Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in ewes

B. W. Brown, P. E. Mattner, P. A. Carroll, R. M. Hoskinson and
R. D. G. Rigby

CSIRO, Division of Animal Production, Locked Bag 1, Delivery Center, Blacktown, NSW 2148,
Australia

Groups of Merino ewe lambs were immunized against GnRH either soon after birth (prepubertal) or around puberty (peripubertal) with a prototype commercial preparation and were studied over the following 2 years to determine the long-term effects on reproductive development, function and hormone concentrations. At least 60% of the GnRH-immunized ewes in either treatment group did not experience oestrus and possessed small uteri and small ovaries that lacked follicular development. Growth rates of immunized and control ewes were similar throughout the study. Compared with the increase in plasma LH and FSH concentrations with age in control ewes, the concentrations of these hormones in immunized animals were lower and remained relatively constant from 46 to 90 weeks of age. Plasma FSH concentrations were particularly suppressed in immunized ewes and were significantly ($P < 0.05$) lower than the control values of 61 and 90 weeks of age. LH release after injection of 1 µg GnRH at 90 weeks of age was either absent or suppressed in immunized ewes compared with controls. The findings suggest that the lack of GnRH stimulation and consequent deprivation of gonadotrophins, early in the life of ewes, may result in some degree of permanent impairment of hypothalamic and/or pituitary function.

Introduction

Immunization of young and adult male and female domestic animals against GnRH prevents the production of gonadotrophins and results in cessation of reproductive function (Jeffcoate *et al.*, 1978, 1982; Robertson *et al.*, 1979, 1982; Schanbacher, 1982; Brown *et al.*, 1994). Although early studies indicated that the suppressive effects of such anti-GnRH immunization on reproductive function were reversible with time in adult sheep and cattle (Keeling and Crighton, 1984), recent data show that anti-GnRH immunization of ram lambs early in life results in long-term suppression of testicular development in many of the treated animals (Brown *et al.*, 1994). Moreover, the mean plasma gonadotrophin concentrations in these immunized rams are lower than those in control animals, even after the anti-GnRH antibody titre has disappeared. This suggests that the immunization induced some degree of impairment to hypothalamic or pituitary function (Brown *et al.*, 1994).

The present study was initiated to examine the long-term effects of an anti-GnRH immunization treatment on subsequent growth, oestrus and reproductive endocrinology in Merino ewes immunized early in life. In addition, plasma concentrations of LH following injection of 1 µg GnRH were compared with those in similarly injected control ewes.

Materials and Methods

Animals and immunization treatments

Sixty Merino ewe lambs born in August were used in a study at Armidale, NSW, until they were approximately 115 weeks of age. They were weaned at 12 weeks of age and maintained at pasture thereafter. Twenty of the lambs each received 1 mg of antigen (see below) in a 2 ml dose (primary dose) at 3–4 weeks of age (prepubertal immunized group) and a boost with the same type and dose at 13–14 weeks of age. Twenty other ewes received their primary and booster doses when they were 20–21 weeks and 30–31 weeks of age, respectively (peripubertal immunized group). The remaining 20 non-immunized ewes served as controls. The primary and booster doses were each injected s.c., at four sites in the neck region.

Immunogen

Details of the prototype commercial immunization preparation, developed in collaboration with Arthur Webster Pty Ltd, Castle Hill, NSW and Peptide Technology Limited, Dee Why, NSW, are given by Hoskinson *et al.* (1990). Briefly, the immunogen was prepared by conjugation of a carboxyl-containing GnRH analogue to ovalbumin using the diimide reaction. This conjugate was emulsified using a mixture of a

solution of 5% DEAE-dextran (pH 7.5) in a 50% water-in-mineral oil emulsion.

Anti-GnRH antibody titre

At frequent (initially 2 week, then 4–6 week) intervals after booster immunization, 5 ml of blood was sampled from the jugular vein of GnRH-immunized ewes to monitor anti-GnRH antibody titre. Antibody titre was determined for all plasma samples from any sheep in the same ^{125}I -radioligand binding assay similar to that reported for steroids (Abraham, 1974) but using ^{125}I -labelled GnRH (specific activity 3700 GBq mmol $^{-1}$, 0.185 KBq per assay tube). The method of iodination of GnRH was essentially as described by Djura and Hoskinson (1986) for somatostatin. Titre is defined as the dilution of antiserum that bound 50% of the available ^{125}I -radioligand.

Body growth, occurrence of oestrus and laparoscopic examination of genitalia

From about 14 weeks of age, the animals were weighed at frequent (4–6 week) intervals to assess rate of body growth. The occurrence of oestrus in control and immunized ewes was determined from crayon markings obtained over 4 and 3 weeks, respectively, in two consecutive breeding seasons when the ewes were run at pasture with raddle-harnessed vasectomized rams. During the first breeding season, jugular blood (5 ml) was taken by venepuncture from all control and GnRH-immunized ewes twice a week, for three weeks, for measurements of plasma progesterone concentrations as further confirmation of the cyclic status of the ewes (see details below for processing of blood samples). As the progesterone data and raddle markings were found subsequently to be in accord, such blood sampling was not performed over the second joining period.

After vasectomized rams had been run with the ewes during the second breeding season, laparoscopic procedures were used to make assessments of the relative size and appearance of the ovaries and genital tracts in all animals, and to determine the presence of any follicles or corpora lutea on the surface of the ovaries.

Plasma concentrations of LH and FSH and responsiveness to GnRH challenge

Ten control, ten prepubertal immunized and ten peripubertal immunized ewes were selected at random and used to determine group mean plasma concentrations of LH and FSH when they were 46, 61 and 90 weeks of age. At each of these ages, jugular blood samples (5 ml) were obtained by venepuncture at intervals of 15 min for 6 h, held on ice until centrifugation (at 5°C and 3000 g for 20 min) within 30 min of collection, and the plasma was stored at –10°C until assay. At 90 weeks of age, 1 µg GnRH (ICI, Melbourne) in 2 ml of 0.9% (w/v) saline was injected into a jugular vein in each of three control ewes and seven GnRH-immunized ewes selected from either immunized group to provide some ewes that were non-cyclic and some that exhibited oestrous behaviour, to assess their LH responses to a GnRH challenge. The blood samples (5 ml) were obtained by jugular venepuncture before the GnRH injection

and afterwards, at 10 min intervals for 1 h and then at 20 min intervals for a further 2 h.

Hormone assays

All hormones were measured by radioimmunoassay. The sensitivity of the assays for LH (Radford *et al.*, 1987a), FSH (Radford *et al.*, 1987b) and progesterone (Avenell *et al.*, 1985) were 0.19, 0.06 and 0.1 ng ml $^{-1}$, respectively. The intra- and interassay coefficients of variation (at 50% displacement of tracer) for LH and FSH were all < 10% and for progesterone, < 8% and 13%, respectively.

Statistical analysis

Data were analysed using standard procedures for repeated measures analysis of variance, one-way analysis of variance and Student's *t* test (Minitab, Release 7.2, Minitab Inc., PA). After GnRH injection, a positive response (rise in LH) was said to occur when (i) the concentration of the hormone increased by at least 50% above the preceding baseline value, (ii) there were at least two points between the peak value and the succeeding baseline, and (iii) the rate of decline in concentration after the peak was no greater than that allowed by the half-life of the hormone (Khalid *et al.*, 1987).

Results

Anti-GnRH antibody titre

In all immunized animals, the peak anti-GnRH antibody titre response occurred 2 weeks after the booster dose, after which time the titre declined at various rates. There were marked differences among animals in the magnitude of the peak antibody titre value after boost (range 1:1500–1:18 300) and the magnitude of these values was neither related to the subsequent rate of decline in antibody titre, nor could it be used as an indicator of those ewes that ultimately experienced oestrous cycles. By the end of the study, antibody titres were detectable in only five of the 40 GnRH-immunized ewes with values ranging from 2% to 13% of the peak titre after the boost (1:350–1:1600).

Body growth, occurrence of oestrus and laparoscopic examination of genitalia

Throughout the study, the rate of body growth for the two groups of GnRH-immunized ewes did not differ significantly from each other or from the control ewes. At 115 weeks of age, the mean $\pm$ SEM body masses of control, prepubertal and peripubertal immunized groups of ewes were 31.8 $\pm$ 0.6, 33.2 $\pm$ 0.8 and 33.5 $\pm$ 1.2 kg, respectively.

Immunization against GnRH was very effective in inhibiting behavioural oestrus in most of the immunized ewes for the duration of the study. Over 4 weeks of the first breeding season, when the ewes were approximately 90 weeks of age, 25% and 40% of the prepubertal and the peripubertal immunized ewes, respectively, were crayon-marked by the vasectomized rams, compared with 95% of the control ewes.

Table 1. Mean $\pm$ SEM concentrations (ng ml⁻¹) of LH and FSH for control and GnRH-immunized ewes at different stages in life and in relation to anti-GnRH antibody titre

Hormone	Age (weeks)	Controls (n = 10)	Prepubertal (n = 10)	Peripubertal (n = 10)
LH	46	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1	0.3 $\pm$ 0
	61	0.6 $\pm$ 0.2	0.4 $\pm$ 0.2	0.4 $\pm$ 0.1
	90	1.1 $\pm$ 0.2	*0.5 $\pm$ 0.1	*0.3 $\pm$ 0.1
FSH	46	0.8 $\pm$ 0.1	0.5 $\pm$ 0.2	*0.2 $\pm$ 0.1
	61	1.1 $\pm$ 0.1	*0.5 $\pm$ 0.2	*0.3 $\pm$ 0.1
	90	1.3 $\pm$ 0.2	*0.6 $\pm$ 0.1	*0.4 $\pm$ 0.1
Range in anti-GnRH antibody titre				
	46		0–1:780 (70)	1:500–1:5900 (100)
	61		0–1:600 (40)	0–1:4300 (70)
	90		0 (0)	0–1:1600 (40)

GnRH-immunized ewes treated either soon after birth (prepubertal immunized) or around puberty (peripubertal immunized). The percentage of ewes with measurable antibody titre is given in parentheses.

*Within rows, values are significantly different ($P < 0.05$) from control value.

The lack of cyclicity in the non-marked GnRH-immunized ewes was confirmed from the low progesterone concentrations (≤ 0.6 ng ml⁻¹) in the plasma of blood samples obtained over 3 weeks of this teasing period. During the same period, all marked ewes, whether GnRH-immunized or control, had plasma progesterone concentrations in at least one sample of the series > 2.0 ng ml⁻¹. Over 3 weeks in the early part of the second breeding season, when the ewes were approximately 115 weeks old, only 20% of either immunized group were marked, compared with 74% of the controls.

Laparoscopic examination of genitalia during the second breeding season confirmed that the uteri in the non-cyclic, GnRH-immunized ewes were regressed and pale in colour, while the ovaries were small and contained few visible follicles ≤ 0.5 mm in diameter. In contrast, in control ewes, and in immunized ewes that exhibited behavioural oestrus, the uteri were normal in size and colour and the ovaries contained many follicles of various sizes as well as corpora lutea that were either functional or regressed (corpora albicantia).

Plasma concentrations of LH and FSH

The mean plasma concentrations for LH and FSH increased with age in control ewes, but the values were relatively constant from 46 to 90 weeks of age in both groups of immunized animals. As a result, the group mean LH values for the two immunized groups at 90 weeks of age, and their group mean FSH values at both 61 and 90 weeks of age, were significantly lower ($P < 0.05$) than the corresponding values for control ewes (Table 1). In addition, the mean FSH value for the peripubertal group at 46 weeks of age was also significantly lower ($P < 0.05$) than that of the controls.

Responsiveness to GnRH injection

In each of the control ewes, there was a marked increase in plasma LH concentration within 10 min after injection of

Table 2. Peak plasma LH concentrations (ng ml⁻¹) in control and GnRH-immunized ewes injected i.v. with 1 μ g GnRH at 90 weeks of age

Ewes	Response to injection	Peak LH ^a	Cyclic status
Control	Positive ^b	12.3	Cyclic
	Positive	19.1	Cyclic
	Positive	20.8	Cyclic
GnRH-immunized	No response	0.2	Non-cyclic
	No response	0.2	Non-cyclic
	No response	0.3	Non-cyclic
	No response	0.7	Non-cyclic
	Positive	3.9	Cyclic
	Positive	6.1	Cyclic
	Positive	6.7	Cyclic

^aPeak values refer to the maximum concentration of hormone subsequent to GnRH injection. ^bPositive response: a rise in concentration of the hormones following injection of GnRH.

GnRH (Table 2). A similar response did not occur in any of the four GnRH-immunized ewes that were non-cyclic. In each of three GnRH-immunized ewes that had been marked by teaser rams, plasma LH concentrations increased markedly within 10 min of GnRH injection, but peak values were considerably lower than those in control ewes (see Table 2). At the time of this treatment, two of the non-cyclic ewes had measurable antibody titres at a very low level (2% and 13% of the peak titre value after the boost). No other ewes injected with GnRH had measurable antibody titres.

Discussion

Immunization of ram lambs against GnRH, either at birth or around the time of puberty, resulted in significantly lower

plasma gonadotrophin concentrations than those of non-immunized controls, and impaired testicular development, which was still in evidence in many of the immunized rams when they were over two years of age (Brown *et al.*, 1994). Such long-term effects were surprising in view of the fact that anti-GnRH antibody titres had not been measurable for some time in most animals (Brown *et al.*, 1994). In the present study, plasma gonadotrophin concentrations, particularly of FSH, were significantly lower in both groups of immunized ewes than in control ewes. Even at 90 weeks of age, the lower mean plasma gonadotrophin concentrations persisted in both immunized groups, despite the lack of measurable anti-GnRH antibody titres in 16 of the 20 immunized ewes studied and very low titre values for the remainder (titre range: 2–13% of the peak titre value after the boost). It appears that in ewes, as in rams (Brown *et al.*, 1994), immunization against GnRH early in life can induce some degree of impairment to hypothalamic and/or pituitary function.

Normal ovarian follicular development, particularly during the maturational stages, depends on adequate gonadotrophin stimulation (Driancourt *et al.*, 1985; McNatty and Henderson, 1987; Webb *et al.*, 1989). In adult ewes, long-term immunoneutralization of LHRH for 30 months (McNeilly *et al.*, 1986), results in suppression of plasma LH and FSH concentrations and prevention of normal follicular development, as evidenced by the absence of histologically healthy ovarian follicles > 2.0 mm in diameter. Since, in the present study, only a few small (≤ 0.5 mm diameter) ovarian follicles were seen in immunized ewes that had been non-cyclic for two years, it is evident that the reduced concentrations of the gonadotrophins, particularly FSH, in these animals were also insufficient to facilitate folliculogenesis. As a result, the ovarian follicles had not reached a size large enough to produce and secrete sufficient oestrogen to induce oestrus.

Although the oestradiol treatments known to induce oestrous behaviour in anoestrous ewes (Goding *et al.*, 1969; Radford *et al.*, 1970) failed to do so in non-cyclic GnRH-immunized animals even when they were 90 weeks of age (B. W. Brown, unpublished observations), their inability to induce such effects in the GnRH immunized animals suggests that other endocrine factors may be important in initiating the process. Indeed, LHRH, prostaglandin E_2 and noradrenaline have been implicated as modulators of sexual behaviour in rats by changing the conformation of previously synthesized oestrogen-induced proteins, through activation of cAMP, into a more active form that may be involved in synaptic transmission (Beyer *et al.*, 1980). The concentrations of GnRH circulating in the hypophyseal portal vessels in the GnRH-immunized ewes are unknown but, as the plasma gonadotrophin concentrations in these animals were very low even after anti-GnRH antibodies were absent, it is assumed that the releasing hormone was also at an extremely low concentration at this time probably below a threshold necessary to facilitate mechanisms involved in initiating sexual behaviour.

As some GnRH-immunized ewes did eventually exhibit regular oestrous cycles, it appears that the long-term detrimental effects of the immunization may not be mediated via direct effects on folliculogenesis or ovarian function. In the cyclic immunized ewes treated with GnRH, LH release was markedly decreased compared with that for control ewes, suggesting that

immunization may have resulted in either reduced sensitivity of the pituitary gland to GnRH stimulation, or a diminished capacity of the pituitary to produce and secrete LH and FSH. However, as the concentration of pituitary GnRH receptors appears to be under positive autoregulatory control of GnRH (Clayton and Catt, 1981; Fraser *et al.*, 1982; Popkin and Fraser, 1985), the diminished LH response to the i.v. injection of GnRH may merely reflect a downregulation of pituitary GnRH receptors, owing to little or no unbound GnRH in the hypophyseal portal circulation of the immunized animals.

The results of the present study show that a primary and boost immunization against GnRH early in life can produce long-lasting effects on reproduction in ewes, with the possibility that some treated animals may never develop normal gonadal function or display any sexual behaviour. While the low plasma concentrations of gonadotrophins in GnRH-immunized rams were sufficient to facilitate sexual behaviour and gonadal function (including spermatogenesis) in most animals by two years of age (Brown *et al.*, 1994), such low concentrations of gonadotrophins in GnRH-immunized ewes were insufficient to support reproductive function in most of the ewes when they reached the same age. It appears that females may be more susceptible to the effects of anti-GnRH immunization on reproductive function than are males.

The authors thank E. J. Holland, D. R. Paull, G. C. Uphill and R. D. Nethery for valuable technical assistance and P. Baker of the CSIRO Biometrics Unit for advice on the statistical analysis. FSH was assayed using NIAMDD reagents kindly supplied by A. F. Parlow, Pituitary Hormones and Antiseria Center, Torrance, CA USA.

References

- Abraham GE (1974) Radioimmunoassay of steroids in biological materials *Acta Endocrinologica* **75** (Supplementa) 7–12
- Avenell JA, Saepudin Y and Fletcher IC (1985) Concentrations of luteinizing hormone, oestradiol-17 β and progesterone in the peripheral plasma of swamp buffalo cows around the time of oestrus *Journal of Reproduction and Fertility* **74** 419–424
- Beyer C, Canchola E, Cruz ML and Larsson K (1980) A model for explaining estrogen–progesterone interactions in the induction of lordosis behaviour *Proceedings 6th International Congress of Endocrinology* Melbourne, pp 615–618
- Brown BW, Mattner PE, Carroll PA, Holland EJ, Paull DR, Hoskinson RM and Rigby RDG (1994) Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in rams *Journal of Reproduction and Fertility* **101** 15–21
- Clayton RN and Catt KJ (1981) Gonadotropin-releasing hormone receptors: characterization, physiological regulation, and relationship to reproductive function *Endocrine Reviews* **2** 186–209
- Djura P and Hoskinson RM (1986) Radioiodination of somatostatin analogues employing Sep-Pak rapid sample purification and label assessment by high-performance liquid chromatography *Journal of Chromatography* **363** 424–427
- Driancourt MA, Gibson WR and Cahill LP (1985) Follicular dynamics throughout the oestrous cycle in sheep: a review *Reproduction Nutrition Développement* **25** 1–15
- Fraser HM, Popkin RM, McNeilly AS and Sharpe RM (1982) Changes in pituitary LHRH receptor levels in situations of increased or decreased gonadotropin secretion in the male rat *Molecular and Cellular Endocrinology* **28** 321–333
- Goding JR, Catt KJ, Brown JM, Kaltenbach CC, Cumming IA and Mole BJ (1969) Radioimmunoassay for ovine luteinizing hormone. Secretion of luteinizing hormone during estrus and following estrogen administration in the sheep *Endocrinology* **85** 133–142

- Hoskinson RM, Rigby RDG, Mattner PE, Huynh VL, D'occhio M, Neish A, Trigg TE, Moss BA, Lindsey MJ, Coleman GD and Schwartzkoff CL (1990) Vaxstrate[®]: an anti-reproductive vaccine for cattle *Australian Journal of Biotechnology* **4** 166–170
- Jeffcoate IA, Foster JP and Crighton DB (1978) Effect of active immunization of ewes against synthetic luteinizing hormone releasing hormone *Theriogenology* **10** 323–335
- Jeffcoate IA, Lucas JMS and Crighton DB (1982) Effect of active immunization of ram lambs and bull calves against synthetic luteinizing hormone releasing hormone *Theriogenology* **18** 65–77
- Keeling BJ and Crighton DB (1984) Reversibility of the effects of active immunization against LH-RH. In *Immunological Aspects of Reproduction in Mammals* pp 379–397 Ed. DB Crighton. Butterworth, London
- Khalid M, Haresign W and Hunter MG (1987) Pulsatile GnRH administration stimulates the number of pituitary GnRH receptors in seasonally anoestrous ewes *Journal of Reproduction and Fertility* **79** 223–230
- McNatty KP and Henderson KM (1987) Gonadotrophins, fecundity genes and ovarian follicular function *Journal of Steroid Biochemistry* **27** 365–373
- McNeilly AS, Jonassen JA and Fraser HM (1986) Suppression of follicular development after chronic LHRH immunoneutralization in the ewe *Journal of Reproduction and Fertility* **76** 481–490
- Popkin RM and Fraser HM (1985) Changes in pituitary and ovarian LHRH receptors after active immunization of female rats against LH or LHRH *Journal of Reproduction and Fertility* **73** 491–496
- Radford HM, Wallace AL and Wheatley IS (1970) LH release, ovulation and oestrus following the treatment of anoestrous ewes with ovarian steroids *Journal of Reproduction and Fertility* **21** 371–372
- Radford HM, Avenell JA and Panaretto BA (1987a) Some effects of epidermal growth factor on reproductive function in Merino sheep *Journal of Reproduction and Fertility* **80** 113–118
- Radford HM, Panaretto BA, Avenell JA and Turnbull KE (1987b) Effects of mouse epidermal growth factor on plasma concentrations of FSH, LH and progesterone and on oestrus, ovulation and ovulation rate in Merino ewes *Journal of Reproduction and Fertility* **80** 383–393
- Robertson IS, Wilson JC and Fraser HM (1979) Immunological castration in male cattle *Veterinary Record* **105** 556–557
- Robertson IS, Fraser HM, Innes GM and Jones AS (1982) Effect of immunological castration on sexual and production characteristics in male cattle *Veterinary Record* **111** 529–531
- Schanbacher BD (1982) Responses of ram lambs to active immunization against testosterone and luteinizing hormone-releasing hormone *American Journal of Physiology* **242** E201–E205
- Webb R, Gauld IK and Driancourt MA (1989) Morphological and functional characterization of large antral follicles in three breeds of sheep with different ovulation rates *Journal of Reproduction and Fertility* **87** 243–255