Variations in testicular androgen receptors and histology of the lamb testis from birth to puberty

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Summary. Changes in testicular androgen receptor numbers were studied in lambs from 25 to 100 days of age. During this period, cytoplasmic receptors increased from 5 to 80 pmol/testis and nuclear receptors from 1 to 12 pmol/testis, while the total volume of Leydig cells increased 7-fold. The total number of Sertoli cells doubled between 25 and 40 days of age. From 40 days onward their number remained constant while their cellular and nuclear sizes increased by a factor of 3 and 1.5 respectively. Cytoplasmic receptor concentration was positively correlated with the number of Sertoli cells per section of seminiferous tubule, and negatively correlated with the number of germinal cells per cross section. One explanation for these results could be that Sertoli cells are the main androgen target cells in lamb seminiferous tubules.

Introduction

Androgens are known to be essential for the completion of spermatogenesis in mammals (for review, see Parvinen, 1982) and particularly in the ram (Courot et al., 1979). We have previously demonstrated the existence of androgen receptors in the ram testis, both in their cytoplasmic (Monet-Kuntz, Terqui & Locatelli, 1979) and nuclear form (Monet-Kuntz & Terqui, 1983). In the present paper, we investigated the development of androgen receptivity from birth to puberty.

There is controversy as to which cells in the testis are the target cells for androgens. In the rat, some investigators have suggested that, besides Sertoli cells, other testicular cells may be targets for androgens, namely Leydig cells (S. Gulizia, B. Sanborn & E. Steinberger, unpublished observations) and germinal cells (Galena, Pillai & Terner, 1974; Tsai, Sanborn, Steinberger & Steinberger, 1977; Wright & Frankel, 1980). However, Grootegoed, Peters, Mulder, Rommerts & Van der Molen (1977) have not been able to detect a nuclear androgen receptor in rat germinal cells. The cell composition of the lamb testis changes steadily from birth to puberty, and therefore a study of the relations between the androgen receptivity and the onset of spermatogenesis or the development of Leydig cells may provide some indication about receptor localization.

In this experiment, we examined the changes in the androgen receptor numbers in the lamb testis from 25 days of age until the appearance of spermatozoa in the seminiferous tubules. We also measured changes in the different categories of tubular and intertubular cells in the same animals.

Materials and Methods

Animals

The 42 Romanov–Ile-de-France crossbred male lambs were born in spring (March–April) and were subjected to natural variations of light and temperature. They were weaned at 45 days of age, randomly allotted to 6 groups and castrated at 25, 40, 55, 70, 85 or 100 days of age.
Testes were excised after an injection of a local anaesthetic (Sylvocaine; Merieux, France) in each spermatic cord. The tunica albuginea was removed and the testicular parenchyma was weighed. After removal of 1–2 cm³ of tissue from each testis for fixation in Bouin–Holland solution, the remainder was immediately frozen in liquid nitrogen. Storage at −196°C did not exceed 1 month.

Chemicals

[1,2,6,7-3H]Testosterone (sp. act. 94 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, U.K.) and unlabelled steroids from Steraloids (U.S.A.). The following buffers were used: TEM (10 mM-Tris, 1.5 mM-EDTA, 1.5 mM-2-mercaptoethanol, pH 7.4); S1 (140 mM-NaCl, 1 mM-MgCl₂, 3 mM-CaCl₂, 0.25 mM-mucose in TEM buffer); S2 (140 mM-NaCl, 1 mM-MgCl₂, 3 mM-CaCl₂, 1.5 mM-mucose in TEM buffer). The dextran-coated charcoal suspension consisted of 1% charcoal and 0.1% dextran in TEM buffer with 0.1% gelatin.

Androgen receptor binding assays

Cytosolic and nuclear fractions preparation. All procedures were carried out at 0–4°C. Testes were quickly thawed, and a sample of approximately 3 g was used for measurements of both cytoplasmic and nuclear receptors. The samples were then homogenized in 10 volumes (w/v) of S1 buffer, with 20 strokes of a Potter–Elvehjem homogenizer at 1000 rev./min. The homogenate was filtered through 200 μm and 30 μm nylon gauze (ZBF, Switzerland) to eliminate fibrous material, and filtrate was centrifuged at 500 g for 5 min. Glycerol was added to the 500 g supernatant to a final concentration of 10%. Centrifugation for 60 min at 105 000 g yielded the cytosolic fraction. Protein concentration ranged between 3 and 5 mg per ml cytosol. The 500 g pellet was resuspended in 8 volumes of S2 buffer, layered on 12 volumes of the same buffer and centrifuged at 1000 g for 20 min. The resulting pellet was resuspended in 4 volumes (weight of fresh tissue/vol) of S2 buffer to obtain a nuclear suspension.

Cytoplasmic receptor assay. This assay was performed essentially as previously described by Monet-Kuntz et al. (1979). Briefly, 0.2 ml samples of cytosol were incubated for 20 h at 4°C with nine different doses of [³H]testosterone, ranging from 0.3 to 16 nM (triplicate assay). Parallel incubations were performed in the presence of a 100-fold excess of unlabelled testosterone (duplicate assay). The long incubation time required for maximal [³H]testosterone binding indicates that an exchange with bound endogenous testosterone has occurred. At the end of incubation, a 1000-fold excess of unlabelled 5α-dihydrotestosterone was added and all tubes were incubated for a further 2 h to dissociate any [³H]testosterone–androgen binding protein complexes. Bound steroids were then separated from free using dextran-coated charcoal: 0.6 ml charcoal suspension was added to all tubes and the samples were mixed and allowed to stand for 20 min. After centrifugation at 2000 g for 15 min, the supernatant was transferred to counting vials containing 10 ml scintillator 299 (Packard Instruments, U.S.A.).

Nuclear receptor assay. The assay was performed as previously described by Monet-Kuntz & Terqui (1983). Samples of 0.2 ml nuclear suspension were added to glass tubes previously coated with bovine serum albumin. An equal volume of protamine sulphate (Grade I, Sigma, U.S.A.) at a concentration of 1.7 mg/ml TEM buffer was then added and immediately mixed. Tubes were left for 5–10 min at 0°C, centrifuged at 800 g for 10 min and the supernatants were discarded.

Precipitates were resuspended in 200 μl TEM buffer containing the same [³H]testosterone concentrations as for the assay of cytosolic receptors in the absence (triplicate assay) or presence (duplicate assay) of a 100-fold excess of unlabelled testosterone. The suspensions were gently shaken for 16 h at 4°C to permit an exchange with bound endogenous testosterone. Tubes were then centrifuged for 60 min at 5000 g and the supernatants were removed. The remaining precipitate was washed three times with 2 ml ice-cold buffer without further centrifugation. The pellets were solubilized in 0.2 ml soxhene 350 (Packard) for 30 min at 60°C and counted for radioactivity in 2 ml scintillation fluid (toluene 1 litre, PPO 5 g, POPOP 0.1 g).
Calculation of androgen receptor numbers

Specific binding to receptors was estimated by the difference between \([3\text{H}]\)testosterone binding in the absence and in the presence of a 100-fold excess of unlabelled testosterone. The linear regression between non-specific binding and total amount of \([3\text{H}]\)testosterone was computed for each experiment. Subsequently, for each Scatchard plot, the regression line was computed taking into account the non-specific binding as proposed by Blondeau & Robel (1975). The linearity was checked and only linear Scatchard plots were retained, i.e. 34 plots for cytoplasmic receptors and 39 plots for nuclear receptors.

To express the amounts of androgen receptors as concentrations, the data from Scatchard analysis were calculated taking into account the equivalent weight of wet tissue incubated. It was then multiplied by the testis weight in order to give the total number of receptors per testis. Under these conditions, the sensitivity of the receptor measurement was 0·1 and 0·04 pmol/g testis for the cytosolic and nuclear fractions respectively. However, the numbers of receptors given herein are numbers of assayable receptor sites and one should consider their variations rather than their absolute values.

Histological analysis

Histological analysis of intertubular and tubular tissue was performed as previously described (Hochereau-de Reviers, Loir & Pelletier, 1976a; Hochereau-de Reviers et al., 1979).

The total number of Leydig cells per testis was calculated from the estimations of Leydig cell individual volume and total volume per testis. The Sertoli cells, gonocytes and A0 and A1 spermatogonia (Hochereau-de Reviers, Ortavant & Courot, 1976b), leptotene primary spermatocytes and round spermatids were counted in 10 cross sections (10 \(\mu\)m thick) respectively at stages 8 (spermatogonia and round spermatids) and 1 (leptotene primary spermatocytes) of the cycle of the seminiferous epithelium (Ortavant, 1959). The true numbers of nuclei of these cells per cross section, including those of Sertoli cells which were assumed to be spheres, were calculated by the formula of Abercrombie (1946) as modified by Ortavant (1959). The total numbers of Sertoli and germ cells per testis were determined as described by Attal & Courot (1963).

Statistical analysis of the results

For receptor number comparisons, the non-parametric Mann–Whitney U test (Siegel, 1956) was used since the normality of this variable has not been demonstrated. For histological data analysis, Student’s \(t\) test was used.

An analysis of the correlations between receptor concentrations and some histological characteristics which reflected the concentrations of the different testicular cells (so that the testicular weight would not interfere with the correlation) was performed. Animals were divided into two groups for this analysis: non-pubertal lambs (presenting only gonocytes or spermatogonia) and prepubertal lambs. The non-parametric Spearman rank correlation coefficients were calculated (Siegel, 1956).

Results

Testis weight

Testicular weight increased slowly during the first 55 days, and then rapidly from 70 to 100 days of age (Text-fig. 1). It exhibited a high variability during the period of rapid testicular growth.
Text-fig. 1. Change with age of the testis weight in Romanov × Ile de France cross-bred lambs born in spring. Values are mean ± s.e.m. (n = 7).

Cytoplasmic and nuclear androgen receptors evolution

Scatchard plots of testosterone binding data indicated the presence of a single class of high affinity binding sites in the cytosolic and nuclear fractions. There was no significant change in the binding affinities of either cytoplasmic or nuclear receptors with age. The mean ± s.d. equilibrium association constants (K_a) were 1·0 ± 0·59 × 10^8 M^{-1} in cytosol (n = 34) and 3·0 ± 2·8 × 10^8 M^{-1} in the nuclear fraction (n = 39). The total number of cytoplasmic androgen receptors increased with age from 5 pmol/testis at 25 days to 80 pmol/testis at 100 days (Text-fig. 2). The total number of nuclear androgen receptors did not vary between 25 and 55 days of age (1 pmol/testis) but thereafter increased to reach 12 pmol/testis by 100 days of age.

Intertubular tissue evolution

Cellular and nuclear size of Leydig cells did not vary with age except at 70 days when cellular size was significantly (P < 0·05) larger than in other groups. Total volume (Table 1) and total

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>No. of animals</th>
<th>Total volume (ml)</th>
<th>Cellular area (µm²)</th>
<th>Nuclear area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>7</td>
<td>0·2 ± 0·03</td>
<td>57 ± 2·7</td>
<td>25 ± 0·7</td>
</tr>
<tr>
<td>40</td>
<td>7</td>
<td>0·3 ± 0·02</td>
<td>60 ± 4·7</td>
<td>28 ± 0·6</td>
</tr>
<tr>
<td>55</td>
<td>8</td>
<td>0·4 ± 0·07</td>
<td>56 ± 5·3</td>
<td>24 ± 2·0</td>
</tr>
<tr>
<td>70</td>
<td>7</td>
<td>0·7 ± 0·10</td>
<td>69 ± 2·8</td>
<td>28 ± 1·4</td>
</tr>
<tr>
<td>85</td>
<td>6</td>
<td>1·3 ± 0·93</td>
<td>59 ± 5·6</td>
<td>25 ± 2·1</td>
</tr>
<tr>
<td>100</td>
<td>7</td>
<td>1·3 ± 0·17</td>
<td>57 ± 3·3</td>
<td>25 ± 1·4</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

Table 1. Characteristics of intertubular tissue in the testes of Romanov × Ile-de-France lambs at different ages.
number of Leydig cells per testis (Text-fig. 2b) increased approximately 7-fold between 25 and 100 days of age. Both showed a slow increase to 55 days of age followed by a more rapid increase thereafter.

**Tubular tissue development**

Between 25 and 100 days of age, the mean ± s.e.m. diameter of seminiferous tubules increased from 58 ± 1 to 162 ± 7 µm, and their total length increased from 590 ± 56 to 1510 ± 93 m (n = 7).

The total number of Sertoli cells per testis approximately doubled between 25 and 40 days of age, and was thereafter constant (Text-fig. 2b). From 40 days onwards, their cellular and nuclear sizes increased significantly (P < 0.05) by factors of 3 and 1.5 respectively (Table 2). Both

**Table 2. Characteristics of cells in the seminiferous tubules of Romanov x Ile-de-France lambs born in spring**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Cellular area (µm²)</th>
<th>Nuclear area (µm²)</th>
<th>Sertoli cells</th>
<th>Germ cells (10⁶/testis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gonocytes</td>
<td>A₀ spermatogonia</td>
</tr>
<tr>
<td>25</td>
<td>51 ± 2.8</td>
<td>28 ± 0.7</td>
<td>0.4 ± 0.05</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>62 ± 3.9</td>
<td>27 ± 0.8</td>
<td>0.7 ± 0.12</td>
<td>0.9</td>
</tr>
<tr>
<td>55</td>
<td>73 ± 3.9</td>
<td>31 ± 1.1</td>
<td>0.4 ± 0.40</td>
<td>1.0 ± 0.34</td>
</tr>
<tr>
<td>70</td>
<td>99 ± 12.8</td>
<td>36 ± 1.4</td>
<td>1.1</td>
<td>1.4 ± 0.50</td>
</tr>
<tr>
<td>85</td>
<td>127 ± 18</td>
<td>40 ± 2.1</td>
<td>0</td>
<td>1.6 ± 0.42</td>
</tr>
<tr>
<td>100</td>
<td>183 ± 16</td>
<td>44 ± 1.8</td>
<td>0</td>
<td>1.5 ± 0.42</td>
</tr>
</tbody>
</table>

The mean ± s.e.m. includes only the animals in which the cells are present. Their proportion is indicated in parentheses.
characteristics were highly correlated ($P < 0.01$) with the numbers of A1 spermatogonia (0.76 and 0.76) and with the numbers of leptotene primary spermatocytes (0.89 and 0.82) per cross section of seminiferous tubule.

In 25-day-old lambs only gonocytes were present but, between 40 and 70 days, they progressively gave place to A0 and A1 spermatogonia (Table 2). In some of the 70-day-old lambs, leptotene primary spermatocytes were present but were very few in number. At 85 days of age, leptotene primary spermatocytes were present in all animals whereas in half of the animals some round spermatids were seen. At 100 days of age, half of the lambs were producing round spermatids.

**Relations between androgen receptor concentrations and histological characteristics**

Cytoplasmic receptor concentration was highly correlated with the relative volume of Leydig cells in the intertubular tissue of the prepubertal lambs (Table 3). It was also positively correlated with the number of Sertoli cells per section of seminiferous tubule in prepubertal lambs. For the germ cells, the sign of the correlation coefficient differed depending on whether the cells were differentiated or not: cytoplasmic receptor concentration was correlated positively with the number of gonocytes and A0 spermatogonia per cross section in non-pubertal lambs, but negatively with the numbers of A1 spermatogonia, spermatocytes and spermatids per cross section in all lambs. The three last cell types are indicators of the development of spermatogenic activity.

A similar correlation analysis showed no significant correlation between any histological characteristic and nuclear receptor concentration.

**Table 3. Coefficients of correlation between histological characteristics and the cytoplasmic androgen receptor concentration per gram of lamb testis**

<table>
<thead>
<tr>
<th></th>
<th>Non-pubertal lambs (N = 19)</th>
<th>Prepubertal lambs (N = 14)</th>
<th>All lambs (N = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative volume of Leydig cells in the intertubular tissue</td>
<td>-0.29</td>
<td>0.83**</td>
<td>0.36*</td>
</tr>
<tr>
<td>Sertoli cell number per cross-section</td>
<td>0.26</td>
<td>0.49*</td>
<td>0.45*</td>
</tr>
<tr>
<td>A0 spermatogonia and gonocytes per cross-section</td>
<td>0.42*</td>
<td>-0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>A1 spermatogonia per cross-section</td>
<td>—</td>
<td>—</td>
<td>-0.41*</td>
</tr>
<tr>
<td>Leptotene primary spermatocytes per cross-section</td>
<td>—</td>
<td>—</td>
<td>-0.38</td>
</tr>
<tr>
<td>Round spermatids per cross-section</td>
<td>—</td>
<td>—</td>
<td>-0.46</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01.

**Discussion**

The apparent binding affinity of androgen receptors in the lamb testis was similar to that which we have previously found in the adult testis, but was one order of magnitude smaller than in other androgen target organs, e.g. hypothalamus and pituitary (Pelletier, 1982). One possible explanation is that endogenous testosterone present in the testis (Attal, 1970) competes with the [³H]testosterone used for the assay, thus lowering the apparent affinity of receptors for [³H]testosterone. The ratio of nuclear to cytoplasmic receptors was about 10% in the testes of animals kept under normal conditions. Similarly, there is good evidence from the literature that only 10% of the total cellular steroid receptors need to interact with chromatin in order to produce physiological responses (for review see Leake, 1981)
Androgen receptors were shown to be present as early as 25 days of age in the testis of Romanov × Ile-de-France lambs. This can be related to the secretion of testosterone which starts from the first week of life in both Romanov (Lafortune et al., 1982) and Ile-de-France (Garnier, Cotta & Terqui, 1978) lambs. From 25 to 100 days of age, there was a continuous increase in the testicular androgen receptor content. In parallel there was a continuous increase in the total number of Leydig cells. This corresponds to the increase in the total interstitial cell mass observed during puberty in Hampshire lambs by Waites, Wenstrom, Crabo & Hamilton (1983). However, the cellular and nuclear sizes of Leydig cells did not vary during this period, unlike those in the boar (Peyrat, Meusy-Dessolle & Garnier, 1980), and, at 100 days of age, they had not reached the size seen in adult intact animals (Courot et al., 1979). Multiplications of the Leydig cells are accompanied by a continuous increase in the testicular content of testosterone (Skinner, Booth, Rowson & Karg, 1968). Moreover, in our prepubertal lambs, the number of cytoplasmic androgen receptors was highly correlated with the volume of Leydig cells. These observations further support the hypothesis of a positive control of testosterone on its own receptors (Kaufman, Pinsky, Hollander & Bailey, 1983).

One explanation for the positive correlation between androgen receptor concentration and Sertoli cell concentration could be that these cells are the main androgen target cells in seminiferous tubules of the lamb. This would agree with many reports for the rat that show the presence of cytoplasmic and nuclear androgen receptors (Mulder et al., 1976; Sanborn, Steinberger, Tcholakian & Steinberger, 1977) which bind to Sertoli cell chromatin (Tsai, Sanborn, Steinberger & Steinberger, 1980). In addition, the progressive dilution of Sertoli cells in the tubules as germinal cells multiply probably accounts for the negative correlations observed between androgen receptor concentration and differentiated germinal cell concentration.

From the end of Sertoli cell division (40 days of age in our lambs) until puberty, the testicular content of androgen receptors increased 12-fold. During the same period in the same species, FSH receptors have been shown to increase 10-fold (Barenton, Hochereau-de Reviers, Perreau & Saumande, 1983) and androgen binding protein 7-fold (Carreau, Drosdowsky & Courot, 1979). From the end of division onwards, cellular and nuclear sizes of Sertoli cells increased. This corresponds to the development of seminiferous epithelium as indicated by the high correlation we observed between Sertoli cell size and germinal cell numbers. An increase in the total masses of Sertoli cell cytoplasm and nuclei has been observed long after the end of division in Hampshire lambs (Waites et al., 1983). In the lamb, therefore, after a period of multiplication, Sertoli cells enter a period of morphological differentiation as well as functional maturation.

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References

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