration of GnRH in the peripheral blood is probably below the limit of assays currently available (Nett & Adams, 1977).

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The present experiment was designed to investigate the role played by GnRH in the control of the synchronous LH and FSH surges and the second FSH surge after the onset of oestrus in the ewe. The response to passive immunization against GnRH was measured in terms of jugular plasma LH and FSH concentrations.

Materials and Methods

Raising antibodies against synthetic GnRH

Two ewes were immunized against synthetic GnRH (Abbott, Lot 41070) by the method of Fraser, Gunn, Jeffcoate & Holland (1974) with slight modifications. Using 125 mg carbodiimide (Sigma), 5 mg GnRH were conjugated to 5 mg bovine serum albumin (Armour Pharm.). After dialysis, the conjugate in saline plus 1 ml Pertussis vaccine (Lilly Laboratories, U.S.A.) was emulsified in Freund's complete adjuvant (Difco, U.S.A.) for the primary immunization. In the later immunizations, Freund's incomplete adjuvant was used. Each ewe was given 2 ml (1 mg GnRH-BSA) subcutaneously. After secondary immunizations pooled plasma giving about 50% specific binding at a dilution of 1:2000 in a double-antibody radioimmunoassay was used in the following studies.

Normal sheep plasma (NSP) was collected from ewes on Day 3 of the oestrous cycle and had concentrations of <0.2 ng progesterone/ml and 4.5 pg oestradiol/ml.

Animals

Mature parous Clun Forest ewes were housed indoors with a vasectomized raddled ram. Experiments were carried out in February, January and August. One day before the beginning of each experiment polythene catheters (p.p. 120: Portex) were introduced into a jugular vein under local anaesthesia (Xylocaine: Astra).

Synchronization of oestrus

An analogue of prostaglandin F-2 α , cloprostenol (Estrumate: I.C.I.), was injected intramuscularly in 2 doses, 125 μ g each, 9–10 days apart. About 40–48 h after the second cloprostenol injection, when the onset of oestrus was indicated by the ram, each ewe was moved into an individual pen.

Experimental design

Group 1. Two ewes were injected with 30 ml NSP i.v. at the onset of oestrus (0 h).

Group 2. Two ewes were injected i.v. with 30 ml NSP 20 h after the onset of oestrus.

Group 3. Four ewes were injected i.v. with 30 ml GnRH antibody at 0 h.

Group 4. Four ewes were injected i.v. with 30 ml GnRH antibody at 20 h.

The ovaries of all these ewes were examined with the aid of a fibre-optic endoscope (Wolf 7134: Knittlingen, West Germany) for evidence of ovulation 48–52 h after the onset of oestrus.

Groups 5 and 6. On Day 3 of the cycle, ewes were injected with 30 ml NSP i.v. (2 ewes, Group 5) or 30 ml GnRH antibody i.v. (3 ewes, Group 6) and 30 min later with 50 μ g GnRH i.v. (a gift from Dr H. Gregory, I.C.I.).

Groups 7 and 8. On Day 3 of the cycle, ewes were injected with 30 ml NSP i.v. (3 ewes, Group 7) or 30 ml GnRH antibody i.v. (3 ewes, Group 8) and 10 min later with 20 μ g GnRH i.v.

Groups 9 and 10. Ewes were infused at 0 h with 3 μ g oestradiol-17 β (Koch-Light, U.K.)/h for 4 h and at 6 h 30 ml NSP (3 ewes, Group 9) or 30 ml GnRH antibody (3 ewes, Group 10) were injected through the jugular catheter.

Blood sampling

Heparinized blood samples (5 ml, 50 i.u. heparin/ml) were collected through the indwelling catheter at 2 h intervals for 24 h in anoestrous ewes and for 48–52 h in oestrous ewes. Every 6 h, 30 ml blood were collected for oestradiol assay. Blood samples (5 ml) were collected from the ewes in Groups 5–8 every 15 min for 5 h. The blood samples were immediately centrifuged at 700 *g* for 15 min and the plasma was stored at -15°C until analysis.

Blood samples (5 ml) were also collected from the ewes in Groups 1–4 by jugular venepuncture every 2 or 3 days for 50 days for progesterone assay.

Radioimmunoassay of hormones

The concentrations of LH, FSH, oestradiol-17 β and progesterone were measured by methods already described (Niswender, Reichert, Midgley & Nalbandov, 1969; Dobson & Ward, 1977; Pant, Hopkinson & Fitzpatrick 1977). Assaying a plasma sample of low hormone concentration, the respective inter- and intra-assay coefficients of variation were 17.8 ($n = 15$) and 13.0% ($n = 6$) for LH, 14.0 ($n = 12$) and 5.8% ($n = 8$) for FSH, 13.3 ($n = 6$) and 16.3% ($n = 8$) for oestradiol, and 17.1 ($n = 10$) and 10.4% ($n = 10$) for progesterone. Assaying a plasma sample of high hormone concentration the respective inter- and intra-assay coefficients of variation were 9.1 ($n = 12$) and 9.0% ($n = 10$) for LH, 16.6 ($n = 12$) and 14.4% ($n = 6$) for FSH, 6.4 ($n = 8$) and 9.4% ($n = 10$) for oestradiol, and 18.2 ($n = 10$) and 23.1% ($n = 10$) for progesterone. The results are expressed as ng equivalents/ml of NIH-LH-S16/ml for the LH assay and as NIH-FSH-S10/ml for the FSH assay. The sensitivity of the method for LH was 0.2 ng/tube and for FSH 5 ng/tube. The water blank values for the oestradiol and progesterone assays were 1.1 ± 0.3 (s.e.m.) ($n = 36$) and 16.3 ± 3.9 ($n = 20$) pg/ml respectively; the plasma steroid values were thus not corrected for 'method blank'. Recovery of radioactive oestradiol from plasma was $85.3 \pm 3.1\%$ ($n = 18$) and of progesterone $74.5 \pm 4.6\%$ ($n = 10$). Addition of 1:1000 GnRH antibody had no effect on the standard curve of either gonadotrophin.

The binding of GnRH antibody in the pooled plasma was determined according to the method of Jeffcoate, Fraser, Holland & Gunn (1974) with slight modifications. ^{125}I -labelled GnRH (50 000 c.p.m.) was added to serial dilutions of antibody and the separation of bound hormone was carried out using a second antibody against sheep- γ -globulin raised in our laboratory. Cross-reaction studies kindly performed by Dr H. M. Fraser (M.R.C. Unit of Reproductive Biology, Edinburgh) revealed high cross-reactions with the 2–10, 3–10 and 4–10 fragments of GnRH.

In the recipient ewes, the total binding capacity of the plasma was also determined. Plasma samples (200 μl) and ^{125}I -labelled GnRN (200 μl) were incubated at 4°C for 48 h and then the unbound hormone was removed with 0.5 ml cold 0.25% charcoal in 0.1% gelatin-0.15 M-PBS. After centrifugation at 3000 *g* for 10 min the supernatant was counted and the total binding capacity was considered as the c.p.m. in the supernatant expressed as a percentage of c.p.m. added initially.

Statistical analyses

All the values are expressed as mean $\pm$ s.e.m. and Student's *t* test was used to compare the treated with both control groups.

Results

Oestrous ewes

In both control groups (Groups 1 and 2), the patterns of plasma gonadotrophin concentrations were similar and for the purpose of statistical analysis they were combined. Considering maximum LH concentration as 0 h, composite graphs calculated for control and treated ewes are shown in Text-fig. 1 (one ewe in Group 3 which showed complete blockade of the LH surge was not considered in this composite graph). Relative to the onset of behavioural oestrus,

Text-fig. 1. Mean $\pm$ s.e.m. jugular plasma concentrations of LH (●) and FSH (○) in ewes treated with (a) normal sheep plasma (NSP), 30 ml i.v. (Groups 1 and 2, 5 ewes), or an antibody (Ab) against synthetic GnRH at (b) 0 h, 3 ewes, or (c) 20 h, 4 ewes, after the onset of oestrus (arrows). One ewe (Ewe 20), which showed complete blockade of the gonadotrophin surge, is not included in (b).

maximum LH concentrations were noted at 10.3 ± 1.3 h in the controls (Groups 1 + 2 combined) and at 26.7 ± 4.4 h in Group 3 ($P < 0.001$). The maximum LH concentration in Group 3 (45.0 ± 5.0 ng/ml) was not significantly different from that of the combined control group (52.5 ± 2.5 ng/ml).

Only one ewe in Group 3 showed an FSH surge synchronous with that of the delayed LH surge; there was no obvious first or second FSH surge in the other 3 ewes. It is the FSH concentration of this ewe which, upon calculation of the mean, appears to show an FSH surge synchronous with the LH surge in the composite graph (Text-fig. 1b). The second maximum

FSH concentration in Groups 1 + 2 was 298.8 ± 46.4 ng/ml and in Group 4 was 327.5 ± 33.0 ng/ml at a mean time of 34.5 h after the onset of oestrus (in both groups).

The binding capacity of the antibody in the recipient ewes declined rapidly within 1 week. Afterwards, there was slow decline which persisted for at least 21 days. The mean $\pm$ s.e.m. binding capacity for ^{125}I -labelled GnRH at Days 0, 1, 7, 14 and 21 was 45.8 ± 0.7 , 99.2 ± 0.2 , 80.9 ± 0.8 , 73.4 ± 1.5 and $67.8 \pm 1.3\%$ respectively.

In Groups 1 + 2, 2 ewes had a jugular plasma oestradiol concentration of 10.2 pg/ml 2 h before the onset of oestrus. In the other 2 ewes, maximum plasma oestradiol concentrations at the onset of oestrus were 6.1 and 4.3 pg/ml. In Group 3, the maximum oestradiol concentration in individual ewes was 9.7, 10.2, 9.7 and 4.8 pg/ml at 0, 12, 0 and 0 h from the onset of oestrus respectively. In blood samples collected between 18 and 30 h after the onset of oestrus, the mean oestradiol concentrations were 6.4 ± 0.6 pg/ml in Group 2 and 5.7 ± 0.6 pg/ml in Group 4. There was no significant rise in plasma oestradiol concentration in control or treated ewes before the second FSH surge.

The ovaries of all the ewes in Group 3 and those of 2 ewes in Group 4 had 1 or 2 ripe follicles with a papilla 6–8 mm diameter at the time of endoscopy (48–52 h after the onset of oestrus). The other 2 treated ewes in Group 4 and all the control ewes showed 1 or 2 fresh corpora lutea haemorrhagica. At 6 days after the start of the experiment plasma progesterone concentrations in Groups 1 + 2, Group 3 (except Ewe 20) and Group 4 were 1.1 ± 0.1 , 1.3 ± 0.5 and 1.5 ± 0.6 ng/ml respectively. There was then sustained rise to ≥ 1.5 ng/ml until values declined before oestrus to reach 0.2 ng/ml. In Ewe 20 in Group 3, which showed complete blockade of the LH surge, the progesterone concentrations were 0.2, 0.4, 0.3, 0.6 and 1.2 ng/ml on Days 0, 6, 8, 10 and 13 respectively, and remained at this level until they declined to 0.4 ng/ml on Day 34, when behavioural oestrus occurred. Another ewe, which showed partial LH blockade, showed behavioural oestrus after 32 days. One ewe in Group 4 had a luteal phase of 45 days and went into anoestrus during mid-March. All the other ewes in the 4 groups became oestrous 19.4 ± 0.4 days after the experiment, and all conceived and subsequently gave birth to 1 or 2 lambs.

Day 3 of the oestrous cycle

In ewes treated with GnRH antibody the maximum LH concentrations noted 45 min after 50 μg GnRH was 31.7 ± 1.7 ng/ml (Group 6) compared with the values of 45.0 ± 5.0 ng/ml in the control ewes (Group 5). In Group 8 maximum LH concentration reached 1 h after 20 μg GnRH was 21.0 ± 4.0 ng/ml compared with the value of 42.5 ± 2.5 ng/ml in the control ewes (Group 7). There was no definite pattern of FSH release in the antibody-treated ewes. Maximum FSH concentration was reached at 2 and 1.5 h in Groups 5 and 7 and was 400 ± 100 and 260 ± 70 ng/ml respectively. In Groups 6 and 8 FSH concentrations at these times were 150.0 ± 37.9 and 151.3 ± 12.9 ng/ml respectively.

Anoestrous ewes infused with oestradiol

In the control ewes 12–14 h from the start of oestradiol infusion, there were synchronous LH and FSH surges (Text-fig. 2a); the maximum concentration of LH was 55.3 ± 3.3 ng/ml and of FSH 350.0 ± 80.4 ng/ml. One of the ewes treated with GnRH antibody showed complete blockade of oestradiol-induced LH and FSH surges (F6 in Text-fig. 2b). In the same ewe, the baseline LH concentration was also reduced by 50% from the pretreatment value of 3.75 ± 0.3 ng/ml ($n = 4$) to a post-treatment value of 1.75 ± 0.25 ng/ml ($n = 9$). In the other two treated ewes there was a delay of 6 and 3 h respectively in the appearance of the LH surge which was itself of decreased magnitude, 35 and 40 ng/ml. One of the treated ewes showed an FSH surge which was asynchronous with the LH surge.

Text-fig. 2. Jugular plasma LH (●) and FSH (○) concentrations in oestradiol-infused (3 µg/h) anoestrous ewes treated with (a) normal sheep plasma (NSP), or (b) GnRH antibody (Ab). The horizontal bar indicates the period of oestradiol infusion and the arrows indicate the time of treatment.

Discussion

In the present study, administration of GnRH antibody before the expected synchronous surge of gonadotrophins at oestrus or after oestradiol infusion to anoestrous ewes produced a decline or a delay of the LH and FSH surges and blocked ovulation. This suggests that neutralization of endogenous GnRH affects normal gonadotrophin release in sheep in accordance with the reports of such inhibition in the rat (Arimura *et al.*, 1974; Kerdelhue *et al.*, 1976), hamster (De La Cruz *et al.*, 1976) and rhesus monkey (McCormack, Plant, Hess & Knobil, 1977). It is probable that the antibody was neutralizing circulating GnRH, as there is no evidence to show that antibodies penetrate nerve endings where the neurohormone is stored. When the antibody falls below a critical concentration, the released free GnRH would be able to exert an effect on pituitary gonadotrophs in order to induce gonadotrophin surges. In ewes which showed complete blockade of gonadotrophin surge (noted during the sampling period), it is likely that the antibody concentration was sufficient to neutralize the continued release of GnRH.

The evidence that our antibody was directed against GnRH comes from the study of the effect of exogenously administered GnRH on gonadotrophin release in antibody-treated ewes. Lack of complete blockade of the GnRH-induced gonadotrophin release in the antibody-treated ewes could have been due to the large dose of GnRH employed (20 µg). As GnRH induces immediate LH release, the time required to attain antigen-antibody equilibrium was probably too great to allow complete block of LH release. Before maximum FSH release was reached greater equilibrium may have been attained, thus enabling the antibody to inhibit the duration and magnitude of the FSH surge to a greater extent. In each experiment except Group 4, FSH release was more sensitive than LH to blockade by GnRH antibody.

Pant *et al.* (1977) found maximum oestradiol concentrations in the jugular blood before the onset of behavioural oestrus. In the present study, there was not a high oestradiol concentration preceding gonadotrophin surge in all the ewes because blood sampling was started at the onset of oestrus. However, the apparent lack of rise in oestradiol concentration preceding the second FSH surge in the control oestrous ewes and the ability of exogenous oestradiol to induce synchronous gonadotrophin surges followed by a second isolated FSH surge in the anoestrous ewe (Dobson & Ward, 1977; Pant, 1977) strongly suggest that the oestradiol stimulus brings about short- and long-term changes in neuronal activity leading to FSH release. That this may be brought about via GnRH is deduced from the alteration of the total pattern of FSH release in the ewes in Group 3. With this hypothesis however it is difficult to account for the second surge of FSH alone and to explain the fact that there was marked inhibition of first FSH surge and GnRH-induced FSH release but not of the second FSH surge after GnRH antibody. Although there is a report of the purification of FSH-releasing hormone (Johansson *et al.*, 1975) there has been no confirmation of its isolation or synthesis.

In spite of ovulation blockade in ewes treated with GnRH antibody at the onset of oestrus, as reflected by the plasma progesterone profile, ovulation could have occurred after the endoscopic examination (48–52 h after the onset of oestrus) or anovulatory luteinization of follicle(s) could have taken place. Surprisingly, in 2 of 4 ewes given antibody 20 h after the onset of oestrus (about 13.5 h after the preovulatory LH and FSH surge) there was no fresh corpus luteum haemorrhagicum.

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