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Effect of active immunization against testosterone on plasma gonadotrophin concentrations, spermatogenic function, testicular blood flow, epididymis mass and mating behaviour in adult rams

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The long-term effects of active immunization against testosterone were studied in rams, with particular reference to blood concentrations of gonadotrophin and testosterone, spermatogenesis, testis blood flow and mating behaviour. Ten 18-month-old Merino rams, kept on pasture, were studied for 1 year. Every 2 months, five rams received injections of BSA in Freund's adjuvant and five other rams were treated with testosterone-3(*o*-carboxymethyl)oxime–BSA as immunogen. Anti-testosterone antibodies (mean titre: 1:4484 ± 582, after boosters) were maintained in the circulation, with the help of regular booster injections. In time, immunization reduced live mass in testosterone-immunized rams; however, there was no effect on testicular volume throughout the whole study. In testosterone-immunized rams, significantly higher concentrations of gonadotrophins were found in jugular venous plasma, as well as increased concentrations of total plasma testosterone. LH pulse frequency, amplitude and nadir were increased significantly in testosterone-immunized rams. After 12 months of immunization, no differences were found in the number of spermatozoa per ejaculate, in daily sperm production or in testis mass between the two groups of rams; however, testicular blood flow (per testis) and epididymis mass were significantly reduced in testosterone-immunized rams. Testosterone immunoneutralization also resulted in a significant reduction in the number of mounts culminating in ejaculation performed during a 10 min trial carried out on a number of occasions during the experiment. Additional information on these rams was collected 3 months after castration. However, there were no significant differences in mean plasma LH and FSH concentrations, either before, or after, a single GnRH injection between the two groups of rams at this time.

Introduction

It has been suggested that immunity against testosterone early in life may confer lifetime reproductive advantages to bulls (D'Occhio *et al.*, 1987). Daily sperm production increases in young bulls actively immunized against testosterone, without negative effects on the epididymis mass, and other androgen-dependent organs important for sperm maturation and storage. Similarly, an increase in sperm production and normal seminal characteristics have been reported for young bulls immunized against testosterone (Walker *et al.*, 1984). In ram lambs, a decreased anabolic effect with no improvement in testicular mass was described by Schanbacher (1982). Immunoneutralization of testosterone did not diminish sexual behaviour in young rams (Haynes and Southee, 1984) or in young boars (Thompson *et al.*, 1985). However, adverse effects of this treatment, such as loss of sexual activity (Nieschlag and Kley, 1974; Nieschlag and Wickings, 1977, 1978) and atrophy of accessory reproductive glands (Hillier *et al.*, 1975a, b; Nieschlag *et al.*, 1975), have been reported in laboratory animals.

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However, no long-term studies using active immunization against testosterone in adult rams have been reported. We therefore carried out an experiment for 1 year with Merino rams to determine whether sperm production could be improved by increasing gonadotrophin secretion induced by immunoneutralization of testosterone. Various components of the male reproductive system (sexual behaviour, testicular blood flow and epididymal mass) were also evaluated to determine whether the treatment affected these variables.

Materials and Methods

Animals

Eighteen-month-old Merino rams were kept on pasture throughout the year and received cereal hay (*ad libitum*) and sheep commercial pellets (approximately 150 g day^{-1}) as supplement during the dry season (February–May). The main study started in August 1988 and ended in July 1989. Additional information on these rams was collected in October 1989, 3 months after castration. Live mass and testicular

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volume were recorded every month until November 1988; measurements were then taken every two months. Assuming that the testis is a prolate spheroid, testicular volume was calculated according to the formula $1/6\ \pi\ a^2b$ (where a = largest width and b = length of the testis) (Setchell and Waites, 1964).

Immunization procedure

Five rams (controls) received a primary injection of BSA in Freund's complete adjuvant (FCA) (Commonwealth Serum Laboratories, Melbourne) in August. Five rams (testosterone-immunized) received a primary injection of testosterone-3(*o*-carboxymethyl)oxime–BSA conjugate (obtained from R. I. Cox, CSIRO, Division of Animal Production, Prospect, NSW) in FCA. The molar ratio of steroid to protein was 18:1 for this conjugate. All rams received a booster injection in November. Additional boosters were given in January, March, May and July. Freund's incomplete adjuvant (Commonwealth Serum Laboratories, Melbourne) was used instead of FCA for each booster injection. A booster injection was also given in October 1989, approximately 10 weeks after castration. For each immunization, 1 mg antigen was emulsified in 2 ml saline:adjuvant (1:1 v:v) and was injected into each ram at two s.c. or i.d. sites under each fore and hind limb.

Antibody titre

Blood samples were collected from each ram immediately before and 14 days after each immunization. The titre was determined by incubating 100 µl of various dilutions of plasma (from 1:100 to 1:10 000) with 100 µl of [1,2,6,7-^{3}H] testosterone (approximately 37 pg $(100\ \mu l)^{-1}$) and 100 µl of 0.05 mol PBS l^{-1} plus 0.2% (w/v) gelatin overnight at 4°C. Free and bound hormone were separated as described by Auclair *et al.* (1995). The titre was defined as the dilution that bound 50% of the radiolabelled testosterone.

Blood collection and hormone assays

Before immunization, the rams were bled intensively (every 20 min for 6 h). These bleedings were repeated 14 days after each booster injection. On the day before sampling, rams were kept indoors in individual pens and one jugular vein was cannulated with an indwelling polyethylene cannula (1.5 mm o.d., 1.0 mm i.d.). On these occasions, the rams were fed with sheep commercial pellets (approximately 300 g day^{-1}) and lucerne hay (approximately 100 g day^{-1}). Mean hormone concentrations were determined by assaying a plasma pool representing the 6 h sampling period. Radioimmunoassay procedures for LH, FSH, prolactin and testosterone, sensitivity and coefficients of variation are as described by Auclair *et al.* (1995). Extraction of samples into toluene:hexane (2:1 v:v) ensured that anti-testosterone antibody titres did not influence recoveries and that total testosterone was measured.

Characterization of LH profiles

Analyses of LH profiles were performed as described by Auclair *et al.* (1995), using a computer algorithm program (Munro; Elsevier-BIOSOFT, Cambridge, UK).

Pituitary responsiveness to GnRH

Pituitary function was assessed by monitoring the response of the anterior pituitary gland to an i.v. bolus of GnRH (5 ng kg^{-1} body mass) (Sigma Chemical Co., St Louis, MO). GnRH was administered immediately after the intensive bleedings were carried out in November, March and July. Blood sampled 10, 20, 30, 40, 60, 90 and 120 min after the injection was assayed for LH. In addition, in October 1989 (approximately 12 weeks after castration and 2 weeks after the final booster), castrated rams were bled at 10 min intervals for 4 h after which they received a single GnRH injection (5 ng kg^{-1} body mass). Additional blood samples were collected at intervals of 10 min during the following 2 h. Pool samples collected before and after the GnRH injection were assayed for LH.

Testicular responsiveness to hCG

Immediately after the intensive bleeding carried out in January, testicular responsiveness to a single hCG injection (20 iu kg^{-1} body mass) (Sigma Chemical Co.) was evaluated (in conscious rams). This dose was chosen because it can produce a maximal short-term testosterone response in male sheep (Chandrasekhar *et al.*, 1985). Blood samples were collected at 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after the hCG injection. Pool samples collected before and after the hCG injection were assayed for testosterone.

Total testicular blood plasma flow, testosterone production and response to hCG challenge

Before castration, all rams were anaesthetized using sodium pentobarbitone (Nembutal; Abbott, Ceva Chemicals Australia Pty, Ltd, Hornsby, NSW). Total testicular blood plasma flow, testosterone production and response to a single hCG injection (20 iu hCG kg^{-1} body mass) (Sigma Chemical Co.) were measured as described by Auclair *et al.* (1995).

Libido trials

The rams had not been used previously as flock sires and were considered inexperienced at the start of the experiment. Sexual activity was determined by recording the number of times that each of the following aspects of behaviour – sniffs, nudges, mounts and intromissions followed by ejaculations – was displayed by each ram during a 10 min exposure to a teaser ewe. These components of mating behaviour were described by Banks (1964) and have been recorded in other libido trials (D'Occhio and Brooks, 1980; D'Occhio *et al.*, 1985). Each trial was carried out in a large yard (5.0 m × 8.5 m) adjacent to a pen where the rams were temporarily held. The teaser (non-ovariectomized) ewe was secured in a collection bail (as used for semen collection with artificial vagina) that was placed in one corner of the service yard. Oestrus was induced in the teaser ewe by daily i.m. injections of 50 mg oestradiol benzoate in 1 ml of peanut oil. This treatment was necessary only for the first (October) and second (January) libido trials as by the third (June) and fourth (July) trials most rams

were mounting the teaser ewes even when not treated with hormones.

Semen collection and evaluation

Semen was collected on four occasions (2 November, 29 May, 4 July and 11 July) with an artificial vagina (Evans and Maxwell, 1987). On these occasions, rams that did not mount the teaser ewe were not included in the study (two testosterone-immunized rams in the first collection and one testosterone-immunized ram in the second, third and fourth collections). The same method was used for the final collection (26 July) except for two rams (one control and one testosterone-immunized) that did not mount the teaser ewe at that time; semen was collected from these rams, by electro-ejaculation. Rams were trained for the artificial vagina method of collection a few weeks before the first collection in October and at various occasions between January and May. The volume of ejaculate was recorded for each ram and the ejaculate was examined under a light microscope. The number of spermatozoa per unit volume of ejaculate was estimated using a haemocytometer.

Castration

All rams were castrated at the end of July 1989. The masses of the epididymis and testes were recorded. Daily sperm production was estimated using homogenization–haemocytometric techniques (Amann, 1970). Small blocks of testicular tissue were fixed in Bouin's solution for 24 h and then transferred to 70% ethanol. Tissues were embedded in paraffin wax and sections, 7 µm thick, were cut and stained with haematoxylin and eosin. Specimens were examined under a microscope to detect abnormalities in the general appearance of the seminiferous tubules and interstitial tissues.

Statistical analyses

Data for characteristics involving repeated observations over time were analysed by analysis of variance for repeated measures. Other variables were analysed using a one-factor analysis of variance to localize differences among, and within, groups. Appropriate transformations of the data were performed whenever necessary. Pairwise comparisons of the means were made using Student's t test (Steel and Torrie, 1980).

Results

Antibody titres

All testosterone-immunized rams responded well to the immunization protocol used. Titres ranged between 1:75 and 1:750 before the first booster; mean titres were 1:1340 ± 234 immediately before the last five boosters and 1:4484 ± 582 14 days after all boosters. The percentage binding of testosterone was negligible (< 5% in plasma diluted 1:100) in controls throughout the experiment.

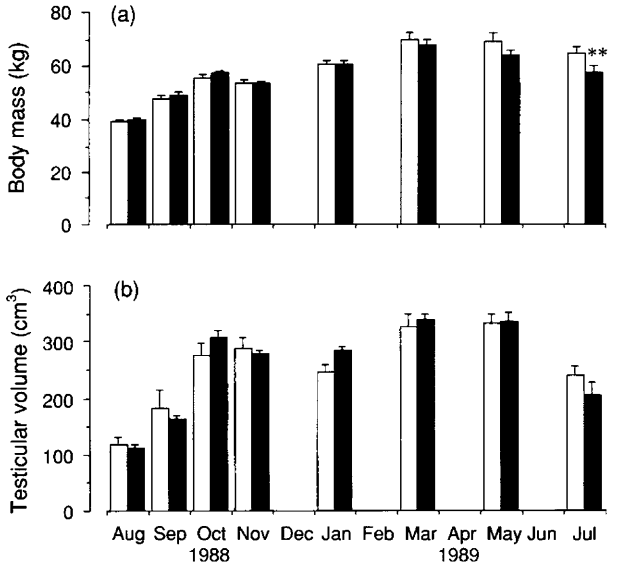

Fig. 1. Changes (a) in body mass (kg ± SEM) and (b) in testicular volume (cm^3 ± SEM) from August 1988 to July 1989 in adult rams actively immunized against (□) BSA ($n = 5$) or against (■) testosterone-3–BSA ($n = 5$). Significantly different from controls $^{**}P < 0.01$.

Live mass and testicular volume

Analysis of variance showed a significant ($P < 0.001$) interaction between immunization and time on live mass (Fig. 1a). There was an increase in live mass from 18 to 24 months of age in both groups of rams, followed by a slight decrease, that was more pronounced in the testosterone-immunized rams than in controls. Thus, by July, live mass was significantly reduced in testosterone-immunized rams compared with controls ($P < 0.01$). Testicular volume was not affected by immunization throughout the experiment; however, there was a significant time effect on this variable ($P < 0.001$) (Fig. 1b). Testicular volume increased during the first three months of study and decreased between May and July, in both groups of rams.

Testosterone concentrations

No significant difference in plasma testosterone concentrations was found between the two groups of rams before treatment. Mean testosterone concentrations were significantly affected by immunization ($P < 0.001$) and time ($P < 0.02$) (Fig. 2a). In controls, mean testosterone concentrations were higher between January and May than during the other months. In testosterone-immunized rams, concentrations of testosterone did not vary significantly with time (Fig. 2a).

FSH concentrations

No significant difference in plasma FSH concentration was found between the two groups of rams before treatment. Interaction between immunization and time significantly affected mean plasma FSH concentrations ($P < 0.05$). Mean FSH concentrations were significantly higher in testosterone-immunized rams than in controls, and differences between

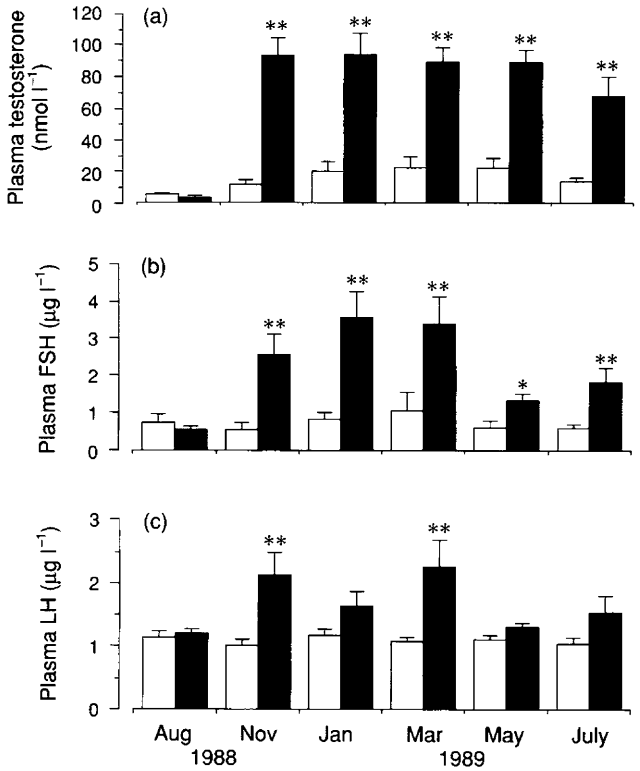

Fig. 2. Mean hormonal concentration (± SEM) in jugular plasma for (a) testosterone, (b) FSH and (c) LH at various occasions in adult rams immunized against (□) BSA ($n = 5$) or against (■) testosterone-3–BSA ($n = 5$). Significantly different from controls $^{*}P < 0.05$; $^{**}P < 0.01$.

means were more pronounced between November and March than during the other months. In controls, mean FSH concentrations did not vary with time (Fig. 2b).

LH concentrations

Mean plasma LH concentrations and LH pulse characteristics were not significantly different between the two groups of rams before treatment (Fig. 2c and Table 1). Interaction between immunization and time significantly affected mean LH concentrations, the number of LH peaks per 6 h sampling period, mean pulse interval and mean pulse amplitude ($P < 0.01$, for all variables). In November and March, mean LH concentrations and the number of LH peaks increased significantly in testosterone-immunized rams compared with controls, while mean pulse interval was significantly reduced ($P < 0.001$, for all variables). In November, mean pulse amplitude was also significantly increased in testosterone-immunized rams ($P < 0.001$). Immunization significantly increased mean pulse nadir ($P < 0.001$). In controls, in which low pulse frequency was observed, mean LH concentrations did not vary significantly with time, although all the other LH pulse characteristics did ($P < 0.05$, for all variables).

Prolactin concentrations

Plasma prolactin concentrations were not significantly different between the two groups of rams before treatment. Mean plasma prolactin concentrations were not affected by immunization; however, they were significantly affected by time ($P < 0.001$). The lowest prolactin concentrations (average: 71.2 ± 7.1 μg l^{-1}) were found during the short-day period (in May, July and August) and the highest concentrations (average: 217.2 ± 11.8 μg l^{-1}) during the long-day period (in January and March).

Pituitary responsiveness to a GnRH challenge

All rams responded to each GnRH injection. Plasma LH concentrations following a GnRH challenge, in November (Fig. 3a) and in March (Fig. 3b), were significantly affected by immunization ($P < 0.01$, for both months) and by duration ($P < 0.001$, for both months). In July (Fig. 3c), plasma LH concentrations associated with the response curve were not significantly affected by immunization but were affected by duration ($P < 0.001$). In testosterone-immunized rams, pituitary responsiveness varied significantly between GnRH challenges ($P < 0.001$), while, in controls, LH responses were not significantly different. Indeed the LH response, in testosterone-immunized rams, was more pronounced in November than in March, and more pronounced in March than in July. The maximal increase in circulating LH was generally observed 10 min after the GnRH injection and LH concentrations returned to pretreatment values within 120 min.

Testicular responsiveness to an hCG challenge in January

In January, mean testosterone concentrations measured in jugular plasma of conscious rams (pool samples) were significantly increased by immunization ($P < 0.01$) and by the hCG injection ($P < 0.001$) (Table 2).

Total testicular blood plasma flow, testosterone production and response to hCG challenge in July

Blood flow was measured successfully in 18 testes (nine testes from five controls and nine testes from five testosterone-immunized rams) in July. Total testicular blood plasma flow per testis (ml min^{-1}) or per unit mass of testis (μl g^{-1} min^{-1}) was not significantly affected by hCG injection (Fig. 4). There was a significant immunization effect on total testicular blood plasma flow per testis ($P < 0.05$) (Fig. 4a); however, when this variable was expressed per unit mass of testis (Fig. 4b), the difference was not significant. Analysis of variance for each of these two variables did not indicate a significant interaction between immunization and time.

At the end of July, mean testosterone production per testis and mean testosterone concentrations measured in jugular plasma of anaesthetized rams (pool samples) were significantly affected by immunization ($P < 0.001$, for both) and by the hCG challenge ($P < 0.001$; $P < 0.08$, respectively) (Table 2). When compared with testosterone responses obtained in January in conscious rams, a significant time effect ($P < 0.01$) was found. The testosterone response was significantly less pronounced in July than in January in testosterone-immunized rams ($P < 0.001$), although testosterone concentrations in the jugular were comparable before the hCG injection (Table 2). In

Table 1. LH pulse characteristics in control ($n = 5$) and testosterone-immunized ($n = 5$) rams at various times

Month	Mean LH ($\mu g\ l^{-1}$)	Number of peaks in 6 h	Pulse interval (min)	Pulse amplitude ($\mu g\ l^{-1}$)	Pulse nadir ($\mu g\ l^{-1}$)
Control					
August	1.14 ± 0.09	0.4 ± 0.3	360.0 ± 0.0	0.78 ± 0.48	1.38 ± 0.08
November	1.02 ± 0.09	0.6 ± 0.3	360.0 ± 0.0	0.48 ± 0.27	1.01 ± 0.06
January	1.18 ± 0.09	1.4 ± 0.5	294.0 ± 42.9	0.62 ± 0.18	0.74 ± 0.08
March	1.07 ± 0.09	0.6 ± 0.3	360.0 ± 0.0	0.44 ± 0.19	0.32 ± 0.10
May	1.11 ± 0.08	1.6 ± 0.3	228.0 ± 56.4	1.09 ± 0.09	0.42 ± 0.13
July	1.06 ± 0.09	2.0 ± 0.6	198.7 ± 67.2	1.44 ± 0.57	0.85 ± 0.19
Testosterone-immunized					
August	1.22 ± 0.06	0.4 ± 0.3	360.0 ± 0.0	0.26 ± 0.19	1.37 ± 0.10
November	2.13 ± 0.34**	3.2 ± 0.6**	113.3 ± 17.3**	2.06 ± 0.34**	1.65 ± 0.29*
January	1.65 ± 0.20	2.2 ± 0.6	215.3 ± 59.7	1.09 ± 0.13	1.12 ± 0.03
March	2.25 ± 0.43**	3.2 ± 0.6**	83.3 ± 15.0**	0.90 ± 0.12	1.38 ± 0.19**
May	1.32 ± 0.06	1.6 ± 0.5	194.0 ± 68.1	0.86 ± 0.30	1.02 ± 0.23**
July	1.53 ± 0.27	1.6 ± 0.3	224.0 ± 64.0	1.04 ± 0.21	1.58 ± 0.19**

Values are means ± SEM. Blood was sampled every 20 min for 6 h at each occasion.
Significantly different from controls *$P < 0.05$; **$P < 0.01$.

controls, testosterone responses to hCG measured in January and July were not significantly different, although plasma concentrations of testosterone following the hCG injection were significantly lower in July ($P < 0.05$).

Spermatozoa per unit volume of ejaculate and per ejaculate

The numbers of spermatozoa per unit volume of ejaculate or per ejaculate collected in November, May and July were not significantly different between the two groups of rams (average: $5.4 \pm 0.2 \times 10^9$ spermatozoa ml^{-1}; $5.7 \pm 0.5 \times 10^9$ spermatozoa per ejaculate). Neither variable was affected by time of collection. The lowest sperm concentration was found in one testosterone-immunized ram (1.9×10^9 spermatozoa ml^{-1}) during the last collection (semen collected by electroejaculation).

Sexual activity

The number of sniffs was not significantly affected by immunization or by time (Fig. 5a). Interaction between immunization and time significantly affected the number of nudges ($P < 0.02$) and the number of mounts ($P < 0.01$). The number of nudges tended to decrease with time in controls, whereas it increased in testosterone-immunized rams. Thus, the number of nudges in July was significantly higher in testosterone-immunized rams than in controls ($P < 0.05$) (Fig. 5b). The number of mounts also increased significantly with time in testosterone-immunized rams, while it remained constant in controls (Fig. 5c). The number of mounts culminating in ejaculation (intromissions and ejaculations) was significantly affected by immunization ($P < 0.01$). Control rams were more successful than testosterone-immunized rams; the mean difference was significant in October ($P < 0.01$) and in June ($P < 0.05$) (Fig. 5d).

A few testosterone-immunized rams did not perform any mount that culminated in ejaculation during the various trials (two in October, one in January, three in June and one in July). One ram was particularly inactive during the first three trials (no nudges or mounts); however, in July, this ram performed its highest number of sniffs, and a high number of nudges and mounts (all incomplete).

Testicular mass and volume, epididymis mass and daily sperm production at time of castration

Immunization did not significantly affect testicular mass or volume at time of castration (Fig. 6a), or daily sperm production per unit mass of testis or per testis at time of castration (Fig. 6b); however, all these variables tended to be lower in testosterone-immunized rams. Epididymis mass was significantly reduced in testosterone-immunized rams ($P < 0.05$) (Fig. 6a). The lowest values for testicular mass (92.9 g), testicular volume (102.9 cm^3), epididymis mass (23.72 g) and daily sperm production (2.68×10^6 spermatozoa g^{-1} testis day^{-1} or 0.25×10^9 spermatozoa per testis day^{-1}) were found in a testosterone-immunized ram.

Spermatogenesis and testicular histology

In control rams, the general aspect of the seminiferous tubules appeared normal and contained numerous meiotic figures and apparently normal spermiogenesis with round or elongated spermatids. Spermatozoa were released into the lumen of many seminiferous tubules in all of these rams. In four of the five testosterone-immunized rams, the general aspect of the seminiferous tubules also appeared normal and spermatozoa were apparent in the lumen of a large number of tubules. In the fifth testosterone-immunized ram, however, disorganization of many seminiferous tubules in which the

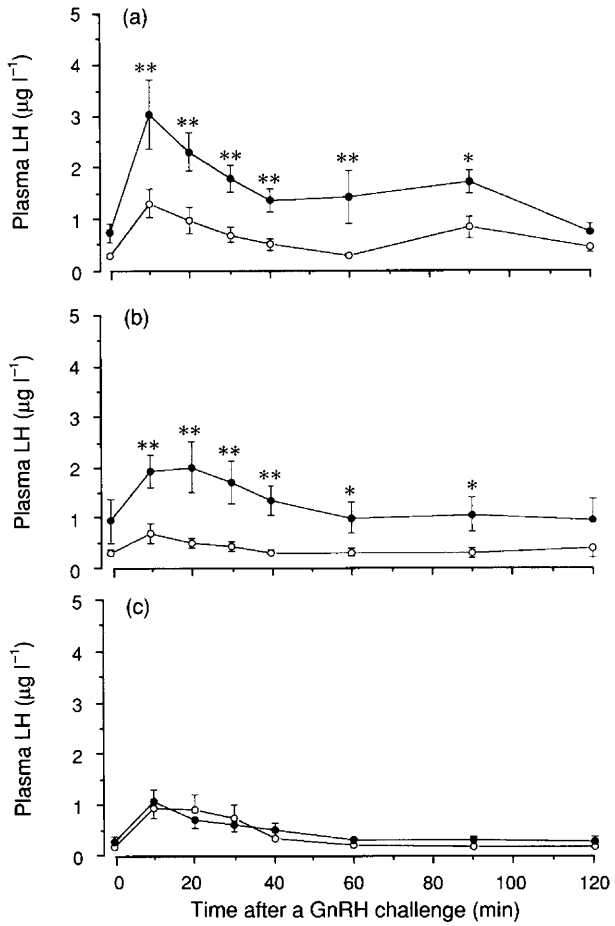

Fig. 3. Time course of LH concentrations (mean ± SEM) measured in jugular plasma, after a single i.v. injection of GnRH (5 ng kg^{-1} body mass), in (a) November, (b) March and (c) July, in adult rams immunized against (○) BSA ($n = 5$) or against (●) testosterone-3–BSA ($n = 5$). Significantly different from controls $^{*}P < 0.05$; $^{**}P < 0.01$.

number of spermatids and spermatozoa was considerably reduced were observed. No abnormalities were observed in the interstitial tissues of the control and testosterone-immunized rams.

Endocrinological evaluation after castration

Three months after castration, all rams responded to the GnRH injection. Mean plasma LH and mean FSH concentrations were not affected by immunization, but were significantly affected by the GnRH injection ($P < 0.001$ for LH; $P < 0.02$ for FSH) (Fig. 7). A 55% increase in LH concentration was measured after a single GnRH injection; however, for FSH the 9% increase in FSH observed was probably not biologically significant, since this value is within the assay variation (that is, 8.4% for FSH).

Discussion

In this study, we have clearly demonstrated that the presence of a high concentration of anti-testosterone antibodies in adult rams can neutralize the biological activity of testosterone. Indeed, gonadotrophin secretion was significantly increased in testosterone-immunized rams, even in the presence of markedly raised concentrations of plasma total testosterone. Furthermore, immunoneutralization of testosterone significantly reduced epididymis mass and affected sexual behaviour. Live mass was also reduced towards the end of the experiment by this treatment. However, sperm production was not altered during the experiment as measured by repeated semen evaluations, and as shown by similar daily sperm production values, in control and testosterone-immunized rams, after 12 months of immunization. These observations differ from those reported by Walker *et al.* (1984) and by D'Occhio *et al.* (1987), who suggested an improvement in sperm production in testosterone-immunized bulls. Our results also differ from those obtained from young rams (Haynes and Southee, 1984), and from young boars (Thompson *et al.*, 1985); no change was found in sexual activity following active immunization against testosterone in these studies. The results reported here also differ from those reported by D'Occhio *et al.* (1987) who reported similar epididymis masses in testosterone-immunized and control bulls. However, since the immunization treatment was initiated before puberty in all these studies, the differences from our results may reflect variation due to the age of the animals. Moreover, differences in results may also reflect species variation, important dissimilarities in the characteristics of the antibodies produced and genetic differences in immune response.

The spermatogenic process remained functional in all but one ram, throughout the experiment, as reflected by the initial increase in testicular volume and by maintenance of normal testicular volume thereafter. Normal spermatogenesis was confirmed in each ram, at the end of the experiment, by histological examination of testicular tissues. Spermatogenesis is considered to be testosterone-dependent; thus, some testosterone must have been available to receptors within the seminiferous tubules. Moreover, some testosterone must have been formed beyond the reach of the antibodies within the Sertoli cells which can also convert progesterone or other substrates into testosterone (Bardin *et al.*, 1988). Thus, our results confirm that testosterone can still exert its biological action within the seminiferous tubules and permit maintenance of spermatogenesis even in the presence of circulating anti-testosterone antibodies (Nieschlag *et al.*, 1975; Nieschlag and Wickings, 1978; Haynes and Southee, 1984; Walker *et al.*, 1984; D'Occhio *et al.*, 1987). Whether spermatogenesis can be maintained by steroids other than testosterone in testosterone-immunized rams, has not yet been demonstrated. Interestingly, observations from previous work (Auclair *et al.*, 1995) indicate that, in male sheep, even in the presence of high concentrations of testosterone, LH and FSH, spermatogenesis may not be fully activated and may require the participation of oestrogens. In support of this view, it has been suggested that oestrogens participate in the autocrine or paracrine regulation of spermatogenesis in mice (Nitta *et al.*, 1993).

A significant reduction in testicular blood flow was found in testosterone-immunized rams at the end of the experiment. Similar changes in testicular blood flow were observed in ram lambs actively immunized against oestradiol in which gonadotrophin and testosterone concentrations were also raised

Table 2. Mean testosterone concentration in jugular vein in January and July, and testosterone production per testis in July, before and after an hCG injection (20 iu kg^{-1} body mass), in rams immunized against BSA (control) or against testosterone-3–BSA (T)

Treatment	Time	Testosterone concentration		Testosterone production per testis in anaesthetized rams† in July (nmol min^{-1})
		Conscious rams* January (nmol l^{-1})	Anaesthetized rams* July (nmol l^{-1})	
Control	Before hCG	20.0 ± 6.6^{a}	12.3 ± 5.1^{a}	4.03 ± 0.92^{f}
Control	After hCG	82.5 ± 32.9^{b}	21.3 ± 5.1^{a}	8.56 ± 0.82^{g}
T-immunized	Before hCG	95.5 ± 14.1^{c}	115.7 ± 16.2^{c}	16.17 ± 3.68^{h}
T-immunized	After hCG	558.9 ± 47.6^{d}	183.9 ± 29.4^{e}	52.27 ± 7.53^{i}

Values are means ± SEM.
*Five rams per group; †nine testes per group.
Values with different superscripts are significantly different ($P < 0.05$).

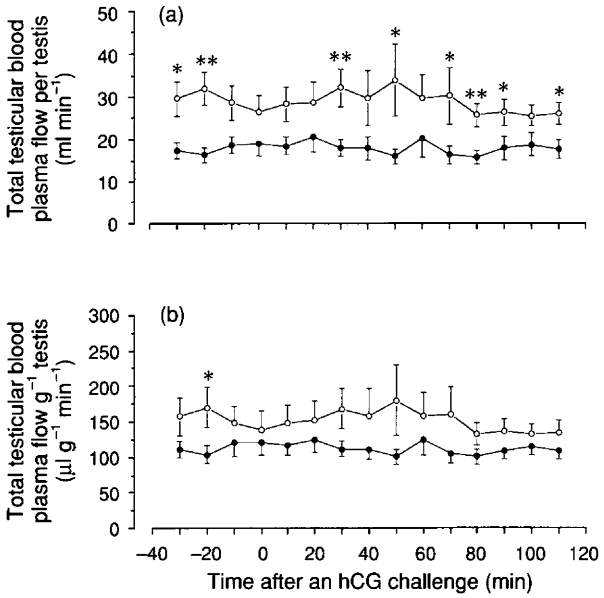

Fig. 4. Total testicular blood plasma flow (a) per testis and (b) per unit mass of testis, before and after an hCG injection (20 iu kg^{-1} body mass), in adult rams immunized against (○) BSA or (●) testosterone-3–BSA. Values are the means ± SEM ($n = 9$ testes from 5 rams in each group). Significantly different from controls *$P < 0.05$; **$P < 0.01$.

(Auclair *et al.*, 1995). As with our previous finding in ram lambs (Auclair *et al.*, 1995), testicular blood flow in adult rams was unaffected, within 2 h, by a single injection of hCG. The conflicting observations of the effects of gonadotrophins on testicular blood flow and the possibility that blood flow could impose an upper limit on the amount of steroid being released into the circulation in rams actively immunized against gonadal steroids are also discussed in our previous paper (Auclair *et al.*, 1995). It is not known whether a reduction in testosterone bioavailability has direct effects on testicular blood flow, vascular permeability or vascular smooth muscle tone.

Our observations on testosterone secretion reflect an overall increase in the steroidogenic capacity of the testis in testosterone-immunized rams, presumably in response to the increased LH concentrations. Other authors have reported hyperplasia in the presence or absence of hypertrophy of Leydig cells following active immunization against testosterone in rabbits (Nieschlag and Wickings, 1977, 1978) and in bulls (Wrobel *et al.*, 1990).

Repeated measurements, over many months, of many variables (circulating LH concentrations, LH pulse characteristics, pituitary responsiveness to GnRH, testicular responsiveness to hCG) indicated that the differences observed between testosterone-immunized and control rams were more pronounced between November and March (3–6 months after the start of the immunization) and less pronounced thereafter, even though the percentage binding of testosterone in plasma remained similarly raised throughout the study. Since seasonal changes in LH pulse characteristics, in plasma testosterone concentrations and in testicular volume (and perhaps in testosterone response to hCG) were also apparent in our control rams, we believe that such temporal changes observed in testosterone-immunized rams reflect seasonal variation. Indeed, a seasonal change in Leydig cells sensitivity to hCG could be, in part, responsible for increased testosterone secretion in January (Barenton and Pelletier, 1983). Moreover, high concentrations of circulating prolactin observed, in both groups of rams, in January, may have had stimulating effects on the steroidogenic function of the testis at that time (see Ravault *et al.*, 1977; Klindt *et al.*, 1985; Yarney and Sanford, 1989). Nevertheless, the fact that the system may have become adjusted to the presence of anti-testosterone antibodies after a long period of immunization (Scaramuzzi, 1976; Martin, 1984) cannot be excluded. For instance, there was no clear seasonal change in pituitary responsiveness to GnRH in controls, but there was a decrease in response to GnRH, from November to July, in testosterone-immunized rams. Thus, there may be some variation in the amount of free testosterone that can act on the hypothalamic–pituitary axis during the course of immunization. Similar transient hormonal changes have also been observed in other animals actively immunized against testosterone over a long period (rabbits: Thorneycroft *et al.*, 1975; Rhesus monkeys: Wickings and Nieschlag, 1978; bulls: D'Occhio *et al.*, 1987).

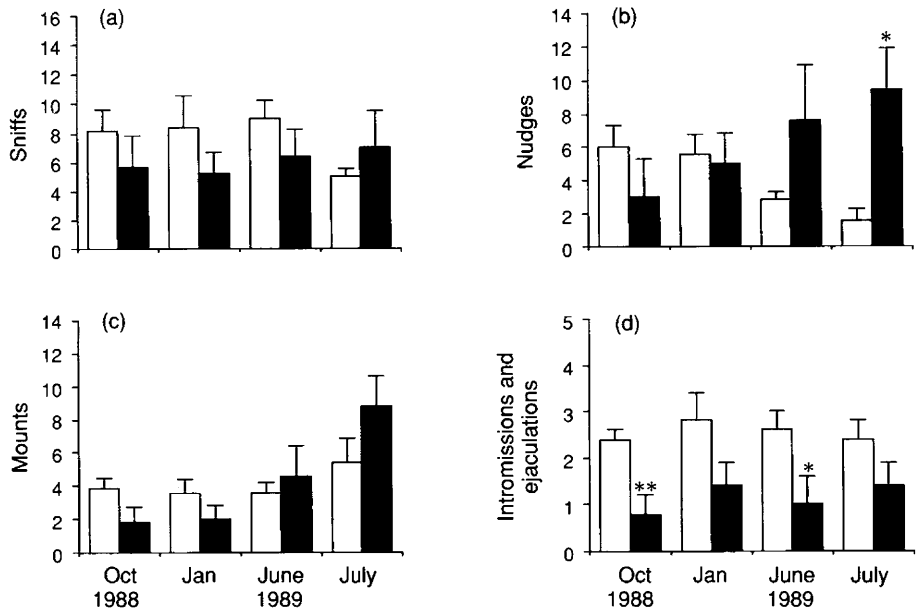

Fig. 5. Number of (a) sniffs, (b) nudges, (c) mounts and (d) intromissions followed by ejaculations in adult rams immunized against (□) BSA ($n = 5$) or against (■) testosterone-3–BSA ($n = 5$) during 10 min exposure to a teaser ewe. Libido trials were repeated at four occasions for each ram. Values are the means ± SEM. Significantly different from controls $^{*}P < 0.05$; $^{**}P < 0.01$.

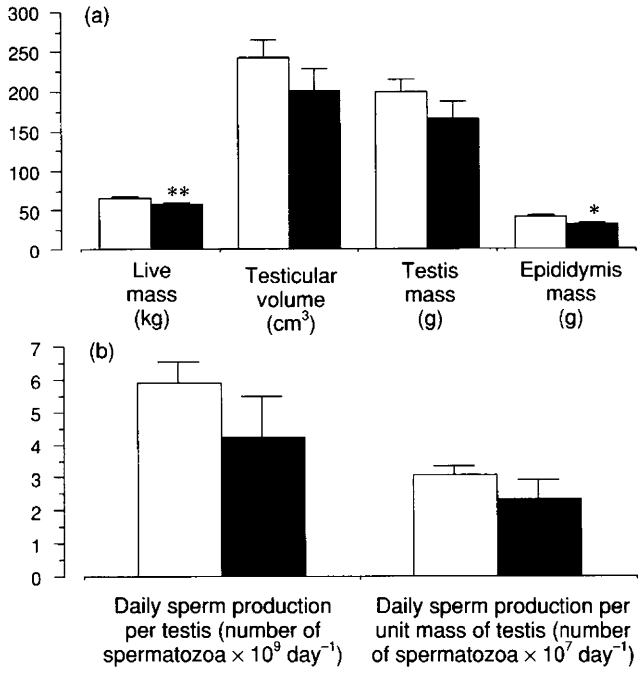

Fig. 6. (a) Mean values (± SEM) for different variables measured at time of castration and (b) daily sperm production (± SEM) per testis (× 10^9) and per unit mass of testis (× 10^7) in adult rams immunized against (□) BSA ($n = 5$) or against (■) testosterone-3–BSA ($n = 5$). Significantly different from controls $^{*}P < 0.05$; $^{**}P < 0.01$.

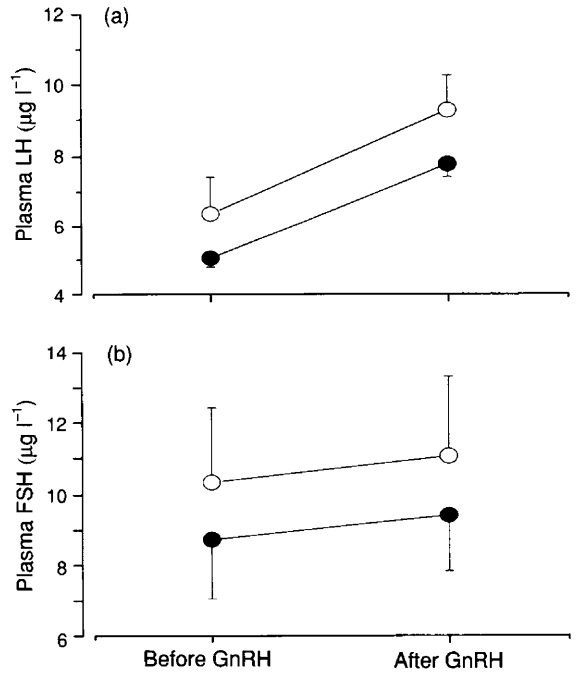

Fig. 7. (a) Mean LH concentration (± SEM) and (b) mean FSH concentration (± SEM) measured in jugular plasma, before and after a single i.v. injection of GnRH (5 ng kg^{-1} body mass), in castrated rams immunized against (○) BSA ($n = 5$) or against (●) testosterone-3–BSA ($n = 5$). Blood samples were taken at 10 min intervals for 4 h before the GnRH injection and for 2 h after the GnRH injection. 'Pre GnRH' and 'post GnRH' pools were made before assessment. GnRH challenge was carried out in October 1989, approximately 3 months after castration.

The fact that the increased concentrations of gonadotrophins measured in testosterone-immunized rams did not reach the values found in castrated rams, indicates that

gonadal hormones exercise some negative feedback on the hypothalamic–pituitary system. Since it is not possible to evaluate the bioavailability of testosterone, and, therefore, the extent of the neutralization of testosterone, within each particular tissue, the possibility that some testosterone was still acting on the hypothalamic–pituitary axis (as within the seminiferous tubules) in the testosterone-immunized rams cannot be excluded. Furthermore, it is well known that the testes secrete hormones other than testosterone (for example oestradiol and inhibin) which could have reduced gonadotrophin secretion in the testosterone-immunized rams. A rise in the concentration of steroids other than testosterone is likely to occur in testosterone-immunized animals as a result of increased substrate availability within Leydig cells, subsequent to an increase in gonadotrophin stimulation (Nieschlag and Wickings, 1978; Haynes and Southee, 1984; Thompson *et al.*, 1985).

It is of interest that 3 months after castration, no significant differences in LH and FSH secretion before and after a GnRH injection, between the two groups of rams was found, even though the capacity of plasma to bind radiolabelled testosterone was still significantly raised in each testosterone-immunized ram. Thus, it seems that no persistent changes in the functioning of the hypothalamic–pituitary axis had occurred in the testosterone-immunized rams, even in the absence of any gonadal influence. This finding supports the view that the increase in gonadotrophin secretion observed before castration in testosterone-immunized rams is due mainly to antibody interference with testicular products (for example, testosterone involved in negative feedback action) rather than intrinsic changes within the hypothalamic–pituitary axis (Nieschlag and Wickings, 1977, 1978; Haynes and Southee, 1984).

Since the specificity of each antisera was not estimated in this study, we have assumed that the antibodies produced by each testosterone-immunized ram crossreacted with other steroids in the same way as the antibodies produced in other animals immunized with testosterone-3–BSA conjugate (that is, a high degree of crossreactivity with dihydrotestosterone and negligible crossreactivity with oestrogens; see Nieschlag and Wickings, 1977, 1978) and, therefore, that the results obtained in these rams could be due to the neutralization of both androgens: testosterone and dihydrotestosterone.

We conclude that major reductions in manifestations of androgen biological activity (such as reductions in epididymis mass, in sexual behaviour, in negative feedback action on the hypothalamic–pituitary axis) confirm that testosterone can be efficiently neutralized in rams actively immunized against testosterone-3–BSA. Furthermore, although persistent increments in gonadotrophin and testosterone secretion were observed in these rams, such hormonal changes are not beneficial to spermatogenic function and can be associated with a significant reduction in testicular blood flow.

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