Effect of active immunization against oestradiol in developing ram lambs on plasma gonadotrophin and testosterone concentrations, time of onset of puberty and testicular blood flow

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Merino ram lambs were actively immunized against oestradiol-6 (*o*-carboxy methyl) oxime-BSA conjugate at 14 weeks of age and received a booster injection 4 weeks later. This treatment led to an increase in plasma concentrations of gonadotrophin and tended to enhance the increase in testicular volume until 26 weeks of age; however, testis size and mass at time of castration (30 weeks of age) were similar to values in BSA-immunized lambs. Detrimental effects were observed in some oestradiol-immunized ram lambs, for example a steep decline in testicular volume towards the end of the experiment, the presence of large vacuoles within the seminiferous epithelium and, in one lamb, few germ cells at 30 weeks of age. Testicular blood plasma flow was significantly reduced in oestradiol-immunized lambs ($P < 0.01$). The steroidogenic function of the testis was markedly enhanced in oestradiol-immunized lambs as reflected by high plasma concentrations of testosterone measured at 22, 26 and 30 weeks of age and by high testosterone production calculated from blood flow and venous-arterial differences at 30 weeks of age. Nevertheless, total live mass gain over the 16 week study was not increased in oestradiol-immunized lambs. Testicular biopsies were taken at 22 and 26 weeks of age in half of the lambs in each treatment group. Testicular volume measured at castration was decreased in control lambs in which biopsies were taken ($P < 0.05$), and plasma concentrations of testosterone measured at 30 weeks of age were significantly lower in oestradiol-immunized lambs in which biopsies were taken ($P < 0.02$) compared with lambs in which no biopsy had been taken. It is concluded that active immunization against oestradiol in ram lambs does not advance the time of onset of puberty and does not confer any reproductive or maturational advantages.

Introduction

Before puberty, the hypothalamic-pituitary axis is extremely sensitive to inhibition by gonadal steroids, resulting in low tonic LH and FSH secretion. As puberty proceeds, there is a decrease in responsiveness to the inhibitory actions of steroids, resulting in increased gonadotrophin secretion, which is sufficient to initiate reproductive activity. This change in responsiveness, known as the 'gonadostat', is particularly apparent in male rats (Ramirez, 1973) and male sheep (Olster and Foster, 1986, 1988). In rams, oestradiol can provide potent negative feedback signals to the hypothalamic-pituitary axis under physiological conditions (Schanbacher, 1979; Schanbacher *et al.*, 1984, 1987; Sanford, 1985, 1987a, b; Monet-Kuntz *et al.*, 1988). Furthermore, oestradiol can inhibit testicular androgen production by affecting the metabolism of interstitial cells of rats (Hsueh *et al.*, 1978; Brinkmann *et al.*, 1980; Kalla *et al.*, 1980; Moger, 1980; van der Molen *et al.*, 1981; Aquilano and Dufau, 1983; Ronco *et al.*, 1988), and men (Daehlin *et al.*, 1985).

Oestrogen receptors have been identified in the hypothalamus (Pelletier and Caraty, 1981; Pelletier, 1982) and pituitary (Thieulant and Pelletier, 1979, 1985) of rams, but have not yet been identified or characterized in the testis. In rats, large amounts of oestrogen receptors have been found in purified Leydig cells, but there is still controversy concerning the presence of oestrogen receptors in Sertoli cells, and oestrogen receptors have not been identified in germinal cells (Brinkmann *et al.*, 1972; Van Beurden-Lamers *et al.*, 1974; Mulder *et al.*, 1974; de Boer *et al.*, 1976; Tsai-Morris *et al.*, 1985).

Considering these lines of evidence, oestradiol could be regarded as a potent steroid that can restrain the maturational process associated with puberty in males. Land *et al.* (1981) found that the rate of growth of the testes increased in Merino lambs that had been passively immunized against oestrogens (between 14 and 26 weeks of age), but no response was observed by Jenkins *et al.* (1986) using a similar approach in younger crossbred lambs (between 2 and 16 weeks of age). The use of active immunization against oestradiol, a longer-term approach involving continuous production of antibodies, has not yet been tested in developing ram lambs. In comparison

with passive immunization, active immunization eliminates all concerns about determining the amount of exogenous antibodies required to neutralize the hormone efficiently, and about the possibility that the recipient will mount an effective antibody-mediated clearance of exogenous antibodies (Nieschlag and Wickings, 1978).

Since the active immunization approach offers many advantages over passive immunization, we have explored the possibility of advancing the time of onset of puberty in Merino ram lambs by active immunization against oestradiol. An additional objective of this study was to evaluate the effect of biopsy sampling on testicular development.

Materials and Methods

Animals

Seventeen Merino ram lambs, born at the beginning of June, were kept outdoors with their mothers until weaning at 12 weeks of age, and were then transferred to a room with controlled light (12 h light:12 h dark). This lighting regimen was chosen because it was reported that ram lambs interpret this constant cycle as short days (Klindt *et al.*, 1985). The lambs were initially fed twice a day with commercial sheep pellets (approximately 400 g day⁻¹) and lucerne chaff (50 g day⁻¹). The number of pellets was increased throughout the experiment to achieve an average live mass gain of 120 g day⁻¹. Pubertal development was studied intensively between 14 and 30 weeks of age. Variables assessed each week included live mass and testicular volume. Assuming that the testis is a prolate spheroid, testicular volume was calculated according to the formula $1/6 \pi a^2 b$ (where a = largest width and b = length of the testis) (Setchell and Waites, 1964).

Immunization procedure

At 14 weeks of age, eight lambs received a primary injection of oestradiol-6(*o*-carboxymethyl)oxime-BSA conjugate (molar ratio of steroid to protein was 19:1) (Sigma Chemical Co., St Louis, MO) in Freund's complete adjuvant (FCA), (Commonwealth Serum Laboratories, Melbourne) while nine lambs (controls) were treated with BSA in FCA. All lambs received a booster injection 4 weeks later using Freund's incomplete adjuvant instead of FCA. The immunogen, 1 mg antigen emulsified in 2 ml saline:adjuvant (1:1 v:v), was injected into each lamb at two s.c. and/or i.d. sites under each fore and hind limb.

Biopsy procedure

Testicular biopsies were taken from four controls and four immunized lambs at 22 weeks (left testis) and 26 weeks (right testis) of age. For practical reasons, the heaviest lambs were selected for this procedure. Except for a few modifications mentioned below, the technique performed was essentially that described by Lunstra and Echtenkamp (1988). The ram lambs were tranquillized with xylazine 2% (Rompum; Bayer Australia Ltd, Botany, NSW) and the subcutaneous tissues around the

spermatic cord were infiltrated with 5 ml lignocaine 2% (lidocaine HCl: Delta Veterinary Laboratories Pty Ltd, Hornsby, NSW). A small triangular incision (approximately 5 mm × 5 mm × 5 mm) was made into the tunica albuginea (extreme care was taken to avoid severing blood vessels in the tunica vasculosa layer of the capsule), and the protruding testicular tissue with the tunica albuginea was removed and immediately placed in Bouin's solution. The tunica albuginea, tunica vaginalis and skin were sutured separately using catgut. After surgery, rams were given an i.m. injection of 3 ml of the penicillin preparation.

Antibody titre and specificity

The titre was determined by incubating 100 µl of various dilutions of plasma (from 1:10 to 1:20 000) with 100 µl of (2,4,6,7,16,17-³H[N])oestradiol (³H)oestradiol: about 18 pg per 100 µl) 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 with 500 µl dextran-coated charcoal (25 mg dextran T70 and 250 mg Norit A charcoal in 100 ml PBS) for 15 min at 4°C. The tubes were then centrifuged at 1000 *g* for 15 min; the supernatant was transferred to polyethylene scintillation vials; and the radioactivity was counted. The titre was defined as that dilution binding 50% of the radiolabelled oestradiol.

Plasma samples from each oestrogen immunized lamb, collected at 30 weeks of age, were tested for crossreactivity. Specificity was examined by incubating diluted plasma (dilution binding 50% of [³H]oestradiol) with [³H]oestradiol in the presence or absence of graded doses of nonlabelled oestradiol, oestrone, oestrone sulfate, testosterone, dihydrotestosterone, or androstenedione (0–1000 pg ml⁻¹). The relative inhibitory activity of each steroid for the antiserum was calculated from the ratio of the mass of oestradiol required to displace 50% of [³H]oestradiol to the mass of the crossreacting steroid required to displace the same fraction of [³H]oestradiol.

Blood collection and hormone assays

The lambs were bled intensively from an indwelling polyethylene cannula in the jugular vein (every 20 min for 6 h) at 14, 22, 26 and 30 weeks of age. Mean hormone concentrations were determined by assaying a plasma pool representing the 6 h sampling period. Plasma LH, FSH and prolactin concentrations were measured using a double-antibody radioimmunoassay procedure (D'Occhio and Setchell, 1984), using materials and protocols supplied by the NIDDK (Torrance, CA). The reference standards used were: NIADDK-oLH-25, NIDDK-oFSH-RP-1, NIDDK-oPRL-I-2 and the tracers used were: ¹²⁵I-labelled NIADDK-oLH-I-3, ¹²⁵I-labelled NIDDK-oFSH-I-1 and ¹²⁵I-labelled NIDDK-oPRL-I-2. The following antisera were used: NIADDK-anti-oLH-I, at dilution 1:2 000 000; NIDDK-anti-oFSH-I, at dilution 1:80 000 and NIDDK-anti-oPRL-2, at dilution 1:600 000. The sensitivity of these assays was 0.2 µg l⁻¹, 0.2 µg l⁻¹ and 1.0 µg l⁻¹ for LH, FSH and prolactin, respectively. The intra-assay coefficients of variation were 5.5%, 8.4% and 9.3% for LH, FSH and prolactin, respectively. The interassay coefficients of variation were 13.4%, 13.9% and 15.0% for LH, FSH and prolactin, respectively. Testosterone

concentrations were determined by extraction radioimmunoassay (using toluene:hexane; 2:1 v:v) as previously described and validated by D'Occhio and Brooks (1983). The testosterone antiserum (no. 457) was a gift from R. I. Cox (Hormone Assay Development Group, CSIRO, Division of Animal Production, Prospect, NSW). The crossreactivity of this antiserum is 98% with dihydrotestosterone, 47% with 4-androsten-3 β ,17 β -diol, 4.7% with androstenedione, 3.6% with 4-androsten-17 β , 19-diol-3-one, and less than 1% with other steroids. The sensitivity of this assay was 0.2 nmol l⁻¹ and the intra- and interassay coefficients of variation were 5.2% and 17.1%, respectively.

Characterization of LH profiles

The LH profiles were analysed for pulses using a computer algorithm program developed by P. L. Taylor (MRC Reproductive Biology Unit, Edinburgh) for the Apple Macintosh microcomputer (Munro: Elsevier-BIOSOFT, Cambridge, UK). The analyses were carried out as described by Martin *et al.* (1987) using the following parameters. The G parameters (the number of standard deviations by which a peak must exceed the baseline in order to be accepted) were 3.98, 2.40, 1.68, 1.24 and 0.93 for G1–G5, these are the requirement for pulses composed of one to five samples that exceed the baseline, respectively. The Baxter parameters, based on the intra-assay variations in replicate determinations (six per sample) of quality control plasma pools at different LH concentrations were 0.09129, 0.01235 and 0.01010 for b_1 , b_2 and b_3 , respectively. Nadirs (minimal LH concentration up to 60 min before the peak of a pulse), pulse intervals and pulse amplitudes (difference between pulse peak and preceding nadir) were calculated for each pulse and mean values for each profile were used in the analysis of treatment effects.

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

Before castration, three controls and three oestrogen-immunized ram lambs were anaesthetized using sodium pentobarbitone (Nembutal, Abbott: Ceva Chemicals Australia Pty Ltd, Hornsby, NSW). The lambs were kept in a supine position during the procedure. Testicular blood flow was measured by *p*-amino-hippuric acid infusion, essentially as described by Laurie and Setchell (1978), Chandrasekhar *et al.* (1985a), Setchell *et al.* (1991a) and Mieusset *et al.* (1992). Cannulae were positioned in one testicular vein (under the caput epididymis) and one internal spermatic vein. A 2% (w/v) solution of *p*-amino-hippuric acid was then infused at the rate of 100 μ l min⁻¹ into the testicular vein using a peristaltic pump (Minipulse 2 Gibson, Villiers). Blood samples were withdrawn from the internal spermatic and jugular veins at 10 min intervals for 140 min. A dose of 20 iu hCG kg⁻¹ body mass (Sigma Chemical Co., St Louis, MO) was injected into the jugular vein 40 min after the start of blood sampling. This dose was chosen because it can produce a maximal short-term testosterone response in ram lambs (Chandrasekhar *et al.*, 1985b). The method of Kaland and MacArthur (1950) was used to determine *p*-amino-hippuric acid concentration in plasma samples. Testosterone production (defined as the product of

total testicular blood plasma flow and the difference in testosterone concentration between each internal spermatic vein and the jugular vein) was also calculated.

Measurements after castration

The ram lambs were castrated at 30 weeks of age, except for the two lightest lambs (one control and one immunized animal which were castrated at 34 weeks of age). The testis and the epididymis were not separated from one another, but weighed together, and then perfused, via the testicular artery, with saline followed by 2% (w/v) glutaraldehyde in 0.1 mol cacodylate buffer l⁻¹ (Johnson *et al.*, 1981). Testicular volume was again calculated using precise measurements on the removed testis. After fixation, the epididymis was separated from the testis and both organs weighed separately. Daily sperm production was estimated using homogenization-haemocytometric techniques (Amann, 1970).

Testicular histology

Small blocks of testicular tissues were fixed in Bouin's solution for 24 h and then transferred to 70% (v/v) ethanol. The tissues were embedded in paraffin wax and sections, 7 μ m thick, were cut and stained with haematoxylin and eosin. The presence of seminiferous tubules, exhibiting complete spermatogenesis at the time of biopsy or castration, was verified under a microscope.

Statistical analysis

Data for characteristics involving repeated observations over time were analysed by analysis of variance for repeated measures. Other variables were analysed using factorial analysis of variance. Appropriate transformations of the data were performed whenever necessary. Differences were then tested by Duncan's new multiple-range test (Steel and Torrie, 1980).

Results

Antibody titres and specificity

In the control animals, the percentage binding of oestradiol in plasma diluted 1:100 was negligible (< 4%) throughout the experiment. In lambs immunized against oestrogen, between 16 and 20 weeks of age, mean percentage binding of oestradiol in plasma diluted 1:100 increased from 8.7 $\pm$ 3.0% to 60.7 $\pm$ 6.8%. Between 22 and 30 weeks of age, mean percentage oestradiol binding in plasma diluted 1:5000 varied between 43.5 $\pm$ 6.1 and 57.1 $\pm$ 5.6%. Before the booster injection, antibody titres in plasma from all immunized lambs were less than 1:100, whereas, at the end of the experiment, mean antibody titre reached 1:8375 $\pm$ 2654 (range 1:2000–1:10 000). At 30 weeks of age, antisera from each immunized lamb demonstrated very low crossreactivity towards testosterone, dihydrotestosterone and androstenedione (< 2.5% at 1000 pg

Fig. 1. Fold increase in testicular volume (unit $\pm$ SEM) in relation to testicular volume at 14 weeks of age, in ram lambs immunized against BSA or against oestradiol-6-BSA, some of which underwent testicular biopsy. (□) Control, no biopsy ($n = 5$); (○) control, biopsy ($n = 4$); (■) oestradiol-immunized, no biopsy ($n = 4$) and (●) oestradiol-immunized, biopsy ($n = 4$).

ml^{-1}). The crossreactivities of the antisera towards oestrone and oestrone sulfate averaged $15.0 \pm 3.1\%$ and $3.2 \pm 0.5\%$, respectively.

Live mass and testicular volume

Mean live masses at 14 weeks of age were: control, no biopsy: 20.1 ± 1.2 kg; control, biopsy: 22.6 ± 0.3 kg; immunized, no biopsy: 19.7 ± 1.5 ; and immunized, biopsy: 23.0 ± 0.2 kg. Live mass increased progressively until the end of the experiment in all groups of lambs. The immunization treatment did not affect total live mass gain over the 16 week study (controls: 20.48 ± 0.89 kg and oestradiol-immunized: 20.45 ± 1.15 kg). In the four groups of lambs, the increase in testicular volume (Fig. 1) was small until 20 weeks of age, thereafter a period of rapid increase was observed until 26 weeks of age. From 27 weeks of age until the end of the experiment, except for the control lambs that were not biopsied, the increase in testicular volume reached a plateau and even regressed. Between 22 and 26 weeks of age, the increase in testicular volume was greater in the immunized lambs that were not biopsied than in the other groups; however, the differences between means were not statistically significant at any age. Immunization alone did not affect testicular volume. Biopsy treatment and interaction between immunization and age tended to affect this variable, although the effects were not statistically significant. In half of the lambs in each group, testicular volume decreased towards the end of the experiment. Testicular volumes of the lamb in each group that exhibited the most pronounced decline in testicular volume towards the end of the experiment is shown (Fig. 2).

Concentrations of hormones

Testosterone concentration in plasma increased significantly with age in all lambs ($P < 0.01$) (Fig. 3a). Analysis of variance showed a significant immunization effect ($P < 0.01$). Testosterone concentrations were significantly higher in oestradiol-immunized lambs than in controls at 22, 26 and 30 weeks of

age ($P < 0.001$). There was a significant interaction between biopsy treatment and age ($P < 0.01$). At 30 weeks of age, a significantly higher concentration of testosterone was found in the immunized lambs that had not undergone biopsy than in the immunized lambs that had undergone biopsy ($P < 0.02$). Testosterone concentrations were still comparable between both groups of control animals at that age.

Mean FSH concentrations were significantly affected by immunization ($P < 0.001$) (Fig. 3b). In oestradiol-immunized lambs, mean FSH concentrations were significantly higher than those in controls at 22, 26 and 30 weeks of age ($P < 0.001$). At 22 weeks of age (before any biopsy procedure) FSH concentrations were higher ($P < 0.02$) in immunized lambs not biopsied than in immunized lambs that underwent biopsy treatment; however, at 26 and 30 weeks of age, differences in mean values between these two groups were not statistically significant. Mean FSH concentrations were comparable between the two control groups at all ages. Age did not significantly affect mean FSH concentrations between 22 and 30 weeks of age.

Immunization treatment significantly affected plasma LH concentrations ($P < 0.01$) (Fig. 3c). Since no significant effects on LH concentration due to biopsy treatment, or due to an interaction between biopsy and age, were found, the results from all control lambs and all oestradiol-immunized lambs were pooled and the LH pulse characteristics within a 6 h sampling period at four different ages were compared (Table 1). In the oestradiol-immunized lambs, mean LH concentrations were significantly higher at 22, 26 and 30 weeks of age ($P < 0.05$). Mean pulse amplitude was the only LH pulse characteristic that was significantly affected by immunization treatment ($P < 0.01$). Mean pulse amplitude was significantly higher in immunized lambs at 26 weeks of age than in controls ($P < 0.02$). Mean pulse interval tended to be reduced in the immunized lambs compared with controls; however, this was not statistically significant. The number of LH peaks per 6 h sampling period, mean pulse amplitude, and mean pulse nadir were significantly affected by age ($P < 0.02$, in all cases), but mean LH concentration was not affected. In all ram lambs, an increase in pulse frequency between 22 and 26 weeks of age was observed, as well as an increase in pulse nadir and a decrease in pulse amplitude throughout the study.

Mean prolactin concentrations in plasma were not affected by immunization or biopsy treatment. Prolactin concentrations in plasma were lower at 14 weeks of age (138.4 ± 18.8 ng ml^{-1}) than at any other age in all groups (255.2 ± 12.9 ng ml^{-1}). Between 22 and 30 weeks of age, there was no significant age effect.

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

Blood flow was successfully measured in five testes from three control lambs and five testes from three oestradiol-immunized lambs, none of which underwent biopsies. Total testicular blood plasma flow per testis (ml min^{-1}) (Fig. 4a) and total testicular blood plasma flow per unit mass of testis ($\mu\text{l g}^{-1} \text{min}^{-1}$) (Fig. 4b) were not significantly affected by the hCG challenge. Analysis of variance did not show a significant

Fig. 2. Changes in testicular volume (cm^3) from 14 to 30 weeks of age in the ram lamb that exhibited the most pronounced decline in testicular volume towards the end of the experiment in each treatment group. The lambs were immunized against BSA or against oestradiol-6-BSA and some underwent testicular biopsy. (a) Control, no biopsy; (b) control, biopsy; (c) oestradiol-immunized, no biopsy and (d) oestradiol-immunized, biopsy. Mean testicular volume ($\text{cm}^3 \pm \text{SEM}$) for the corresponding group is represented by the dashed line.

Table 1. LH pulse characteristics in control ($n = 9$) and oestradiol-immunized ($n = 8$) ram lambs* at various ages

Treatment	Mean LH concentration ($\mu\text{g l}^{-1}$)	Number of peaks in 6 h	Pulse interval (min)	Pulse amplitude ($\mu\text{g l}^{-1}$)	Pulse nadir ($\mu\text{g l}^{-1}$)
Control					
14 weeks	1.00 ± 0.23	1.9 ± 0.3	188.1 ± 47.7	4.26 ± 1.45	0.39 ± 0.10
22 weeks	0.75 ± 0.14	2.7 ± 0.6	172.9 ± 40.7	1.86 ± 0.27	0.44 ± 0.14
26 weeks	0.54 ± 0.17	2.4 ± 0.7	194.7 ± 47.1	0.90 ± 0.15	0.80 ± 0.09
30 weeks	0.70 ± 0.22	1.8 ± 0.5	242.8 ± 46.7	1.03 ± 0.25	0.96 ± 0.19
Oestradiol-immunized					
14 weeks	0.75 ± 0.15	2.3 ± 0.3	155.0 ± 31.7	3.10 ± 0.69	0.33 ± 0.08
22 weeks	1.30 ± 0.10^a	3.5 ± 0.5	109.7 ± 15.8	2.42 ± 0.32	0.67 ± 0.12
26 weeks	1.12 ± 0.07^a	3.0 ± 0.3	95.4 ± 11.4	1.60 ± 0.14^a	1.07 ± 0.03
30 weeks	1.16 ± 0.20^a	2.9 ± 0.4	145.0 ± 37.6	1.40 ± 0.18	1.02 ± 0.07

*Blood was sampled every 20 min for 6 h on each occasion.

Values are means $\pm$ SEM.

^aSignificantly different from controls ($P < 0.05$).

immunization effect on total testicular blood plasma flow per testis; however, a significant immunization effect was found when this variable was expressed per unit mass of testis ($P < 0.01$). For both variables, there was a significant interaction between immunization and time ($P < 0.01$).

Testosterone production per testis was significantly affected by immunization ($P < 0.05$) and by the hCG challenge ($P < 0.001$). Before stimulation with hCG, testosterone production per testis and testosterone concentration in jugular plasma tended to be higher in oestradiol-immunized, not biopsied lambs than in control, not biopsied lambs but the differences in means were not statistically significant (Table 2). After the

hCG injection, these variables were significantly higher in immunized lambs than in control lambs ($P < 0.05$, for both).

Testis plus epididymis mass, testicular volume and daily sperm production at castration

Testicular volume at castration was significantly affected by biopsy ($P < 0.03$). Immunization tended to increase this variable; however, this effect was not statistically significant. Interaction between biopsy and immunization was not significant. Testicular volume was significantly smaller in control

Fig. 3. Mean hormonal concentration ($\pm$ SEM) in jugular venous plasma for (a) testosterone (nmol l^{-1}), (b) FSH ($\mu\text{g l}^{-1}$) and (c) LH ($\mu\text{g l}^{-1}$) at various ages in ram lambs immunized against BSA or against oestradiol-6-BSA, some of which underwent testicular biopsy. (□) control, no biopsy ($n=5$); (▨) control, biopsy ($n=4$); (■) oestradiol-immunized, no biopsy ($n=4$) and (▩) oestradiol-immunized, biopsy ($n=4$). Values with different superscripts are significantly different ($P < 0.05$).

lambs that underwent biopsy ($P < 0.05$) than in control and immunized lambs that had not, although the larger lambs were used for biopsy (Fig. 5a). Analysis of variance did not show any significant immunization or biopsy effect on testis mass, epididymis mass or testis plus epididymis mass and there was no significant interaction between immunization and biopsy, in any case (Fig. 5a). Immunization or biopsy treatment did not significantly affect daily sperm production per testis or per unit mass of testis (Fig. 5b). Again, for each of these two variables, there was no significant interaction between immunization and biopsy treatment. The minimum daily sperm production value (7.0×10^5 spermatozoa g^{-1} testis) was found in an oestradiol immunized lamb that had not undergone biopsy; this lamb had exhibited the most pronounced decline in testicular volume towards the end of the experiment (see Fig. 2c). The maximum daily sperm production value (4.9×10^7 spermatozoa g^{-1} testis) was found in a control lamb that had not undergone biopsy.

Fig. 4. Total testicular blood plasma flow (a) per testis (ml min^{-1}) and (b) per unit mass of testis ($\mu\text{l g}^{-1} \text{min}^{-1}$), before and after an hCG injection (20 iu kg^{-1} body mass), in ram lambs immunized against (□) BSA or against (■) oestradiol-6-BSA. No animals underwent testicular biopsy. Values are means $\pm$ SEM ($n=5$ testes from three lambs in each group).

Testicular histology and spermatogenesis

At 22 weeks of age, three of four control lambs and two of four oestradiol-immunized lambs, all of which had undergone biopsies, had spermatozoa present in the lumen of the seminiferous tubules. At 26 weeks of age, all immunized and all control lambs that had undergone biopsies had achieved puberty. By the end of the experiment, spermatozoa were present in the testes of all lambs. At 22 and 26 weeks of age, large vacuoles were observed in the epithelium of the seminiferous tubules of one biopsied, immunized lamb. At 30 weeks of age, one lamb (immunized, not biopsied) showed few germ cells, with other apparent signs of degeneration in the seminiferous tubules such as those observed in another lamb at 22, 26 and 30 weeks of age. In the other lambs, the general aspect of the seminiferous tubules appeared normal and contained numerous meiotic figures and apparently normal spermiogenesis with round or elongated spermatids at 30 weeks of age.

Discussion

To our knowledge, this is the first time that active immunization against oestradiol has been used to study pubertal development in ram lambs. The main advantage of active immunization is that it involves continuous production of antibodies that are always likely to be in excess of endogenous antigen production (Nieschlag and Wickings, 1978). In the experiment reported here, we showed that prepubertal Merino ram lambs responded well to the immunization procedure used

Table 2. Mean testosterone concentration in jugular vein and testosterone production per testis, before and after an hCG injection (20 iu kg^{-1} body mass), in ram lambs immunized against BSA (control, no biopsy) or against oestradiol-6-BSA (oestradiol-immunized, no biopsy)

Treatment	Time	Testosterone concentration* (nmol l ⁻¹)	Testosterone production† (nmol min ⁻¹)
Control, no biopsy	Before hCG	3.0 ± 0.3^a	1.82 ± 0.90^d
Control, no biopsy	After hCG	56.0 ± 3.0^b	17.56 ± 7.24^c
Oestradiol-immunized, no biopsy	Before hCG	24.6 ± 16.3^a	5.21 ± 2.38^d
Oestradiol-immunized, no biopsy	After hCG	246.3 ± 64.6^c	52.51 ± 11.31^f

Values are means $\pm$ SEM.

*Three animals per group; †five testes per group.

Values with different superscripts are significantly different ($P < 0.05$).

Fig. 5. (a) Mean values ($\pm$ SEM) for different variables measured at time of castration and (b) daily sperm production (number of spermatozoa) per testis ($\times 10^9$) and per unit mass of testis ($\times 10^7$) in ram lambs immunized against BSA or against oestradiol-6-BSA, some of which underwent testicular biopsy. (□) Control, no biopsy ($n = 5$); (▨) control, biopsy ($n = 4$); (■) oestradiol-immunized, no biopsy ($n = 4$) and (▩) oestradiol-immunized, biopsy ($n = 4$). Values with different superscripts are significantly different ($P < 0.05$).

and produced a considerable amount of oestradiol antibodies. Our results do not support the hypothesis that puberty in ram lambs can be advanced by active immunization against oestradiol.

Pubertal development in the rams used in this study was characterized by increases in LH pulse frequency, LH pulse

nadir, plasma testosterone concentrations, testicular volume and by a decrease in LH pulse amplitude. Similar maturational changes have been described for rams by Olster and Foster (1986). A significant increase in plasma gonadotrophin concentrations, which was accompanied by a significant increase in plasma testosterone concentrations, was observed in lambs immunized against oestrogen. The fact that LH pulse interval was reduced and that LH pulse amplitude increased significantly in the oestradiol-immunized ram lambs compared with controls, supports the view that the hypothalamus and the pituitary are important sites for oestradiol action (Thiery and Martin, 1991). The increase in FSH concentrations in the oestradiol-immunized rams also supports the view that this pituitary hormone is, in part, regulated by oestrogens during pubertal development. In male sheep, oestradiol and inhibin appear to regulate FSH secretion (Price, 1991). Simultaneous increases in FSH and testosterone secretion have frequently been observed in oestradiol-immunized, adult rams (Schanbacher, 1979; Sanford, 1987b; Schanbacher *et al.*, 1987; Monet-Kuntz *et al.*, 1988). Similarly, in adult rams, immunoneutralization of circulating oestradiol had led to an increase in plasma LH concentrations (Schanbacher, 1979, 1984; Sanford, 1985, 1987b; Schanbacher *et al.*, 1987; Monet-Kuntz *et al.*, 1988). The fact that high concentrations of testosterone in plasma did not reduce gonadotrophin secretion in the immunized ram lambs, even after 16 weeks of immunization, suggests that the negative feedback system regulating the hypothalamic-pituitary axis has lost its efficiency or that peripheral oestradiol is required for optimal functioning of this regulatory system.

The increase in gonadotrophin and testosterone secretion observed in the oestradiol-immunized ram lambs seemed to confer no reproductive advantages. At the end of the study, the spermatogenic function was comparable between control and immunized ram lambs. The steroidogenic function of the testes of the immunized lambs was markedly enhanced; thus, it seems unlikely that all the circulating testosterone was unavailable to target cells because of the presence of oestradiol antibodies. Androgen binding was shown to be negligible *in vitro*; even high concentrations of non-labelled testosterone, dihydrotestosterone or androstenedione (1000 pg ml^{-1}) did not displace labelled oestradiol from the antibodies in diluted plasma. In addition, metabolic clearance rate of testosterone (related to the unbound form of testosterone) is unaffected by

active immunization against oestradiol in rats (Nishihara and Takahashi, 1983) and in rams (Schanbacher *et al.*, 1987), suggesting that the oestradiol antibodies do not interfere with the metabolism and activities of testosterone. The differences between the results presented here and those obtained by Land *et al.* (1981) may be due partly to the magnitude of the endocrine changes subsequent to neutralization of oestradiol. Land *et al.* (1981) observed an increase in testicular growth, without significant changes in LH, FSH or testosterone concentrations (measured at 18 weeks of age), in Merino lambs that received 5 ml of anti-oestradiol antiserum at 14, 16 and 18 weeks of age (that is, at a much lower titre than in the present study). Other factors should also explain some of the differences between the results obtained in these two studies, for example different sensitivity of the rams to oestrogen, the degree to which oestradiol was neutralized, photoperiod, nutrition and immunization factors.

One of the most striking observations in the present work is the decline in testicular volume observed towards the end of the study, in about half of the lambs. This decline was more pronounced in the oestradiol-immunized lambs, particularly in two lambs in which major defects in spermatogenesis were observed. These testicular abnormalities were also reflected by the very low daily sperm production in one lamb and, by the significantly lower testicular blood flow found at the end of the experiment in other immunized lambs that did not undergo biopsies. This last observation contrasts with that made by other investigators who reported a larger volume of blood and lymphatic vessels within the testis interstitium in oestradiol-immunized rams (Monet-Kuntz *et al.*, 1988), and a higher percentage volume of blood vessels in the testes of oestradiol immunized rabbits (Nieschlag *et al.*, 1975). It is unclear whether the hormonal changes that occurred after immunization against oestradiol were responsible for the change in testicular blood flow. A decrease in testicular blood flow in response to a long-term increase in gonadotrophin secretion following immunoneutralization of testosterone in adult rams was observed (Auclair *et al.*, 1995). It is perhaps relevant that testis blood flow is lower in surgically hypophysectomized rams treated with high doses of pituitary extract, sufficient to produce LH and FSH concentrations about five times higher than in intact controls (Setchell *et al.*, 1991a). The present data and data obtained in testosterone-immunized rams (Auclair *et al.*, 1995) clearly show that testicular blood flow is unaffected, in the short term, by a single hCG injection. This lack of immediate response of testis blood flow to hCG is similar to the situation in rats, although, in rats, there is initially a fall and then an increase in testicular blood flow after hCG injection (see Setchell, 1990, for review). Since a reduction in testicular blood flow would impose an upper limit on the amount of steroid being released into the circulation (Setchell 1986; Setchell *et al.*, 1991b), such a mechanism could play an important role in rams, immunized against gonadal steroid, in reducing the amount of gonadotrophins that reach Leydig cells. More work is required to determine exactly how reproductive hormones control this blood flow limitation process and whether a reduction in bioavailability of oestradiol has direct effects on testicular blood flow (Setchell 1986, 1990), vascular permeability (Sowerbutts *et al.*, 1986) and vascular smooth muscle tone (Walsh, 1994). What effect reduced blood flow has

on testicular development is not yet known, but severe alteration of the spermatogenic function, such as that observed in some of the oestradiol-immunized lambs would certainly influence this variable. It is generally accepted that the amount of blood flowing through the testis is determined largely by the mass of the tubules (Setchell *et al.*, 1991b).

It is not known whether the high concentration of testosterone produced in the oestradiol-immunized lambs contributed to the decline in testicular volume observed towards the end of the experiment (for example, direct inhibitory effects on testicular function). However, Sanford (1987b) suggested that a considerable testosterone response to immunization could be detrimental to testicular function in the long term. Sanford (1987b) reported that, for the adult crossbred rams living in Canada under natural lighting, the testicular regression occurring in early winter was more apparent in rams that were passively immunized against oestradiol. The presence of intratesticular oestradiol, even in small amounts, may also be a requisite for the normal functioning of the testis. The recent finding that germ cells of mice express P450 aromatase activity suggests that oestrogen could participate in the autocrine or paracrine regulation of spermatogenesis (Nitta *et al.*, 1993). Our results also support the view that oestrogens play such a role in the testes of developing lambs. Another possible explanation for the decline in testicular volume is that environmental conditions, perhaps the lighting regimen, were not suitable for optimal testicular development in these winter-born lambs never exposed to decreasing day length (Colas *et al.*, 1987). The prolactin data indicate that the lambs interpreted the 12 h light:12 h dark photoperiod as long days; this is in contrast to the autumn-born crossbred ram lambs, studied by Klindt *et al.* (1985), which interpreted the same artificial cycle as short days.

The high concentration of circulating testosterone observed in the oestradiol-immunized lambs did not have any positive effect on total live mass gain over the 16 week study period. It is possible that a particular amount of oestrogens might be required with testosterone to obtain a measurable anabolic effect and the continuous maturation of the testes in ram lambs. Nevertheless, since there is no growth spurt during pubertal development in sheep, a relatively lower (or normal) testosterone concentration may already have maximal effect on body growth. Biopsy sampling did not significantly impair testicular development or daily sperm production. Successful repetitive testicular biopsies have already been reported in rams during pubertal development (Lunstra and Echterkamp, 1988) and in adult bulls (Pimentel *et al.*, 1984). A procedure that minimizes damage to the vascular layer of the tunica albuginea and minimizes postoperative inflammation appear to be the key to successful biopsies. Nevertheless, we cannot conclude that this procedure is totally without consequences, as a reduction in testicular volume (precisely measured at castration) in control lambs, in which biopsies had been taken, was observed, as well as a reduction in plasma testosterone concentration in oestradiol-immunized lambs submitted to biopsy sampling. These observations suggest that the use of this surgical procedure in an endocrinological study on pubertal development should be avoided.

In conclusion, this study has provided additional evidence that oestradiol in the blood circulation plays an important role in the regulation of LH and FSH secretion in ram lambs during

pubertal development. Moreover, it has been shown that active immunization against oestradiol in ram lambs does not advance puberty and does not confer any reproductive or maturational advantages.

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