Effect of immunization of rams against bovine inhibin $\alpha 1-26$ on semen characteristics, scrotal size, FSH, LH and testosterone concentrations

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The effects of inhibin immunization on inhibin antibody titres, semen characteristics, scrotal size, fertility, FSH, LH and testosterone concentrations were determined by immunizing adult rams against bovine inhibin $\alpha 1-26$ -Gly-Tyr conjugated to human serum albumin ($n = 16$) in non-ulcerative Freund's adjuvant and DEAE:dextran (1:1) or adjuvant alone ($n = 16$) on days 0 (29 June), 30, 60, 191, 303 and 394. Blood samples were collected and bovine inhibin $\alpha 1-26$ -Gly-Tyr antibody titres and serum testosterone concentrations were determined. Each month, between days 174 and 417, semen was collected every 30 min to a maximum of 15 ejaculates over 7 h and scrotal circumference was measured. Ram fertility was recorded during natural service. FSH, LH and testosterone concentrations and GnRH-induced FSH and LH release were measured in a subgroup of immunized ($n = 5$) and control ($n = 5$) rams at frequent intervals. Antibody titres were variable among immunized rams (0–46% ¹²⁵I-labelled bovine inhibin $\alpha 1-26$ -Gly-Tyr at 1:1600 serum dilution) but mean titres were consistently higher than in control rams ($P \leq 0.001$). Immunization did not alter the semen volume, output or quality of spermatozoa or ram fertility, but increased the mean scrotal circumference (37.6 ± 0.8 cm versus 34.4 ± 0.7 cm, $P < 0.001$). Mean FSH concentrations were higher in immunized rams during two intensive blood sampling periods (in June and August) (5.8 ± 0.7 ng ml⁻¹ versus 3.0 ± 0.3 ng ml⁻¹, $P < 0.001$ in June; and 4.8 ± 0.9 ng ml⁻¹ versus 2.0 ± 0.3 ng ml⁻¹, $P < 0.02$ in August), and were correlated with antibody titres ($r^2 = 0.3$, $P < 0.05$ in June; and $r^2 = 0.8$, $P < 0.001$ in August). Discrete FSH pulses were not detected. Immunization did not alter mean or basal testosterone or LH concentrations, or LH pulse frequency; LH pulse amplitude was increased (1.6 ± 0.2 ng ml⁻¹ versus 0.8 ± 0.2 ng ml⁻¹, $P < 0.02$) and was correlated with antibody titres ($r^2 = 0.6$, $P < 0.01$). Immunization enhanced GnRH-induced FSH ($P < 0.05$) but not LH release. In conclusion, immunization of adult rams against bovine inhibin $\alpha 1-26$ Gly-Tyr increased scrotal circumference, mean FSH concentrations and LH pulse amplitude, without altering semen characteristics, fertility, mean LH concentrations, LH pulse frequency or mean testosterone concentrations.

Introduction

Although the roles of gonadotrophins and androgens in the initiation, regulation and maintenance of spermatogenesis are currently being elucidated, their definitive roles are still unclear. The differentiation of A₀ to A₁ spermatogonia appears to be sensitive to LH, while multiplication from intermediate to B₂ spermatogonia, and therefore the rate of spermatogenesis, is dependent on FSH; meiotic divisions and spermiogenesis are maintained by testosterone (Courot *et al.*, 1979; Kilgour *et al.*, 1993). Although testosterone alone can qualitatively support spermatogenesis in the rat (Bocabella, 1963), gonadotrophins

are also required for quantitative support of spermatogenesis (Barlett *et al.*, 1989; Awonyi *et al.*, 1990; Kilgour *et al.*, 1993). Hormonal profiles following unilateral castration indicate that FSH is a key determinant of the rate of spermatogenesis (Walton *et al.*, 1978; Waites *et al.*, 1983; Schanbacher, 1988). Furthermore, FSH plays a primary role in determining testicular size in rams (Lincoln and McNeilly, 1989; Lincoln *et al.*, 1990), while scrotal circumference is highly correlated with testicular mass and sperm production (Willett and Ohms, 1957; Foote, 1978; Coulter, 1980; Cameron *et al.*, 1984a, b).

Inhibins are glycoprotein hormones consisting of two dissimilar subunits, α and β , joined by disulfide bonds, which preferentially inhibit the production or secretion of FSH or both processes (Burger and Igarashi, 1988). Inhibins occur in various forms with different molecular masses and bioactivity;

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Fig. 1. Experimental protocol. Rams ($n=16$) received a primary immunization against bovine inhibin $\alpha 1-26$ -Gly-Tyr (bINH) conjugated to human serum albumin on day 0 (29 June) and boosters on days 30, 60, 191, 303 and 394. Control rams ($n=16$) received adjuvant alone on the same days. Blood samples were collected throughout the experimental period for determination of bINH antibody titres (▼) and serum testosterone concentrations (T). Semen (□) was collected each month between day 174 (December) and day 417 (August) and scrotal circumference (■) was measured between day 196 (January) and day 417 (August). Rams were mated (⊠) in August (day 44) and October (day 109). Intensive blood samples were collected (□) in June (days 358–360) for FSH determination and in August (day 407) for FSH, LH and testosterone determination.

the principal bioactive form in the circulation is 30–32 kDa inhibin (de Kretser and Robertson, 1989; Tilbrook *et al.*, 1992). FSH stimulates the secretion of inhibins from Sertoli cells *in vitro* (Le Gac and de Kretser, 1982; Verhoeven and Franchimont, 1983; Ultee van-Gessel *et al.*, 1986; Bicsak *et al.*, 1987; Gonzales *et al.*, 1988) and *in vivo* (Au *et al.*, 1984) by a cAMP-dependent mechanism (Bicsak *et al.*, 1987). Inhibins, in turn, suppress FSH release from the anterior pituitary *in vitro* (Muttukrishna and Knight, 1990) and *in vivo* (Tilbrook *et al.*, 1993a, b); this interaction is consistent with a closed-loop, endocrine feedback system. In addition to this systemic role, there is increasing evidence that inhibins inhibit spermatogonial development in a paracrine manner (van-Dissel-Emiliani *et al.*, 1989; Hakovitra *et al.*, 1993) and modulate LH-stimulated testosterone secretion (Hsueh *et al.*, 1987). Therefore, it can be hypothesized that inhibins play a substantial role in regulating testicular function and the rate of spermatogenesis through selective suppression of the concentration of FSH in peripheral blood.

However, there are conflicting results from experiments in which males have been immunized against different inhibin immunogens. Active immunization against inhibin-enriched follicular fluid or inhibins themselves has been reported to increase the daily sperm output in the urine of ram lambs (Al-Obaidi *et al.*, 1987) and the testicular sperm density in bulls (Martin *et al.*, 1991; Schanbacher, 1991) but to have no significant effect on the epididymal sperm reserves of adult rams (Voglmayr *et al.*, 1990) or the daily sperm output of bulls (Schanbacher, 1991). Testis size is increased (Al-Obaidi *et al.*, 1987) or unaffected (Martin *et al.*, 1991; Schanbacher, 1991), while LH and testosterone concentrations have either been altered (Voglmayr *et al.*, 1990; Martin *et al.*, 1991) or unchanged (Al-Obaidi *et al.*, 1987; Schanbacher, 1991) by inhibin immunization. In females, immunization against synthetic peptides from the carboxy-terminal region of the inhibin α subunit has successfully increased ovulation rate in rats

(Rivier and Vale, 1989), heifers (Morris *et al.*, 1993; Scanlon *et al.*, 1993) and ewes (Wrathall *et al.*, 1990; Boland *et al.*, 1994). Although there are sex-related differences with respect to the functions of inhibins (van Dijk *et al.*, 1986), collectively these data augur well for the use of inhibin immunization to increase the rate of sperm production in males.

We hypothesized that inhibins have a role in the regulation of sperm production and in the determination of testis size by their ability to suppress circulating FSH concentrations. This hypothesis was tested by actively immunizing adult rams against the $\alpha 1-26$ subunit of bovine inhibin. The main objectives of this experiment were to determine the antibody titre response, to monitor changes in scrotal circumference, daily sperm output and sperm quality over an extended period, and to observe the fertility of immunized rams. The secondary objectives of this study were to examine the effects of inhibin immunization on detailed FSH, LH and testosterone blood profiles, and on GnRH-induced FSH and LH release.

Materials and Methods

Animals and treatments

Adult rams ($n=32$, comprising Suffolk, Texel, Blue-faced Leicester and Dorset Horn) were paired on the basis of breed, weight and age, and randomly allocated within pairs to one of two treatment groups. One group of rams ($n=16$) was immunized against bovine inhibin $\alpha 1-26$ -Gly-Tyr (bINH) conjugated to human serum albumin (HSA) by the glutaraldehyde method (Reichlin, 1980). The ratio of weight of bINH:HSA after conjugation was approximately 1:1. The rams received a primary immunization (on 29 June, day 0; Fig. 1) consisting of 0.33 mg bINH–HSA conjugate emulsified separately in 1.25 ml DEAE–dextran (D1162; Sigma-Aldrich Co. Ltd, Poole) and 1.235 ml non-ulcerative Freund's adjuvant (NUFA, F010;

Guildhay Antisera Ltd, Guildford), with 0.1 ml *Corynebacterium parvum* vaccine (Wellcome Ireland Ltd, Dublin) in the DEAE-dextran emulsion. Rams received identical booster injections on days 30, 60, 191, 303, 394 but without *C. parvum* added. All immunizations were given subcutaneously in two sites behind the shoulder. Control rams ($n = 16$) received 625 μ l DEAE-dextran and 625 μ l NUFA emulsified in a single injection.

Scrotal circumference

Measurements of scrotal circumferences were carried out monthly between day 196 (January) and day 417 (August) using a flexible measuring tape to measure the widest point of the scrotum with the ram standing. Body weight and body condition score (Russell *et al.*, 1969) were recorded on the same day. All measurements were performed by the same operator.

Semen collection and evaluation

Semen was collected from each ram using an artificial vagina once per month between day 174 (December) and day 417 (August) at 30 min intervals over 7 h, to a maximum of 15 ejaculates or until rams were sexually exhausted. Rams that failed to ejaculate within 5 min of being introduced to the ewe were excluded until the following collection time. Ejaculate volume, sperm concentration and wave motion were evaluated for each ejaculate immediately following collection according to recognized procedures (Evans and Maxwell, 1987). Ejaculate volume was measured to the nearest 0.1 ml using a 2 ml syringe. The concentration of spermatozoa was measured in a colorimeter previously calibrated using a haemocytometer (checked against standard solutions on each day of collection), and the output of spermatozoa for each ejaculate was estimated as the product of ejaculate volume and sperm concentration. The wave motion of undiluted semen was assessed using a heated stage (37°C) under a microscope at a magnification of $\times 100$, scored on a scale of 0–5. Spermatozoa were stained by mixing 0.1 ml semen with 0.7 ml nigrosin–eosin solution (10:1.6% w/v; Hancock, 1951) in a 6 mm $\times$ 75 mm polystyrene tube and incubating them in a water bath for 3 min at 30°C. Smears were made and air-dried on prewarmed slides (30°C) from the second, eighth, and fourteenth ejaculates. The smears were examined under a microscope at a magnification of $\times 400$ as dry preparations. A total of 100 spermatozoa from five separate fields were counted on each slide, and the percentage of dead spermatozoa and the incidence of primary and secondary abnormalities were recorded.

Fertility

Four groups of rams, (a) immunized Suffolk ($n = 4$), (b) immunized Texel ($n = 8$), (c) control Suffolk ($n = 4$) and (d) control Texel ($n = 8$), were mated with separate groups of synchronized crossbred ewes during the first breeding season after immunization (on 12 August, day 44, and 16 October, day 109). Immunized and control rams were mated with a total of 212 and 213 ewes, respectively. The proportion of ewes pregnant and lambs per ewe to first service were calculated.

Blood samples for inhibin antibody titres and hormone concentrations

Blood samples were collected by jugular venepuncture at 14–30 day intervals from day 0 until the end of the experiment (day 470; Fig. 1) to determine bINH antibody titres. Blood samples were not collected in November as the rams were breeding and therefore unavailable. Titres were determined as described by Martin *et al.* (1991). bINH radiolabelled with 125 I was incubated with 200 μ l serum diluted 1:100, 1:400, 1:1600 and 1:6400. The percentage of radiolabelled bINH bound to serum diluted 1:1600 (on the linear portion of dilution curve) was used in the presentation of bINH antibody titres. Serum testosterone concentrations were measured by radioimmunoassay once per month except during November. FSH concentrations were determined from blood samples collected every 3 h for 12 h on 3 consecutive days in June (days 358–360).

FSH and LH concentrations were measured in a subgroup of immunized rams ($n = 5$) selected on the basis of titre response in December (day 171) and control rams ($n = 5$) selected at random, to determine whether active immunization against inhibins alters blood hormone profiles. Rams were housed individually and fitted with an indwelling jugular cannula 24 h before blood sampling began. Blood samples were collected every 12 min for 12 h (on 10 August, day 407, 13 days after booster 5). Testosterone concentrations were subsequently measured in the same sera every 12 min for a specific 2–4 h period selected to span two LH pulses in each ram.

A single injection of the GnRH analogue Buserelin (PL0086/4125; Hoechst AG, Frankfurt) was given intravenously (40 ng kg $^{-1}$) at the end of this 12 h sampling period, and blood samples were collected every 12 min for a further 3 h, to establish whether active immunization against inhibins altered GnRH-induced FSH and LH release.

After collection, blood samples were incubated at room temperature for 1 h and overnight at 4°C, samples were centrifuged at 700 g for 20 min, and the serum was then decanted and stored at -20°C until assayed.

Serum FSH concentrations were determined by a radioimmunoassay (Crowe *et al.*, 1995) modified for ovine serum (Sweeney, 1995). The interassay coefficients of variation ($n = 5$) for three serum pools of 1.4, 3.0 and 8.1 ng ml $^{-1}$ were 10.8%, 9.3% and 7.2%, respectively. The intra-assay coefficients of variation ($n = 5$) for the same serum pools were 9.4%, 10.5% and 9.1%, respectively. The sensitivity of the ovine FSH assay, as defined by 95% binding, was 0.4 ng ml $^{-1}$.

Serum LH concentrations were determined by a radioimmunoassay using the method described by Matteri *et al.* (1987) and modified for use in sheep (Sweeney, 1995). The interassay coefficients of variation ($n = 5$) for three serum pools of 0.4, 2.1 and 4.5 ng ml $^{-1}$ were 8.6%, 6.2% and 2.9%, respectively. The intra-assay coefficients of variation ($n = 5$) for the same serum pools were 9.5%, 6.9% and 2.4%, respectively. The sensitivity of the ovine LH assay, as defined by 95% binding, was 0.2 ng ml $^{-1}$.

Testosterone concentrations were determined by a direct radioimmunoassay described by Schanbacher and D'Occhio (1982), validated by Ronayne *et al.* (1993) and rechecked for the measurement of testosterone in ovine serum. Briefly, 10 μ l

serum was incubated for 24 h at 4°C in 390 µl phosphate buffer (pH 7.4), 100 µl anti-testosterone solution (1:400 000 dilution GDN506; Ronayne *et al.* (1993) and 100 µl [125 I]testosterone solution (10 000 c.p.m. in 100 µl; Amersham). A charcoal suspension (500 µl; 5% (w/v) activated charcoal and 0.5% (w/v) dextran) was added to each tube for 5 min and tubes were centrifuged for 10 min at 2000 g. The supernatant fluid was decanted into vials and the amount of radioactivity measured in a gamma counter. The standard testosterone used was T1500 (Sigma-Aldrich Co. Ltd). Serum samples from adult rams during the breeding and non-breeding seasons and from ram lambs all produced displacement curves parallel to the standard curve. The recovery rate was 91% for a sample (1 ng ml $^{-1}$) with 1 ng testosterone ml $^{-1}$ added, assayed in duplicate. The interassay coefficients of variation ($n = 8$) for three serum pools of 1.0, 2.7 and 4.0 ng ml $^{-1}$ were 15.9%, 9.3% and 4.9%, respectively. The intra-assay coefficients of variation ($n = 8$) for the same serum pools were 13.4%, 10.4% and 11.1%, respectively. The sensitivity of the testosterone assay, as defined by 95% binding, was 0.1 ng ml $^{-1}$.

Statistical analysis

Unless otherwise stated, all values shown are means $\pm$ SEM. Semen parameters, scrotal circumference and testosterone concentration (monthly samples) were subjected to repeat measures ANOVA. Chi-squared analysis was used to compare the effect of immunization on the proportion of dead and morphologically abnormal spermatozoa with that in control rams. Fertility data were analysed separately to compare the effects of the experiment on different ram breeds and mating dates. As there were no differences between Suffolk and Texel breeds, nor between matings on days 44 and 109, all data were pooled to determine differences between immunized and control rams. The proportion of pregnant ewes was analysed by χ^2 analysis and litter size by ANOVA. The parameters of FSH, LH and testosterone hormone profiles were determined by the PC-Pulsar pulse detection algorithm (Merriam and Wachter, 1982). The G parameters used were $G(1) = 5.00$, $G(2) = 1.90$, $G(3) = 1.80$, $G(4) = 1.30$ and $G(5) = 1.20$. Basal concentrations described in this study are equivalent to the 'smoothed mean' values from PC-Pulsar. The amplitude of each hormone pulse was the difference between the peak value and this basal concentration. The assay SD terms for PC-Pulsar were obtained as follows: the SD of replicate standards at seven doses (five replicates per dose for the FSH assay and eight replicates per dose for the LH and testosterone assays) were calculated. The SD of each dose was then fitted to the corresponding mean values using least squares regression. This gave constant, linear and quadratic values for each assay for the PC-Pulsar program. Pulse frequency was not determined for testosterone as only one pulse was measured in each ram. GnRH-induced FSH and LH release were determined as the area under the response curve above basal concentrations (calculated by triangulation). Comparisons between groups on all hormone parameters were made using ANOVA. When a significance level of $P < 0.05$ was obtained, the analysis was followed by the PLSD Fisher test and Scheffe F-test. Linear regression analysis was used to examine the degree of correlation between different variables as mentioned in the text.

Fig. 2. (a) Mean ($\pm$ SEM) antibody titre (% radioiodinated bovine inhibin $\alpha 1$ -26-Gly-Tyr ([125 I]bINH) bound at 1:1600 of dilution) of immunized ($n = 16$; $\circ$) and control ($n = 16$; $\blacksquare$) rams. Primary immunization (P) was given on 29 June (day 0), booster 1 (B1) on day 30, booster 2 (B2) on day 60, booster 3 (B3) on day 191, booster 4 (B4) on day 303, and booster 5 (B5) on day 394. (b) Output of spermatozoa, scrotal circumference and serum testosterone concentrations of immunized ($n = 16$; $\circ$) and control ($n = 16$; $\blacksquare$) rams. Values are means ($\pm$ SEM) and asterisks above points indicate whether means for immunized rams are significantly different (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) from controls.

Results

Inhibin antibody titres

Antibody titres were detected in bINH-immunized rams 30 days after the primary immunization and these became larger following each booster (Fig. 2a). However, titres were highly variable among immunized rams (0–46% [125 I]-labelled bINH at 1:1600 dilution): four of the 16 rams had greater than 40% maximal binding; six had 30–40%; three had 20–30%; and three had less than 20% throughout the experiment. Titres remained high from December (day 174) until October (day 478) during the main experimental period. Mean bINH antibody titres were consistently higher in immunized compared with control rams ($P \leq 0.001$).

Table 1. The effects of immunization of adult rams against bovine inhibin $\alpha 1-26$ -Gly-Tyr on semen characteristics between days 174 (December) and 417 (August) and on scrotal circumference between days 196 (January) and 417 (August); values are totals from all ejaculates or means ($\pm$ SEM) over a 9-month experimental period

	Control	Immunized
Number of rams	16	16
Total number of ejaculates	1076	1316
Mean semen volume per ejaculate (ml)	0.54 ± 0.04^a	0.55 ± 0.04^a
Mean sperm output per ejaculate ($\times 10^9$)	1.54 ± 0.15^a	1.61 ± 0.11^a
Mean sperm concentration (number per ml $\times 10^9$)	2.82 ± 0.07^a	2.98 ± 0.06^a
Mean sperm motility (scale 0–5)	2.77 ± 0.09^a	2.90 ± 0.05^a
Dead spermatozoa (%)	10.34 ± 1.00^a	10.52 ± 1.02^a
Morphologically abnormal spermatozoa (%)	6.00 ± 0.50^a	6.16 ± 0.48^a
Primary abnormalities (%)	1.72 ± 0.26^a	1.94 ± 0.22^a
Secondary abnormalities (%)	4.38 ± 0.31^a	3.71 ± 0.56^a
Scrotal circumference (cm)	34.36 ± 0.68^a	37.59 ± 0.77^b

^{a,b}Values in a row with different superscripts are significantly different ($P < 0.001$).

Scrotal circumference and seasonal testosterone changes

Immunized rams had a greater scrotal circumference compared with controls (Fig. 2b) throughout the measurement period ($P < 0.05$) except during January ($P = 0.18$). From June to August the difference between the scrotal circumference of immunized and control rams was highly significant ($P < 0.001$). However, scrotal circumference was not correlated with antibody titre ($P > 0.05$).

Changes in serum testosterone concentrations showed a distinct seasonal pattern in rams assigned to both treatment groups (Fig. 2b). Testosterone decreased from the concentration recorded in July to a nadir in January, and increased from March to a peak in July before declining again. Mean testosterone concentrations were not different in immunized rams compared with controls ($P > 0.05$), and neither were they correlated with antibody titre ($P > 0.05$).

Semen characteristics

With the exception of transient effects, immunization did not alter semen volume, spermatozoa output, spermatozoa concentration or spermatozoa quality over the 9 month collection period (Table 1). Immunized rams had a lower total semen volume in February (2.1 ± 0.3 ml versus 3.4 ± 0.6 ml, $P < 0.05$) but a higher concentration of spermatozoa in August ($2.5 \pm 0.1 \times 10^9$ spermatozoa ml⁻¹ versus $2.2 \pm 0.06 \times 10^9$ spermatozoa ml⁻¹, $P < 0.001$) compared with controls. There was a seasonal change in semen volume and output of spermatozoa (Fig. 2b) similar to the cycle in scrotal circumference.

Fertility

There was no difference in the proportion of ewes pregnant to first service (75.0% versus 70.4%, $P > 0.05$) or litter size (1.9 ± 0.1 lambs per ewe versus 1.8 ± 0.1 lambs per ewe, $P > 0.05$) in ewes mated with immunized or control rams.

Serum FSH concentrations

Mean FSH concentrations in June (days 358–360) and August (day 407) were significantly higher in immunized compared with control rams (Table 2) and were correlated with antibody titres ($r^2 = 0.32$, $P < 0.05$ for June; $r^2 = 0.77$, $P < 0.001$ for August) and scrotal circumference ($r^2 = 0.36$, $P < 0.05$). Pulses of FSH were not detected in either treatment group (e.g. see Fig. 3); neither was there any consistent pattern of change in FSH concentrations in rams over the 60 h blood sampling period (days 358–360).

Serum LH concentrations

There was no effect ($P > 0.05$) of inhibin immunization on mean or basal LH concentrations or LH pulse frequency (Table 2). However, the mean amplitude of LH pulses was higher in immunized rams compared with controls, and this was correlated with antibody titre ($r^2 = 0.64$, $P < 0.01$).

Serum testosterone concentrations

Testosterone pulses were observed in all rams in response to an LH pulse. There was no effect ($P > 0.05$) of bINH immunization on mean or basal testosterone concentrations or on testosterone pulse amplitude (Table 2), and none was correlated to antibody titre ($P > 0.05$).

GnRH-induced FSH and LH release

GnRH-induced FSH release (area under response curve above basal concentrations obtained from samples taken during the 12 h period before GnRH injection) was higher (643.2 ± 130.9 versus 257.3 ± 40.2 arbitrary area units, $P < 0.05$) in immunized rams compared with controls and this was correlated to antibody titres ($r^2 = 0.67$, $P < 0.005$). However, when expressed as a percentage of the mean

Table 2. Effect of immunization of adult rams against bovine inhibin $\alpha 1$ -26-Gly-Tyr on mean FSH concentrations sampled every 3 h for 12 h on three consecutive days in June (days 358–360), on FSH and LH concentrations measured in a subgroup every 12 min for 12 h in August (day 407), and on testosterone concentrations subsequently measured in the same sera every 12 min for a specific 2–4 h period selected to span two LH pulses in each ram; values are means ($\pm$ SEM)

	<i>n</i>	Control	Immunized
Mean FSH concentration (ng ml ⁻¹) (June)	15	2.99 $\pm$ 0.34 ^a	5.81 $\pm$ 0.72 ^c
Mean FSH concentration (ng ml ⁻¹) (August)	5	2.01 $\pm$ 0.30 ^a	4.81 $\pm$ 0.92 ^b
Mean LH concentration (ng ml ⁻¹)	5	1.01 $\pm$ 0.26 ^a	1.35 $\pm$ 0.27 ^a
Basal LH concentration (ng ml ⁻¹)	5	0.83 $\pm$ 0.21 ^a	0.94 $\pm$ 0.23 ^a
LH pulse amplitude (ng ml ⁻¹)	5	0.76 $\pm$ 0.20 ^a	1.59 $\pm$ 0.23 ^b
LH pulse frequency (pulses h ⁻¹)	5	0.49 $\pm$ 0.05 ^a	0.45 $\pm$ 0.07 ^a
Mean testosterone concentration (ng ml ⁻¹)	5	4.32 $\pm$ 0.44 ^a	3.95 $\pm$ 0.50 ^a
Basal testosterone concentration (ng ml ⁻¹)	5	3.23 $\pm$ 0.59 ^a	2.87 $\pm$ 0.24 ^a
Testosterone pulse amplitude (ng ml ⁻¹)	5	6.43 $\pm$ 0.97 ^a	5.43 $\pm$ 1.29 ^a

Values in a row with different superscripts are significantly different: ^{a,b}*P* < 0.02; ^{a,c}*P* < 0.001.

Fig. 3. Serum FSH (---), LH (—) and testosterone (....) concentrations, and GnRH-induced FSH and LH release in a representative immunized and control ram. Blood samples were collected on days 358–360 at 3 h intervals for 12 h, and on day 407 at 12 min intervals for 12 h before, and for 3 h after, GnRH administration.

FSH concentration before the GnRH challenge, there was no difference between groups (*P* > 0.05), nor was this correlated with antibody titre (*P* > 0.05). GnRH-induced

LH release was unaltered by immunization against bINH (6304.3 $\pm$ 1184.8 versus 5464.2 $\pm$ 1110.4 arbitrary area units, *P* > 0.05).

Discussion

This study demonstrates that active immunization of adult rams against the synthetic $\alpha 1$ -26-Gly-Tyr bovine inhibin peptide conjugated to HSA raised inhibin antibody titres, increased FSH concentrations, LH pulse amplitude and scrotal circumference, without measurable effects on semen characteristics or ram fertility following natural service of synchronized ewes. After immunization, antibodies that bound the ^{125}I -labelled $\alpha 1$ -26-Gly-Tyr peptide were present in the serum of immunized rams, but were not detected in control rams. The antibody titre response was approximately similar in magnitude and duration to that observed in other studies using the same inhibin fragment in ewes (Boland *et al.*, 1994) and heifers (Scanlon *et al.*, 1993). However, it is not clear why such a wide range in antibody titres was observed between individual animals. This might be due to variations in the immune status or the sensitivity of the immune system to an extrinsic antigen between individual animals (Simpson, 1984).

The epididymal sperm reserves of rams were depleted using a frequent semen collection procedure, to examine thoroughly the effects of inhibin immunization on sperm production. The lack of an effect of bINH immunization on any of the semen characteristics examined, despite an increase in both the concentration of FSH and scrotal circumference, was surprising, since it has been demonstrated that FSH (Walton *et al.*, 1978; Waites *et al.*, 1983; Schanbacher, 1988) and scrotal circumference (Willett and Ohms, 1957; Foote, 1978; Coulter, 1980; Cameron *et al.*, 1984a, b) are key determinants of spermatogenic rate. Furthermore, it has been demonstrated that the rate of spermatogenesis can be reduced by administering bioactive inhibins to rodents (van Dissel-Emiliani *et al.*, 1989; Hakovitra *et al.*, 1993) or by immunizing rams against FSH (Kilgour *et al.*, 1993). The fact that scrotal circumferences were positively correlated with mean FSH concentrations, which were, in turn, highly correlated to antibody titres, strongly suggests that immunoneutralization of endogenous inhibins results in higher circulating FSH concentrations and thus increases testicular growth. This suggestion is supported, at least in part, by results indicating that FSH is a principal factor regulating testicular size (Lincoln and McNeilly, 1989; Lincoln *et al.*, 1990) and by the increase in testicular diameter reported in ram lambs actively immunized against inhibin-enriched preparations from bovine follicular fluid (Al-Obaidi *et al.*, 1987).

There is conflicting evidence from studies *in vivo* and *in vitro* with respect to the putative role of inhibins as selective regulators of FSH secretion, without effect on LH. It is clear from this study that active immunization of adult rams against bINH increases the peripheral concentrations of FSH twofold in June and August. In addition, there was a significant correlation, within immunized rams, between FSH concentrations and inhibin antibody titres. It is interesting to note that the difference in FSH concentrations between immunized and control rams in this study is similar in magnitude to that reported between castrated and intact rams (Tilbrook *et al.*, 1993a), suggesting that inhibin is a potent systemic inhibitor of FSH in rams. FSH pulses were not detected during either sampling period, supporting previous reports that FSH is non-pulsatile in rams (Fraser and Lincoln, 1980; Tilbrook *et al.*, 1993b). Mean serum FSH concentrations were approximately

1 ng ml⁻¹ higher in both immunized and control rams during June than in August. It is likely that the higher FSH concentration in June represents a temporary seasonal increase during the developing phase of the testes (Lincoln *et al.*, 1990).

Mean and basal LH concentrations and LH pulse frequency (August) were not different between immunized and control rams. This observation supports the contention that inhibins are selective regulators of FSH and have little or no effect on LH. In support of this finding, previous studies have reported that mean and basal LH concentrations are unaltered by immunization against inhibins in rams (Al-Obaidi *et al.*, 1987), bulls (Schanbacher, 1991), ewes (Wrathall *et al.*, 1992) and heifers (Scanlon *et al.*, 1993). Furthermore, infusion of rams with human recombinant inhibin A does not alter LH concentrations (Tilbrook *et al.*, 1993a) and basal LH secretion from ovine pituitary cell cultures is not influenced by bovine follicular fluid (Huang and Miller, 1984; Muttukrishna and Knight, 1990) or highly purified bovine inhibin (Muttukrishna and Knight, 1990). However, Voglmayr *et al.* (1990) demonstrated that immunization of rams against a human recombinant inhibin α subunit increases mean LH concentrations, while in bulls mean LH concentrations are reduced following immunization against bovine inhibin $\alpha 1$ -26 (Martin *et al.*, 1991) – although alterations in testosterone concentrations may explain these discrepancies.

Immunization against inhibin $\alpha 1$ -26-Gly-Tyr increased LH pulse amplitude twofold in immunized rams compared with controls, and pulse amplitude was significantly correlated with antibody titre. This result was unexpected and contradictory to results from castrated rams (Tilbrook *et al.*, 1993a) and ovariectomized ewes (Findlay *et al.*, 1987). Inhibins have been shown to exert a paracrine effect on LH-stimulated testosterone release in the testes (Hsueh *et al.*, 1987); however, immunization in this study did not significantly affect basal, mean testosterone concentrations or testosterone pulse amplitude. Interestingly, the relationship between LH pulse amplitude and the mean testosterone concentrations was altered by bINH immunization; the biological significance of this finding is unclear.

GnRH-induced FSH release was significantly higher in bINH-immunized rams than in controls and was correlated with antibody titres. GnRH-induced LH release was unaltered by immunization. These data are consistent with results from inhibin immunization of ewes (Findlay *et al.*, 1989), heifers (Scanlon *et al.*, 1993) and bulls (Schanbacher, 1991), but contradict studies *in vitro* in which inhibin enhanced GnRH-stimulated LH release from ovine pituitary cells (Huang and Miller, 1984; Muttukrishna and Knight, 1990).

The results in this study might be explained by the hypothesis that an upper limit of spermatogenic rate is set by the number of Sertoli cells (determined during the prepubertal period) and that each Sertoli cell can only support a certain number of developing germ cells, adequate gonadotrophin support thus being necessary to approach maximal spermatogenic efficiency (Culler and Negro-Vilar, 1988). This seems to be true for rats (Orth *et al.*, 1988) and, recently, the administration of FSH has been shown to increase the mitotic rate of Sertoli cells, seminiferous tubule length and the relative mass of spermatocytes and spermatids in prepubertal boars (Swanlund *et al.*, 1995). In support of these studies, manipulation of the

FSH-inhibin endocrine axis by immunization against inhibins early in life has been reported to increase sperm output in the urine of rams (Al-Obaidi *et al.*, 1987) and testicular sperm density of bulls (Martin *et al.*, 1991; Schanbacher, 1991), but to have no significant effect on epididymal sperm reserves when rams were immunized as adults (Voglmayr *et al.*, 1990). However, this hypothesis does not explain why bINH immunization does not affect sperm output during the non-breeding season when the spermatogenic rate is submaximal. An alternative explanation is that inhibins inhibit spermatogonial development in a paracrine manner (van Dissel-Emiliani *et al.*, 1989; Hakovitra *et al.*, 1993), and that the antibodies raised by bINH immunization are excluded from the adluminal compartment of the seminiferous tubules by the blood-testis barrier, as suggested by Schanbacher (1991). The relative importance of paracrine and endocrine actions of inhibins and the precise functional roles of inhibins and FSH in the regulation of spermatogenesis and testicular function need to be determined.

In summary, active immunization of adult rams against bovine inhibin $\alpha 1$ -26-Gly-Tyr conjugated to HSA resulted in antibody titres, but did not alter semen volume or the concentration, output, quality or fertility of spermatozoa; however, scrotal circumference, serum FSH concentrations and GnRH-induced FSH release were increased. FSH is non-pulsatile in the adult ram; immunization against inhibin increased LH pulse amplitude but did not affect mean or basal LH concentrations, LH pulse frequency or GnRH-induced LH release.

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