Effect of immunization against synthetic peptide sequences of bovine inhibin α -subunit on ovulation rate and twin-calving rate in heifers

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Three peptide sequences from the bovine inhibin α -subunit (P1: 18–30; P2: 63–72 and P3: 107–122) were synthesized and conjugated to human serum albumin (HSA). Hereford cross-heifers ($n = 5$ per group) were injected with 3 mg of one of the peptide conjugates, followed by three booster injections at intervals of 11 weeks. Control heifers ($n = 5$) were injected with HSA only. Antibodies recognizing both the individual peptides and 32 kDa bovine inhibin were generated and ovulation rate was increased in peptide immunized heifers. In group P1, 1 of 5 heifers responded with an increased ovulation rate whereas in groups P2 and P3, 5 of 5 and 4 of 5 heifers, respectively, had an increased ovulation rate. In group P2, in the first oestrous cycle following booster injections 2 and 3, 4 of 5 and 3 of 5 heifers, respectively, responded with twin ovulations, whereas a fourth heifer had three ovulations following booster injection 3. After breeding following booster injection 3, 3 of 5 heifers in group P2 and 1 of 5 in group P3 gave birth to twin calves. This study demonstrates the potential of immunizing against synthetic peptide sequences of the α -subunit of bovine inhibin to increase ovulation and twinning rates in cattle.

Introduction

Inhibin is a dimeric protein hormone composed of two dissimilar, disulfide-linked subunits (termed α and β), and is involved in the negative feedback regulation of gonadotrophin secretion, preferentially follicle-stimulating hormone (FSH) (Burger and Igarashi, 1988; Ying, 1988). Interference with this negative feedback by active immunization against either partly purified inhibin (O'Shea *et al.*, 1984; Morris *et al.*, 1991), recombinant inhibin (Forage *et al.*, 1987; Mizumachi *et al.*, 1990) or synthetic inhibin peptides (Wrathall *et al.*, 1990; Boland *et al.*, 1991) resulted in significant and consistent increases in ovulation rate in sheep. An increased ovulation rate has also been recorded following immunization of cattle against either partly purified inhibin (Price *et al.*, 1987; Sreenan *et al.*, 1987) or a synthetic inhibin peptide (Glencross *et al.*, 1990; Sunderland *et al.*, 1991). An increase in litter size has also been reported following immunization of sheep against either partly purified (O'Shea *et al.*, 1984) or recombinant bovine inhibin (Tsonis *et al.*, 1989). There are no published data on calving or twinning rates in cattle following immunization against inhibin.

This paper describes the effect of active immunization of heifers against each of three synthetic peptide sequences from the bovine inhibin α -subunit on peptide antibody titre, inhibin binding, ovulation rate, calving rate and twin-calving rate.

Materials and Methods

Sequence identification

The amino acid sequence of the bovine inhibin α -subunit (Forage *et al.*, 1986) was analysed by the methods of Chou and Fasman (1978) and Kyte and Doolittle (1982), using a computer program assembled by Mammi (1989). This program combines algorithms for the prediction of hydrophilicity/hydrophobicity and secondary structure, and enables the rapid identification of likely immunological epitopes. Three peptide sequences (Fig. 1) were identified as potential antigenic sites. The natural sequences were modified during synthesis by the addition of either tyrosyl or cystenyl residues, linked through a glycine spacer where appropriate, to facilitate iodination and conjugation, respectively.

Peptide synthesis

The crude peptides were synthesized on methylbenzhydrylamine resin using an ACT (Advanced Chemical Technology, Louisville, KY) series 200 automated system. Free peptide amides were cleaved from the resin by treatment with hydrogen fluoride/anisole or hydrogen fluoride/metacresol and purified by gel filtration on Sephadex G-25 (Pharmacia LKB, Milton Keynes) followed by preparative high pressure liquid chromatography (HPLC) on 1.5×45 cm columns of silica (10–15 μ m, Vydac C18, The Separations Group, Hesperia, CA). Peptide purity was assessed by analytical HPLC, thin layer chromatography (TLC)

and amino acid analysis of acid hydrolysates. The purity of each peptide was greater than 90%.

Peptide conjugation

Each peptide was conjugated to human serum albumin (HSA) and conalbumin (Sigma, Poole, Dorset) as follows. HSA or conalbumin (100 mg) was dissolved in 0.5 ml 0.2 mol sodium borate l^{-1} , 0.15 mol NaCl l^{-1} , pH 8.3 and reacted at 23°C for 5 min with 9.0 mg of *N*- γ -maleimido-butyryloxysuccinimide (GBBS; Calbiochem, Lucerne) dissolved in 20 μl of dry freshly distilled dimethylformamide. Samples were then centrifuged for 1 min in an Eppendorf microfuge (15 000 g) and desalted immediately on a column (0.9 $\times$ 30 cm) of Sephadex G-25, equilibrated with de-gassed 0.1 mol Hepes l^{-1} , pH 7.0. The modified protein eluting in the void volume was transferred to a stoppered vial, and 10 mg inhibin peptide, dissolved in 0.1 mol Hepes l^{-1} just before addition, was added a drop at a time. The mixture was stirred at 23°C for 4 h and at 4°C overnight before dialysis against 2 l of 0.01 mol sodium phosphate l^{-1} , pH 7.4, containing 0.15 mol sodium chloride l^{-1} (three changes), using Spectra Por 1 membrane (cut off *M*, 6000–8000; Spectrum Med. Ind., Los Angeles, CA). The extent of protein substitution by peptide was estimated by analysing the complexes on an 8–25% gradient sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Phastsystem; Pharmacia LKB). For each complex approximately 6–8 mol of peptide were bound per mole of carrier. Peptide conjugates were stored as lyophilized powders at –25°C until required for immunization.

Immunization

Twenty nulliparous Hereford cross-heifers, approximately 18 months of age and 367 ± 7 kg (mean $\pm$ SEM), were randomly allotted in equal numbers ($n=5$) to three treatment groups and a control group. Inhibin peptide–HSA conjugates (3 mg) were dissolved in 2 ml saline (0.85% NaCl), emulsified in an equal quantity of Freund's complete adjuvant (Difco, Detroit, MI) and injected into four sites in the brisket region. Three booster injections (1.5 mg of conjugate) were similarly prepared but emulsified in non-ulcerative Freund's adjuvant (NUFA; Guildhea Antisera, Guildford, Surrey) and administered at intervals of 11 weeks. The control group was immunized with HSA carrier only. The second and third booster injections were given to all groups between day 7 and day 19 of the oestrous cycle. Blood samples were taken immediately before (pre-immune) and at regular intervals after immunization (immune) by jugular venepuncture. The blood samples were taken into 10 ml tubes without additive (Vacutainer; Beckton and Dickinson, UK) and allowed to clot for 1 h at 37°C. After centrifugation, the serum was removed and stored at –25°C until assay for anti-peptide antibodies using a solid phase enzymeimmunoassay and for anti-bovine 32 kDa inhibin antibodies using a solution phase radioimmunoassay.

Enzymeimmunoassay procedure

Microtitre plate wells (No. 3590; Costar, High Wycombe, Bucks) were coated with 100 μl of a 2 $\mu\text{mol l}^{-1}$ solution (as peptide) of peptide–conalbumin conjugate in 0.05 mol sodium bicarbonate l^{-1} , pH 9.6. After incubation for 2 h at 37°C, the

- 1 P1: bI- α -Tyr¹⁶Gly¹⁷(18–30): YGQRPEEPAAHADC
- 2 P2: bI- α -(63–72)Gly⁷³Tyr⁷⁴: CGLSPQDLPGY
- 3 P3: bI- α -Cys¹⁰⁵Gly¹⁰⁶(107–122): CGHVRTTSDGGYSFKYEM

Fig. 1. Sequences of the synthetic peptides derived from the bovine α -inhibin subunit.

plates were washed three times with 100 mmol sodium phosphate l^{-1} , pH 7.4 (PB) containing 0.15 mol NaCl l^{-1} (PBS). Plate wells were blocked with 1% (w/v) ovalbumin (BDH, Poole, Dorset) in PBS (PBSO) and incubated for 4 h at 37°C and overnight at room temperature. The plates were then washed with PB containing 1.0 mol NaCl l^{-1} and 0.3% (w/v) Tween-20 (PBST); 100 μl of pre- and immune-serum, diluted in PBSO containing 0.05% (w/v) Tween-20, was added to each well in duplicate and incubated for 2 h at 37°C. After washing as before, the wells were further incubated for 1 h at 37°C with 100 μl horseradish peroxidase labelled rabbit anti-bovine immunoglobulin (Dakopatts, Glostrup, DK) diluted 1:8000 in PB containing 0.3 mol NaCl l^{-1} , 0.05% (w/v) Tween-20 and 2% normal rabbit serum. After a further washing, the wells were incubated for 30 min at room temperature with 150 μl of 1.5 mg *o*-phenylenediamine ml^{-1} , 5 mmol H_2O_2 l^{-1} in 0.05 mol phosphocitrate l^{-1} , pH 5.0. The reaction was stopped by the addition of 50 μl 4 mol H_2SO_4 l^{-1} and the absorbance of each well was measured at 492 nm on an enzyme-linked immunosorbent assay (ELISA) reader (MR580; Dynatech, Billingshurst). Titre was expressed as the reciprocal dilution of serum required to give an absorbance of 0.5 under the assay conditions used.

Radioimmunoassay procedure

Inhibin iodination. Bovine 32 kDa inhibin was radiolabelled with ¹²⁵I to a specific activity of approximately 24 $\mu\text{Ci } \mu\text{g}^{-1}$ by a modification of the chloramine-T method of Greenwood *et al.* (1963). Briefly, 5 μg of essentially pure biologically active bovine inhibin (Peninsula Laboratories, UK) was dissolved in 10 μl of 0.1% (v/v) trifluoroacetic acid in distilled water and added to a 1 ml Eppendorf tube containing 20 μl 0.5 mol PB l^{-1} , pH 7.5. To this, 1 mCi (10 μl) of Na¹²⁵I] (IMS 30; Amersham Int., Bucks) was added followed by 10 μl chloramine T in PB (0.2 mg ml^{-1}). After vortexing for 1 min, 50 μl of tyrosine in PB (0.4 mg ml^{-1}) was added and vortexed for 15 s. The mixture was then applied to a Sephadex G-75 (Pharmacia LKB) column (50 $\times$ 1 cm) pre-equilibrated with 10 mg BSA, and eluted with 0.05 mol Tris–HCl l^{-1} , pH 7.5 containing 8 mol urea l^{-1} and 5 mmol 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfonate (Chaps) l^{-1} . Suitable dilutions of the inhibin containing fractions eluted were assessed for binding by incubating with chicken anti-inhibin antiserum (H95/2; Knight *et al.*, 1989) in an optimized radioimmunoassay, and were characterized with regard to molecular weight by 8–18% gradient SDS-PAGE (ExcelGel SDS; Pharmacia LKB) and autoradiography on Hyperfilm β -max (Amersham Int.). Maximally binding fractions were associated with a 32 kDa protein that migrated as two subunits of 20 and 12 kDa under reducing conditions, which is consistent with the known structure for bovine inhibin.

Inhibin binding. [¹²⁵I]-labelled inhibin (100 μl ; 10 000 d.p.m.) diluted in 50 mmol sodium phosphate l^{-1} pH 7.4 containing

0.15 mmol NaCl l⁻¹ and 0.1% ovalbumin was incubated with 100 µl of serum (final dilution 1:40) from immunized heifers for 24 h at 20°C. Antibody bound label was then precipitated by adding 400 µl of ice-cold 20% polyethylene glycol (PEG, MW-6000; BDH) to each tube. After 30 min at 4°C, 3 ml of 13% PEG was added to each tube and the tubes were centrifuged for 30 min at 4000 g. The radioactivity in the pellet was counted in a γ -counter (Multigamma 1261; Pharmacia LKB). Binding was expressed as the percentage of radiolabel bound relative to total counts added (%B:T) after correction for nonspecific binding by pre-immune serum.

Oestrous and ovarian responses

Five days after each booster injection, the oestrous cycles of the heifers were synchronized with a short-term (9-day) progestagen (Sreenan and Mulvehill, 1975). Twenty-four hours before pessary removal, each heifer received a luteolytic dose (15 mg) of Luprosetol (Prosolvine; Intervet, Cambridge, UK) intramuscularly. At progestagen removal, heifers were tail-painted to aid detection of oestrus and were observed four times a day for standing oestrus. Ultrasonography (DSL 300, 5 MHz probe; Diagnostic Sonar, Livingston, Scotland) was carried out between day 6 and day 10 on all heifers during each oestrous cycle to determine the number of corpora lutea present.

Conception and calving rate

At the synchronized and first repeat oestrus following booster injection 3, heifers were mated. The number of fetuses present at 45 days after mating was counted by ultrasonography and at parturition the number of calves born was recorded.

Statistical analysis

The data were analysed using least squares ANOVA procedures (Harvey, 1987). Before analysis, peptide titre and percentage inhibin binding were transformed by log and angular transformations, respectively, to approximate normal variates. The model recognized that repeated measurements were made on the same heifers over time and included the effect of immunization treatment, heifers within treatment, booster injection number, cycle number and the interaction of treatment with cycle number. The treatment by cycle interaction was analysed in more detail, by comparing both cycle number after booster injection within treatment, and treatment within cycle number after booster injection using the Student–Newman–Keuls test. Because ovarian measurements during a fourth oestrous cycle were made on only a small number of heifers, these observations were assigned to cycle number three for analysis of variance. As inhibin binding, but not peptide titre, was common to all groups, it was the only between group parameter of the immune response that was analysed statistically. Correlations between ovulation rate in the first oestrous cycle following a booster injection, peptide titre and inhibin binding at 14 days after a booster injection (2–3 days before oestrus) were examined by regression analysis. The significance of the differences among these correlations was examined by testing the homogeneity of the regression of ovulation rate on titre. Inhibin binding is presented after back-transformation.

Fig. 2. Peptide antibody titres (means $\pm$ SEM) in serum from heifers immunized against inhibin peptides (■) P1, (●) P2 and (▼) P3. Heifers were injected at intervals of 11 weeks (arrows). P: primary immunization; B₁–B₃: consecutive booster injections.

Fig. 3. Percentage binding (means $\pm$ SEM) of ¹²⁵I-labelled 32 kDa bovine inhibin by serum from heifers immunized against inhibin peptides (■) P1, (●) P2 and (▼) P3. Heifers were injected at intervals of 11 weeks (arrows). P: primary immunization; B₁–B₃: consecutive booster injections.

Results

Immune response

Antibodies were generated to each of the peptides and titres reached a maximum value between 7 and 21 days following a booster injection (Fig. 2). The highest peptide titres recorded for individual animals within groups P1, P2 and P3 were 316 000, 284 000 and 59 000, respectively. Peptide titres measured in the HSA-immunized control heifers and in pre-immune sera were less than 100.

All heifers immunized with peptides generated antibodies that bound to inhibin, and binding reached a maximum between 7 and 21 days after a booster injection (Fig. 3). The highest

Table 1. Specific inhibin binding (%) 14 days after immunization of heifers with inhibin peptides

Group	Inhibin binding (%)	
P1	21.5 ^a (15)	16.5–27.1 (4.6–35.0)
P2	27.5 ^b (15)	22.0–33.5 (6.4–42.2)
P3	5.5 ^c (15)	2.9–8.8 (1.1–13.4)

Values are means followed by 95% confidence limits of the mean of three booster injections with numbers of observations in parentheses followed by the range. $b > a$ $P < 0.05$; $a, b > c$ $P < 0.01$.

[¹²⁵I] 32 kDa bovine inhibin binding recorded was 35%, 42% and 13% (1:40 dilution) for individual heifers within groups P1, P2 and P3, respectively. Inhibin binding in HSA immunized heifers and in pre-immune sera was less than 2%.

Booster injection number did not affect ($P > 0.05$) the percentage of radiolabelled inhibin bound 14 days after a boost. There were significant differences among the peptide immunized groups in the percentage of radiolabelled inhibin bound at 14 days after a booster injection (Table 1) with P2 exhibiting the highest binding. Peptide and inhibin titres were correlated for groups P1 ($r = 0.46$, d.f. = 68, $P < 0.01$) and P2 ($r = 0.74$, d.f. = 68, $P < 0.01$) but not for group P3 ($r = -0.14$, d.f. = 68, $P > 0.05$).

Oestrous and ovarian response

The proportion of heifers showing oestrus was not affected ($P > 0.05$) by peptide immunization. Duration of oestrous cycle (least squares mean $\pm$ SEM) for groups P1, P2, P3 and the control group was 19.8 ± 0.5 , 20.3 ± 0.6 , 19.2 ± 0.3 and 20.6 ± 0.3 days, respectively ($P > 0.05$).

Peptide immunization increased ($P < 0.05$) the ovulation rate overall; however, there was no difference among peptide groups ($P > 0.05$) (Table 2). In the control group the ovulation rate was 1.0 at each cycle throughout the immunization period. Ovulation rate was increased in 1 of 5, 5 of 5 and 4 of 5 of the heifers and in 6 of 38 (16%), 13 of 37 (35%) and 6 of 33 (18%) of the ovulatory cycles observed following immunization against P1, P2 and P3, respectively. Of the 25 multiple ovulatory cycles recorded, 19 (76%) were twin ovulatory. Booster injection number did not affect ovulation rate ($P > 0.05$). However, there was a significant ($P < 0.05$) effect of cycle number on ovulation rate and also a significant ($P < 0.005$) treatment by cycle interaction (Table 3). One heifer (No. 13, P3) became anovulatory for 60–70 days after booster injection 2. Within groups, peptide titre and ovulation rate were not correlated ($P > 0.05$). The within group correlations between inhibin binding and ovulation rate were $r = 0.39$, d.f. = 14, $P > 0.05$ for group P1, $r = 0.56$, d.f. = 13, $P < 0.05$ for group P2 and $r = -0.57$, d.f. = 11, $P < 0.05$ for group P3, revealing significant heterogeneity ($P < 0.05$).

Table 2. Number of ovulations in successive oestrous cycles following each of three booster injections for peptide immunized and control groups

		Booster injection number									
		1				2				3	
Group	Heifer number	Cycle number									
		1	2	3	4	1	2	3	4	1	2
P1	1	1	1	1	—*	1	—	1	—	1	—
	2	2	1	4	—	1	3	2	—	3	4
	3	1	1	1	—	1	1	1	—	1	—
	4	1	1	1	1	1	1	1	—	1	—
	5	1	1	1	—	1	1	1	1	1	1
P2	6	2	1	1	1	2	1	1	—	3	2
	7	1	1	1	1	2	1	1	—	1	—
	8	0†	1	1	—	1	1	2	1	2	—
	9	3	1	1	—	2	1	1	—	2	—
	10	1	0	1	—	2	2	1	—	2	—
P3	11	2	1	1	—	2	1	1	—	1	1
	12	2	—	—	1	2	1	1	1	1	—
	13	0	1	1	1	0	0	0	—	2	—
	14	1	1	1	—	1	1	1	—	2	1
	15	1	1	1	—	0	1	1	—	1	—
Control	16	1	1	1	—	1	0	1	—	1	—
	17	1	1	1	—	1	1	1	1	1	—
	18	1	1	1	1	1	1	1	—	1	1
	19	1	1	1	1	1	1	1	—	1	—
	20	1	1	1	—	1	1	1	—	1	—

*No observations were made; †anovulation.

Table 3. Ovulation rate at each oestrous cycle after booster injection and the treatment group $\times$ cycle number interaction from the analysis of variance

Group	1	2	3
P1	(1.22 $\pm$ 0.20) ^a (n = 15)	(1.41 $\pm$ 0.24) ^a (n = 11)	(1.48 $\pm$ 0.23) ^a (n = 12)
P2	(1.85 $\pm$ 0.21) ^b (n = 14)	(1.24 $\pm$ 0.25) ^a (n = 10)	(1.17 $\pm$ 0.22) ^a (n = 13)
P3	(1.47 $\pm$ 0.22) ^{ab} (n = 12)	(1.04 $\pm$ 0.24) ^a (n = 10)	(1.07 $\pm$ 0.24) ^a (n = 11)

Values are least squares means $\pm$ SEM. Number of observations in parentheses. Within rows or columns, means with different superscripts differ significantly $P < 0.01$.

Conception and calving rates

Ten of the 15 peptide immunized heifers and four of the five control heifers conceived to first service, as determined by ultrasonography at day 45 (Table 4). The remaining six heifers conceived to the second service. Of the six heifers with multiple pregnancies at day 45, five had two ovulations; four of these

Table 4. Conception and calving data following breeding after booster injection three

Group	Heifer number	Number of corpora lutea	Number of fetuses at 45 days	Calves born
P1	1	1	1	1
	2	4 (R)	4	0
	3	1	1	1
	4	1	1	1
	5	1 (R)	1	1
P2	6	2 (R)	2	2
	7	1	1	1
	8	2	2	2
	9	2	2	2
	10	2	2	0
P3	11	1 (R)	1	0
	12	1	1	1
	13	2	2	2
	14	1 (R)	1	0
	15	1	1	1
Control	16	1	1	1
	17	1	1	1
	18	1 (R)	1	1
	19	1	1	1
	20	1	1	1

R: observation from the first repeat oestrus.

produced twin calves at term, whereas the fifth heifer subsequently lost both fetuses. The remaining heifer had four ovulations and had four fetuses at day 45, all of which were subsequently lost. Two of the nine peptide-immunized, single-ovulating heifers, pregnant at day 45, subsequently lost pregnancies. All five single-ovulating control heifers pregnant at day 45 subsequently calved.

Discussion

This study demonstrates that active immunization of heifers against synthetic peptide sequences of the α -subunit of bovine inhibin can increase ovulation rate and twin-calving rate.

The three inhibin peptides used for immunization differed in the ability of the antisera generated to bind to inhibin. Binding was highest for P2, followed by P1, whereas binding for P3 was five times lower than P2. The lower binding for P3 may have been due to conformational differences of this site in the free peptide and native inhibin or to steric hindrance by an iodine atom after inhibin iodination. Peptide 3 has tyrosine residues at positions 116 and 120, which, if subsequently iodinated in the native molecule, could reduce inhibin binding by antibodies generated against this region of P3. However, the lower binding is most likely the result of an immune response with a lower affinity for P3 compared with either P1 or P2. This is borne out by the fact that the relative magnitude of the immune response is similar among groups, whether measured by enzymeimmunoassay or radioimmunoassay. We are not aware of other studies

comparing the immune response in cattle following immunization using different inhibin peptides.

Immunization did not affect either the proportion of heifers showing oestrus or the duration of the oestrous cycle. Price *et al.* (1987) reported a decrease of one day in duration of the oestrous cycle in heifers immunized against partly purified ovine inhibin, but this may have been due to immunization against other components in the impure inhibin preparation used in their study.

The highest ovulation rate increase was recorded in group P2 in which all heifers responded with an increased ovulation rate at some time. At each of the first oestrous cycles following the second and third booster injections, 4 of 5 (80%) heifers responded with an increased ovulation rate in P2. A total of 11 of 22 (50%) ovulatory cycles with multiple ovulations were recorded in group P2 following the second and third booster injections of which 10 (91%) were twin ovulatory. Price *et al.* (1987) reported an increased ovulation rate at the first oestrus after a second booster injection in 3 of 5 heifers immunized with 4 mg of a partly purified ovine inhibin; however, this increase was not repeated after a subsequent booster injection. Schanbacher (1989) reported a mean ovulation rate of 1.9 ± 0.4 following immunization of heifers ($n = 20$) with an α -(1-30) porcine inhibin peptide, but there was no indication of the proportion with twin ovulations. Glencross *et al.* (1990) reported that 4 of 6 heifers immunized with an α -(1-29) bovine inhibin peptide responded with twin ovulations at some time following immunization. An increased ovulation rate (2.5 ± 0.4) in heifers immunized with an α -(1-26) bovine inhibin has also been reported by Sunderland *et al.* (1991); however, the response was variable, and the proportion of animals with twin ovulations only was not reported. A superovulatory response with high variability has also been recorded by Bindon *et al.* (1988) in pubertal heifers (11.6 ± 3.9) and by Hillard *et al.* (1990) in cows (5.4 ± 2.7) immunized against a 300-fold inhibin enriched preparation of ovine follicular fluid.

The relationship between inhibin binding and ovulation rate in the experiment reported here is not clear. Ovulation rate was positively correlated with inhibin binding for group P2 and negatively correlated for group P3. As peptide titre and inhibin binding were correlated for groups P1 and P2, but not P3, it is possible that the measurement of inhibin binding within individual heifers in group P3 may have been compromised for technical reasons discussed above. It is also clear, however, that the increased ovulation rate cannot be explained by inhibin binding alone, and it is possible that antibodies may have been raised to other α -inhibin subunits likely to be involved in the control of ovulation. Ovulation rate was increased in all heifers in group P2 and in only one heifer in group P1, yet inhibin binding was similar for both groups. In addition, 4 of 5 heifers responded with an increase in ovulation rate in group P3, whereas inhibin binding was five times lower than for group P2. Schanbacher (1989) reported a correlation ($r = 0.44$, $P < 0.05$) between ovulation rate in heifers and titre to the α -(1-30) porcine peptide, whereas Price *et al.* (1987) could find no detectable correlation between ovulation rate and antibody titre to the partly purified ovine inhibin used in their study. The failure of heifers with high inhibin binding in group P1 to respond with an increased ovulation rate may be due to the inability of the antibodies in these heifers to neutralize biologically active

inhibin. Forage *et al.* (1987) found inhibin neutralizing antibodies in only 60% of ewes immunized against bovine inhibin α -subunit, all of which had a higher ovulation rate than the controls. The increase in ovulation rate reported here occurred for the most part in the first cycle after a boost with the incidence of twin ovulations decreasing in subsequent cycles even though inhibin binding was still relatively high, indicating that physiological compensation was occurring as free antibody titres decreased.

The neutralization of biologically active inhibin (increasing pituitary FSH secretion) in cattle by immunization against synthetic peptides from the α -subunit may be attenuated for a number of reasons. The ovary secretes large amounts of 'free' inhibin α -subunit (Knight *et al.*, 1989; Sugino *et al.*, 1989) and other precursor forms of inhibin containing the α -inhibin sequence (Robertson *et al.*, 1989; Sugino *et al.*, 1989). Dimeric and biologically active inhibin may represent only a very small fraction of the total immunoreactive inhibin present in follicular fluid (Beard *et al.*, 1991; Betteridge and Craven, 1991). Groome *et al.* (1990) found that a polyclonal antibody raised in sheep against the α -amino-terminal end [α -(1-29)YG] of bovine inhibin demonstrated a 29-fold greater reactivity (on a molar basis) with the immunizing peptide than with the native 32 kDa form. Knight *et al.* (1989) and Vaughan *et al.* (1989) have reported that the 25 kDa free α -subunit reacts about 10-fold better than native 32 kDa inhibin, with polyclonal antibodies raised against the α -amino-terminal peptide. FSH concentrations have not yet been measured in the present experiment, and must await detailed analysis at a later date.

There are also indications of a possible role for inhibin and the α -subunit of inhibin as autocrine or paracrine regulators of FSH action in the ovary. For example, bovine inhibin has been shown to inhibit rat oocyte meiosis (O *et al.*, 1989), whereas porcine 31 kDa inhibin (Ying *et al.*, 1986) and the amino-terminal fragment of the α -chain of porcine inhibin (Hillier *et al.*, 1987) can inhibit FSH-induced rat granulosa cell aromatase activity *in vitro*. Schneyer *et al.* (1991) identified a likely competitor of FSH receptor binding (FRBC) in follicular fluid as the 57 kDa α -inhibin precursor protein. Immunization in this experiment may have increased pituitary FSH secretion by immunoneutralization of inhibin or by increased FSH receptor binding at the ovary, thus preventing follicular atresia and an increase in ovulation rate.

Immunization against the peptides had no apparent effect on conception or calving rates. Although the small number of heifers involved in this study does not allow definitive conclusions to be made regarding the effects of immunization on pregnancy rate, the production of four sets of twin calves from six twin ovulatory cycles suggests that peptide immunization is a promising approach to the induction of twinning in cattle. This is the first report of an increase in the twin calving rate following immunization against inhibin.

Although the increase in ovulation rate in this experiment was primarily due to the generation of antibodies to the α -inhibin subunit, the exact mechanism(s) by which this increase was mediated is unclear. It is possible that antibodies to inhibin neutralized its negative feedback effect on FSH secretion by the anterior pituitary resulting in increased FSH secretion and an increase in follicular growth. However, an effect of immunization by interfering with the complex local control of ovulation at the ovarian level cannot be excluded.

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