Effect of immunization against the alpha N (α N) and alpha C (α C) peptides of the alpha₄₃ subunit of inhibin on antral follicular growth and atresia and the patterns of gonadotrophin secretion in ewes

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The aims of this study were to investigate the role of inhibin in the distribution of healthy and atretic antral follicles and the secretion patterns of gonadotrophins. Ewes were actively immunized against either α N or α C of the inhibin α subunit with a primary injection and three booster injections. The control ewes received adjuvant only. The ovaries were removed either before or at 24 h after hCG administration in a synchronized follicular phase 48 h after removal of intravaginal progesterone pessaries. Morphological observations were made on every fifth section of the complete ovary (one per ewe) stained with haematoxylin and eosin. The mean number of corpora lutea observed per ewe with corpora lutea was not significantly different in ewes immunized against α N (2.4; α N-immunized ewes) or α C (2.6; α C-immunized ewes), and control (2.4) ewes, although some corpora lutea appeared cystic in the immunized ovaries. Compared with luteal phase concentrations, mean basal FSH concentrations in the early follicular phase were significantly increased in the α C-immunized ewes, similar in α N-immunized ewes and reduced in control ewes. No

differences were observed in any of the LH parameters. Before hCG treatment, healthy antral follicles > 1 mm in diameter were not observed in any of the 52 follicles in the α C-immunized ewes and were observed in one of 37 follicles from α N-immunized ewes compared with 19 of 28 follicles in control ewes ($P < 0.0001$). For healthy antral follicles < 1 mm in diameter, there were 72 of 85 follicles in the α C-immunized ewes, 79 of 81 follicles in the α N-immunized ewes and 81 of 82 follicles in the control ewes. Similar results were obtained in healthy antral follicles < 1 mm in diameter at 24 h after hCG administration. In contrast to the control ewes, no healthy preovulatory follicles (> 6 mm in diameter) were observed in α N- and α C-immunized ewes either before or 24 h after hCG administration. Two newly formed corpora lutea from α C-immunized ovaries contained retained oocytes compared with none in control and α N-immunized ovaries. In conclusion, immunization against α N and α C may result in disruption of the normal processes of antral follicular growth and maturation independent of the concentrations of FSH and LH.

Introduction

The follicular phase in ewes is characterized by the selection and establishment of a dominant follicle or follicles at the expense of other large antral follicles, which undergo atresia and are incapable of ovulating (Scaramuzzi *et al.*, 1993; Campbell *et al.*, 1995). The gonadotrophins, together with growth factors including inhibin and inhibin-related proteins (Findlay *et al.*, 1993; Findlay, 1994), have been implicated in final differentiation of the preovulatory follicle. Inhibin or its subunits have intraovarian regulatory actions in rats (Hillier *et al.*, 1991; Schneyer *et al.*, 1991),

and *in vivo* infusion of sheep ovaries with recombinant human inhibin decreases oestradiol and androstenedione secretion (Campbell *et al.*, 1996).

The amino terminal peptide (α N) of the 43 kDa inhibin α -subunit precursor isolated from bovine follicular fluid (Robertson *et al.*, 1989) may have an intraovarian role in modulating fertility (Findlay *et al.*, 1989a, 1994; Russell *et al.*, 1994). Ewes actively immunized against recombinant bovine α N had a reduced pregnancy rate without any apparent endocrine disturbances that could explain the reduced fertility (Findlay *et al.*, 1989a; Russell *et al.*, 1994). The mechanisms by which immunoneutralization of α N and α N-containing inhibins leads to infertility are unclear. Russell *et al.* (1995) observed aberrant corpus luteum formation in ewes immunized against α N. In a preliminary study, atresia was observed in all antral follicles > 1 mm in

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diameter in ewes actively immunized against α N (Dhar *et al.*, 1998), indicating that α N may be essential for viability of antral follicles. If α N or α N-containing inhibins prevent atresia in large antral follicles such as the ovulatory follicle, their absence or restriction could compromise the ovulatory cascade induced by the gonadotrophin surge, thereby leading to restricted tissue remodelling without preventing luteinization.

Active immunization against the α C portion of inhibin α -subunit precursor increased the ovulation rate in different species, including gilts (Brown *et al.*, 1990) and mares (McCue *et al.*, 1992; Terhaar *et al.*, 1997). The increase in ovulation rate was accompanied by an increase in FSH concentrations in sheep and cattle (O'Shea *et al.*, 1989; Findlay *et al.*, 1989b; Wrathall *et al.*, 1990; Glencross *et al.*, 1992), although antibodies raised against different preparations of immunogen and different portions of the α C fragment elicit inconsistent results in terms of FSH response and ovulation rate (O'Shea *et al.*, 1989, 1991). In sheep, long term immunization against the α C portion of inhibin was associated with an increase in intrafollicular concentrations of inhibin and activin and an increase in ovulation rate without a sustained increase in FSH concentrations (Tannetta *et al.*, 1998), thereby implying a potentially FSH-independent intraovarian role of inhibin-related peptides.

Follicle growth and atresia after immunization against α C have not been described in detail in sheep ovaries. On the basis of the response of ovulation rate after immunization against α C, it was hypothesized that this treatment would result in lower atresia and more follicular development. Hence, the first aim of the present study was to examine the effects of immunization of ewes against α N and α C on the populations of healthy and atretic antral follicles in the preovulatory and periovulatory periods. The second aim was to compare the secretion patterns of FSH and LH during the luteal and follicular phases in each of the treatment groups.

Materials and Methods

Animals and immunization

Adult Corriedale (age 3–5 years) parous ewes were kept on pasture or maintained on an *ad libitum* diet in pens. The ewes were immunized against either α N, a recombinant fusion protein containing the full-length bovine α N sequence ($n = 10$: Findlay *et al.*, 1989a; Dhar *et al.*, 1998), or α C, a recombinant fusion protein containing the full length bovine α C sequence ($n = 10$: Forage *et al.*, 1987). The control ewes ($n = 10$) from the same flock received the appropriate adjuvant only. Ewes received a primary i.m. injection of immunogen ($300 \mu\text{g ml}^{-1}$, day 1) in Freund's complete adjuvant (1 ml dose divided between the two hind legs) and three booster injections in Montanide–Marcol (SEPPIC, Paris and ESSO, Sydney; 1:9) given at 4 week intervals.

Laparoscopy was performed to count the number of

corpora lutea on the ovarian surfaces, and a jugular venous blood sample for antibody titres was collected at the time of primary immunization and the three booster injections in α N-immunized ($n = 9$), α C-immunized ($n = 10$) and control ($n = 9$) ewes (Table 1). The experiment was approved by the Institutional Animal Ethics Committee as conforming to NH and MRC guidelines.

Experimental procedures

The experiment was conducted during the breeding season. The oestrous cycles of the ewes were synchronized with progestin inserts (CIDRs; Lyppard Veterinary Supplies, Victoria) for 2 weeks, 84 days after the primary immunization. Laparotomy was performed 48 h after removal of the CIDRs ($t = 0$ h) from α N-immunized ($n = 3$), α C-immunized ($n = 6$) and control ($n = 3$) ewes. At the same time, 750 iu hCG (Intervet, Sydney) was injected i.m. into the remaining ewes and after a further 24 h laparotomy was performed in α N-immunized ($n = 2$), α C-immunized ($n = 3$) and control ($n = 3$) ewes. The final numbers of ewes in the three groups were lower than those mentioned above due to complications after laparoscopy and low antibody titres ($< 10\%$). The reproductive tracts were exposed at laparotomy and the major structures on the surface of the ovaries were recorded as described by Findlay *et al.* (1994). The ovaries were then removed and fixed by perfusion of 10% (v/v) buffered formalin into the ovarian artery for 7 min. The ovaries were post-fixed in 10% (v/v) buffered formalin for a further 24 h, washed three times for 10 min in Tris-buffered saline (TBS) and stored at 4°C for 24 h. The washes were repeated and the ovaries were stored in TBS at 4°C until processed. Fixed ovaries were dehydrated in serially increasing concentrations of ethanol and then embedded in paraffin wax.

Assessment of follicle status

Serial sections (10 μm thickness) were cut from the complete ovary containing the largest follicle obtained from each ewe at 0 and 24 h. The sections were mounted on poly-L-lysine-coated slides and stained with haematoxylin and eosin. Every fifth section was analysed morphologically and each follicle was measured when its oocyte appeared to be at maximum diameter; this prevented an over-estimation of the number of follicles. The mean follicle diameter was obtained as described by Dhar *et al.* (1998) and preovulatory follicles were defined as having a mean diameter > 6 mm (McIntush *et al.*, 1996).

Each follicle was classified as healthy or atretic (Hay *et al.*, 1976; Turnbull *et al.*, 1977; Tsafiri and Braw, 1984; Dhar *et al.*, 1998). A healthy follicle was defined as having fewer than five pyknotic nuclei, mitotic figures in the granulosa layer, no disruption of the basement membrane and a highly vascularized theca interna. A group of borderline healthy follicles was identified that had irregular basement membrane and 5–10 granulosa cells with hyperchromatic nuclei or apoptotic cells isolated in the

antrum. Atresia was classified into primary, secondary and tertiary. The early primary atretic follicles were characterized by an irregular wavy basement membrane (apparent separation of the basement membrane from the thecal layer), few mitotic figures and 5–15 pyknotic nuclei. Primary atretic follicles had more pyknotic nuclei, fewer mitotic figures, and degeneration and discontinuity of the granulosa cells protruding through the basement membrane. As the atresia progressed, there was a gradual degeneration of the granulosa layer, formation of more atretic bodies or apoptotic bodies defined as discrete membrane-bound structures containing chromatin or variable amounts of cytoplasm, basement membrane breakdown and thecal cell degeneration. Apoptotic cells were defined as having nuclei with condensed chromatin (Jolly *et al.*, 1997a). These appeared as single pyknotic cells or a fragmented cluster from a single cell that had not been phagocytosed by the neighbouring cells. Two independent observers also examined a subset of the material in a blind study to assess the classification of atresia made by the author and there was excellent agreement among all observers.

All antral follicles in the representative cross-section were analysed. A total of 248 antral follicles < 1 mm in diameter (control: $n = 82$; α N-immunized: $n = 81$; α C-immunized: $n = 85$) and 117 follicles > 1 mm in diameter (control: $n = 28$; α N-immunized: $n = 37$; α C-immunized: $n = 52$) were obtained at 0 h. Of the follicles analysed from the 24 h group, there were 167 follicles < 1 mm in diameter (control: $n = 44$; α N-immunized: $n = 40$; α C-immunized: $n = 83$) and 85 follicles > 1 mm in diameter (control: $n = 24$; α N-immunized: $n = 14$; α C-immunized: $n = 47$). The study included the borderline healthy follicles but excluded the tertiary atretic follicles. Sections consecutive to the haematoxylin and eosin-stained sections were selected from immunized and control ovaries at 0 h to confirm the atretic classification by analysis for apoptosis by 3' end-labelling technique of the fragmented DNA (TUNEL).

Gonadotrophin concentrations in the follicular and luteal phases

FSH and LH concentrations were measured in blood samples in the luteal and follicular phases of the oestrous cycle to determine the effect of immunization against α N and α C on LH profiles and to compare FSH concentrations among the three groups. The jugular veins of subgroups of ewes with CIDR intravaginal implants, selected on the basis of antibody titre (immunized > 15% and control < 1%; $n = 4$ control ewes, $n = 4$ α N-immunized ewes and $n = 5$ α C-immunized ewes), were cannulated and blood samples were collected at 10 min intervals for 8 h starting 52 h before (luteal phase) the removal of CIDRs and again 20 h after (follicular phase) the removal of CIDRs.

LH concentrations in ewe plasma was measured as described by Lee *et al.* (1976) using NIH-oLH-S18 as standard. The mean sensitivity was 0.18 ± 0.01 ng ml⁻¹ and the interassay coefficient of variation was 8.1% for a

plasma pool run in all assays. An LH pulse was defined when the value of the assay of a given sample was more than the assay value of the previous sample by three times the assay standard deviation of the previous sample (Scott, 1993). The plasma concentration of LH also had to remain unchanged or decrease over two successive samples before another pulse was counted. Interpulse interval was calculated as the time between two successive peaks of defined pulses and pulse amplitude as the difference between the peak height for a defined pulse and the plasma concentration of hormone immediately before initiation of the pulse (Scott, 1993).

FSH was assayed by the method of Bremner *et al.* (1980) using NIADDK reagents for ovine FSH and USDA-oFSH-SIAFD-RP-2 as standard. The mean sensitivity was 0.22 ± 0.01 ng ml⁻¹ and the interassay coefficient of variation was 5.1%. Hormone concentrations were calculated based on the programme of Burger *et al.* (1972) and the intra-assay coefficient of variation was < 15% of the assay coefficient of variation range of the dose-response curve for both radioimmunoassays.

Antibody-binding assays

The antibody titres in plasma from α N-immunized ewes were measured by binding of [¹²⁵I] α N_{1–26} to plasma at a dilution of 1 in 4000 as described by Dhar *et al.* (1998). The sample for the α C binding assay was incubated with [¹²⁵I] 32 kDa bovine inhibin to plasma at a dilution of 1 in 2000 (Findlay *et al.*, 1989b).

In situ 3' end-labelling (TUNEL)

Tissue sections were processed for TUNEL staining using the original method of Gavrieli *et al.* (1992) with modification. Sections were deparaffinized, rehydrated and washed in 0.01 mol PBS l⁻¹ (containing 0.15 mol NaCl l⁻¹ at pH 7.4). The sections were then incubated with proteinase K (10 μ g ml⁻¹ in 20 mmol Tris l⁻¹ and 2 mmol CaCl₂ l⁻¹) in a humidified chamber at 37°C for 30 min, followed by washing in 0.01 mol PBS l⁻¹ for 45 min. The sections were labelled at 3' ends by incubating in a mixture of TdT buffer, digoxigenin DNA labelling mix (10 $\times$), cobalt chloride (25 mmol l⁻¹), TdT enzyme (25 U μ l⁻¹) (Boehringer Mannheim, GmbH, Mannheim) and milli Q water in a humidified chamber at 37°C for 60 min and the reaction was stopped by washing slides in 0.01 mol PBS l⁻¹ for 45 min. The sections were then equilibrated in buffer 1 (0.09 mol maleic acid l⁻¹ and 0.15 mol NaCl l⁻¹ at pH 7.5) for 10 min, blocked in 1% (v/v) buffer 2 (Boehringer Mannheim blocking solution) for 30 min and then incubated in sheep anti-digoxigenin antibody (150 U (200 μ l)⁻¹) at 1 in 2000 dilution in buffer 2 at room temperature for 1 h in a humidified chamber. The slides were washed in 0.01 mol PBS buffer l⁻¹ for 45 min and equilibrated in buffer 3 (Tris-HCl, pH 9.5, containing 0.02 mol NaCl l⁻¹ and 0.01 mol MgCl₂ l⁻¹) for 10 min. The colour reaction was obtained with nitroblue tetra

(337.5 µg ml⁻¹) and 5-bromo-4 chloro-3-indoly/phosphate (Boehringer Mannheim; 175 µg ml⁻¹) in buffer 3 (with additives) and 1.5 µl levamisole ml⁻¹ (240 µg ml⁻¹; Sigma Chemical Company, St Louis, MO) for 4–5 min to quench the endogenous alkaline phosphatase. The reaction was stopped by washing the slides in distilled water for 9–10 min and later in 95% (v/v) ethanol for another 5 min, after which the slides were dehydrated and mounted in DPX. The negative control slides were incubated in labelling mixture as above but TdT enzyme was omitted.

Data analysis

Hormone concentrations are expressed as mean ± SEM and were compared within each treatment group by one-way ANOVA. FSH data were log transformed before analysis. The mean FSH and LH concentrations in immunized and control groups were compared by paired *t* test. LH pulse, mean LH pulse interval, pulse amplitude and mean basal LH concentration in either group were compared by one-way ANOVA. The mean number of corpora lutea observed at the time of laparoscopy was analysed by Mann–Whitney two-tailed test. The numbers of healthy and atretic antral follicles were compared between control and immunized groups by chi-squared tests. The antibody titres at 0 h and 24 h were compared between control and immunized groups by two-sample *t* test. Student’s *t* test was used to compare the data within each treatment group. Differences were significant at *P* < 0.05.

Results

Antibody titres

Antibody titres in αN- and αC-immunized ewes were significantly different from those of control ewes by the time of the second booster injection (Fig. 1a,b; *P* < 0.05) and were in the range of values described previously for αN (Dhar *et al.*, 1998) and αC (Findlay *et al.*, 1989b) immunized ewes. At the time of the third booster injection, antibody binding in the αN-immunized group was 45.41 ± 15.00 and 43.46 ± 10.76% at 0 and 24 h after hCG administration, respectively (*P* < 0.05), and in the αC-immunized group antibody binding was 15.03 ± 2.24 and 15.88 ± 1.42, respectively (*P* < 0.001). All these values were significantly higher than the control values. In control ewes the mean titres at all times were around the non-specific binding levels of < 1% (Fig. 1a,b).

Corpora lutea observed at laparoscopy

The mean numbers of corpora lutea observed at the time of first, second and third laparoscopy were not significantly different among control, αN-immunized and αC-immunized ewes (Table 1). At the third laparoscopy, six of 17 corpora lutea in αN-immunized ewes and eight of 26 corpora lutea in αC-immunized ewes appeared pale and cystic compared with none of 17 corpora lutea in control ewes.

Fig. 1. Antibody titres in ewes at 0 and 24 h after hCG administration at the times of the primary immunization, and first, second and third booster injections. (a) ■: αN control group at 0 h (*n* = 4). ●: αN-immunized group at 0 h (*n* = 3). ▲: αN control group at 24 h (*n* = 4). ◆: αN-immunized group at 24 h (*n* = 4). (b) ■: αC control group at 0 h (*n* = 4). ●: αC-immunized group at 0 h (*n* = 5). ▲: αC control group at 24 h (*n* = 4). ◆: αC-immunized group at 24 h (*n* = 5). Titres in αN- and αC-immunized ewes were significantly different from control titres by the time of the second booster injection, but began to plateau by the third booster injection just before surgery. Antibody titres are mean ± SEM. ^aAntibody titre is significantly different from corresponding control value (αN group at 0 h; *P* < 0.05). ^bAntibody titre is significantly different from corresponding control value (αN control group at 24 h; *P* < 0.05). ^cAntibody titre is significantly different from corresponding control value (αC control group at 0 h; *P* < 0.001). ^dAntibody titre is significantly different from corresponding control value (αC control group at 24 h; *P* < 0.001).

Table 1. Number of corpora lutea observed per ewe with corpora lutea at the time of first, second and third laparoscopy

Laparoscopy	Number of corpora lutea		
	Control (<i>n</i> = 7)	αN-immunized (<i>n</i> = 7)	αC-immunized (<i>n</i> = 10)
1	1.4 ± 0.57	1.3 ± 0.57	3.2 ± 1.3
2	1.1 ± 0.55	1.9 ± 0.51	1.3 ± 0.63
3	2.4 ± 0.61	2.4 ± 0.78	2.6 ± 0.58

Values are mean ± SEM.

Ovarian mass

There was no significant difference between and within the treatment groups in mean ovarian masses at the time of ovariectomy (data not shown).

Follicle status

Atresia in follicles < 1 mm in diameter obtained at 0 h was significantly lower in control (1/82) and α N-immunized ewes (2/81) ($P < 0.001$) compared with α C-immunized ewes (13/85) (Table 2). Similarly, at 24 h atresia was lower in follicles < 1 mm in diameter in control (6/44) and α N-immunized ewes (6/40) compared with α C-immunized ewes (24/83), although this difference was not significant (Table 3). In all antral follicles > 1 mm in diameter examined at 0 h, none of 52 in α C-immunized and one of 37 in α N-immunized ewes were healthy compared with 19 of 28 in control ewes ($P < 0.0001$, Table 2). Similar to the 0 h group, no healthy follicles > 1 mm in diameter were observed in α N- and α C-immunized ovaries compared with control ovaries at 24 h after hCG administration (Table 3). Three newly formed corpora lutea from two α C-immunized ovaries showed retained oocytes (Fig. 2). Retained oocytes were not observed in any of the six newly formed corpora lutea from the control ewes and α N-immunized ewes had no newly formed corpora lutea at 24 h after hCG administration. There was no morphological evidence of differences in the patterns of atresia among the control, α N- and α C-immunized groups (see Fig. 3).

Morphological and in situ 3' end-labelling of ovine granulosa and thecal cells

Apoptotic cells and apoptotic bodies were identified by haematoxylin–eosin staining of the membrana granulosa and thecal cells of all antral follicles > 1 mm in diameter collected from α N- and α C-immunized ewes. Apoptosis was confirmed by TUNEL staining. A follicle containing a cell with single density staining nucleus (pyknotic body) and apoptotic bodies formed from the fragmentation of the membrana granulosa is shown (Fig. 4a). As described morphologically, TUNEL staining clearly showed evidence of DNA fragmentation in the apoptotic cells containing pyknotic bodies (Fig. 4b). The above labelling was specific because none was visible in the negative control sections (Fig. 4c). A borderline atretic follicle displayed faint nuclear labelling in a few cells close to the antrum with other granulosa cells indicating no signs of apoptosis (Fig. 4d). Some of the preantral follicles from immunized ovaries also showed evidence of hyperchromatic nuclei in the granulosa cells bordering the ovum and the basement membrane, as well as the thecal cells (Fig. 4e). The same cells showed TUNEL staining (Fig. 4f). Morphological and DNA fragmentation of granulosa and theca cells of a tertiary atretic follicle are shown (Fig. 4g and h, respectively). In secondary and tertiary follicles, the apoptotic cells were distributed throughout the membrana granulosa and theca cells,

Table 2. Effect of immunization against α N or α C antigen on the number of healthy and atretic antral follicles < 1 mm and > 1 mm in diameter at 0 h in control and immunized ewes

Group	Follicles < 1 mm in diameter			Follicles > 1 mm in diameter		
	Atretic	Healthy	Total	Atretic	Healthy	Total
Control	1	81	82	9	19	28
α N	2	79	81	36	1*	37
α C	13*	72	85	52	0*	52

$n = 3$ ovaries were examined per group, except for α C-immunized group ($n = 6$ per group).

*Value is significantly different from control value ($P < 0.001$; chi-squared test).

Table 3. Effect of immunization against α N or α C antigen on the number of healthy and atretic antral follicles < 1 mm and > 1 mm in diameter at 24 h after hCG administration in control and immunized ewes

Group	Follicles < 1 mm in diameter			Follicles > 1 mm in diameter		
	Atretic	Healthy	Total	Atretic	Healthy	Total
Control	6	38	44	22	2 (6)	24
α N-immunized	6	34	40	14	0 (0)	14
α C-immunized	24	59	83	47	0 (2)	47

$n = 3$ ovaries for control and α C-immunized groups. $n = 2$ ovaries for α N-immunized group.

Values in parentheses are newly formed corpora lutea.

although only the tertiary atretic follicle had a rim of membrana granulosa cells bordering the antrum showing the presence of apoptosis and DNA fragmentation (Fig. 4g,h). Pyknotic cells and TUNEL staining in the membrana granulosa with no evidence of atresia in the cumulus and the oocyte are shown (Fig. 4i,j). All the above TUNEL positive sections were accompanied by a negative control. Thus, on the basis of morphology (haematoxylin–eosin) and 3' end-labelling data, apoptosis appeared to be the predominant form of cell death with occasional cellular debris showing features of oncosis present along the antral border of the membrana granulosa that were not detected by 3' end-labelling (Fig. 4b). There was no apparent influx of leucocytes in atretic follicles.

Gonadotrophin concentrations in the follicular and luteal phases

There were no significant differences in any of the LH parameters (Table 4). FSH concentrations were similar in each of the groups during the luteal phase and showed the normal decrease in concentration in the follicular phase

Fig. 2. Light micrographs showing oocytes (o) retained in the newly formed corpus luteum (NFCL) from two individual ewes ((a) and (b) are from the same ovary). (d) Luteinized granulosa cells (Lu g cells) of an adjoining follicle (shown in (c)). (e) Lip of a NFCL with a large number of macrophages. (f) Presence of hypertrophied theca cells of a tertiary atretic follicle with pyknotic nuclei (arrowhead). a: antrum; t: theca. Scale bar represents 45 μ m.

compared with the luteal phase in control ewes. In contrast, there was a significant increase in follicular FSH concentrations ($P < 0.05$) in the α C-immunized ewes but not in the α N-immunized ewes (Fig. 5). However, the fact that FSH concentrations remained unchanged between the luteal and follicular phases in the α N-immunized ewes was an equally important observation. The FSH:LH ratio in each of the groups was similar in the luteal phase, and was maintained in the follicular phase in α C-immunized ewes,

reflecting an increase in FSH concentrations. Consequently a decrease in ratio in the control group reflected the decrease in FSH concentrations in the follicular phase. The ratio in α N-immunized ewes in the follicular phase was intermediate between the other two groups, but below the FSH:LH ratio in the luteal phase. Overall, these findings indicate that follicles in the α N and α C groups were exposed to a relatively higher FSH milieu than follicles in ovaries of control ewes in the follicular phase (Table 4).

Fig. 3. Light micrographs illustrating parts of (a) a healthy follicle (arrowhead showing the basement membrane) and (b) an atretic follicle from the control ewes. Atretic follicles from ovaries of (c) α N- and (d) α C-immunized ewes. The arrowheads (b,c,d) show apoptotic bodies from control, α N- and α C-immunized ovaries. a: antrum; g: granulosa; t: theca. Scale bar represents 45 μ m.

Discussion

The results of the present study indicate that virtually all of the antral follicles > 1 mm in diameter in the pre- and periovulatory periods of α N- and α C-immunized ewes were classified as atretic by morphological analysis and confirmed by TUNEL staining. This extensive atresia occurred despite the fact that peripheral FSH concentrations were either unchanged from the luteal to the follicular phase in α N-immunized ewes or increased in α C-immunized ewes compared with control ewes. A further observation was an increase in the proportion of corpora lutea with a cystic appearance at laparoscopy in α N- and α C-immunized ewes and evidence of retained oocytes in follicles undergoing luteinization and corpus luteum formation with ovulatory stigma formation after hCG treatment. Collectively, these data indicate that both α N- and α C-containing inhibins or α N alone may be involved in preventing atresia in antral follicles and facilitating the ovulatory cascade induced by LH leading to tissue remodelling. This finding confirms our earlier work showing that immunization of ewes against α N resulted in lower rates of oocyte release (Findlay *et al.*, 1994), atresia of antral

follicles > 1 mm in diameter (Dhar *et al.*, 1998) and compromised tissue remodelling at the time of ovulation and luteinization (Russell *et al.*, 1995). This finding can now be extended to ewes immunized against the α C portion of inhibin α subunit.

These proposed actions of the inhibin-related proteins on atresia are hypothesized to occur locally in the ovarian follicle independent of the concentrations of circulating FSH. Furthermore, it is hypothesized that the compromised tissue remodelling and retained oocytes in α N- and α C-immunized ewes are a consequence of atresia in antral follicles rather than a direct role of inhibins in these processes.

The process of follicular atresia in ewes occurs through the mechanism of apoptosis (Jolly *et al.*, 1997a,b). The atretic follicles from the immunized ovaries, classified morphologically by well-established criteria (Hay *et al.*, 1976; Turnbull *et al.*, 1977; Tsafiriri and Braw, 1984), displayed the presence of DNA fragmentation as detected by TUNEL staining in granulosa and theca cells. Apoptosis was also present in the theca cells more predominantly in the preantral and early primary atretic follicles. Apoptosis is responsible for the process of atresia in the theca interna

Fig. 4. Light micrographs of portions of sheep ovarian tissue. Haematoxylin and eosin stained atretic follicle from α C-immunized ovary, with the presence of pyknotic nuclei (arrows). (b) *In situ* 3' end-labelling of an adjacent section with arrows showing the presence of fragmented DNA. (c) A consecutive section from the same follicle with the absence of TdT enzyme (negative control). Arrows show fragmented DNA. (d) An early primary atretic follicle with faint chromatin stains in granulosa

Fig. 5. FSH concentrations in control ewes ($n = 4$) and ewes immunized against α N ($n = 4$) and α C ($n = 5$) in the luteal (□) and follicular (■) phases of the oestrous cycle. Values are mean $\pm$ SEM. ^{ab}Values with different letters are significantly different ($P < 0.05$) between the follicular and luteal phase. ^{xy}Values with different letters are significantly different ($P < 0.01$; ANOVA).

cells (O'Shea *et al.*, 1978). Huet *et al.* (1998) reported that the first signs of atresia appear in the theca cells in ewes, thus implying that follicular atresia may be initiated by alteration in function of the theca cells. The cumulus and the oocyte did not show any sign of DNA fragmentation in an advanced secondary atretic follicle. The presence of

necrotic cells along the antral border of follicles in advanced stages of atresia is a common observation and may be a secondary event that occurs in cells after apoptotic cell death (Jolly *et al.*, 1997a) or after the granulosa cells undergo terminal differentiation (Van Wezel *et al.*, 1999).

It is not known which inhibin molecules the respective antibodies recognize and it may vary among ewes in the same treatment group. It can be surmised that α N directed antibodies would recognize all forms of free α subunit and high molecular mass dimeric inhibins that contain α N as well as free α N, but would exclude the biologically active dimeric 31 kDa inhibin A. In contrast, α C directed antibodies would recognize all forms of free α subunit and dimeric inhibins containing α C, including 31 kDa inhibin A but excluding free α N. These predicted antibody profiles would be consistent with the observed effects on gonadotrophin concentrations in peripheral plasma. Active immunization against α N (Findlay *et al.*, 1989a, 1994; Russell *et al.*, 1994) and α C (McLeod *et al.*, 1992) had little or no influence on the circulating patterns and concentrations of LH, as observed in the present study. FSH concentrations undergo either no change or a slight increase after active immunization of ewes with α C (Forage *et al.*, 1987; Findlay *et al.*, 1989b; McLeod *et al.*, 1992) and α N (Russell *et al.*, 1994). In the present study, a significant increase in FSH concentrations in α C-immunized ewes above that of control and α N-immunized ewes was observed in the follicular phase. FSH is known for its ability to stimulate folliculogenesis and reduce the proportion of atretic follicles, leading to an increase in ovulation rates (Scaramuzzi *et al.*, 1993). However, the slight increase (α N

Table 4. Effect of immunization against α N and α C on pulsatile LH release, basal LH concentrations and the FSH:LH ratio during the follicular and luteal phases of the oestrous cycle in control and immunized ewes

Group	<i>n</i>	LH pulse (pulses h ⁻¹)	LH pulse amplitude (ng ml ⁻¹)	Mean pulse interval (min)	Mean basal LH concentration (ng ml ⁻¹)	FSH:LH ratio
Luteal phase						
Control	4	9.00 $\pm$ 1.08	0.57 $\pm$ 0.12	57.18 $\pm$ 5.38	0.79 $\pm$ 0.19	0.90
α N-immunized	4	7.75 $\pm$ 1.11	1.00 $\pm$ 0.29	59.08 $\pm$ 7.55	0.93 $\pm$ 0.10	0.98
α C-immunized	5	10.00 $\pm$ 1.10	0.74 $\pm$ 0.11	50.93 $\pm$ 7.08	1.28 $\pm$ 0.32	0.88
Follicular phase						
Control	4	7.50 $\pm$ 2.10	0.61 $\pm$ 0.03	48.89 $\pm$ 2.52	1.23 $\pm$ 0.23	0.50
α N-immunized	4	10.75 $\pm$ 1.55	1.05 $\pm$ 0.06	45.37 $\pm$ 5.57	1.53 $\pm$ 0.15	0.64
α C-immunized	5	9.60 $\pm$ 0.68	1.20 $\pm$ 0.33	46.91 $\pm$ 2.00	2.12 $\pm$ 0.66	0.87

Values are mean $\pm$ SEM, except for the ratio.

bordering the antrum (shown by arrows) and in the theca cells. (e) Haematoxylin–eosin-stained preantral follicle from an α N-immunized ovary. (f) *In situ* 3' end-labelling of an adjacent section showing DNA fragmentation in granulosa and theca cells (arrows). (g,h) Adjacent sections from α N-immunized ovary (g) with haematoxylin–eosin stain and (h) 3' end-labelling (arrows show apoptotic nuclei with fragmented DNA). (i) Healthy cumulus and oocyte (o), as well as pyknotic nuclei (arrows). (j) No DNA fragmentation in the cumulus (cu) and oocyte (o) but there is DNA fragmentation in membrana granulosa (arrows). a: antrum; g: granulosa; t: theca. Scale bar represents 45 μ m.

group) and significant increase (α C group) in FSH concentrations observed in the follicular phase of ewes in the present study were not able to prevent the atresia observed in antral follicles > 1 mm in diameter, which are normally responsive to FSH (McNatty *et al.*, 1992). This finding is consistent with the hypothesis that the direct effects of immunization against α N and α C on follicular atresia are independent of peripheral actions of inhibin on pituitary secretion of gonadotrophins.

Both α N- and α C-immunized ewes are capable of an LH surge, ovulation and luteinization (McLeod *et al.*, 1992; Russell *et al.*, 1994), although some of the processes may be compromised, such as oocyte release and tissue remodelling (Russell *et al.*, 1995). This finding implies that at the preovulatory stage, despite being morphologically atretic, these follicles are capable of producing sufficient oestradiol to induce an LH surge. However, atresia has been characterized by a decrease in capacity to produce oestradiol (Moor *et al.*, 1978; Jolly *et al.*, 1997b). There are no direct data on oestradiol production rates in α N- and α C-immunized ewes *in vivo*. It is known that inhibin increases LH- and FSH-mediated androgen substrate and oestradiol production from sheep theca and granulosa cells, respectively (Campbell and Webb, 1995). McLeod *et al.* (1992) showed that compared with controls, the oestradiol secretion rates increased in non-oestrogenic but not in oestrogenic follicles of α C-immunized ewes, implying no apparent influence of α C-related inhibins on oestrogen production by oestrogenic follicles.

One explanation for the observations described above would be the existence of different functional, as opposed to morphological, atresias. The functional atresia in α N- and α C-immunized ewes would be related to events in the periovulatory period associated with ovulation and tissue remodelling and not with the hormone producing capacity of the follicle. It is worth noting that in α C-immunized ewes, the observed increases in number of corpora lutea have not always led to increased fertility (Tsonis *et al.*, 1989; Wrathall *et al.*, 1992) and in the α N-immunized ewes, fertility is compromised (Russell *et al.*, 1994). However, it is not known how the inhibin-related proteins act locally to differentiate functional from morphological atresia. It is unlikely to involve a local autoimmune response (Fray *et al.*, 1994) resulting in an inflammatory response leading to increased macrophage infiltration and cytokine production (Fukumatsu *et al.*, 1992), as no massive infiltration of macrophages was observed.

Follicular development in ovaries of immunized ewes could be affected by a change in the stroma or extracellular matrix as is also observed in the stroma of inhibin α knockout mice (Matzuk *et al.*, 1992). Russell *et al.* (1995) observed a lack of increase in matrix metalloproteinase 2 (MMP-2) and MMP-9 content in periovulatory follicles from α N-immunized ewes compared with controls, which could compromise tissue remodelling and oocyte release. There are no data on the direct effects of inhibin-related proteins on MMPs. It is possible that an earlier atretic event induced

by α N immunization led to the effects on MMPs observed by Russell *et al.* (1995). The decrease in MMP activity was not associated with an increase in tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) expression in periovulatory follicles of α N-immunized ewes (Dhar *et al.*, 1998). Studies on the influence of α C immunization on MMPs and TIMPs have not been reported.

Mice with inhibin α deficiency gradually develop one sided or both sided mixed or incompletely differentiated gonadal stromal tumours leading to infertility (Matzuk *et al.*, 1992) and increased concentrations of FSH, activin and oestradiol (Matzuk *et al.*, 1996). The latter changes are also observed in ewes immunized actively against the α C portion of α inhibin subunit (McLeod *et al.*, 1992; Tannetta *et al.*, 1998), but stromal tumours have not been reported. Some ovaries from inhibin α deficient mice occasionally show follicles with prominent antra but no corpora lutea (Matzuk *et al.*, 1996), indicating a possible role of inhibin in regulating stroma besides the early and late follicular growth. In compound homozygous mutant mice with mutations in both the inhibin α and GnRH genes, the ovaries were small. The morphology showed relatively little follicular development through to the secondary follicle stage (Kumar *et al.*, 1996), indicating that inhibin (α N/ α C) may be needed for follicular development beyond the primary to the secondary stage.

In conclusion, it is hypothesized that immunization against α N and α C resulted in disruption of the normal processes of antral follicular growth and maturation independent of the concentrations of FSH and LH, which leads to a compromise in the cascade of events induced by the LH surge. This study raises an issue about the functional capability of an early atretic preovulatory follicle in terms of hormone output versus subsequent ovulation, tissue remodelling and oocyte fertilizability. Overall, these observations support the possibility that α -inhibin precursor, including parts of the precursor that contain α N, may have paracrine or autocrine activity distinct from the endocrine role of inhibin.

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