Reproductive hormone secretion and testicular growth in bull calves actively immunized against testosterone and oestradiol-17 β

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Summary. Groups of bull calves received a primary immunization against testosterone (Group T; N = 7) or oestradiol-17 β (Group E; N = 9) at 3 months of age and booster injections on four occasions at ~2 month intervals. Controls (Group C, N = 7) 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 T (730 ± 231 ; reciprocal of titre) and Group E ($12\,205 \pm 4366$) bulls; however, peak antibody titres generally declined with successive booster injections. Mean plasma concentrations of LH, FSH and testosterone during the period from 3 to 10 months of age were higher ($P < 0.05$) in Group T bulls than in Groups C and E. Group T bulls had larger testes compared with controls from 6 months of age onwards. At castration at 14 months of age, testes of Group T bulls were heavier ($P < 0.05$) than those of Groups C and E (179 ± 13 , 145 ± 8 and 147 ± 6 g, respectively). At 10 months of age, there were no differences among treatment groups in LH responses to LHRH, but the testosterone responses were greater ($P < 0.05$) in bulls in Group T (26.2 ± 4.9 ng/ml) and Group E (16.6 ± 1.8 ng/ml) compared with those in Group C (6.9 ± 0.6 ng/ml). Testosterone responses to hCG determined at 13 months of age were also greater ($P < 0.05$) in Groups T and E relative to controls. At 14 months of age daily sperm production rates per bull ($\times 10^{-9}$) were higher ($P < 0.10$) in Group T bulls (2.2 ± 0.1) than those in Groups C (1.6 ± 0.2) and E (1.6 ± 0.1). These results indicate that early immunity against testosterone is associated with increased gonadotrophin secretion and accelerated growth of the testes in prepubertal bulls. Also, chronic immunity against testosterone or oestradiol-17 β enhances the steroidogenic response of bull testes to gonadotrophic stimulation. If the above responses observed in young bulls are shown to be sustained, then immunity against gonadal steroids early in life may confer some reproductive advantage in mature animals.

Introduction

Growth and maturation of the testes in prepubertal bulls are acutely dependent on gonadotrophic stimulation (Schanbacher, 1981; Schanbacher *et al.*, 1982). An increase in the frequency of pulsatile secretion of LH at around 3 months of age provides an endocrine signal which initiates testicular

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development in bull calves (Pelletier *et al.*, 1981; Amann & Walker, 1983; Schanbacher, 1984a). Coincident with testicular maturation there is elevated output of gonadal steroids which, in turn, imposes increasing feedback on LH secretion (Lacroix *et al.*, 1977; Lacroix & Pelletier, 1979). The serum LH profile in developing bull calves can therefore be described in general terms by low circulating concentrations during the first few months of life, a transient rise between 3 and 6 months of age, followed by relatively low concentrations until puberty (Pelletier *et al.*, 1981). Based on the temporal relationships between LH secretion and testis development, approaches aimed at accelerating pubertal maturation in bulls might include (1) advancing the transient increase in pulsatile secretion of LH which appears to initiate maturation of the testes, (2) amplifying the transient rise in LH, or (3) reducing the decline in LH associated with increased secretion of gonadal steroids. Follicle-stimulating hormone (FSH) is also considered important for testicular development (Courot *et al.*, 1984); however, circulating concentrations of FSH appear to show little change during the prepubertal period in bulls (McCarthy *et al.*, 1979; Amann & Walker, 1983; D'Occhio *et al.*, 1986).

Changes in the pattern of gonadotrophin secretion during various stages of development in prepubertal bulls are considered due, in part, to shifts in sensitivity of the hypothalamo-pituitary axis to gonadal steroid feedback (Amann & Walker, 1983). Immunization against gonadal steroids which participate in feedback mechanisms modifies their actions at the hypothalamus and anterior pituitary gland and alters the hormonal interrelationships between the gonads and the brain (Haynes & Southee, 1984; Scaramuzzi & Hoskinson, 1984). In the present study, bull calves were immunized against testosterone and oestradiol-17 β (oestradiol) in an attempt to influence, as a first step, the reproductive endocrinology of the prepubertal period.

Materials and Methods

Animals. Shorthorn bull calves were used in this study. Animals were maintained under field conditions and were weaned at 7 months of age. Body weights were determined at monthly intervals during the study. The diameter of each testis was also measured monthly using finely calibrated calipers.

Immunogens and immunization procedures. For immunization, testosterone-3-(O-carboxymethyl)oxime 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 (0.005 mM for testosterone-3-CMO; 0.01 mM for oestradiol-6-CMO). Conjugation occurred in phosphate buffer (0.05 M, pH 7.8) over 16 h at ambient temperature. The tetrahydrofuran was evaporated from the reaction mixture under reduced pressure at 40°C. The residue, comprising both soluble and insoluble products, was lyophilized and used as conjugate. Control bulls were immunized against human serum albumin alone. Steroid-protein conjugates were emulsified in Freund's complete adjuvant and administered subcutaneously along the brisket. Treatments included control (Group C, N = 7), testosterone-immunized (Group T, N = 7) and oestradiol-immunized (Group E, N = 9) bull calves. Bull calves received a primary immunization at 3 months of age and booster immunizations on 4 occasions at ~2 month intervals thereafter. The same amounts of immunogen (equivalent to 5 mg protein) and complete adjuvant were used for all immunizations.

Blood sampling and LHRH and hCG tests. Single blood samples were taken by jugular venepuncture weekly, but not always at the same time of day, to follow longitudinal changes in circulating concentrations of LH, FSH and testosterone. Pituitary and testicular responses after a bolus intravenous injection of native LHRH (5 ng/kg body-weight; Sigma, St Louis, MO, U.S.A.) were ascertained at 10 months of age for 6 bulls selected at random from each treatment group. On this occasion bulls 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. Response of the testes to hCG (3000 i.u.; Sigma) was determined at 13 months of age for the same animals that had received LHRH. Bulls were similarly equipped with jugular vein cannulae and blood samples were taken 20 min before injection of hCG and at 20-min intervals for 3 h after injection. Additional blood samples were taken by jugular venepuncture on Days 1, 2, 3, 4, 8 and 15 after hCG.

Testicular and epididymal weights and daily sperm production rates. At 14 months of age all bulls were castrated and testicular and epididymal weights recorded. Samples of testicular parenchyma were fixed in glutaraldehyde buffer (Forssmann *et al.*, 1977) for determination of daily sperm production (DSP) rates using homogenization-haemocytometric techniques (see Amann, 1970). Samples (20–25 mg) of testicular parenchyma were minced for 3 min in 4 ml homogenizing fluid (Johnson *et al.*, 1981) using a Janke and Kunkel Ultra-Turrax fitted with a N8 probe. A time

divisor of 5.32 days was used to calculate DSP from the number of spermatid nuclei in testicular homogenates (Amann *et al.*, 1974).

Antibody titres and hormone assays. Circulating antibody titres were determined using procedures outlined in detail by Abraham (1974). Antibody titres for the respective steroid were taken as the plasma dilution which bound 50% of a constant mass of radiolabelled steroid (≈ 10 pg; $\approx 20\,000$ d.p.m.). Background binding, represented by the dilution of plasma from non-immunized bulls which bound 50% of radiolabelled steroid, was around 1:10 for both testosterone and oestradiol. Serum concentrations of LH and FSH were determined by double-antibody radioimmunoassays previously described (D'Occhio *et al.*, 1986). An extraction single-antibody radioimmunoassay was used to measure plasma testosterone concentrations (D'Occhio & Brooks, 1983). Vigorous extraction of samples into a toluene:hexane (2:1 v/v) cocktail ensured that anti-testosterone antibody titres did not influence recoveries and that total testosterone was measured. 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 testosterone/ml.

Statistical analyses. Data were analysed by one-way or two-way analysis of variance procedures. Variables included treatment group, animals within treatment and, when appropriate, time. When necessary data were transformed to natural logarithms before analysis. If the analysis of variance showed significant effects then differences amongst treatment groups were assessed using the least significant difference test (Sokal & Rohlf, 1969).

Results

Antibody titres

Antibody titres were relatively low in bulls in Groups T and E (Table 1), but were of the same order of magnitude as reported previously for cattle (Wise & Schanbacher, 1983; Sreenan, 1984). Successive booster injections failed to induce titres that exceeded the initial secondary response. Also, booster injections induced a transient rise in antibody levels which was followed by a prompt decline in titres (see results for Weeks 8, 9 and 12 in Table 1).

Table 1. Antisteroid antibody titres (mean $\pm$ s.e.m.) in bulls immunized against testosterone (Group T; N = 7) or oestradiol (Group E, N = 9)

Age (months)	Week of treatment	Vaccination treatment	Antibody titre†	
			Group T	Group E
3	0	Primary	0	0
5	8	Boost 1	96 $\pm$ 42	1235 $\pm$ 520
	9		730 $\pm$ 231	12205 $\pm$ 4366
	12		396 $\pm$ 124	7690 $\pm$ 2828
7	16	Boost 2	243 $\pm$ 101	2017 $\pm$ 547
	17		306 $\pm$ 160	2453 $\pm$ 732
9	24	Boost 3	65 $\pm$ 26	1065 $\pm$ 329
	25		182 $\pm$ 61	1847 $\pm$ 641
12	32	Boost 4	55 $\pm$ 36	779 $\pm$ 245
	33		216 $\pm$ 117	967 $\pm$ 318

†Titres are presented as reciprocal antisteroid antibody titres.

Body weight

There were no differences in body weights between treatment groups for the duration of the study (Fig. 1). There was a decrease in bodyweight gains associated with weaning at 7 months and the decline in body weights observed from 9 to 11 months of age for all groups resulted from poor quality summer pastures.

Fig. 1. Increases in bodyweights (means) and testicular diameter (mean $\pm$ s.e.m.) in control bulls (Group C) and bulls actively immunized against testosterone (Group T) or oestradiol (Group E) from 3 months of age. For testicular diameter, when no s.e.m. is shown the variation was smaller than the size of the symbol. * $P < 0.05$ compared with values for Group C bulls.

Table 2. Plasma concentrations of LH, FSH and testosterone in control bulls (Group C) and bulls actively immunized against testosterone (Group T) and oestradiol (Group E)

Group	No. of bulls	LH (ng/ml)	FSH (ng/ml)	Testosterone (ng/ml)
C	7	0.75 ± 0.07^a	14.7 ± 0.3^a	3.9 ± 0.3^a
T	7	1.13 ± 0.09^b	15.9 ± 0.3^b	21.2 ± 1.3^b
E	8	0.88 ± 0.07^a	14.5 ± 0.3^a	6.6 ± 0.5^a

Values are mean $\pm$ s.e.m. hormone concentrations during the period from 3 to 10 months of age.

Within columns, means without a common superscript differ ($P < 0.05$; ANOVA, least significant difference test).

LH, FSH and testosterone concentrations

During the period from 3 to 10 months of age, bulls in Group T had higher ($P < 0.05$) mean plasma LH concentrations than did those in Groups C and E (Table 2). On a weekly basis, LH concentrations were consistently higher ($P < 0.05$) in Group T bulls only between 5 and 6 months of age. Plasma FSH did not differ amongst treatment groups at any single sampling time, but over the period from 3 to 10 months of age mean FSH concentrations were higher ($P < 0.05$) in Group T bulls than in those in Groups C and E (Table 2). Profiles of testosterone from 3 to 10 months of

age are shown in Fig. 2. Testosterone concentrations in control bulls showed a gradual rise between 3 and 6 months of age and remained relatively constant thereafter. In Group T bulls, testosterone increased sharply to reach maximum serum concentrations between 5 and 6 months of age, and then showed a progressive decline. A similar but significantly less pronounced trend was observed in Group E bulls. During the period from 3 to 10 months mean testosterone concentrations were higher ($P < 0.001$) in Group T bulls than in those in Groups C and E (Table 2).

Fig. 2. Serum testosterone concentrations (mean $\pm$ s.e.m.) in control bulls (Group C) and bulls actively immunized against testosterone (Group T) 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.

Table 3. Measurements in 14-month-old control bulls (Group C), and bulls actively immunized against testosterone (Group T) or oestradiol (Group E)

Group	No. of bulls	Body wt (kg)	Testis diam. (mm)	Testis wt (g)	Epididymal wt (g)	Daily sperm production	
						Per gram testis parenchyma ($\times 10^{-6}$)	Per bull† ($\times 10^{-9}$)
C	7	229 $\pm$ 4 ^a	49 $\pm$ 1 ^a	145 $\pm$ 8 ^a	12.0 $\pm$ 0.5 ^a	6.3 $\pm$ 0.9 ^a	1.6 $\pm$ 0.2 ^c
T	7	230 $\pm$ 4 ^a	55 $\pm$ 1 ^b	179 $\pm$ 13 ^b	12.2 $\pm$ 1.1 ^a	6.9 $\pm$ 0.4 ^a	2.2 $\pm$ 0.1 ^d
E	8	219 $\pm$ 8 ^a	48 $\pm$ 1 ^a	147 $\pm$ 6 ^a	10.5 $\pm$ 0.5 ^a	6.4 $\pm$ 0.4 ^a	1.6 $\pm$ 0.1 ^c

Values are means $\pm$ s.e.m.

Within columns, means without a common superscript differ (^{a,b} $P < 0.05$; ^{c,d} $P < 0.10$; ANOVA, least significant difference test).

†Tunica albuginea was assumed to constitute 10% of weight of the testis (see Amann & Almquist, 1962).

Testicular size, testicular and epididymal weights, and daily sperm production rates

From 6 months of age onwards, Group T bulls had larger ($P < 0.05$) testes than did Group C bulls (Fig. 1). Testicular diameter in Group E bulls was intermediate between that of bulls in Groups T and C from 6 to 10 months. At castration at 14 months, testes of Group T bulls were heavier ($P < 0.05$) than those of bulls in Groups C and E which did not differ from one another (Table 3). Epididymal weights did not differ amongst treatment groups (Table 3). There were also no differences amongst treatment groups in DSP rates per unit weight of testicular parenchyma. However, when DSP was calculated for both testes, bulls in Group T had a higher ($P < 0.10$) DSP rate compared with those in Groups C and E (Table 3). Daily sperm production rates across all treatments were generally low for 14-month-old bulls; however, this was associated with low body weights and it can be concluded that both DSP and bodyweight were adversely influenced by the poor pasture conditions which prevailed during summer.

Responses to LHRH and hCG

LH and testosterone concentrations after administration of LHRH are presented in Table 4. There were no differences amongst treatment groups in LH responses to LHRH although the Δ LH values tended to be greater in Group E animals. Increases in testosterone in the 2 h after LHRH were lower ($P < 0.05$) in Group C than in Group T and Group E bulls which did not differ from

Table 4. LH and testosterone responses after bolus intravenous injections of LHRH (5 ng/kg bodyweight), at 10 months of age and hCG (3000 i.u.) at 13 months of age, in control bulls (Group C) and bulls actively immunized against testosterone (Group T) or oestradiol (Group E)

Group	No. of bulls	LHRH		hCG	
		LH (0–20 min)	Testosterone (0–2 h)	Testosterone	
				0–3 h	Day 4
C	6	4.1 ± 0.3 ^a	6.9 ± 0.6 ^a	8.4 ± 1.5 ^a	13.4 ± 2.8 ^a
T	6	3.7 ± 0.4 ^a	26.2 ± 4.9 ^b	31.9 ± 6.7 ^b	37.0 ± 4.1 ^b
E	6	5.3 ± 0.9 ^a	16.6 ± 1.8 ^b	20.5 ± 2.7 ^b	20.5 ± 1.9 ^a

Values represent peak hormone concentrations during the respective times after injection of LHRH or hCG and are presented as means ± s.e.m. Within columns, means without a common superscript differ ($P < 0.05$; ANOVA, least significant difference test).

Fig. 3. Testosterone responses to a bolus intravenous injection of hCG (3000 i.u. arrow) in control bulls (Group C) and bulls immunized from 3 months of age against testosterone (Group T) or oestradiol (Group E). Values are mean ± s.e.m. for 6 bulls; when no s.e.m. is shown the variation was smaller than the size of the symbol.

one another. Increases in circulating concentrations of testosterone after intravenous injection of hCG are depicted in Fig. 3 and the results are summarized in Table 4. Acute testosterone responses to hCG (0 to 3 h) were lower ($P < 0.05$) in Group C bulls than in those in Groups T and E which did not differ from one another. For all treatment groups, testosterone concentrations continued to increase between Days 1 and 4 after administration of hCG, suggesting that maximal secondary responses may have occurred between Days 4 and 8. By Day 8 testosterone concentrations had begun to decline. Nevertheless, by 4 days after hCG injection testosterone concentrations in Group T bulls were higher ($P < 0.05$) than in those in Groups C and E which did not differ from one another.

Discussion

The present study sought to determine whether active immunization against gonadal steroids early in life may provide an approach for influencing the reproductive endocrinology of the prepubertal period in the bull. It was found that bull calves actively immunized against testosterone had higher circulating concentrations of LH, FSH and testosterone during the prepubertal period and these animals also showed increased rates of testicular growth. We suggest that immunization against testosterone reduced feedback on the hypothalamo-pituitary axis resulting in increased gonadotrophic stimulation of the testes (Nieschlag & Wickings, 1978). Differences in LH concentrations between testosterone-immunized and control bulls were most apparent between 5 and 6 months and less evident thereafter. Similar transient changes in LH concentrations in the face of sustained high antibody titres have previously been demonstrated in rabbits (Thornycroft *et al.*, 1975) and monkeys (Wickings & Nieschlag, 1978) immunized against testosterone. It is possible that, with continued testosterone immunity, circulating concentrations of metabolites of testosterone such as 5 α -dihydrotestosterone and oestradiol might increase, and these metabolites may in turn assume increased importance in feedback on LH (Wickings & Nieschlag, 1978). Both 5 α -dihydrotestosterone and oestradiol suppress gonadotrophin secretion in the bull (Gettys *et al.*, 1984). The results of the present study agree with other findings for monkeys (Wickings & Nieschlag, 1978), rams (Schanbacher, 1982), rabbits (Thornycroft *et al.*, 1975), and rats (Hillier *et al.*, 1975), but contrast with the results of Walker *et al.* (1984) who observed no increases in gonadotrophin secretion in prepubertal bulls immunized against testosterone. An unexpected decrease in plasma concentrations of LH and FSH was observed in prepubertal boars actively immunized against testosterone (Thompson *et al.*, 1985). The lack of effect of oestradiol immunization on LH concentrations observed in the present study was consistent with results of oestradiol immunization in the rat (Hillier *et al.*, 1975) and oestrone immunization in the stallion (Thompson & Honey, 1984). The latter findings do not necessarily exclude a role for oestrogens in control of gonadotrophins in the male since feedback would presumably be maintained in oestrogen-immunized individuals by normal or slightly elevated testosterone concentrations. However, Schanbacher (1984b) reported increased circulating concentrations of both LH and testosterone in rams actively immunized against oestradiol.

The initial sharp rise in plasma testosterone concentrations between 5 and 6 months in bulls immunized against testosterone, followed by a gradual decline, also was of interest. Such a testosterone pattern may have been related to a general decline in peak antibody response to booster immunization. Testosterone concentrations are a function of secretion and clearance rates, the latter being influenced by testosterone binding capacity in plasma (Wickings *et al.*, 1976). A direct relationship between circulating antibody titres and testosterone might therefore be anticipated. Similar longitudinal changes in circulating testosterone in response to testosterone immunization have also been observed in developing rams (Haynes & Southee, 1984).

Increased rates of testicular growth in testosterone-immunized prepubertal animals similar to those observed in the present study have been reported for bulls (Walker *et al.*, 1984) and rams (Haynes & Southee, 1984) but were not observed for boars (Thompson *et al.*, 1985). In prepubertal

stallions, increased testicular growth was induced by active immunization against oestrone (Thompson & Honey, 1984) whilst passive immunization against oestrone and oestradiol produced the same responses in young rams (Land *et al.*, 1981). From studies of adult rats (Hillier *et al.*, 1975) and rabbits (Nieschlag *et al.*, 1975) it would appear that increased testicular size in testosterone-immunized animals involves both hyperplasia and hypertrophy of the interstitial tissue.

The testes of bulls immunized against testosterone secreted larger amounts of testosterone than did those of controls in response to administration of both LHRH and hCG. This was no doubt due, in part, to the larger testicular size of these animals and possible proliferation of the Leydig cells. However, it is also likely that testes of such bulls had an increased sensitivity to gonadotrophin as has been demonstrated for immunized rabbits (Nieschlag *et al.*, 1975). It could also be argued that increased testosterone concentrations in testosterone-immunized bulls after LHRH and hCG injection were due to greater plasma binding capacity and decreased testosterone clearance rate in these animals (see Thompson *et al.*, 1985). However, the initial sharp rise in plasma testosterone in such bulls in response to gonadotrophic stimulation argues for an increased capacity for testosterone secretion. Bulls immunized against oestradiol also secreted more testosterone compared with controls in response to LHRH and hCG. Testicular size of oestradiol-immunized bulls was similar to that of controls, suggesting different mechanisms for increased sensitivity to gonadotrophin between bulls immunized against oestradiol and testosterone. Oestrogens have been proposed as having a direct negative influence on steroid biosynthesis in the testes of some species (see D'Occhio *et al.*, 1984). Immunity against oestradiol may therefore alter possible direct actions of this steroid within the bovine testis, allowing increased steroidogenic responses to gonadotrophin input. Unstimulated testosterone concentrations were elevated in oestradiol-immunized rats (Hillier *et al.*, 1975) and rams (Schanbacher, 1984b) and oestrone-immunized young stallions (Thompson & Honey, 1984), and a slight increase in circulating testosterone was observed in the bulls immunized against oestradiol in the present study.

In summary, the present study has demonstrated that active immunity against testosterone early in life increases gonadotrophin secretion in the bull and accelerates growth of the testes. Whether this approach can be used to enhance overall reproduction performance of mature bulls remains to be determined. However, encouraging observations were that total daily sperm production rates are increased in young testosterone-immunized bulls and there are no effects of this treatment on the weight of the epididymis, an androgen-dependent organ important for sperm maturation and storage. The latter observation was consistent with normal seminal characteristics reported for young testosterone-immunized bulls (Walker *et al.*, 1984). Since immunity against gonadal steroids appears not to diminish sexual behaviour in the male (Haynes & Southee, 1984), it is possible that immunity against testosterone early in life may confer some lifetime reproductive advantages in the bull.

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