Age-related changes in blood concentrations of FSH, LH and testosterone and testicular morphology in a new rat sterile mutant with hereditary aspermia

J. Noguchi^{1*}, M. Yoshida¹, H. Ikadai¹, T. Imamichi¹, G. Watanabe²
and K. Taya²

¹Imamichi Institute for Animal Reproduction, Dejima, Niihari, Ibaraki 300-01, Japan; and

²Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan

A new rat mutant showing aspermia was investigated. Groups of 4–7 mutant male rats were killed at 3, 5, 10, 15 and 25 weeks of age. Examination by microscope showed apparent abnormalities in the seminiferous epithelium from 3 weeks of age onward. Inclusion-like bodies were observed in the cytoplasm of pachytene spermatocytes from 3 weeks old and instead of spermiation, polynuclear giant cells were formed within the seminiferous epithelium after 5 weeks of age. Histological analysis of seminiferous epithelium of adult mutant rats also showed a marked decrease in the number of preleptotene, leptotene and pachytene spermatocytes and tubules containing only spermatogonia and Sertoli cells in the seminiferous epithelium increased with age. However, the combination of other cellular elements of germ cells in the seminiferous epithelium was similar to that in normal rats and the distribution rate of these seminiferous tubules was close to that of normal rats, indicating that cyclicity of seminiferous epithelium was still maintained in the mutant rats despite the lack of spermiation. Plasma concentrations of FSH and LH were significantly higher in the mutant male rats than in normal male rats at 5 and 10 weeks of age onward, respectively. Plasma concentrations of testosterone were lower in the mutant male rats than in normal male rats. Silastic capsules containing testosterone were implanted into the unilateral testis of adult mutant male rats and animals were autopsied 5 weeks later. However, intratesticular administration of testosterone did not affect restoration of spermatogenesis. These results indicate that the arrest of spermatogenesis had occurred from the first cycle of spermatogenesis and the primary defect in the mutant is not gonadotrophin and testosterone deficiency.

Introduction

Mutations affecting spermatogenesis in laboratory animals are valuable tools for analysing the different processes of spermatogenesis and human male infertility. In rats, several investigations have used sterile mutations affecting spermatogenesis, caused by for example *grc* (growth and reproductive complex, Gill III and Kunz, 1979; Greiner *et al.*, 1980; Gill III *et al.*, 1983; Gunsalus *et al.*, 1985) and *H^e* gene (Gumbreck *et al.*, 1972; Russell and Gardner, 1974; Musto *et al.*, 1978; Spitz *et al.*, 1983; Anthony *et al.*, 1984; Heindel *et al.*, 1988). We have recently found a new rat mutant which shows a disorder of spermatogenesis without deficiency of body growth. This mutant male rat is infertile because of the lack of sperm production, but females of the strain show no detectable abnormality. Genetical

analysis revealed autosomal recessive transmission of this defect and the locus was designated *As* representing 'Aspermia' (Ikadai *et al.*, 1992). The rats had been maintained by sister-brother matings of the carriers in the Imamichi Institute for Animal Reproduction. This study describes morphological and hormonal findings of the new mutant rat with advancing age.

Materials and Methods

Animals

Rats with bilateral small testes were detected in 1986 in a colony of an inbred Wistar strain, which had been maintained in the Imamichi Institute. Since these rats were infertile, male littermates having normal sized testes were mated with female littermates and successive inbreedings within littermates of the mutant have been made for 12 generations. Animals were kept under conventional conditions and were fed a commercial diet (EM; Oriental-Kobo, Co. Ltd, Tokyo) and tap water *ad libitum*.

*Present address and address for correspondence: Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan.

Received 7 April 1992.

Morphological observation and blood collection

Age related changes in morphological and endocrinological features of gonads were examined by decapitating four to seven mutant rats at 3, 5, 10, 15 and 25 weeks old. Normal male littermates were also decapitated at the same age as controls. Blood samples were collected and serum was stored at -20°C for radioimmunoassay of LH, FSH and testosterone. Testes were removed immediately after death and were weighed. Epididymides, prostates and seminal vesicles were also removed from animals at 10, 15 and 25 weeks of age and were weighed. Testes were fixed in Bouin's solution and then embedded in paraffin wax. Middle portion of testis was cross-sectioned and stained with haematoxylin and eosin or periodic acid-Schiff's haematoxylin for observation by microscope. Two-hundred sections of seminiferous tubules of the testis of each animal were examined for the identification of various stages of the seminiferous cycle (Leblond and Clermont, 1952). The number of each type of germ cell and Sertoli cell in seminiferous epithelium was counted in 30 tubules, ten from each of three different rats.

Pituitary contents of FSH and LH

Immediately after blood collection, anterior pituitary glands were removed and homogenized in 5 ml cold (4°C) NaCl solution (9 g NaCl l^{-1}). After centrifugation at $36\,000\text{ g}$ for 30 min at 4°C , the supernatant fraction was obtained and stored at -20°C until assayed for FSH and LH.

Testicular responsiveness to exogenous testosterone

Testosterone implants were constructed from 5 mm lengths of Silastic tubing (1.0 mm inner diameter $\times$ 2.0 mm outer diameter; Dow Corning, Midland, MI). Under ether anaesthesia, a small incision was made in the scrotal sac and tunica albuginea of the left testis of three mutant males at 20 weeks of age; a Silastic capsule filled with 2 mg crystalline testosterone (Sigma Chemical Co., St Louis, MO) was inserted into the testis and the incisions were closed with sutures. After 5 weeks, the testis with the testosterone implant and its contralateral, untreated testis were removed and weighed. The capsule was dissected free and all testes were prepared for histology.

Hormone assays

Serum and pituitary concentrations of FSH and LH were measured using NIDDK RIA kits (NIH, MD) for rat FSH and LH. Iodinated preparations were rat FSH-I-5 and LH-I-5. The antisera used were anti-rat FSH-S-11 and anti-rat LH-S-8. Results were expressed in terms of the NIDDK rat FSH-RP-1 and LH-RP-1 preparations. The sensitivity of the assays for LH and FSH were 0.25 and 1.2 ng per tube, respectively, and the intra- and interassay coefficients of variation were 2.5 and 5.0%, respectively for FSH and 3.3 and 15.7% for LH, respectively.

Serum concentrations of testosterone were measured by a double-antibody radioimmunoassay system using a ^{125}I -labelled radioligand testosterone-3-(O-carboxymethyl)oximino-(2- ^{125}I iodohistamine) (IM 128, Amersham International plc, Bucks) as described previously (Taya *et al.*, 1985). The antiserum against

Fig. 1. Changes in testicular weight of the (●) mutant and (○) normal male rats from 3 to 25 weeks of age. Results are expressed as means $\pm$ SEM of four to five animals. * $P < 0.05$ and *** $P < 0.001$ represent statistical significance compared with respective controls.

testosterone (GDN 250; Gay and Kerlan, 1978) was provided by G. D. Niswender (Colorado State University, Fort Collins, CO); the sensitivity of the assay was 0.625 pg per tube and the intra- and interassay coefficients of variation were 6.0% and 15.5%, respectively.

Statistical analysis

Values were expressed as means $\pm$ SEM. Statistical comparisons were made with Student's *t* test. A *P* value of <0.05 was selected as the limit of statistical significance.

Results

Body and organ weights

The increase in body weight with age of normal and mutant male rats was similar (data not shown). However, the weight of the testes was significantly depressed in the mutant rats relative to the normal male rats from 5 weeks of age onward (Fig. 1). The weight of the epididymis of the mutant rats was also significantly lower than in normal rats at 10, 15 and 25 weeks of age (923.3 ± 6.1 , 1331.0 ± 17.9 and 1603.4 ± 45.8 in normal rat versus 664.6 ± 19.3 , 949.8 ± 24.8 and 1192.3 ± 38.8 in the mutant rat). *P* values were $P < 0.05$ at 10 weeks of age and $P < 0.01$ at 15 and 25 weeks of age. However, the weights of seminal vesicle and prostate of the mutant rats were similar to those of normal rats (data not shown).

Seminiferous tubules

Morphological abnormalities were already observed in the seminiferous epithelium of the mutant rats at 3 weeks of age. In

Fig. 2. Cross-section of seminiferous tubules of testes from normal rats at (a) 3, (c) 5 and (e) 25 weeks of age and from the mutant rats at (b) 3, (d) 5 and (f) 25 weeks of age. Arrows indicate inclusion-like bodies in the pachytene spermatocytes. Arrowheads indicate polynuclear giant cells. Scale bar = 100 μ m for each panel.

the normal male rats, spermatogenesis advanced up to pachytene spermatocytes by 3 weeks old (Fig. 2a). The diameter of seminiferous tubules increased with age and spermiation was observed by 5 weeks of age (Fig. 2c). Although spermatogenesis advanced up to pachytene spermatocyte in the mutant rats at 3 weeks of age, inclusion-like bodies were seen in the cytoplasm of several pachytene spermatocytes at 3 weeks of age (Fig. 2b). In the mutant rats, at 5 weeks of age, no seminiferous tubules showed spermiation and spermatogenesis was almost arrested at the stage of pachytene spermatocyte (Fig. 2d). Only a small number of seminiferous tubules contained round spermatids. Polynuclear giant cells appeared in seminiferous epithelium

of the tubules at 5 weeks of age. These morphological characteristics of seminiferous epithelium were also observed in the testes of adult mutant rats (Fig. 2f). Several tubules containing only spermatogonia and Sertoli cells in the seminiferous epithelium were observed from 5 weeks of age.

Spermatogenesis

It was difficult to classify the cross-sections of seminiferous tubules of the mutant rats into 14 stages of the seminiferous cycle seen in normal rats because of the lack of spermatids which are used for the identification of the stages. The cross-sections

Fig. 3. Cross-section of seminiferous tubules from testes of the mutant rats at 25 weeks of age. Each seminiferous tubule includes spermatocytes at stage of (a) pachytene, (b) from preleptotene to leptotene and pachytene, (c) zygotene and from pachytene to diplotene and (d) pachytene, diplotene and spermatocyte in cell division, respectively. Each tubule of (a), (b), (c) and (d) corresponds to the stages of rat spermatogenesis I–VIII, IX–XI, XII–XIII and XIV, respectively. Scale bar = 100 μ m for each panel.

of seminiferous tubules were therefore identified according to the types of spermatocyte. Most sections of the mutant rats were classified into four stages of which epithelium contained the spermatocytes. The types classified were pachytene only (Fig. 3a), preleptotene or leptotene in the basal layer and pachytene in upper layer (Fig. 3b), zygotene in middle layer and pachytene in upper layer (Fig. 3c) and pachytene, diplotene and spermatocyte in cell division (Fig. 3d). These four stages were comparable to the stages from I to VIII, IX to XI, XII to XIII and XIV in the classification of normal rat spermatogenesis by Leblond and Clermont (1952), respectively. The frequency of these four stages in the testes were $66.2 \pm 1.9\%$, $21.2 \pm 0.5\%$, $6.9 \pm 1.7\%$ and $5.6 \pm 0.4\%$ in normal rat, but $56.8 \pm 8.5\%$, $28.9 \pm 6.3\%$, $9.9 \pm 1.5\%$ and $4.4 \pm 1.2\%$ in the mutant rat, respectively. No significant difference in the frequency of these four stages was observed between the normal and the mutant rats for each stage.

Plasma concentrations of FSH, LH and testosterone

Plasma concentrations of FSH increased from 3 to 5 weeks of age for both groups of animals, whereas the concentrations

were significantly higher in the mutants than in the normal rats at 5, 10 and 15 weeks of age (Fig. 4a). Similarly, LH concentrations were significantly greater in the mutant rats than in normal rats at 10, 15 and 25 weeks of age (Fig. 4b). Plasma concentrations of testosterone in the mutant rats from 10 to 25 weeks of age were lower than those of the normal rats, although the difference was not significant (Fig. 4c).

Pituitary contents of FSH and LH

Pituitary contents of FSH and LH increased with age in both groups of rats (Fig. 5). Pituitary contents of FSH and LH were not significantly different between the two groups except at two points. Significantly higher contents of FSH and LH were observed in the pituitary of the mutant rats than in normal rats at 5 and 15 weeks of age, respectively.

Effects of intratesticular administration of testosterone on spermatogenesis in mutant rats

Intratesticular implants containing testosterone did not improve sperm production in the mutant rats. There was no

Fig. 4. Plasma concentrations of (a) FSH, (b) LH and (c) testosterone of the mutant (●) and normal male (○) rats from 3 to 25 weeks of age. Results are expressed as means $\pm$ SEM of four to seven animals. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ represent statistical significance compared with the normal rats.

morphological difference in the seminiferous epithelium between testes implanted with testosterone and contralateral untreated testes. Seminiferous tubules did not contain either spermatozoa or elongated spermatids. The incidence of round spermatids in the seminiferous epithelium was not different between testosterone implanted testes ($5.2 \pm 1.9\%$) and contralateral untreated testes ($4.6 \pm 3.3\%$). The numbers of pre- or leptotene and pachytene spermatocytes were significantly lower in both testes of the mutant rats than in normal rats, whereas there was no difference between testosterone implanted and contralateral untreated testes of the mutant rats (Table 1). There were no marked differences in the number of Sertoli cells between testes of normal and mutant rats. In addition, implantation of testosterone did not affect the stages of the seminiferous epithelium (data not shown).

Discussion

The results reported here show that the adult sterile mutant male rat had normal body growth and accessory gland weights

Fig. 5. Pituitary contents of (a) FSH and (b) LH of mutant (●) and normal (○) male rats from 3 to 25 weeks of age. Results are expressed as means $\pm$ SEM of four to seven animals. * $P < 0.05$ represent statistical significance compared with the normal rats.

but smaller testicular and epididymal weights. Histological examination demonstrated that the defect of spermatogenesis of the mutant rat appeared before completion of spermatogenesis and that the lack of sperm production lasted throughout life. Formation of inclusion-like bodies in the cytoplasm of pachytene spermatocytes was observed at 3 weeks of age and polynuclear giant cells appeared in the seminiferous epithelium instead of the elongated spermatids at 5 weeks of age, when spermiogenesis was present in normal rats. Although the origin of these polynuclear cells was unclear, a significant decrease in pachytene spermatocytes suggested that degenerating spermatocytes could partially result in formation of these giant cells. Diplotene spermatocytes and spermatocytes in cell division were also detected, indicating that several of the germ cells could develop to more advanced stages than the pachytene stage, whereas they could not develop beyond the early spermatid stage. However, the combination of other cellular elements of germ cells in the seminiferous epithelium was similar to that in normal rats and the distribution rate of these seminiferous tubules was close to that of normal rats. These results suggested that cyclicity of seminiferous epithelium was still maintained in the mutant rats despite the lack of spermiogenesis. This defect of spermatogenesis in the mutant was controlled by an autosomal recessive locus designated *As*, representing aspermia (Ikada *et al.*, 1992).

In the study reported here, endocrinological analysis demonstrated that the mutant male rats have high concentrations of circulating FSH and LH but slightly reduced concentrations of circulating testosterone. Pituitary contents of FSH and LH were also slightly higher in the mutant male rats. These results suggest that the impaired feedback action of testosterone on the hypothalamus and pituitary gland may be present in the mutant

Table 1. Number of cells per cross-section of tubule at stage IX–XI in testes of normal male and testosterone implanted mutant male rats

Treatment		Tubular cells at stage IX–XI			
		A gonio	Pl-L	P	Sertoli
Normal		1.0 ± 0.2	23.4 ± 1.1	42.2 ± 1.5	13.2 ± 0.6
Mutant	Untreated	0.7 ± 0.1	16.9 ± 1.0 ^b	22.8 ± 1.1 ^b	12.3 ± 0.6
	Testosterone implanted	0.6 ± 0.1	20.0 ± 1.1 ^a	25.3 ± 1.4 ^b	13.3 ± 0.8

Values are means ± SEM of 30 tubules; ten tubules per testis were examined.

A gonio: type A spermatogonia; Pl-L: preleptotene or leptotene spermatocyte; P: pachytene spermatocyte.

^aP < 0.05, ^bP < 0.01 compared with normal rats (Student's *t* test).

rats. However, it is well established that testicular inhibin is one of the major factors controlling the secretion of FSH in male rats (de Jong, 1988). The hypersecretion of FSH in the mutants may be related to insufficient secretion of inhibin by Sertoli cells. In addition, the hypersecretion of LH was also demonstrated in the mutant rats from 10 weeks of age, indicating that a slight decrease in plasma concentrations of testosterone may be involved in the regulation of feedback control of LH secretion in mutant rats. These results also suggest that the secretory response of Leydig cells of the mutant rats slightly decreased, since plasma concentrations of testosterone did not increase in response to high concentrations of plasma LH. These results exclude hypogonadotrophic–hypogonadism as the cause of the disorder of spermatogenesis in the mutant rats. This contention is also supported by the findings of the experiment of intra-testicular implant of Silastic tube containing testosterone. This treatment was designed to produce high local concentrations of testosterone in the testes of the mutant rats, as the same treatment could maintain spermatogenesis in the testes implanted with testosterone but not in the contralateral testes implanted with empty capsules in hypophysectomized rats (Ahmad *et al.*, 1973). However, the treatment with testosterone did not show any changes in morphological features of the seminiferous epithelium in either testosterone implanted or untreated testes. These data indicate that the testes of the mutant rats did not respond to exogenous testosterone, although the binding ability of testosterone to specific sites in testicular cells is unknown.

Some cases of genetically sterile male rats, such as $H^{+}/+$ (Gumbreck *et al.*, 1972), *grc/grc* (Gill and Kunz, 1979), *Tfm* (Stanley *et al.*, 1973), pseudohermaphrodite (Bardin *et al.*, 1973; Ikada *et al.*, 1988), *hd/hd* (Sabourdy and Bozic, 1960) and *hgn/hgn* (Hakamata *et al.*, 1988) have been described previously and their conditions have been compared with human genetic diseases. The hormonal status of these male hypogonadic rats, except *grc/grc* and $H^{+}/+$ rats, have markedly lower testosterone and higher FSH and LH concentrations in the serum compared with intact animals, indicating a general feature in males with primary Leydig cell dysfunction. In male *grc* homozygote rats, normal concentrations of serum LH, FSH and testosterone have been observed (Greiner *et al.*, 1980). Hence, the origin of the sterility is related to the regulation of the activity of an enzyme in the testicular germ cells. However, plasma concentrations of LH and FSH of the restricted rats was higher but

concentrations of prolactin and testosterone were normal, and weights of the sex accessory glands were normal (Heindel *et al.*, 1988). Thus, Heindel *et al.* (1988) have proposed that alterations of both Leydig and Sertoli cell functions account for spermatogenic arrest. The results reported here demonstrated that hormonal status in the present aspermic rats is similar to that of the $H^{+}/+$ rats (Heindel *et al.*, 1988).

In conclusion, although it is difficult to provide a clear explanation for the spermatogenic failure in the mutant rat, the status of the mutant rat might be due to an intratubular defect such as primary Sertoli cell dysfunction or defected function of germ cells in meiosis. This mutant will therefore be useful for studies of the relationship between Sertoli cells and germ cells or investigations of the process of meiosis, responsible for sperm production.

We thank A. F. Parlow and the Rat Pituitary Hormone Distribution Program, NIDDK, NIH, MD, USA for radioimmunoassay materials; G. D. Niswender, Department of Physiology and Biophysics, Colorado State University, Colorado, USA for antiserum to testosterone; K. Wakabayashi, Hormone Assay Center, Institute of Endocrinology, Gunma University, Maebashi, Japan for antiserum to rabbit γ -globulin; Teikoku Hormone MFG Co., Ltd, Tokyo, Japan for testosterone.

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