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Pituitary and ovarian function in ewes immunized against the amino-terminal peptide (αN) of the inhibin α_{43}-subunit

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Immunization of ewes against the amino-terminal peptide (αN) of the pro-α-subunit of inhibin has been shown to reduce fertility, thought to be due to disruption of ovulation. The aims of this study were to examine the effects of active immunization of ewes against αN on circulating concentrations of FSH, LH and on ovarian inhibin and progesterone, and to relate these observations to number of corpora lutea and oocyte recovery rates. Ewes were immunized against one or both of two recombinant full length bovine-αN immunogens (FP_1 and FP_2). Three experiments were performed in which jugular venous plasma was sampled from control and immunized ewes: (1) hourly across the oestrous surge of gonadotrophins (Expt 1); (2) daily for one entire oestrous cycle, and in the subsequent cycle, oviducts were flushed to recover ovulated eggs (Expt 2); and (3) samples were taken at 10 min intervals during the follicular and luteal phases (Expt 3). Binding of ^{125}I-labelled αN_{1-26} to serum was greater ($P < 0.05$) in immunized groups than in controls for all experiments. The number of eggs per corpus luteum recovered from the oviducts was lower ($P < 0.05$) in the αN-immunized groups (39%) than in controls (88%). There were more ($P < 0.05$) corpora lutea per ewe in FP_2 immunized groups 4 (1.8 ± 0.45) and 5 (1.75 ± 0.5) than in the control group (1.13 ± 0.13), but no increase in group 3 (FP_1; 1.4 ± 0.24). The oestrous surge of LH, basal values across cycle and the frequency of LH pulses were similar in treated and control groups. Circulating FSH concentrations were higher ($P < 0.05$) than those of controls in all immunized groups that had binding greater than 10% at all stages of the cycle, except during the oestrous surge. A corresponding decrease ($P < 0.05$) in circulating inhibin concentrations was observed in most immunized groups, with a significant negative correlation between inhibin values and αN-binding in the follicular phase of the cycle. The pattern of progesterone production during the cycle was similar, with a slight non-significant increase in immunized compared with control ewes. These data confirm the previous observation that ovulation is impaired in ewes immunized against the amino-terminal peptide of inhibin α_{43} and also suggest that the mechanism of this effect does not involve disruption of pituitary function, implying a role for αN in the intraovarian events of ovulation.

Introduction

Inhibin is a gonadal glycoprotein hormone consisting of two dissimilar subunits, α and β, joined by disulfide bonds, and is characterized by its ability to suppress FSH production and release (Burger, 1988). The α-subunit of inhibin is secreted in a pro-form comprising three discrete peptides released at Arg–Arg sites by proteolytic cleavage. Post-translational processing of the pro-α inhibin subunit is considered to occur in serum, or possibly in the ovarian follicle (Forage *et al.*, 1986), giving rise to a 6 kDa pro sequence, a 24 kDa peptide from the amino-terminal (αN), and the 20 kDa carboxy-terminal peptide which, when associated with the β-subunit, forms mature 32 kDa inhibin. The 58 kDa inhibin form, which also has inhibin bioactivity (Robertson *et al.*, 1985; Sugino *et al.*, 1992), is the product of a β-subunit linked to a 43 kDa α-subunit comprised of both the carboxy-terminal and amino-terminal peptides. All the above-mentioned inhibin-related peptides have been isolated from follicular fluid (Robertson *et al.*, 1989) as have several much larger inhibin-like forms (Sugino *et al.*, 1992).

Several recent observations have suggested an autocrine or paracrine role for α-inhibin precursor subunits in fertility regulation, distinct from the endocrine bioactivity of inhibin. First, expression of the inhibin α- and β-subunits is regulated separately (Meunier *et al.*, 1988; Woodruff *et al.*, 1988). Second, large α-inhibin precursors have been reported as possible

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intra-ovarian regulators of FSH-receptor binding and bioactivity, at high concentrations (Schneyer *et al.*, 1991). Finally, immunoneutralization of ewes against recombinant bαN results in impaired fertility (Findlay *et al.*, 1989a), despite a small but significant increase in the number of corpora lutea per ewe (Findlay *et al.*, 1989b). Furthermore, the number of oocytes recovered from the Fallopian tubes after ovulation was reduced in αN immunized ewes, and corpora lutea of these ewes had the appearance of luteinized, unruptured follicles (Findlay *et al.*, 1989b). Possibly, αN has an intraovarian role in the processes of ovulation.

In these studies, active immunization of ewes was used to investigate the effect of αN immunoneutralization on circulating gonadotrophins and progesterone throughout the oestrous cycle, to investigate the mechanism by which αN regulates fertility. In addition, these experiments provided an opportunity to study the effect of αN immunization on circulating inhibin concentrations. This was of interest as the αN antibodies may bind to large inhibin-like forms such as 58 kDa inhibin or α_{43}-subunit, which contain both αN and αC. Most current inhibin assay systems are directed against the αC peptide and are unsuitable for discriminating between the many protein species containing αC that may circulate (Knight *et al.*, 1989).

The results indicate that αN immunoneutralization suppresses ovulation while increasing FSH concentrations and number of corpora lutea and decreasing concentrations of immunoreactive inhibin in plasma, with no effect on LH secretion, further supporting an intraovarian role for αN.

Materials and Methods

Immunogens

Two recombinant fusion protein immunogens, termed FP_1 and FP_2, containing the full length bovine αN sequence plus linkers and residues of the β-galactosidase protein, were expressed in *Escherichia coli*. FP_1 has been described by Findlay *et al.* (1989a). FP_2 incorporated a shorter region of the β-galactosidase protein.

Animals and immunizations

All experiments were conducted during the breeding season (February–June) and were approved by the Institutional Animal Ethics Committee as conforming to the guidelines of the NH and MRC. Ewes were run with vasectomized rams bearing crayon mating markers throughout the experimental period. In Expt 1, adult parous Corriedale ewes were either untreated, or were immunized against the FP_1 immunogen only. For Expts 2 and 3, ewes were randomly allocated to five treatment groups. Group 1 ewes were non-immunized controls; group 2 were immunized against the MM adjuvant alone, and served as a second control group. Groups 3 and 4 were given primary immunizations of the FP_1 immunogen. Group 3 continued to receive booster immunizations of FP_1, while group 4 was boosted with FP_2. A further six ewes were given primary and booster immunizations of FP_2 only (group 5). Plasma antibody binding was measured at the time of each experiment.

All immunizations were of 1 ml of the indicated immunogen (300 $\mu g\ ml^{-1}$) in Montanide 888 (SEPPIC, Paris):Marcol 52 (ESSO, Sydney) (MM; 1:9), injected into the hind leg muscle. One primary and one (group 5) or two (groups 3 and 4) booster immunizations were administered to raise antibody concentrations before the first experiment; regular boosts were continued at intervals of 25–30 days to maintain high titres.

Experiment 1: oestrous gonadotrophin surge

Oestrous cycles were synchronized in six FP_1-immunized and seven control ewes, by treatment for 12 days with intravaginal progesterone controlled internal drug release (CIDR) implants (Riverina Artificial Breeders, Albury). An external jugular vein of each ewe was cannulated, and 24 h after CIDR withdrawal, hourly sampling of jugular venous blood began and was continued for 36 h. Blood was heparinized and centrifuged at 500 *g* for 10 min and plasma was collected and stored at − 20°C until assayed for FSH, LH and inhibin.

Experiment 2: hormone concentrations during the oestrous cycle and recovery of oocytes

Control ewes were either untreated (group 1, $n = 2$) or immunized against adjuvant only (group 2, $n = 2$) and were pooled for subsequent analysis. Immunized ewes were randomly selected from group 3 ($n = 4$), group 4 ($n = 3$), or group 5 ($n = 5$). Oestrus was synchronized in the five groups as described above. From 24 h after removal of the CIDR implant, blood samples were collected every 3 h for 24 h to measure the profile of the LH surge. Blood samples were collected daily and were then taken by venepuncture for 18 days or for one oestrous cycle. Plasma samples were handled as for Expt 1. Only animals for which an entire LH surge was observed were used for data analysis because assessment of stage of cycle was based on the time of the LH peak.

Synchronized ewes from groups 1–5 bearing fresh mating marks 2 days after CIDR removal, indicating recent onset of oestrus, were selected for oocyte recovery. Four days after CIDR removal, or approximately 2 days after the expected time of ovulation, ovulated oocytes were recovered from the Fallopian tubes. Reproductive tracts of the ewes were externalized by mid-ventral laparotomy, and the number of corpora lutea on each ovary was recorded. Fallopian tubes were flushed in a retrograde fashion with 2.5 ml saline at 37°C injected with a 21-gauge blunt-ended needle inserted through the utero–tubule junction. Flushings were collected in watch-glass dishes and examined under a dissecting microscope for the presence of oocytes.

Experiment 3: follicular and luteal phase hormone concentrations and LH pulsatility

Ewes in groups 1–5 as described above ($n = 3$), were treated with CIDR intravaginal implants to synchronize oestrus, and a jugular vein of each ewe was cannulated on the day of CIDR withdrawal. Twenty-four hours after removal, that is, during the follicular phase, jugular venous plasma samples were taken

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Table 1. Effects of immunization against the amino terminal peptide (αN) of the inhibin α_{43} subunit on binding titres for αN and parameters of the FSH and LH surges in control and αN-immunized ewes, sampled hourly from 24 h after removal of progesterone controlled internal drug release (CIDR) implant

Treatment	αN binding %	Surge amplitude (ng ml^{-1}) FSH	LH	Total LH release units	Timing from CIDR removal to surge onset (h)
Control ($n = 7$)	< 1	9.23 ± 2.0	111.1 ± 17.3	66.8 ± 6.2	36.6 ± 3.4
Immunized ($n = 6$)	18.0 ± 2.9*	7.32 ± 2.0	92.4 ± 7.6	50.7 ± 5.1	31.0 ± 2.7

Values are means ± SEM.
*$P < 0.01$ compared with control; one-way ANOVA.

every 10 min for 4 h. Ewes were then returned to pasture until day 10 of the cycle (luteal phase) when samples were taken every 10 min for a further 8 h.

Hormone assays

Plasma LH concentrations were measured as described by Lee *et al.* (1976), using NIH-oLH-S18 as standard. The mean sensitivity was 0.31 ± 0.12 ng ml^{-1} for a total of six assays; the interassay coefficient of variation was 6.8% for a plasma pool run in all assays.

Concentrations of FSH in plasma were assayed using the NIADDK reagents for ovine FSH (Bremner *et al.*, 1980), and using NIADDK-oFSH-RP1 standard. The mean sensitivity was 0.34 ± 0.2 ng ml^{-1} for six assays; interassay coefficients of variation were 4.7 and 17% for two quality control plasma pools.

The inhibin assay method used was that described for sheep plasma by Findlay *et al.* (1990). The standard, a pool of charcoal-stripped ovine follicular fluid diluted in ovariectomized ewe plasma, contained 1.04 nmol equivalent inhibin ml^{-1}, by calibration with a reference standard preparation of pure 31 kDa ovine inhibin (Leversha *et al.*, 1987). This antibody also recognizes the inhibin pro-αC subunit, but not αN. Mean sensitivity of the inhibin assay was 2.34 ± 0.8 pmol for six assays and the interassay coefficients of variation were 7.8 and 15% for two plasma pools.

Progesterone was assayed in plasma samples extracted with ethyl acetate (mean recovery 62%), using radioimmunoassay reagents supplied by the WHO matched reagent programme (Geneva). The assay blank was undetectable in all assays, mean sensitivity was 22.06 ± 7.4 fmol per tube for four assays and the interassay coefficient of variations were 18.2 and 20.4% for two plasma pools coextracted with samples.

All assay data were calculated by the method of Burger *et al.* (1972) and all samples were assayed at between 10 and 200 μl such that they were within the < 15% intra-assay coefficient of variation region of the dose–response curve.

Antibody binding assays

Antibody concentrations were measured in duplicate samples of plasma from control and immunized ewes by incubating ^{125}I-radiolabelled pure bαN or αN_{1-26} synthetic peptide with plasma at a 1:4000 final dilution. Bound and free tracer were separated using a precipitating donkey anti-sheep IgG antiserum and 6% (w:v) polyethylene glycol. Samples were centrifuged at 1000 ***g*** for 30 min and supernatants aspirated; radioactivity of antibody-bound tracer contained in the pellet was measured using a gamma counter. Specific binding of tracer to immune serum was measured and expressed as a percentage of total counts added.

Data analysis

Hormone concentrations were compared between treatment groups by one-way analysis of variance (ANOVA). Where control or treatment groups were pooled for analysis, these groups were first shown not to be statistically different. In Expt 2, inhibin concentrations were compared across the whole sampling period, or across the period before the beginning of the LH surge (defined as the first sample > 10 ng ml^{-1}) by accumulated analysis of variance on repeated measures. Regression analysis was performed on the relationship between αN binding and plasma inhibin concentration for individual immunized ewes. Pulse analysis of LH data from samples collected at 10 min intervals was performed using the 'turboPulsar' pulse analysis program of Dong and Handelsman (1990), a modification of the algorithm devised by Merriam and Watcher (1982). The G parameter settings were $G(1) = 4.23$, $G(2) = 2.56$, $G(3) = 1.82$, $G(4) = 1.36$, $G(5) = 1.05$ and the coefficient of variation distributed across the standard curve was expressed as $y = 0.148\,x^2 - 0.32\,x + 0.21$, the peak-splitting cutoff was four coefficients of variation.

Results

Experiment 1: oestrous gonadotrophin surge

Plasma binding of αN at the beginning of Expt 1 (Table 1) was significantly ($P < 0.01$) greater in all immunized ewes (18 ± 7%; mean ± SD) than in controls (< 1%). No significant difference was observed in amplitudes of surge of either FSH or LH at oestrus (Table 1). In addition, the time from CIDR removal to the beginning of the surges and total LH release during the oestrous surge were similar in all treatment groups (Table 1). Inhibin concentrations during the time of oestrus were at peak values in controls (40–45 pmol l^{-1}) before the beginning of the LH surge (late follicular phase), and then

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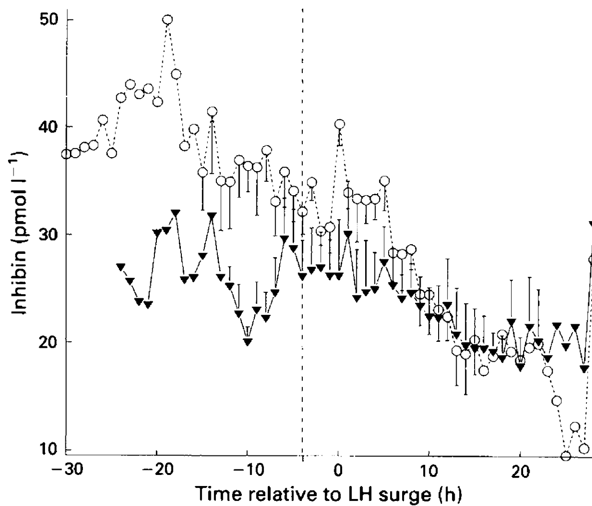

Fig. 1. Inhibin concentration in peripheral plasma samples taken hourly from control ewes (○; $n = 7$) and ewes immunized against the amino terminal peptide of the inhibin α_{43} subunit (▼; $n = 6$) during the time of oestrus (mean ± SEM). Time 0 represents the peak of the LH surge for each ewe. The dotted line represents the time of the beginning of the LH surge.

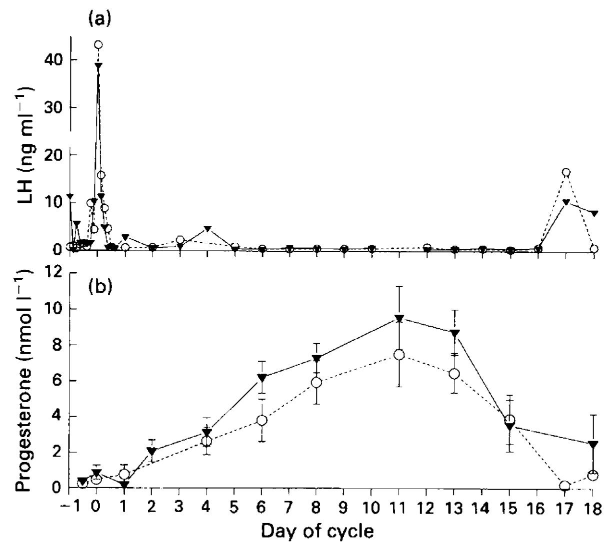

Fig. 2. Peripheral concentrations of (a) LH and (b) progesterone (mean ± SEM) in daily serum samples of control ewes (○; $n = 4$) and ewes immunized against the amino terminal peptide of the inhibin α_{43} subunit (▼; $n = 12$) during the oestrous cycle.

declined after the surge to a minimum of 10–15 pmol l^{-1} (Fig. 1). The mean inhibin concentrations in the αN-immunized group (25.9 ± 3.3 pmol l^{-1}) were significantly ($P < 0.025$; accumulated ANOVA) lower than those in controls (37.7 ± 3.8 pmol) during the pre-surge period. There was no significant difference between the groups during the entire sampling period.

Experiment 2: hormone concentrations during the oestrous cycle and recovery of oocytes

Antibody binding was around the detection limit of the assay in control groups; group 1 (1.0 ± 0.7%) and group 2 (0.7 ± 0.4%) were not significantly different and were pooled for further analysis. All αN-immunized groups had significantly ($P < 0.05$) higher binding than did controls; group 3 (27.8 ± 11.3%) and group 4 (47.9 ± 8.4%) also had significantly higher binding than did group 5 (13.5 ± 8.7%), which had received one fewer booster injection. Concentrations of LH in immunized groups during the cycle were not different from controls, and progesterone concentrations during the luteal phase showed a slight but not significant increase (Fig. 2). Mean FSH values throughout the luteal and follicular phases (excluding surge periods) were higher ($P < 0.05$) in all immunized groups (6.5 ± 2.5 ng ml^{-1}, 4.8 ± 0.4 ng ml^{-1} and 4.7 ± 0.4 ng ml^{-1}, respectively) than in controls (groups 1 and 2; 3.5 ± 0.3 ng ml^{-1}; Fig. 3). Inhibin concentrations were significantly lower than controls (33.4 ± 2.7 pmol) in groups 3 (22.9 ± 5.4 pmol) and 4 (25.2 ± 4.6 pmol), but not in group 5 (36.6 ± 5.4 pmol l^{-1}; Fig. 3).

Structures resembling corpora lutea were observed on the ovaries of all ewes examined, but no attempt was made to distinguish these as either normal or luteinized, unruptured follicles. The number of corpora lutea observed on the ovaries of immunized ewes increased significantly ($P < 0.05$) compared with controls (1.13 ± 0.4) in groups 4 (1.8 ± 0.4) and 5 (1.75 ± 0.5), but not group 3 (1.4 ± 0.5). The number of oocytes recovered from the oviducts of ewes was reduced in all immunized groups: this reduction approached significance in group 3 (29% oocytes per corpus luteum; $P = 0.051$) and group 4 (33%; $P = 0.053$) but not in group 5 (57%); and was significant when data from all immunized groups were pooled ($P < 0.05$). Recovery efficiency (oocytes per corpus luteum) in controls was 88% per corpus luteum (Table 2). Very large, unruptured follicles, greater than 2 cm in diameter, were observed on the ovaries of three of the immunized ewes.

Experiment 3: follicular and luteal phase hormone concentrations and LH pulsatility

Plasma binding of αN, although reduced in comparison to previous experiments, was still higher ($P < 0.05$) than in controls (1.6 ± 0.8%) in groups 3 (9.11 ± 4.3%), 4 (42.5 ± 9.6%) and 5 (4.7 ± 2.6%). One ewe from group 1 was not included in data analysis as inhibin, FSH and progesterone values were all abnormal (subsequent observation during laparotomy revealed small, pale ovaries devoid of follicles or corpus luteum structures, consistent with an anovulatory status). Pulses of LH detected in samples taken at 10 min intervals (Table 3), had increased ($P < 0.05$) frequency, amplitude and basal LH concentrations in the follicular compared with the luteal phase of cycle. However, between immunized and control groups there was no significant difference in any of the LH secretion parameters tested. In the follicular phase, mean FSH concentrations were not significantly different from those of controls (1.8 ± 0.7 ng ml^{-1}), in groups 3 and 5, but were increased ($P = 0.05$) in group 4 (3.0 ± 0.5 ng ml^{-1}), which had the highest αN binding. Inhibin concentrations were not affected in groups 3 and 5, but were significantly lower in group 4

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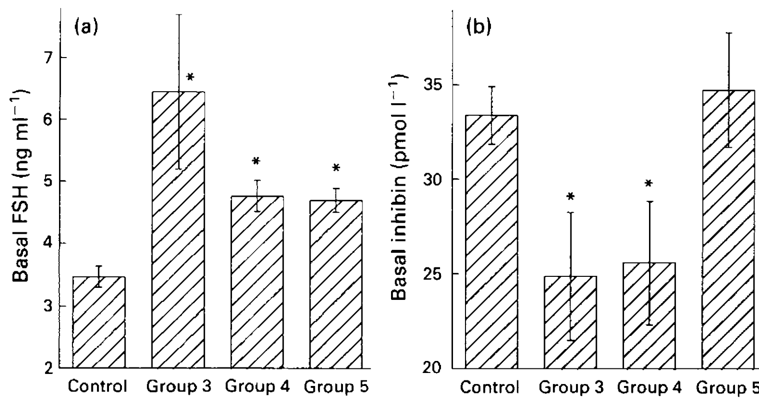

Fig. 3. Mean peripheral concentrations of (a) FSH and (b) inhibin in daily serum samples between day 2 and 16 of the oestrous cycle (means ± SEM) for control ewes ($n = 4$) and groups of ewes immunized against the amino terminal peptide of the inhibin α_{43} subunit. Groups received one or both of two recombinant immunogens: FP_1 or FP_2. Group 3: FP_1 only; ($n = 4$). Group 4: FP_1 and FP_2; $n = 3$. Group 5: FP_2 only; $n = 5$. *$P < 0.05$ compared with control (one-way ANOVA).

Table 2. Number of corpora lutea observed on ovaries and number of oocytes recovered from oviducts of control ewes and groups of ewes immunized against the amino terminal peptide (αN) of the inhibin α_{43} subunit

Group	Number of corpora lutea		Oocyte recovery	
	Total	Per ewe	Total	(% per corpora lutea)
Control ($n = 8$)	9	1.13 ± 0.13	8	88
Group 3 ($n = 5$)	7	1.40 ± 0.24	2	29
Group 4 ($n = 5$)	9	1.80 ± 0.20*	3	33
Group 5 ($n = 4$)	7	1.75 ± 0.25*	4	57
Pooled αN-immunized groups ($n = 14$)			9	39*

Groups were immunized against one or both of two recombinant full length bovine αN immunogens (FP_1 or FP_2). Group 3: FP_1 only; group 4: FP_1 and FP_2; group 5: FP_2 only.
Values are means ± SD.
*$P < 0.05$ compared with controls χ^2 or one-way ANOVA.

(9.9 ± 0.19 pmol l^{-1}; $P < 0.005$) than in controls (26.0 ± 8.07 pmol). In the luteal phase, mean FSH concentrations were higher only in group 4 (6.27 ± 3.1 ng ml^{-1}) than in controls (2.9 ± 1.1 ng ml^{-1}) and inhibin concentrations were significantly ($P < 0.05$) lower than in controls (29.0 ± 2.9 pmol) in groups 3 and 4 (18.8 ± 3.8; and 16.1 ± 3.2 pmol l^{-1}, respectively) but not in group 5 (22.9 ± 8.8 pmol; Table 3). Concentrations of progesterone in the luteal phase showed a slight, but non-significant increase over controls; progesterone concentrations were very low in the follicular phase and no difference was observed between groups. In follicular and luteal phases, FSH concentrations were significantly ($P < 0.05$) increased and inhibin concentrations significantly decreased in pooled data from all ewes with binding greater than 10% (data not shown). Regression analysis of the relationship between inhibin values and αN binding revealed a highly significant negative correlation in the follicular phase ($P < 0.001$; $r = 0.885$, $n = 9$), whereas the correlation in the luteal phase ($r = 0.577$, $n = 9$), was not significant (Fig. 4).

Discussion

The results reported here support the observation that immunization of ewes against αN, the amino-terminal peptide of the pro-inhibin α-subunit, results in fewer oocytes present in the oviducts 2 days after ovulation, while either not affecting, or increasing the number of corpus luteum-like structures on the ovary. Circulating progesterone concentrations were normal in the immunized animals, and it was demonstrated that ewes immunized against αN had normal LH profiles during the cycle, including pulsatile LH secretion and the preovulatory surge of LH at oestrus. These data suggest that immunoneutralization of αN caused failure of ovulation possibly by disrupting intra-ovarian events involved in the ovulatory process.

Oocyte recovery from the oviducts of αN-immunized ewes was low, approaching significance in all groups with the exception of the group with the lowest αN binding. This reduction may be due to loss of oocytes into the peritoneal cavity, or accelerated transport of oocytes through the

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Table 3. Circulating concentrations of gonadotrophins, inhibin and progesterone and binding of the amino-terminal peptide (αN) of the inhibin α_{43} subunit in serum from ewes immunized against αN during the follicular and luteal phases of the oestrous cycle

Treatment	αN binding (%)	LH pulse frequency (pulses h^{-1})	LH pulse amplitude (ng ml^{-1})	Basal LH (ng ml^{-1})	FSH (ng ml^{-1})	Inhibin (pmol l^{-1})	Progesterone (nmol l^{-1})
Follicular phase							
Control	1.3 ± 0.7	1.79 ± 0.1	1.27 ± 0.8	1.26 ± 0.7	1.8 ± 0.7	26.0 ± 8.1	0.3 ± 0.1
Group 3	8.8 ± 5.1*	1.75 ± 0.3	1.73 ± 0.9	1.06 ± 0.5	2.1 ± 0.4	20.4 ± 1.5	0.3 ± 0.2
Group 4	44.6 ± 9.6**	1.83 ± 0.3	1.10 ± 0.5	1.16 ± 0.5	3.0 ± 0.45*	9.9 ± 0.2*	0.1 ± 0.03
Group 5	5.0 ± 2.6*	1.58 ± 0.3	1.05 ± 0.4	1.02 ± 0.45	2.7 ± 1.1	20.5 ± 5.0	0.1 ± 0.06
Luteal phase							
Control	1.8 ± 0.8	0.83 ± 0.3	0.86 ± 0.6	0.24 ± 0.03	2.9 ± 1.1	29.0 ± 2.9	3.4 ± 1.9
Group 3	9.4 ± 4.4*	0.92 ± 0.1	0.73 ± 0.1	0.24 ± 0.02	2.8 ± 0.5	18.8 ± 3.8*	3.8 ± 1.1
Group 4	40.4 ± 11.1**	0.75 ± 0.2	1.15 ± 0.2	0.26 ± 0.1	6.3 ± 3.1*	16.1 ± 3.2**	4.3 ± 1.0
Group 5	4.3 ± 3.5*	0.56 ± 0.1	0.89 ± 0.1	0.22 ± 0.01	3.1 ± 0.7	22.9 ± 8.8	3.6 ± 1.8

Groups were immunized against one or both of two recombinant full length bovine αN immunogens (FP_1 or FP_2). Group 3: FP_1 only; group 4: FP_1 and FP_2; group 5: FP_2 only.
All values are means ± SD. Controls: $n = 5$; treatment groups: $n = 3$.
*$P < 0.05$ compared with controls.
**$P < 0.005$ compared with controls.

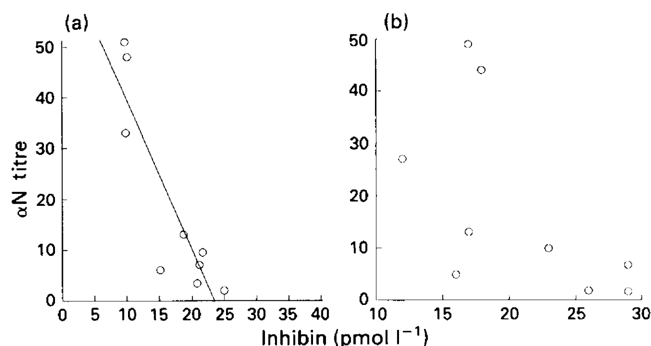

Fig. 4. Regression analysis of peripheral inhibin concentration and binding of the amino terminal peptide (αN) of the inhibin α_{43} subunit in individual ewes from each immunized treatment group. In (a) the follicular phase, the regression is described by: αN binding = 68.8 − 2.93 inhibin ($P < 0.001$); the correlation coefficient $r = 0.885$, $n = 9$. In (b) the luteal phase, the regression is described by: αN binding = 52.9 − 1.70 inhibin, $r = 0.577$, $n = 9$.

Fallopian tube. However, in several earlier experiments (Findlay *et al.*, 1989b), structures consistent with luteinized, unruptured follicles were observed on the ovaries of αN-immunized ewes, and no oocytes were recovered from the uterine cavity. These observations are consistent with incomplete or failed ovulation. Similar reduced rates of oocyte recovery per corpus luteum were obtained from immunized ewes stimulated with pregnant mares' serum gonadotrophin (PMSG) (Findlay *et al.*, 1989b). The results of the present study suggest that an interactive effect between αN immunization and PMSG treatment is unlikely to have been the cause of reduced oocyte recovery in the immunized ewes of the earlier study.

It is widely accepted that the surge of LH secretion occurring at oestrus is the sole pituitary signal required to initiate the intraovarian process of ovulation, and that a peak amplitude as low as 5 ng ml^{-1} is sufficient to cause ovulation and luteinization in mature follicles (Baird and McNeilly, 1981). Our observations of LH surges of normal duration and amplitude in control and immunized ewes, together with a previous report that follicles of immunized ewes luteinize (Findlay *et al.*, 1989b), suggests that a sufficient LH surge occurs in these ewes to induce ovulation.

Basal LH secretion, pulse amplitude and frequency all increased in the follicular compared with the luteal phase of the cycle. However, none of these parameters of LH secretion were affected by αN immunization. LH pulse frequency and amplitude in the follicular phase have been correlated with preovulatory growth of follicles (Picton *et al.*, 1990) and atresia (McNatty *et al.*, 1982). Furthermore, normal oestrous behaviour was repeatedly observed in these ewes (D. L. Russell, unpublished observation), confirming further that preovulatory follicles were mature and capable of responding in terms of oestrogen production, to trigger oestrus and the LH surge. These data suggest that regulation of LH secretion in αN-immunized ewes was normal. We, therefore, suggest that αN immunization does not suppress ovulation via an effect on hypothalamic or pituitary function.

Ovaries of control and immunized ewes observed 2 days after ovulation had structures resembling corpora lutea. However, the possibility that the corpus luteum-like structures on the ovaries of immunized ewes were luteinized, unruptured follicles was not addressed in this study. Peripheral concentrations of progesterone in immunized ewes displayed a similar pattern to that of controls and to values reported during the luteal phase in the ewe by Hauger *et al.* (1977). These observations confirm that follicles of immunized ewes luteinized normally in response to the stimulus of the LH surge. The slight increase in circulating progesterone observed in the luteal phase of immunized ewes was probably due to the increase in the number of corpora lutea.

Circulating inhibin concentrations were significantly reduced in most groups of αN-immunized ewes compared with controls at all stages of the oestrous cycle measured. We interpret this reduction as a decrease in measurable inhibin due to the

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binding of αN antibodies to large inhibin-like protein species such as 58 kDa inhibin that contain both the αN and αC subunits. The presence of αN antibodies could prevent the binding of the αC-directed inhibin antiserum used in the radioimmunoassay, resulting in a reduced measurement of immunoreactive inhibin, rather than a reduction in actual circulating inhibin concentration. The highly significant negative correlation between follicular phase inhibin concentrations and αN binding further supports this hypothesis. The lack of a significant correlation during the luteal phase may indicate lower circulating concentrations of the inhibin forms of higher molecular mass recognized by αN antibodies at this time of the cycle. However, luteal phase inhibin concentrations were significantly reduced in immunized ewes compared with controls. Alternatively, ewes in the luteal phase may have been less closely synchronized than those in the follicular phase as 10 days had elapsed since CIDR removal, making comparison of individual ewes difficult. Antisera raised against the FP_1 and FP_2 immunogens have been shown to bind purified 58 kDa inhibin (Findlay *et al.*, 1989a, b). Further studies to identify the molecular size of serum proteins recognized by the αN antisera and to confirm that binding of αN antibodies displaces αC-directed antisera are required to confirm this hypothesis. One alternative explanation is that immunization against αN directly influences follicle maturation, subsequently reducing follicular inhibin production; however, as progesterone and oestrogen stimulation of follicles appeared normal and follicles were able to luteinize, it is unlikely that they were immature.

The observed increase in circulating FSH in most αN immunized groups is probably a consequence of reduced negative feedback regulation of inhibin on FSH secretion. Binding of antibodies to the αN portion of 58 kDa inhibin may cause a reduction in total inhibin-like bioactivity, due either to prevention of cleavage of the αN subunit by serum proteases that give rise to mature 32 kDa inhibin, or to direct immunoneutralization of inhibin-like bioactivity of the high molecular mass forms. McLachlan *et al.* (1986) reported that spontaneous cleavage of amino-terminal inhibin α-peptide from 58 kDa inhibin occurs in serum. It is therefore possible that 58 kDa inhibin is secreted from the follicle and that cleavage occurs in serum to produce αN and the 32 kDa mature form. The relative proportion of total, circulating inhibin that is the larger form will depend on the half-life of this proteolytic event, and may be important in the maintenance of peripheral inhibin concentrations. There are no previous reports describing concentrations of inhibin proteins of high molecular mass in serum; this observation may therefore provide important evidence that inhibin proteins of larger molecular mass circulate and contribute a considerable component of total inhibin bioactivity in peripheral plasma.

Similar studies in which ewes were immunized against regions of the αC peptide of inhibin gave similar increases in FSH concentrations and number of corpora lutea, while lambing rates (Forage *et al.*, 1987; Wrathall *et al.*, 1992), follicular development and corpus luteum function (McLeod *et al.*, 1992) were not adversely affected. This finding suggests that only the αN peptide, and not αC, is involved in the ovulatory process. However, it remains to be determined whether αN acts as a free peptide, as a part of the larger α-subunit, or as 58 kDa or larger inhibin forms. Each of these inhibin-related proteins are present in bovine follicular fluid (Robertson *et al.*, 1989). A possible function of the inhibin α-subunit or its components has been suggested by Meunier *et al.* (1988) on the basis that the α-subunit is regulated separately from inhibin β. Knight *et al.* (1989) reported that the majority of immunoassayable inhibin produced by the corpus luteum in cows is the monomeric α-subunit. This has also been reported in marmosets by Knight *et al.* (1992) and provides further support for the contention that α-subunit inhibin has a separate function.

There was some inconsistency in the effect of αN immunization on circulating FSH and inhibin concentrations. This was probably due to the relatively small treatment groups in some experiments and to variation in the immune response of individual ewes. Large differences in the plasma binding of αN were noted and the data clearly suggest that high binding is a requisite for significant effects. In addition, there may be variation in the epitopes recognized by antisera raised in each ewe or against the two different immunogens that may result in divergent effects of immunization on the putative bioactivity of the peptide.

In summary, the results of these experiments suggest that antibodies directed against αN, the amino terminal peptide of inhibin α_{43}, may bind to circulating inhibin forms of large molecular mass, causing a reduction in immunoassayable inhibin concentrations. Despite an apparent increase in the number of corpora lutea, the number of oocytes recovered from the oviducts of immunized ewes a short time after the expected time of ovulation was reduced, whereas LH concentrations at all stages of the cycle were unaffected. We conclude that immunization against αN results in failure of oocyte release from the follicle and that αN may be an intraovarian regulator involved in control of the ovulatory process.

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