Gonadotrophin secretion and ovarian responses in prepubertal heifers actively immunized against androstenedione and oestradiol-17 β

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Summary. Groups of heifer calves received a primary immunization against androstenedione (Group A; N = 11) or oestradiol-17 β (Group E; N = 10) at 3 months of age and booster injections on 5 occasions at 2- to 3-month intervals. Controls (Group C, N = 11) were immunized against human serum albumin alone using the same protocol. Immunity was achieved against both steroids as judged by the secondary antisteroid antibody titres in Group A (1126 ± 261 ; reciprocal of titre) and Group E ($10\,357 \pm 4067$) heifers. In Groups A and E there was a general decline in the respective peak antibody titres after successive booster injections. From 3 to 9 months of age mean plasma concentrations of LH were higher ($P < 0.05$) in Group E heifers (0.89 ± 0.08 ng/ml) than in Group C (0.46 ± 0.03 ng/ml) and Group A (0.59 ± 0.05 ng/ml) heifers which did not differ from one another. There were no differences between groups in plasma FSH concentrations. At 10 months of age the LH response to exogenous LHRH was of higher ($P < 0.05$) amplitude for heifers in Group E (2.59 ± 0.56 ng/ml) than for those in Groups C (0.61 ± 0.07 ng/ml) and A (1.04 ± 0.22 ng/ml). Elevated plasma progesterone concentrations at 5 months of age were shown by 2 heifers in Group C, 10 in Group A, and 6 in Group E. From 8 to 14 months of age a consistently higher proportion of Group A heifers exhibited elevated progesterone compared with Group C and Group E heifers. After ovarian synchronization and booster injection at 15 months of age a corpus luteum was present in 2 heifers in Group C, 7 in Group A and none in Group E. The ovaries of Group A heifers were different from those of Groups C and E and were characterized by greater numbers of 2–4 mm follicles. It is concluded that active immunization against gonadal steroids influences both LH secretion and ovarian function in prepubertal heifers. Early increases in ovarian activity in androstenedione-immunized heifers are maintained after puberty and may therefore confer some lifetime reproductive advantages.

Keywords: heifer; gonadal steroid immunization; gonadotrophins; puberty; ovary

Introduction

Onset of pubertal development in heifers is thought to be associated with changes in sensitivity of the hypothalamo–pituitary axis to ovarian steroid feedback (Schillo *et al.*, 1982; Day *et al.*, 1984),

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although this has yet to be clearly established (see Moseley *et al.*, 1984). Oestradiol-17 β (oestradiol) was more effective in suppressing luteinizing hormone (LH) secretion in ovariectomized heifers at 4 months of age than at 8 and 12 months (Schillo *et al.*, 1982). Also, ovariectomized prepubertal heifers receiving constant oestradiol replacement showed an increase in circulating concentrations of LH at about the time when normal, age-matched heifers first showed signs of cyclic ovarian activity (Day *et al.*, 1984). These studies identified oestradiol as an important regulator of LH in young heifers and further suggested that a decrease in the 'negative' component of oestradiol feedback precedes, and might be a requirement for, the onset of pubertal maturation.

Assuming that changes in feedback sensitivity influence the timing of puberty in heifers, then it might be argued that the transition to pubertal development could be induced by removing steroid feedback. Presumably, in the absence of steroid feedback there would be an increase in gonadotrophin secretion which would stimulate earlier onset of ovarian activity. Immunization is considered as one approach for neutralizing endogenous steroids (Scaramuzzi & Hoskinson, 1984). Post-pubertal heifers and cows actively immunized against androstenedione showed enhanced LH secretion (Campbell *et al.*, 1985) and increased ovulation rates (Wise & Schanbacher, 1983). In the present study, therefore, observations were made on gonadotrophin secretion and ovarian responses in prepubertal heifers actively immunized against androstenedione and oestradiol. Effects of early steroid immunization on post-pubertal ovarian activity were also studied.

Materials and Methods

Animals. Shorthorn heifers born in late autumn were maintained under field conditions in a mediterranean environment and were weaned at 7 months of age. Body weights were determined at approximately monthly intervals during the study.

Immunogens and immunization procedures. For immunization, androstenedione-7 α -carboxyethylthio ether and oestradiol-6-(O-carboxymethyl)oxime were conjugated to human serum albumin using tetrahydrofuran in a mixed solvent procedure to activate the steroid with diimide reagent (Lindner *et al.*, 1972). Proportions of reactants were: steroid acid (1 mM), ethyldimethylaminopropyl carbodiimide hydrochloride (1 mM) and human serum albumin (0.01 mM). Further details concerning the conjugation have been described (D'Occhio *et al.*, 1987). Steroid-protein conjugates were emulsified in Freund's complete adjuvant or prepared in 5% DEAE-dextran (pH 7.5) and injected subcutaneously along both sides of the neck and brisket. Treatments included control (Group C; N = 11), androstenedione-immunized (Group A; 7 Freund's, 4 DEAE-dextran) and oestradiol-immunized (Group E; 6 Freund's, 4 DEAE-dextran) heifers. The immunological and physiological responses were similar for heifers treated with Freund's and DEAE-dextran and so data for respective heifers in Group A and Group E were pooled. Heifers received a primary immunization at 3 months of age and booster immunizations on 5 occasions at 2- to 3-month intervals. For the 5th booster injection steroids were conjugated to pig thyroglobulin in place of human serum albumin. The same amounts of immunogen (equivalent to 4.5 mg protein, 0.5 mg steroid) and Freund's complete adjuvant or DEAE-dextran were used for all immunizations.

Blood sampling and LHRH tests. Single blood samples were taken weekly by jugular venepuncture to follow longitudinal changes in circulating concentrations of LH and FSH. Progesterone was determined in weekly samples from 5 months of age onwards, and heifers with concentrations ≥ 1 ng/ml during any month were judged to have ovarian luteal tissue. At 10 months of age (3 weeks after 3rd booster injection) pituitary responses to a bolus intravenous injection of native LHRH (5 ng/kg body weight; Sigma, St Louis, MO, U.S.A.) were ascertained for 6 heifers from each treatment group selected at random. On this occasion heifers were equipped with an indwelling jugular vein cannula which was used both for blood sampling and LHRH injection. Relative to LHRH injection blood samples were taken at -20, 0, 10, 20, 30, 40, 60 and 120 min.

Ovarian responses to synchronization. At 15 months of age ovarian activity was synchronized using the synthetic prostaglandin cloprostenol (Coopers Animal Health, North Ryde, NSW, Australia) given as two injections 11 days apart. Heifers were slaughtered 10 days after the second injection of cloprostenol and reproductive tracts were recovered to determine ovarian status. The 5th booster injection was administered at the time of the first cloprostenol injection. A blood sample was taken on the day before slaughter for progesterone determination.

Antibody titres and hormone assays. Procedures for determining antibody titres have been described (D'Occhio *et al.*, 1987). Serum concentrations of LH and follicle-stimulating hormone (FSH) were determined by double-antibody radioimmunoassays also previously described (D'Occhio *et al.*, 1986b). An extraction single-antibody radioimmunoassay was used to measure plasma progesterone concentrations. Progesterone was extracted into heptane by vigorous vortexing and after freezing of the aqueous phase, the heptane was transferred to assay tubes

and evaporated under a stream of nitrogen gas at 40°C. Tritiated progesterone (35 pg, [1,2,6,7-³H]progesterone, sp.act. 3.03 TBq/mmol; Amersham, U.K.) and antiserum (sheep anti-progesterone-11 α -hemisuccinate-bovine serum albumin) were then added and the tubes vortexed and left at 4°C for 16 h. Dextran-coated charcoal was used to separate bound and free steroid and radioactivity in the bound fraction was determined using a liquid scintillation spectrometer. Cross-reactivity of the antiserum with various steroids was as follows: 11 β -hydroxyprogesterone, 27.0%; 11-deoxycorticosterone, 6.9%; corticosterone, 5.8%; 5 β -pregnane-3,20-dione, 1.4%; other steroids, <0.5%. Intra- and inter-assay coefficients of variation for all radioimmunoassays were <10% based on duplicate samples. Sensitivities were 4 ng FSH/ml, 0.2 ng LH/ml and 0.1 ng progesterone/ml.

Statistical analyses. Longitudinal data for bodyweights and LH and FSH concentrations were subjected to analysis of variance using a split-plot design with immunization treatments as the main-plot effects and the time periods as sub-plot effects. One-way analysis of variance was used to analyse the LH responses to LHRH. Data for LH and FSH were transformed to natural logarithms before analysis. Conservative F values were calculated according to the procedure recommended by Greenhouse & Geisser (1959) and differences amongst treatment groups were assessed by the protected least significant difference test (Snedecor & Cochran, 1980). For analysis of the progesterone data, the absence (<1 ng/ml) of plasma progesterone in a particular heifer, in a particular month, was expressed as zero and the presence ($\geq$ 1 ng/ml) of progesterone was expressed as one. The zero-one data for each heifer were transformed using orthogonal polynomials to derive means and linear, quadratic and cubic effects which were then analysed for treatment differences (avoiding correlation problems with repeated measurements).

Results

Antibody titres

When compared with antisteroid antibody titres observed in other species using similar immunogens, antibody titres in the present study were relatively low, particularly for heifers in Group A (Table 1), but were of the same order of magnitude reported previously for prepubertal and post-pubertal cattle (D'Occhio *et al.*, 1987). The failure of successive booster injections to induce titres that exceeded the initial secondary response, and the transient rise in antibody levels after booster injection followed by a prompt decline in titres (see results for Weeks 8, 9, 12 and 16, 17, 20 in Table 1), were also consistent with previous observations in cattle (D'Occhio *et al.*, 1987). The progressive decline in titres with successive booster injections was not reversed by the use of steroid-pig thyroglobulin conjugates for the 5th booster. Walker *et al.* (1984) reported that, in bull calves, substitution of testosterone-human serum albumin with testosterone-horse serum albumin at the 5th booster of a monthly injection schedule resulted in titres that approximated initial responses. Antibody titres in control heifers for both androstenedione and oestradiol were <100 (reciprocal, see Table 1).

Body weight

There were no differences in body weights between treatment groups during the course of the study (Fig. 1) or at 14 months of age (Group C, 263 $\pm$ 4 kg, mean $\pm$ s.e.m.; Group A, 277 $\pm$ 4 kg; Group E, 279 $\pm$ 6 kg). The arrest of bodyweight gains between 7 and 9 months of age was probably due to a combination of weaning, transport to a new locality and the decline in pastures during summer.

LH and FSH concentrations

During the period from 3 to 9 months of age, heifers in Group E had higher ($P < 0.05$) mean plasma LH concentrations (0.89 $\pm$ 0.08 ng/ml) than did heifers in Group C (0.46 $\pm$ 0.03 ng/ml) and A (0.59 $\pm$ 0.05 ng/ml) which did not differ from one another (Fig. 2). This resulted mainly from consistently higher LH concentrations in Group E heifers between 5 and 8 months. There appeared to be transient rises in circulating LH in Group E heifers after booster injections, and similar responses may also have occurred in Group A heifers (Fig. 2). In contrast to LH, there were no differences between treatment groups in plasma FSH concentrations from 3 to 9 months (Group C, 12.3 $\pm$ 0.4 ng/ml; Group A, 12.6 $\pm$ 0.4 ng/ml; Group E, 12.1 $\pm$ 0.3 ng/ml).

Table 1. Antisteroid antibody titres (means $\pm$ s.e.m.) in heifers immunized against androstenedione (Group A; N = 11) or oestradiol (Group E; N = 10)

Age (months)	Week of treatment	Vaccination treatment	Antibody titre†	
			Group A	Group E
3	0	Primary	< 100	< 100
5	8	Boost 1	< 100	715 $\pm$ 333
	9		1126 $\pm$ 261	10357 $\pm$ 4067
	12		689 $\pm$ 185	4606 $\pm$ 1372
7	16	Boost 2	152 $\pm$ 58	3241 $\pm$ 1039
	17		836 $\pm$ 217	5139 $\pm$ 1463
	20		441 $\pm$ 107	2850 $\pm$ 646
9	24	Boost 3	208 $\pm$ 62	1633 $\pm$ 315
	25		2104 $\pm$ 725	5325 $\pm$ 1356
12	32	Boost 4	222 $\pm$ 211	1910 $\pm$ 1011
	33		1082 $\pm$ 358	4326 $\pm$ 1913
15	40	Boost 5	116 $\pm$ 46	1899 $\pm$ 1381
	41		405 $\pm$ 140	3598 $\pm$ 1511

†Titres are presented as reciprocal antisteroid antibody titres.

Fig. 1. Increases in body weights (means) in control heifers (Group C) and heifers actively immunized against androstenedione (Group A) or oestradiol (Group E) from 3 months of age.

Responses to LHRH

LH responses to exogenous LHRH at 10 months of age are shown in Fig. 3. Heifers in Group E showed a greater ($P < 0.05$) LH response (2.59 ± 0.56 ng/ml, Δ LH) than did heifers in Group C (0.61 ± 0.07 ng/ml) and Group A (1.04 ± 0.22 ng/ml) which did not differ from one another. The number of heifers with elevated (≥ 1 ng/ml) progesterone on the day of the LHRH tests and progesterone concentrations for these heifers were: Group C: 5/6, 1.9 ± 0.1 ng/ml; Group A: 4/6,

Fig. 2. Plasma LH concentrations (means $\pm$ s.e.m.) in control heifers (Group C) and heifers immunized against androstenedione (Group A) or oestradiol (Group E) from 3 months of age. When no s.e.m. is shown the variation was smaller than the size of the symbol.

Fig. 3. LH responses to i.v. injection of LHRH (5 ng/kg body weight), at 10 months of age in control heifers (Group C) and heifers immunized from 3 months of age against androstenedione (Group A) or oestradiol (Group E). Values are means $\pm$ s.e.m. for 6 heifers; when no s.e.m. is shown the variation was smaller than the size of the symbol.

6.4 ± 1.3 ng/ml; Group E: $4/6$, 2.0 ± 1.4 ng/ml. Differences between groups in LH responses to LHRH appeared, therefore, not to be related to differences in circulating progesterone.

Plasma progesterone and ovarian function

All heifers in Groups A and E had plasma progesterone concentrations ≥ 1.0 ng/ml at some stage during the study, whereas 2 heifers in Group C failed to show any signs of luteal activity. At 5 months of age 10 Group A heifers and 6 Group E heifers had elevated progesterone concentration compared with 2 Group C heifers (Fig. 4). At this time body weights were still relatively low (Group C, 140 ± 6 kg; Group A, 147 ± 4 kg; Group E, 148 ± 5 kg). At 7 months (directly after weaning and re-location) only 1 Group A heifer, and no Group C or Group E heifers, showed evidence of luteal tissue. Across the study greater ($P < 0.001$) numbers of Group A heifers showed

Fig. 4. The percentage of heifers with plasma progesterone concentrations ≥ 1.0 ng/ml in Group C (controls), A (immunized against androstenedione) and E (immunized against oestradiol). Vertical arrows indicate times of booster injections. W = weaning.

elevated progesterone concentrations compared with Group C heifers (Fig. 4). For all treatment groups there was a decrease ($P < 0.01$) with time in heifers showing cycles of progesterone secretion; however, at 14 months 7 Group A heifers had elevated progesterone compared with 2 Group C and 3 Group E heifers. A feature of the progesterone profiles was the consistently high plasma concentrations in Group A heifers (Fig. 5). All Group C and Group A heifers showing clear evidence of ovarian activity before synchronization at 15 months also had elevated plasma progesterone concentrations on the day before slaughter and, at slaughter, all these heifers had a corpus luteum. In contrast, the 3 heifers in Group E with elevated progesterone concentrations before cloprostenol did not have increased progesterone after synchronization, nor did they have corpora lutea at slaughter. Failure of these heifers to respond to synchronization may have been due to increased oestradiol antibody titres after booster immunization at the time of the first cloprostenol injection. It was not possible to obtain ovaries from all heifers at slaughter, but, from the tissues that were collected, Group A heifers tended to have increased numbers of 2–4 mm follicles.

Discussion

In the present study a high proportion of heifers immunized against androstenedione had elevated plasma progesterone concentrations as early as 5 and 6 months of age. In the absence of data for oestrous behaviour and actual ovulations it is not possible to conclude whether the heifers were pubertal at this time. At least two phases of increased progesterone secretion have been reported for heifers before puberty (Gonzalez-Padilla *et al.*, 1975; Berardinelli *et al.*, 1979) and it is thought that this progesterone derives from luteal tissue (possibly luteinized follicles) within the ovary (Berardinelli *et al.*, 1979). Irrespective of whether heifers were pubertal at 5 and 6 months, it is clear that immunization against androstenedione, and also oestradiol, influenced ovarian activity early in life. It is considered unlikely that there would have been differences between groups in ovarian

Fig. 5. Longitudinal profiles of plasma progesterone for individual control heifers (Group C) and heifers immunized against androstenedione (Group A) or oestradiol (Group E). Plasma progesterone concentrations > 3 ng/ml are depicted by broken vertical bars.

activity before 5 months of age since antisteroid antibody titres for both androstenedione and oestradiol were relatively low in response to primary immunization.

For the control and oestradiol-immunized groups the highest proportion of heifers with elevated progesterone values occurred at 8 months of age and there was a general decline thereafter up to 14 months. This might have been associated in both groups with a transition from prepubertal sources of progesterone to onset of cyclic ovarian activity. Androstenedione-immunized heifers showed a similar trend although there were always more heifers in this group (never < 60%) showing elevated progesterone concentrations compared with control and oestradiol-immunized heifers. Immunization against androstenedione was therefore associated with a sustained increase in ovarian activity. Immunization against oestradiol may facilitate early reproductive development, but after puberty a delicate balance is necessary to ensure that oestradiol antibody titres do not interfere with the roles of oestradiol in oestrous behaviour and in provoking the preovulatory LH surge (Martin *et al.*, 1978). This was exemplified in the present study by the failure to ovulate after receiving a booster injection and cloprostenol of 3 oestradiol-immunized heifers showing elevated progesterone values at 14 months.

The general decrease between 8 and 14 months in the proportion of heifers with elevated progesterone concentrations occurred at a time when photoperiod was also decreasing. Cattle are considered to be positively photoperiodic and young animals in particular seem sensitive to light (Hansen, 1985; Hansen *et al.*, 1983; Schillo *et al.*, 1983; Nelsen *et al.*, 1985). The apparent overall decline in ovarian activity after 8 months may therefore have been related to the decreasing photoperiod, although other unidentified endogenous or exogenous factors may also have been involved.

Previous evidence of a role for oestradiol in feedback on LH in prepubertal heifers has come from studies in which ovariectomy was combined with oestradiol replacement (Schillo *et al.*, 1982; Day *et al.*, 1984; see also Kinder *et al.*, 1987). The potential of ovarian steroids other than oestradiol to influence LH secretion could obviously not be gauged in these studies. In the present study LH concentrations were elevated in heifers immunized against oestradiol and these heifers also showed an increased LH response to exogenous LHRH. The particular significance of our observations was that an increase in LH occurred in oestradiol-immunized heifers even though other ovarian steroids were presumably available for feedback. We conclude, therefore, that oestradiol is the major ovarian steroid regulating LH secretion before puberty in the heifer. It is considered that group differences in LH responses to LHRH represented treatment effects, particularly since there was a significant difference in the LH response between control and oestradiol-immunized heifers, even though the respective numbers of heifers with elevated progesterone concentrations were similar. The rise in LH after oestradiol immunization was consistent with results of other studies which have demonstrated that the pituitary in prepubertal heifers can secrete more LH (McLeod *et al.*, 1984; MacDonald & Page, 1986; Skaggs *et al.*, 1986). Heifers secreted significantly more LH in response to exogenous LHRH, suggesting that in the prepubertal heifer feedback on LH might be directed primarily at the level of the hypothalamus (McLeod *et al.*, 1984). An effect of oestradiol on the pituitary should also be considered based on the increased LH response to exogenous LHRH in oestradiol-immunized heifers. However, an increased LH response could have resulted if the pituitaries of oestradiol-immunized heifers were tonically exposed to increased endogenous LHRH. In contrast to LH, FSH concentrations remained unchanged in oestradiol-immunized heifers. This provided further support for a role for inhibin in the prepubertal heifer (Johnson *et al.*, 1985; see also Findlay & Clarke, 1987). Immunization against androstenedione was not associated with any apparent long-term changes in LH, although transient increases may have occurred after booster injections.

A transient rise in circulating LH concentrations has been observed in heifers approaching puberty (Hansen *et al.*, 1983). In the present study all groups showed an increase in LH between 7 and 8 months of age and the highest incidence of heifers with elevated progesterone concentrations occurred at 8 months. The latter sequence of events may have been coincidental since it would appear unlikely that puberty should have been so closely synchronized.

Heifers immunized against androstenedione tended to show increased folliculogenesis. In mature heifers, increased follicular development was noted after passive (Sreenan, 1984) and active (D'Occhio *et al.*, 1986a) immunization against testosterone and active immunization against oestradiol (Martin *et al.*, 1978). Collectively these findings indicate that ovarian activity in cattle can be enhanced by immunization against both androgens and oestrogens. Immunization against oestradiol should, however, be approached with caution since it can lead to cystic follicles (Martin *et al.*, 1978; M. J. D'Occhio & D. R. Gifford, unpublished). In this regard, immunization against oestrone may prove more practical (Scaramuzzi & Hoskinson, 1984). The consistently high plasma progesterone concentrations in androstenedione-immunized heifers in the present study, and in androstenedione- (Wise & Schanbacher, 1983) and testosterone- (D'Occhio *et al.*, 1986a) immunized heifers in previous studies, provided further support for the conclusion that immunization against androgens enhances ovarian function in cattle. The mechanisms whereby steroid immunization results in enhanced ovarian activity have yet to be clearly defined. Changes in gonadotrophin secretion are thought to be involved (Scaramuzzi & Hoskinson, 1984; Campbell *et al.*, 1985) although direct intraovarian responses may also occur.

In summary, the present study has demonstrated that active immunization against ovarian steroids influences both LH secretion and ovarian function in prepubertal heifers. Early increases in ovarian activity in androstenedione-immunized heifers are maintained after puberty, suggesting that prepubertal immunization against androstenedione may confer some lifetime reproductive advantages in heifers.

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