Effects of active immunization against a synthetic peptide sequence of the inhibin α -subunit on plasma gonadotrophin concentrations, ovulation rate and lambing rate in ewes

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Summary. Thirty adult Mule (Blue-faced Leicester $\times$ Swaledale) ewes were actively immunized against a synthetically produced peptide corresponding to the N-terminus of the α -subunit of bovine inhibin conjugated to tuberculin purified protein derivative (PPD). Primary immunization in the late anoestrous period was followed by two booster injections at 5 week intervals. Control groups were either not immunized ($n = 15$) or received PPD only ($n = 15$). Ten days after the second booster, oestrus was synchronized using progestagen sponges and ovulation rate was assessed by laparoscopy on days 9–10 of the cycle. Blood samples were taken at the time of each immunization and immediately before laparoscopy. Ewes were mated with fertile rams in mid-November and the resulting conception, pregnancy and lambing rates monitored. All inhibin-immunized ewes generated antibodies that bound ^{125}I -labelled native bovine inhibin (M_r 32 000), and their plasma follicle-stimulating hormone (FSH) concentrations after the second booster were significantly higher than the preimmunization values (30%; $P < 0.001$) and the corresponding value in the controls (25%; $P < 0.025$). Inhibin immunization was associated with a 90% increase in ovulation rate ($P < 0.005$) and had no adverse effect on conception rate (100%), pregnancy rate (100%) or length of gestation (146 days). However, only a 37% increase ($P < 0.05$) in lambing rate was recorded for inhibin-immunized ewes, indicating a higher incidence of wastage of ova, or embryos, or both, in these ewes. As a consequence of the increased litter size, mean live birthweight of lambs was significantly lower (18%; $P < 0.001$) in the inhibin-immunized group and a higher proportion of the lambs born to inhibin-immunized ewes (15.4% compared with 0.02% in controls) were stillborn. Stillborn lambs weighed considerably less (38%; $P < 0.001$) than viable lambs. In terms of the number of viable lambs produced, there was no significant difference between the inhibin-immunized and control group. Although this study confirms the effectiveness of inhibin immunization using a synthetic peptide-based vaccine as a reliable method for increasing ovulation rate in sheep, the results indicate the limitations of this technique for further increasing viable litter size in 'improved' breeds of relatively high inherent prolificacy.

Keywords: inhibin; immunization; sheep; ovulation; litter size; FSH

Introduction

Various strategies have been used to increase prolificacy in sheep and other domesticated species. In addition to selective breeding programmes (Bradford, 1985), a number of nongenetic methods have

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received considerable attention including administration of exogenous gonadotrophins such as equine chorionic gonadotrophin (Gherardi & Lindsay, 1980), autoimmunization against ovarian steroids by active (Scaramuzzi & Hoskinson, 1984) or passive (Land *et al.*, 1982) immunization and, more recently, autoimmunization against inhibin. Inhibin is a gonadal glycoprotein hormone which exerts a selective, suppressive effect on the secretion of pituitary FSH (Ying, 1988). As such, it would be anticipated that immunoneutralization of endogenous circulating inhibin would lead to an increase in plasma FSH concentrations which could promote ovarian hyperstimulation, perhaps culminating in an increased ovulation rate. Experiments involving active immunization of sheep against various inhibin-containing preparations including partially purified bovine follicular fluid (Henderson *et al.*, 1984; O'Shea *et al.*, 1984; Cummins *et al.*, 1986; Al-Obaidi *et al.*, 1987), recombinant DNA-derived preparations of the inhibin α -subunit (Forage *et al.*, 1987; Tsonis *et al.*, 1989; Mizumachi *et al.*, 1990) and chemically synthesized fragments of inhibin (Wrathall *et al.*, 1990) have provided convincing evidence to support this theory and have proved conclusively that immunoneutralization of endogenous inhibin can promote a marked increase (2–4-fold) in ovulation rate. However, very little information (Tsonis *et al.*, 1989) is available regarding the impact of inhibin immunization on other critical stages of the reproductive process (including the ability of the ewe to display oestrus, mate, conceive and gestate) and whether the observed increase in ovulation rate is actually reflected by an increase in viable litter size.

The objectives of the present study were (i) to confirm the effectiveness of a synthetic peptide-based inhibin vaccine in generating antibodies to inhibin, raising plasma FSH and increasing ovulation rate in a different (and more typical UK lowland) breed of sheep from that used in our initial study (Wrathall *et al.*, 1990); and (ii) to establish the subsequent effect of inhibin immunization on conception, pregnancy and lambing rates in sheep maintained, as closely as possible, under normal husbandry conditions.

Materials and Methods

Preparation of immunogen

A synthetic peptide, corresponding to the N-terminal sequence (1–29) of the α -subunit of bovine inhibin (bI α (1–29)-Tyr30, custom-synthesized by Protein and Peptide Research Consultants, Reading, Berks, UK) was conjugated to tuberculin purified protein derivative (PPD; MAFF Central Veterinary Laboratory, Weybridge, Surrey, UK) at a ratio of 1:3 (w/w) as described by Wrathall *et al.* (1990). The resulting conjugate was divided into 1.5 ml (4 mg conjugate) aliquots and stored at -20°C .

Animals and treatments

The animals used in the study formed part of the University of Reading Animal Production Research Unit flock and were subject to the same general management regimen as the main flock. Ewes were kept on pasture with free access to water at all times. Sixty adult Mule (Blue-faced Leicester $\times$ Swaledale) ewes (mean liveweight 71.7 ± 0.73 kg) were divided into three treatment groups in the late anoestrous period (early August). Group 1 ewes ($n = 30$) were given primary immunization (2 s.c. sites in the hind axillary regions) with 600 μg bI α (1–29)-Tyr30-PPD conjugate per ewe emulsified with 2 volumes of Freund's complete adjuvant. Booster immunizations (200 μg conjugate per ewe emulsified with Freund's incomplete adjuvant) were given 5 and 10 weeks later. A further 15 ewes (Group 2) were immunized with PPD alone (450 μg per ewe emulsified in Freund's complete adjuvant for primary immunization; 150 μg per ewe in Freund's incomplete adjuvant for booster injections 5 and 10 weeks later) and thus represented carrier-immunized controls. The remaining 15 animals (Group 3) served as nonimmunized controls. Synchronization of oestrus was effected 10 days after the second booster immunization by insertion of intravaginal progestagen-containing polyurethane sponges (Veramix: Upjohn Limited, Animal Health Division, Crawley, West Sussex, UK) for 13 days, and 12 days after sponge removal, assessment of ovulation rate was carried out by laparoscopy. Blood samples (5 ml) were taken from all ewes by jugular venepuncture immediately before primary and booster injections, and just before laparoscopic examination.

Immediately after laparoscopy, fertile Suffolk rams ($n = 6$) fitted with raddle harnesses equipped with wax crayon blocks were introduced to the ewes' enclosure. Ewes were inspected every 2 days for raddle marks and approximate dates of oestrus recorded. Owing to lack of pasture, the diet was supplemented at this time by feeding barley (200–300 g per ewe day $^{-1}$) for two weeks, commencing 2–3 days before laparoscopy. Conception rates (the proportion of

ewes that did not show a return to oestrus) and pregnancy rates (the proportion of ewes bearing at least one fetus when scanned ultrasonographically 11 weeks later) were also recorded. In late March, ewes were transferred to indoor lambing enclosures to await parturition. For each group, the proportion of ewes lambing, total number of lambs born per ewe, number of dead lambs born per ewe and lamb birthweight were recorded.

Assessment of antibody titres

Plasma samples were tested for their ability to bind ^{125}I -labelled bovine inhibin (M_r 32 000) isolated by Knight *et al.* (1987) and radiolabelled as described elsewhere (Knight *et al.*, 1989). Incubation of diluted (1:1000) plasma samples with the tracer (approximately 10 000 c.p.m. per tube), in a final volume of 200 μl , was carried out at room temperature for a period of 24 h. Separation of antibody-bound tracer from free tracer was achieved using a preprecipitated second antibody reagent containing donkey anti-sheep IgG serum, normal sheep serum and 4% (w/v) polyethylene glycol. Samples were centrifuged, and after aspiration of the supernatant, the precipitated antibody-bound ^{125}I -labelled tracer was counted using a multigamma counter. After correcting for nonspecific binding (recorded in the absence of sheep serum/antiserum), results were expressed as percentage binding relative to the total counts per tube.

Determination of hormone concentrations

Plasma concentrations of FSH were assessed using a homologous ovine FSH radioimmunoassay kit provided by NIDDK (Bethesda, MD, USA). Intra- and interassay coefficients of variation were 7% and 8%, respectively, and the detection limit of the assay expressed as the amount of FSH standard required to inhibit the binding of the iodinated tracer (NIADDK-oFSH-11) to the antibody (NIADKK-anti-oFSH-1; final dilution 1:16 000) by 20% was 37 pg per tube (equivalent to 0.37 $\mu\text{g l}^{-1}$).

Determination of plasma concentrations of LH was made using the radioimmunoassay described by Walton *et al.* (1977). Intra- and interassay coefficients of variation were 9% and 13%, respectively. The limit of detection of the assay (expressed as the amount of the LH standard (NIH-LH-S16) needed to inhibit the binding of ^{125}I -labelled LH to the antibody by 20%) was 114 pg per tube (equivalent to 1.14 $\mu\text{g l}^{-1}$).

Statistical analysis

Chi-squared analysis and Student's unpaired *t* test were both used to evaluate the effect of immunization on ovulation rate and number of lambs born per ewe. Between-group comparisons examining the effects of immunization on gestation period, lamb birthweight and plasma concentration of FSH and LH were carried out using Student's unpaired *t* test. Where indicated, Student's paired *t* test was used to make within-group comparisons of time-dependent changes in mean plasma concentrations of FSH and anti-inhibin titres during the immunization programme.

Results

In December, one ewe from the inhibin-immunized group died (cause of death not established) and one of the PPD-immunized animals suffered a fractured leg and hence was excluded from the study.

Plasma anti-inhibin titres and concentrations of FSH and LH

Antibodies that bound ^{125}I -labelled bovine inhibin (M_r 32 000) were produced by all ewes receiving the bI α (129)-Tyr30-PPD immunogen, titres rising from very low levels after the first booster to a mean value of $16.3 \pm 1.4\%$ (at 1:1000 dilution) after the second booster injection (Fig. 1). In neither PPD-immunized nor nonimmunized ewe plasma was any binding of ^{125}I -labelled bovine inhibin detectable above the level seen in nonspecific binding (buffer only) tubes.

The data for PPD-immunized and nonimmunized animals have been combined as there were no significant differences in FSH concentration between these two control groups. A significant increase (30%; $P < 0.001$) in mean plasma concentrations of FSH over mean preimmunization levels was seen 10 days after the second booster immunization in inhibin-immunized ewes but not in control animals (Fig. 1). Concentrations of plasma FSH in inhibin-immunized ewes both 5 weeks after the first booster and 10 days after the second booster immunization were significantly higher (25%, $P < 0.025$) than in the corresponding plasma samples from control ewes. In contrast,

Fig. 1. Plasma concentrations of follicle-stimulating hormone (FSH) in 30 ewes during active immunization against a synthetic peptide corresponding to the N-terminal sequence ((1–29)-Tyr30) of the α -subunit of bovine inhibin (○) and in 30 control ewes (●). Fifteen of the control ewes were immunized with the carrier protein alone, while the remaining ewes received no treatment. Plasma anti-inhibin titres in inhibin-immunized (□) and control (■) ewes were assessed by incubating diluted (1:1000) plasma samples with ^{125}I -labelled bovine inhibin (M_r 32 000). Values are means $\pm$ SEM.

*** $P < 0.001$ compared with preimmunization value.

† $P < 0.025$ compared with corresponding values in controls.

mean concentrations of plasma LH did not differ significantly between inhibin-immunized and control animals at any time during the experiment although there was a tendency for LH concentration to be lower (approximately 16%) in the immunized ewes (data not shown). Mean plasma LH concentrations in the two control groups were not significantly different.

Ovulation rates

There was no significant difference between mean ovulation rates in the PPD-immunized (2.21 ± 0.24 , range 1–4) and the nonimmunized (1.93 ± 0.21 , range 1–3) control animals and the values obtained for these two groups were therefore combined (Table 1). The inhibin-immunized group had a mean ovulation rate significantly higher (3.90 ± 0.33 , range 1–8; $P < 0.005$) than that of the combined control group (2.07 ± 0.16 , range 1–4). No correlation ($r = 0.23$, $P = 0.14$) was found to exist between ovulation rate and inhibin antibody titre in individual inhibin-immunized ewes.

Conception, pregnancy and lambing rates

The results for PPD-immunized (Group 2) and nonimmunized (Group 3) ewes were combined since they did not differ significantly from each other. In both inhibin-immunized and control groups, all ewes were fertilized and subsequently deemed to be pregnant approximately 11 weeks later; only one animal (in the control group) failed to produce at least one lamb (Table 2). There was no difference between the two groups in the mean length of gestation. The mean number of lambs born per ewe in the inhibin-immunized group (2.69 ± 0.27 , range 1–8) was significantly higher ($P < 0.05$) than in controls (1.97 ± 0.12 , range 0–3) (Table 3), although a higher proportion of lambs in the inhibin-immunized group were born dead (15.4% of the total, compared with 0.02% in control animals). Consequently, there was no significant difference between the two groups in the number of viable lambs (inhibin-immunized: 2.28 ± 0.21 ; controls: 1.93 ± 0.11 ; $P > 0.05$). In

Table 1. Mean ($\pm$ SEM) ovulation rates and frequency of ovulation rates in inhibin-immunized and control ewes after synchronization of oestrus

	Number of ewes ovulating ^a	Total number of corpora lutea	Mean ovulation rate ^b	Frequency ^a of ovulation rate [†]							
				1	2	3	4	5	6	7	8
Inhibin immunized (Group 1, $n = 29$)	29 (100)	112	3.90 $\pm$ 0.33***	1 (3.5)	8 (27.6)	4 (13.8)	6 (20.7)	6 (20.7)	0 (0)	3 (10.3)	1 (3.4)
Controls (Groups 2 and 3, $n = 29$)	29 (100)	60	2.07 $\pm$ 0.16	8 (27.6)	12 (41.4)	8 (27.6)	1 (3.4)	0 (0)	0 (0)	0 (0)	0 (0)

^aNumber in parentheses indicates percentage; ^bNumber of corpora lutea per ewe. *** $P < 0.005$ compared with controls (unpaired t test). [†]Immunized compared with controls, $P < 0.002$ (χ -squared analysis).

Table 2. Conception rates and outcome of pregnancy for inhibin-immunized and control ewes

Number of ewes (%) conceiving ^a		Gestation period (days) ^c	Number of lambs born		Number of lambs born per ewe ^c			Birthweight (kg) ^c				
			At first service	In total	Number of ewes (%) pregnant ^b	Number of ewes (%) lambing	Total			Alive	Dead	Total
Inhibin immunized (n = 29)	28 (96.6)	29 (100)	29 (100)	145.6 ± 0.26	78	66	12	2.69 ± 0.27*	2.28 ± 0.21	0.41 ± 0.28	4.28 ± 0.12**	2.65 ± 0.2†
Controls (n = 29)	28 (96.6)	29 (100)	29 (100)	146.0 ± 0.34	57	56	1	1.97 ± 0.12	1.93 ± 0.11	0.03 (0.03)	5.21 ± 0.15	(3.0)

^aNonreturn to oestrus. ^bUltrasonographic scanning at approximately 11 weeks gestation. ^cMeans $\pm$ SEM. * $P < 0.05$ compared with controls. ** $P < 0.001$ compared with controls. [†] $P < 0.001$ compared with live birthweight.

Table 3. Frequency of lambing rates and mean lambing rates in inhibin-immunized and control ewes

	Number of lambs (%) per ewe								Mean $\pm$ SEM
	0	1	2	3	4	5	6	7	
Inhibin immunized [†] (Group 1, $n = 29$)	0	5 (17.2)	10 (34.5)	8 (27.6)	4 (14.0)	1 (3.5)	0	0	2.69 $\pm$ 0.27*
Controls (Groups 2 and 3, $n = 29$)	1 (3.5)	3 (10.3)	21 (72.4)	4 (13.8)	0	0	0	0	1.97 $\pm$ 0.12

[†] $P < 0.05$ compared with controls (χ -squared). * $P < 0.05$ compared with controls (t test).

addition, mean live birthweight was significantly lower ($P < 0.001$) in the inhibin-immunized group (4.28 ± 0.12 kg) than in controls (5.21 ± 0.15 kg), whereas within the immunized group itself, lambs born dead weighed significantly less ($P < 0.001$) than those born alive (Table 2).

Discussion

This study confirms and extends previous reports that active immunization of sheep against inhibin-containing preparations including partially purified bovine follicular fluid (Henderson *et al.*, 1984; O'Shea *et al.*, 1984; Cummins *et al.*, 1986), recombinant DNA-derived inhibin subunits (Forage *et al.*, 1987; Tsonis *et al.*, 1989; Mizumachi *et al.*, 1990) and chemically synthesized fragments of inhibin (Wrathall *et al.*, 1990) can promote substantial (2–4-fold) increases in ovulation rate. It was demonstrated that the plasma of ewes immunized against a synthetic fragment of the α -subunit of inhibin contained antibodies that bound ^{125}I -labelled native bovine inhibin (M_r 32 000), such antibodies being absent in nonimmunized and carrier-protein-immunized control ewes. Plasma anti-inhibin titres recorded here were very similar to those observed in our previous study involving administration of the same immunogen to ewes of a different (Romney) breed (Wrathall *et al.*, 1990), demonstrating the consistency of the immune response to this synthetic peptide-based vaccine. As in this earlier study, no correlation was found between inhibin antibody titre and ovulation rate of individual ewes, an observation which is consistent with the report of Forage *et al.* (1987) but contradicts the recent findings of Tsonis *et al.* (1989).

In view of the conflicting evidence about whether active immunization against inhibin actually raises FSH secretion (Henderson *et al.*, 1984; O'Shea *et al.*, 1984; Cummins *et al.*, 1986; Al-Obaidi *et al.*, 1987; Findlay *et al.*, 1989; Wrathall *et al.*, 1990), it is still not possible to state unequivocally that the increased ovulation rate observed in inhibin-immunized ewes is due to a rise in plasma FSH levels, which would be a predicted consequence of inhibin immunoneutralization. The present study, however, provides further evidence to support this explanation since plasma FSH levels were significantly higher (25%) than in control ewes after the second booster immunization, whereas plasma LH levels did not differ significantly between immunized and control ewes. The present findings are therefore consistent with our earlier study (Wrathall *et al.*, 1990), which found a similarly modest (approximately 22%) increase in plasma FSH after inhibin immunization. In this earlier study, more detailed examination of plasma hormone profiles revealed that plasma FSH concentration in inhibin-immunized ewes remained higher than in controls throughout the follicular phase of the cycle, a finding subsequently confirmed by Mizumachi *et al.* (1990) and Wheaton & Carlson (1990). It is noteworthy that both the magnitude of the increase in plasma FSH concentration (approximately 25%) and the accompanying increase in ovulation rate (approximately twofold) observed by us following immunization against the same immunogen were very similar in Romney (Wrathall *et al.*, 1990) and Mule (this study) ewes despite the twofold difference in spontaneous ovulation rate characteristic of these breeds. This observation suggests that the ovulatory response to inhibin immunoneutralization may, to some extent, be independent of spontaneous ovulation rate.

The present study revealed that active immunization against a synthetic inhibin fragment had no adverse effects on fertility (conception rate) and did not alter mean gestation length (146 days). After introduction of fertile rams, all but two ewes (one from each group) conceived at first mating with the remainder conceiving at second mating. It may be assumed, therefore, that all ewes showed behavioural oestrus indicative of a normal preovulatory positive feedback relationship between oestradiol and LH. Ultrasonic scanning at approximately 11 weeks' gestation provided confirmation that all immunized and control ewes were pregnant although it was not possible to ascertain the number of fetuses per ewe when this was judged to exceed two. All ewes, with the exception of one from the control group, gave birth to at least one lamb, mean lambing rate being 37% greater ($P < 0.05$) in the inhibin-immunized group than in the controls. It is clear, however, that the proportion of corpora lutea that resulted in lambs was considerably less for inhibin-immunized (70%)

than for control (95%) ewes (see Table 2). This indicates a greater incidence of wastage of ova, embryos, or both, in the inhibin-immunized group, which is presumed to be a direct consequence of the substantially higher (90%) ovulation rate shown by these ewes and not due to any adverse effect of inhibin immunoneutralization *per se*. In support of this view, Ashworth *et al.* (1989) have shown that embryo survival (to day 30) in ewes of the prolific 'Damline' breed is inversely related to spontaneous ovulation rate, declining steeply from about 80% when ovulation rate is 1–3 to about 30% when ovulation rate is 4–5. Moreover, Tsonis *et al.* (1989) reported that active immunization against inhibin in Border Leicester $\times$ Merino ewes (spontaneous mean ovulation rate = 1.4) increased ovulation rate 3.6-fold, but resulted in only a twofold increase in lambing rate, again indicating an increased incidence of wastage of ova, embryos, or both. Thus, there may be relatively little scope for increasing mean lambing rates much beyond about 2.5 lambs per ewe, making the present technique involving inhibin immunoneutralization more applicable to breeds of lower prolificacy such as the Border Leicester $\times$ Merino ewes studied by Tsonis *et al.* (1989).

A discouraging finding of the present study was the greater mortality of lambs born to inhibin-immunized ewes, with 12 out of 78 lambs (15.4%), compared with 1 out of 57 in the control group, either born dead or found to be dead within 1 h of birth. However, of the 12 dead lambs born to immunized ewes, eight were from a single ewe. As a consequence of their increased litter size, mean live birthweight of lambs was significantly less (18%; $P < 0.001$) for inhibin-immunized ewes than controls, while, within the immunized group, lambs born or found dead weighed considerably less (38%; $P < 0.001$) than viable lambs. No details of lamb mortality or birthweight were given by Tsonis *et al.* (1989) in their report of a comparable experiment involving Border Leicester $\times$ Merino ewes immunized against a recombinant inhibin-based vaccine. However, it might be anticipated that the incidence of lamb mortality would be less than that recorded in the present study with ewes of the Mule breed owing to the inherently lower prolificacy (about 1.15 lambs per ewe) of the Border Leicester $\times$ Merino ewe.

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