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# Effect of active immunization to luteinizing-hormone-releasing hormone on the fertility and histoarchitecture of the reproductive organs of male rat

E. Rovan[1], E. Fiebiger[1], N. R. Kalla[2], G. P. Talwar[3], W. Aulitzky[2], and J. Frick[2]

[1]Institute of Zoology, University of Salzburg, Salzburg, Austria
[2]Department of Urology, General Hospital, Salzburg, Austria
[3]National Institute of Immunology, New Dehli, India

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**Summary.** The feasibility of using a vaccine against luteinizing-hormone-releasing factor for supression of pituitary and gonadal functions has been indicated for some time. Antibody production against this low-molecular-weight, naturally occurring decapeptide, however, requires to be coupled to a carrier protein to enhance its immunogenicity. LHRH was coupled to diphtheria toxoid (DT). Adult male Sprague-Dawley rats with a mean basal body weight of 200 g were immunized with anti-LHRH-DT (20 µg/injection/rat) at four-week intervals. An equal number of unexposed animals served as controls. Six animals were killed every two weeks up the end of the week 43. The vaccination schedule did not have any effect on the gain in body weight, nor was any adverse effect of vaccination observed in the course of the investigations. The pituitary, prostate, epididymis, testes, seminal vesicles, adrenal and thyroid were excised for determination of organ weight and histological examination. The adrenal, pituitary and thyroid showed no remarkable weight changes during the observation period, whereas the weights of the reproductive organs demonstrated significant reductions compared to those of the control group. The histopathology revealed marked to significant changes in the gonads and the accessory sex organs including the prostate. A progressive phase of regeneration of spermatogenesis was evident 98 days after vaccination. Total recovery of spermatogenesis was observed 300 days after vaccination. The mating studies showed the return of fertility 300 days after vaccination. The litters borne were normal. Prostate showed recovery after 154 days of vaccination. Our observations lend strong support to the hypothesis that anti-LHRH vaccine can be effectively used on the management of prostate carcinoma. If the vaccination is given together with a suitable dose of long-acting androgen, contained in an adequate delivery system, the regimen may be used for the regulation of male fertility.

**Key words:** Anti-LHRH vaccination – Histology – Reproductive organs – Male rat

Blocking of gonadal testosterone synthesis has been effectively used in the management of the advanced prostate carcinoma, and in recent years this principle has repeatedly been considered as a possible means of fertility control [6]. The possibilities for medical castration are manifold and can be realized by means of estrogens, gestagens and as recent experience has shown, the biologically effective luteinizing-hormone-releasing hormone (LHRH) analogues [12, 13]. Due to cardiovascular side effects, estrogens and gestagens are now hardly used; LHRH analogues are currently the world's most commonly used substances for blocking testicular testosterone synthesis. This is achieved by pituitary desensitization or down-regulation, which occurs when LHRH analogues are administered chronically [3, 5]. The dream of coupling the LHRH molecule to immunocompetent substances to permit it to act as an antigen and to induce antibodies to block the hormone axis, is also several years old [1].

The possibility of using immunological reactions to block the effect of LHRH on the target organs has been demonstrated in experimental animals, e.g. rabbits, rats and rhesus monkeys [2]. The prerequisite for such experiments was adequate immunogenicity of the LHRH molecule [4]. As a decapeptide, LHRH is a relatively small molecule and for this reason has very low immunogenicity [4, 15]. In order to achieve a longer biological half-life and adequate immunogenicity for biologically effective antibody titers, synthetic LHRH has been coupled to various carrier substances and/or albumin fractions. Various adjuvants, such as Freund's complete adjuvant (FCA), were used to improve immunological reactions. Unfortunately, the pathogenicity of FCA makes it unsuitable for human use. However, make it possible to use the LHRH molecule as an antigen with appropriate immunogenicity, it was coupled to diphtheria toxoid. Other adjuvants used were SPLS, MPD and/or alums [11, 20]. This study on rats was set up to investigate whether LHRH diphtheria toxoid vaccine should block the hormone axis by competitive binding and evoke corresponding inhibitory effects on the hormone-dependent organs in male rats (testis, prostate gland, seminal vesicles and epididymis).

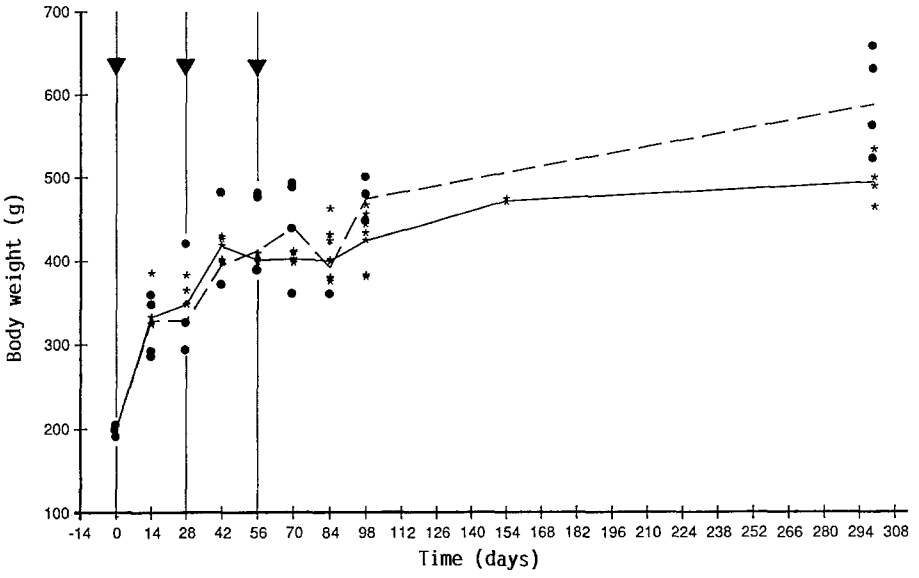


**Fig. 1.** Mean values for body weight. Over the experimental period, the mean values for bodyweight of treated male did not differ significantly from that of controls (*arrowed lines* indicate vaccination times). ●——●, Controls; *——*, immunized

## Material and methods

### *Experimental animals*

For active immunization, 74 adult male Sprague-Dawley rats with an initial weight of 180–200 g (191 ± 12 g) were used. The animals were kept under conditions of controlled temperature, light and diet. Twenty-five animals with the same weight served as controls.

### *Preparation of Vaccine*

Details of synthesis of the LHRH analogue and preparation of the vaccine (LHRH-diphteria toxoid conjugate) have been published elsewhere [9].

### *Immunization*

The test animals were immunized with three consecutive i.m. injections at intervals of 4 weeks. The single dose per animal was 20 µg suspended in 200 µl of a sterile medium (physiological saline with 5% squalene and 0.2% Tween 80). For the first injection, SPLPS from *Salmonella enteritidis* served as the adjuvant. For the two subsequent injections, the LHRH-diphteria toxoid conjugate was adsorbed on alums. The control animals received i.m. injections of the sterile medium in equivalent amount (200 µl) at the same time intervals. Anti-LHRH vaccine was provided by the National Institute of Immunology (New Dehli, India).

### *Histopathology*

Following the first injection on day 0, blood was taken for hormone analysis by heart puncture at 14-day intervals from six treated and six control animals, after which the pituitary, thyroid, adrenal glands, testes, epididymis, seminal vesicles and the ventral prostate were removed, weighed and subsequently prepared for light and electron microscopy. For histological examinations the organs were fixed, either in Bouin's solution (Zenker, Helly and Karnovsky). The paraffin sections were stained with Azan (Heidenhain) and the iron hematoxylin stain (Weigert). For specific aspects, Kresazan (Romeis), Papanicolaous', Masson trichromatic (Goldner) and the periodic acid/Schiff orange G staining techniques (Pearse) were employed.

### *Electron microscopy*

For analysis of cell structures by electron microscopy, the organs were perfused in a 2.5–4% glutardialdehyde buffered with 175 m*M* cacodylate (pH 7.3), and subsequently fixed in 2% aqueous osmium tetroxide by the method described [16]. Ultrathin sections were observed under a Phillips 300 electron microscope.

### *Fertility studies*

For fertility study and observation of sexual behaviour, one treated male was paired with two females for one week on days 0, 98, 154 and 300 of the experiment schedule of the vaccination. The females were allowed to deliver and the number of pups born, if any, were counted in each female. Some of the females were killed for gross observations.

## Results

The effects of active immunization against LHRH on the hormone-dependent organs of the reproductive tract of male rats were examined at two-week intervals following the first injection (day 0). Organ weights are expressed in g/100 g body weight. Within the first 40 days following the commencement of the study, both the treated as well as the control animals showed a continuous increase in body weight (mean 209 ± 6.11 g). After 42 days the weight gain in treated animals was slower than that of the control animals; however by day 98, it was again approximately the same as that of the control animals (Fig. 1). The curve traced by the body weight of the treated animals as well as their general external appearance and eating habits, rule out any adverse effect of the vaccination on the general health of the animals with the exception of the decline in libido, a factor that was to be expected.

From day 28, after the second injection with the anti-LHRH-DT vaccine, the weights of the reproductive organs, such as testis (Fig. 2), epididymis, seminal vesicles and prostate gland (Fig. 3), showed a marked decrease in

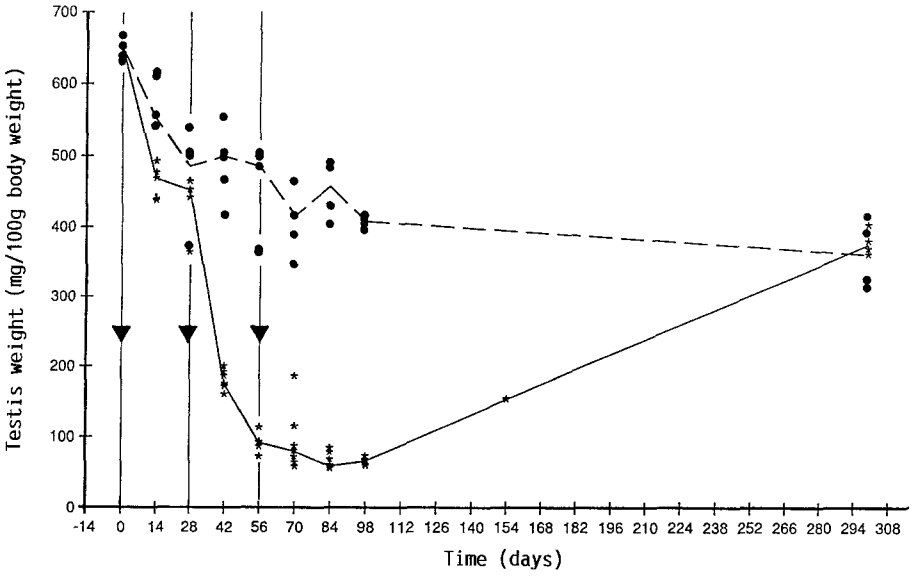


**Fig. 2.** Mean values for testis weights (*arrowed lines* indicate vaccination times). ● – – ●, Controls; *———*, immunized

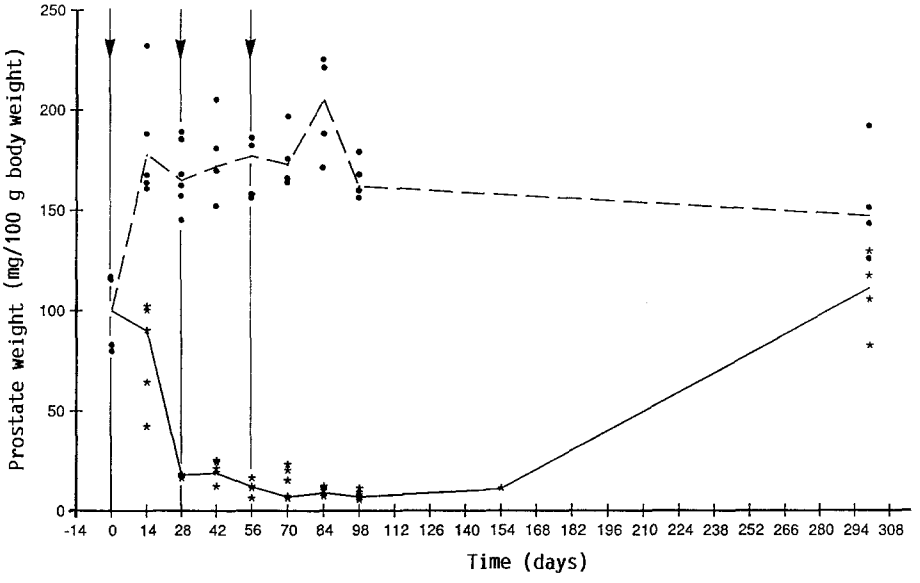


**Fig. 3.** Mean values for prostate weights (*arrowed lines* indicate vaccination times). ● – – ●, Controls; *———*, immunized

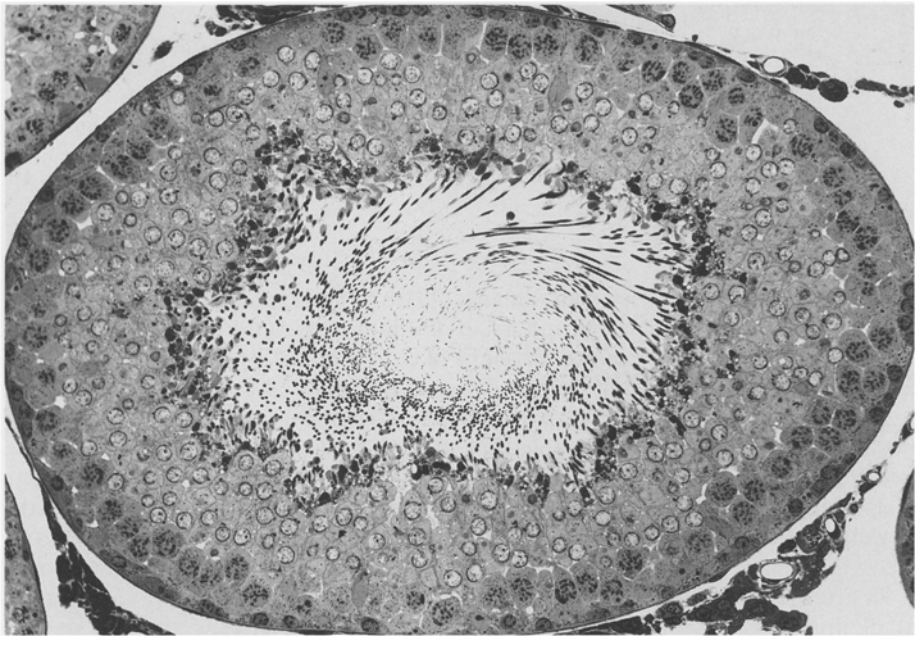

**Fig. 4.** Control testis tubule (semithin section): the normal arrangement of the germinal epithelium. ×1040

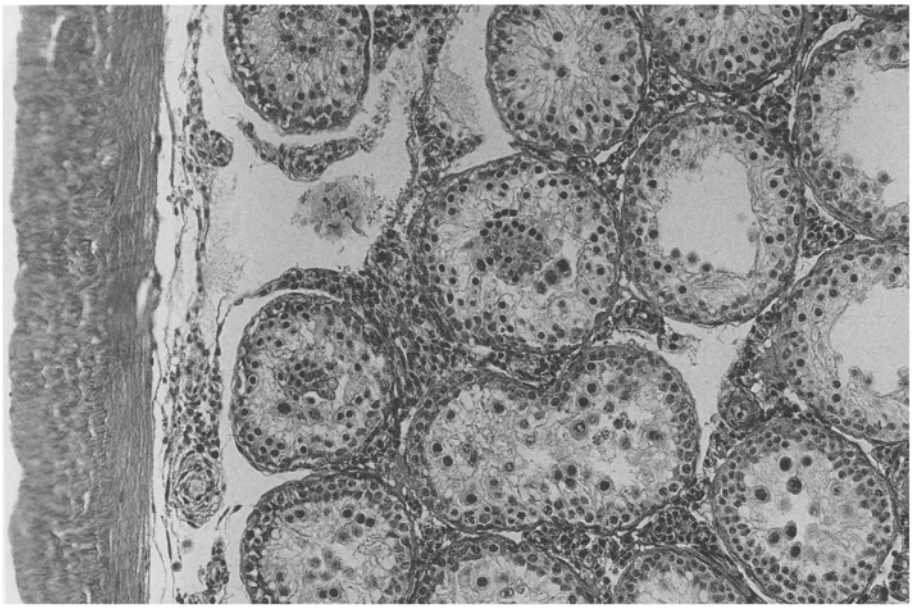

**Fig. 5.** Histopathology of testis on day 98 is showing a coarse, fibrotic tunica albuginea and diameters of tubuless significantly reduced to half that of controls. ×650

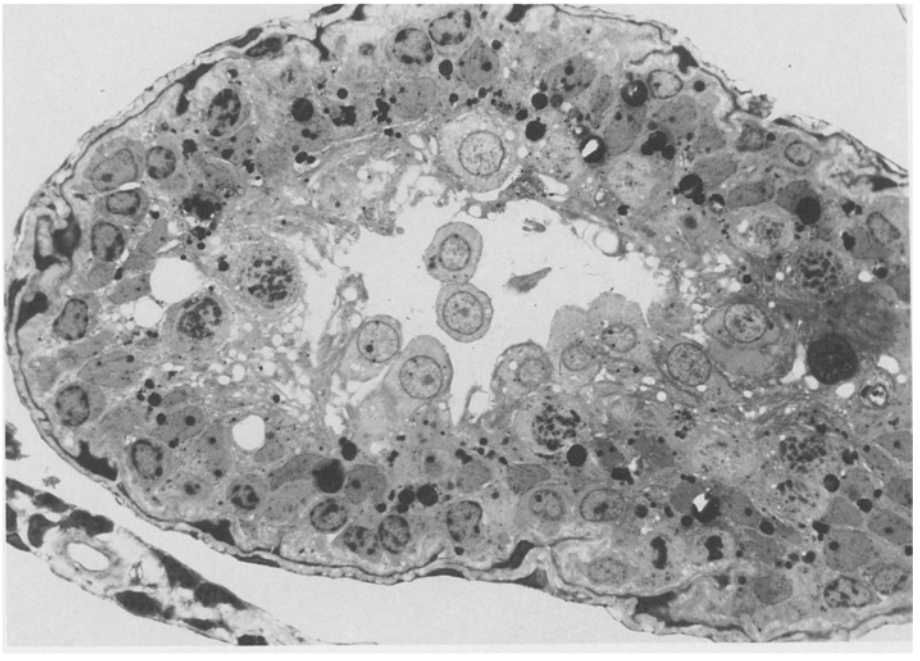

**Fig. 6.** Germinal epithelium on day 54 is characterized by an arrest of spermatogenesis at the stage of spermatid differentiation. The tubular lumen is devoid of spermatozoa. ×2600

weight; the weights of the pituitary, thyroid and adrenal glands were however in the normal range. After the second immunization (day 28), the weights of all the reproductive organs decreased significantly; the maximum inhibition in the testis and prostate weight (Figs. 2, 3) was reached 2 weeks after the third injection (about day 70) and continued till day 98 (6 weeks after third injection) (Fig. 2). In contrast, the adrenal glands, pituitary and thyroid glands showed no change in their weights after the third injection.

Histopathology of the testes on day 98 after the first injection showed, in contrast to the control animals (Fig. 4), a dense, markedly fibrotic and coarse tunica albuginea. The periphery of the lamina propria testis also showed pronounced signs of collagenous fibrosis. Diameter of the seminiferous tubules and thickness of the germinal cell height were reduced to approximately half (Fig. 5). Our study shows that spermatogenesis is clearly arrested at the stage of spermatid differentiation from day 54 after commencement of immunization. The seminiferous tubules were completely devoid of germinal elements and the majority of the tubules showed multinucleated giants cells (Fig. 5). By day 98, the seminiferous tubules (Fig. 6) predominantly consisted of Sertoli cells and one or two

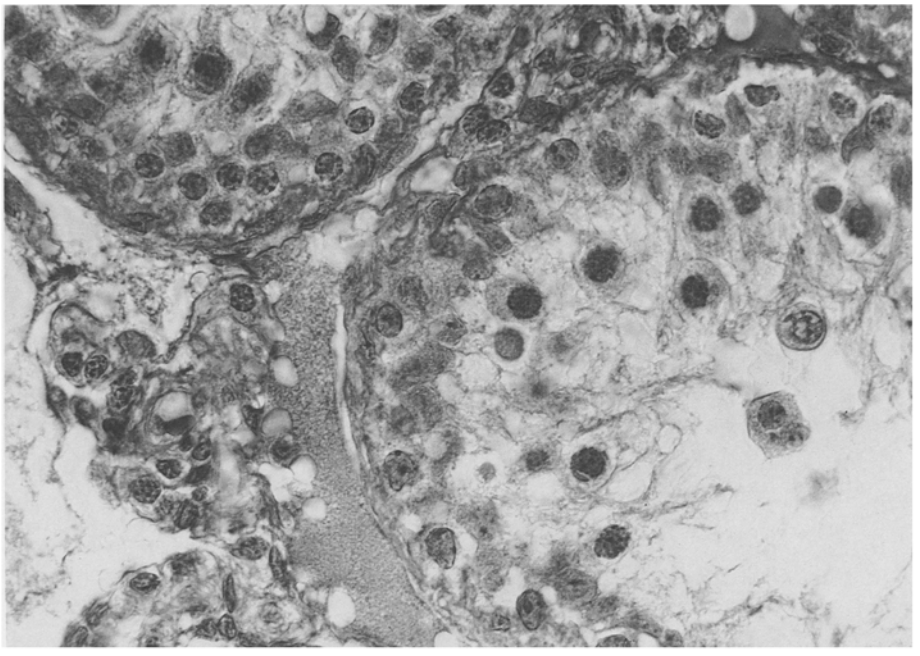

**Fig. 7.** Histology of testis on day 98: the testis tubules are predominantly formed by Sertoli cells and one or two layers of spermatogonia. The spermatocytes appear necrotic, spermatids are exfoliated into the lumen. ×2600

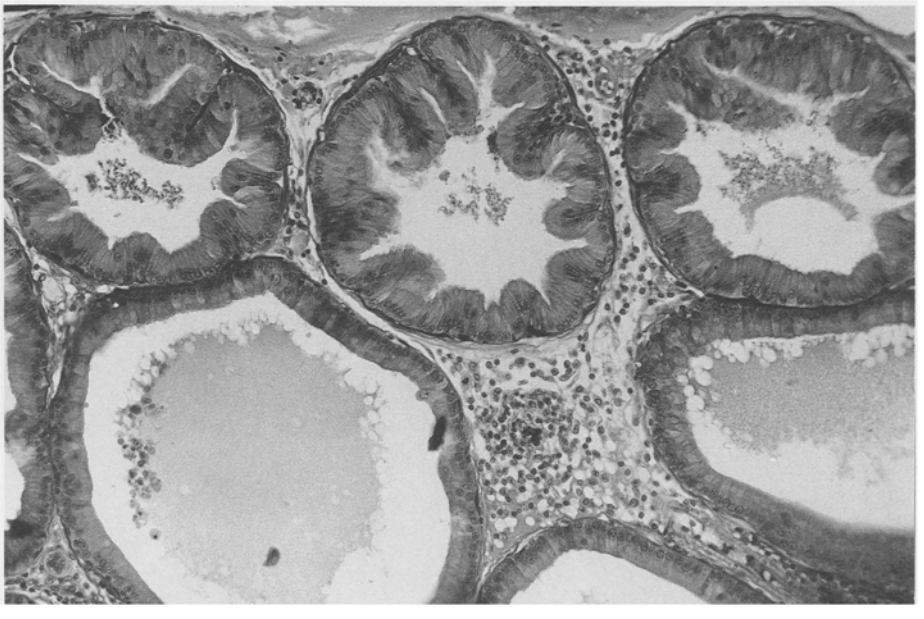

**Fig. 8.** Histology of ventral prostate lobe on day 14: the stroma shows accumulations of lymphocytes as a primary immune response. ×1040

layers of spermatogonia. Necrotic spermatocytes and spermatids, as single cells or clusters of giant cells, were found within the luminal space (Fig. 7).

The histopathology of the prostate gland showed that during the entire study, this organ hat the most severe reactions to the LHRH block. The primary immunological reaction in the form of occasional lymphatic infiltrates (Fig. 8), already occurring 14 days after commencement of immunization, was more pronounced as the vaccination schedule progressed. At day 54, this immune reaction was evidently supported by the appearance of macrophages and granulocytes (Fig. 9). The increased occurrence of mastocytes (Fig. 10) can be regarded as a second step of the immunogenic reaction, including the vasomotor effective release of serotonin, histamine and tissue factors. As a sequel of down-regulation of testosterone synthesis, a general gland atrophy occurred (Fig. 11): the originally highly prismatic or multi-rowed isoprismatic epithelium of the tubulo-alveolar glandular terminals (Fig. 12) were transformed to a necrotic cluster of cells that revealed no signs of secretory activity under light or electron microscopy (Figs. 13, 14). The lumen of the glandular terminals and the collecting tubes were largely free of secretion or filled with inspissated remnants of the

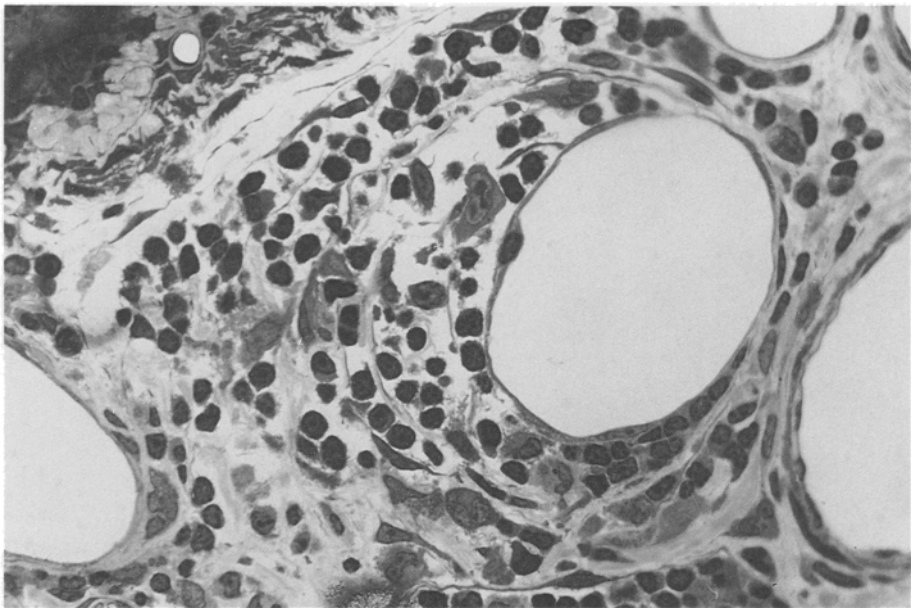

**Fig. 9.** Histology of ventral prostate lobe on day 54: the infiltration of lymphocytes was followed by invasions of macrophages and leucocytes. ×1040

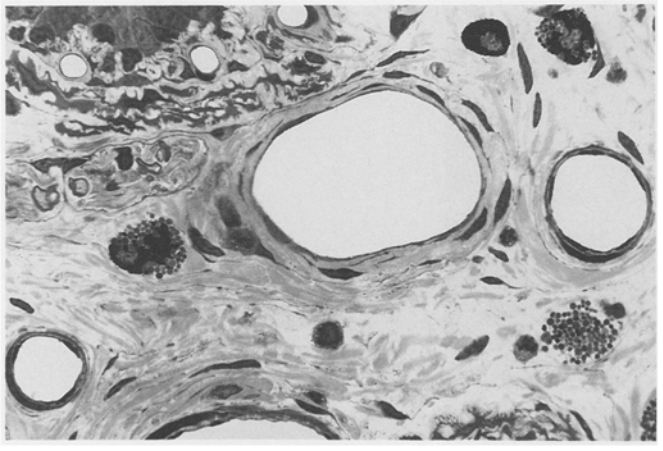

**Fig. 10.** Interstitial prostate tissue on day 98: an increased occurrence of mastocytes delivering their granular contents can be noted. ×2600

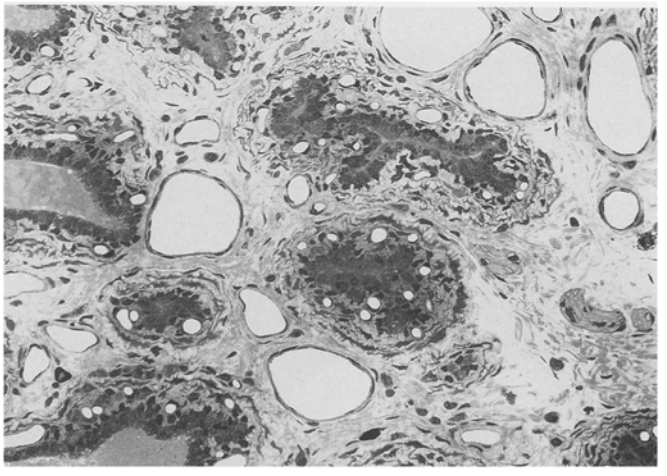

**Fig. 11.** General view of ventral prostate atrophy on day 98. ×260

formerly serous mucous secretion. The basal lamina was collagenous and showed deep, labyrinth-like invaginations into the epithelium (Fig. 13). The originally loose reticular arrangement of the normal interstitial tissue was extensively transformed to a collegenous fibrotic network with only restricted tracts for blood vessels (Fig. 16), and as anticipated, massive accumulations of lympho-, granulo-, mono- and mastocytes were observed in response to immunization (Figs. 9, 15). Atrophic transformations were also found in the seminal vesicles and epididymis, accompanied by a complete arrest of functional activities and interstitial fibrosis (Fig. 17).

Histological examination of the testis 154 days after vaccination showed progressive regeneration of the germinal epithelium and the interstitial tissue. The spermatogenetic cycle was completed and spermatozoa were already present in the tubular lumen (Fig. 18). In parallel with these observations in recovering testis, a distinct recovery in the secretory units and connective tissue of prostate gland was evident by day 154 after commencement of immunization (Fig. 19). Furthermore, the epididymal epithelium acquired its typical cellular arrangement and few spermatozoa could be found in the ductus of the caput region (Fig. 20).

The regeneration progressed in testis and accessory sex organs, so that by day 300 all organs reached their normal function and histological architecture (Figs. 21, 22). Correlating with the histological analysis, the mating studies revealed that, from day 54, immunized males became azoospermic and infertile. This infertility period

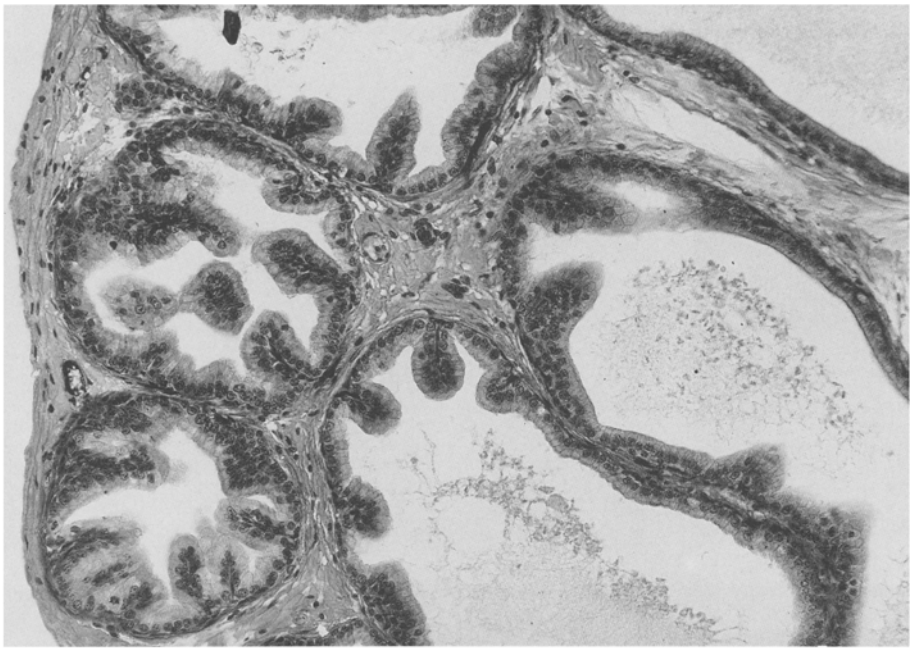

**Fig. 12.** Glandular terminals of control prostate gland. ×1040

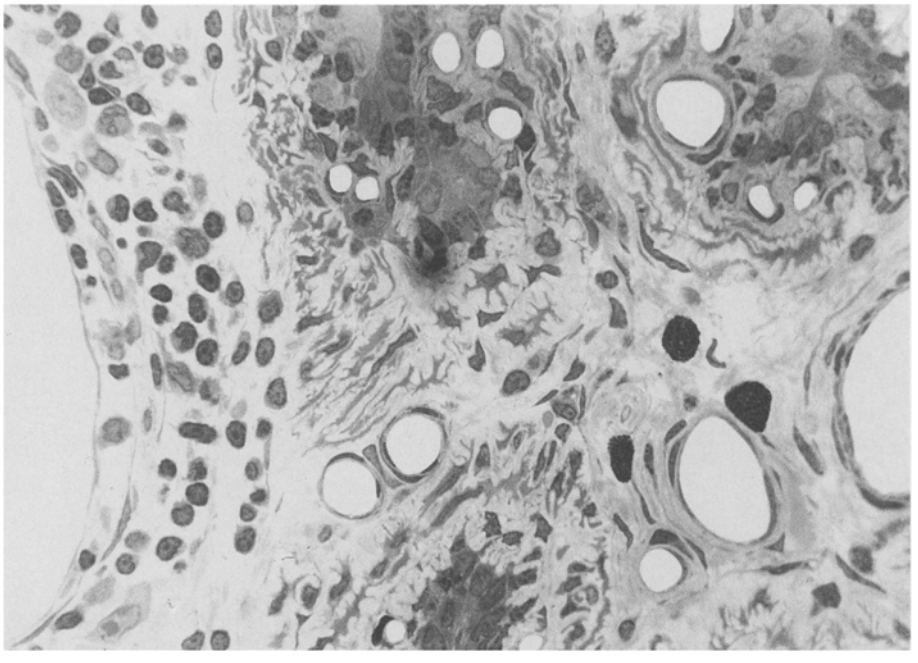

**Fig. 13.** Atrophied secretory terminals of prostate gland with massive fibrosis of lamina propria. ×1040

lasted longer than 100 days, although testis biopsies showed a complete spermatogenic cycle by day 154. Histological evaluations of the epididymis indicated that only in the caput region were a few spermatozoa found, and the following subsequent parts, including the vas deferens, were devoid of mature spermatozoa. By day 300, three out of four treated males were fertile. On autopsy the fourth male had bilateral cryptorchism. All six mated females had normal litters.

## Discussion

Administration of the anti-LHRH-DT vaccine to rats induced complete down-regulation of the hormone axis until at least day 94 after the first injection of the vaccine, and was accompanied by complete inhibition of gonadal testosterone synthesis similar to surgical castration [17]. Talwar et al., in studies conducted in New Delhi, observed complete down-regulation of the hormone axis following treatment with LHRH vaccine [7, 9, 19, 20]. The decrease of gonadal testosterone caused the hormone-dependent organs of the reproductive tract to change correspondingly, with regard to both weight loss and histology. As soon

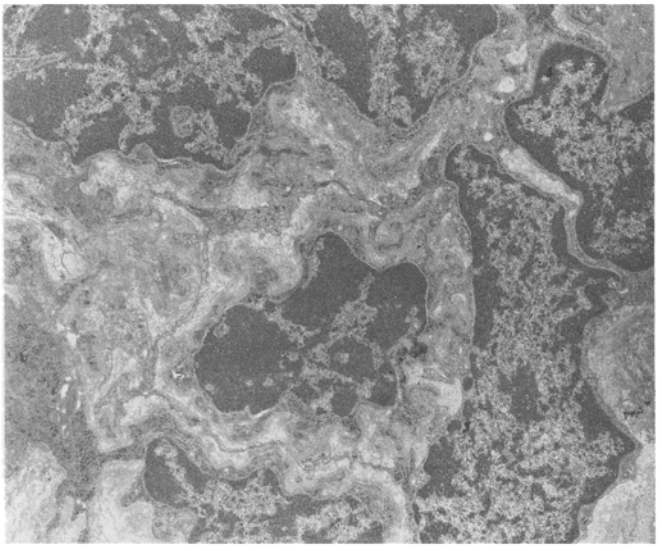

**Fig. 14.** Fine structure of necrotic secretory prostate cells on day 98. ×8800

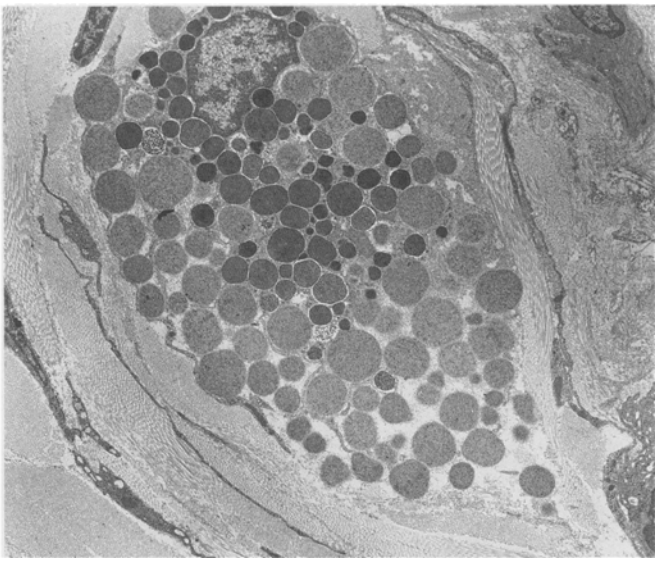

**Fig. 15.** Fine structure of mastocytes with different steps of granule formation. ×15000

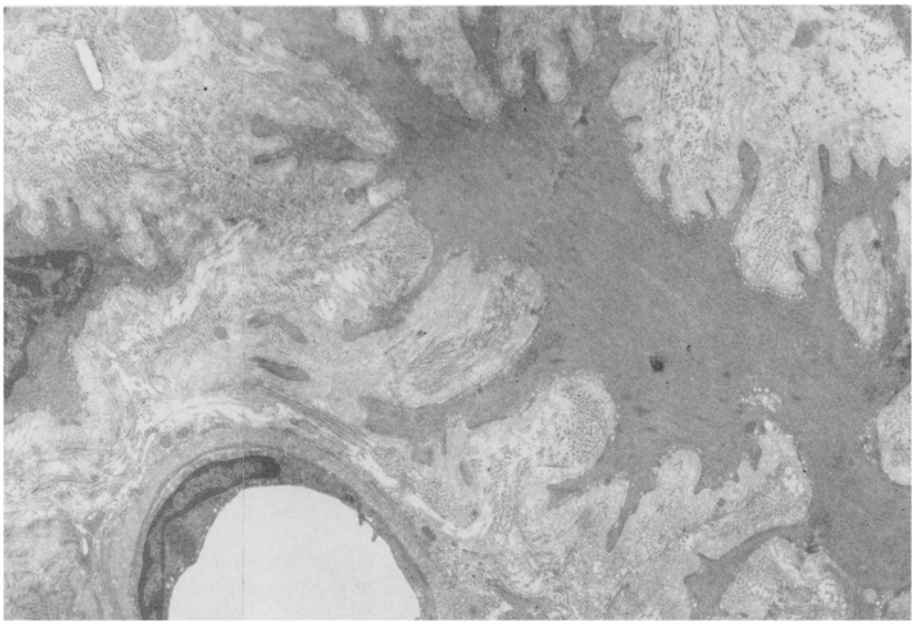

**Fig. 16.** Detail of stroma fibrosis of ventral prostate on day 98. ×15000

as testosterone dropped to castration level, the animals displayed a complete loss of libido, but no other side effects. There was no change in food intake, nor any other reaction advocating hypersensibility. The animals showed no signs of an immune illness. The efficacy of the LHRH-diphtheria toxoid vaccine to induce atrophy of the ventral prostate has been clearly demonstrated in our study. It is a well established observation that structural and functional integrity of accessory sex organs are under the control of androgens. Dihydrotestosterone (converted from testosterone) acts as intracellular androgen for the regulation of metabolic activity of the prostate [14]. From our observations, one can conclude that involution of prostate is due to deprivation of circulating levels of testosterone, an observation supported by a number of studies [7, 9, 11, 19]. In the light of the observations made by Sheth et al. that LHRH regulates the testosterone metabolism in the prostate directly [18], possibilities of LHRH vaccine having a direct effect on prostate, in addition to gonadal testosterone, cannot be ruled out. Although we have carried out studies with normal healthy rats, the data generated from our animal model study can be extrapolated to the management of prostate carcinoma.

Complete arrest of spermatogenesis has been observed by day 98 in our study; it is believed that anti-LHRH immunization is exercising its atrophic effect by blocking

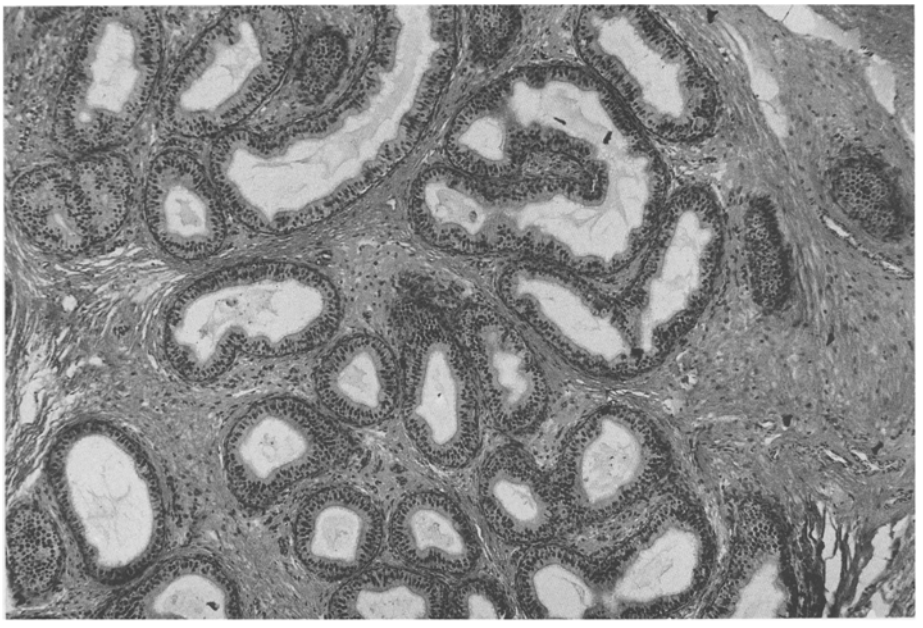

Fig. 17. Advanced atrophy of epididymis on day 98. ×650

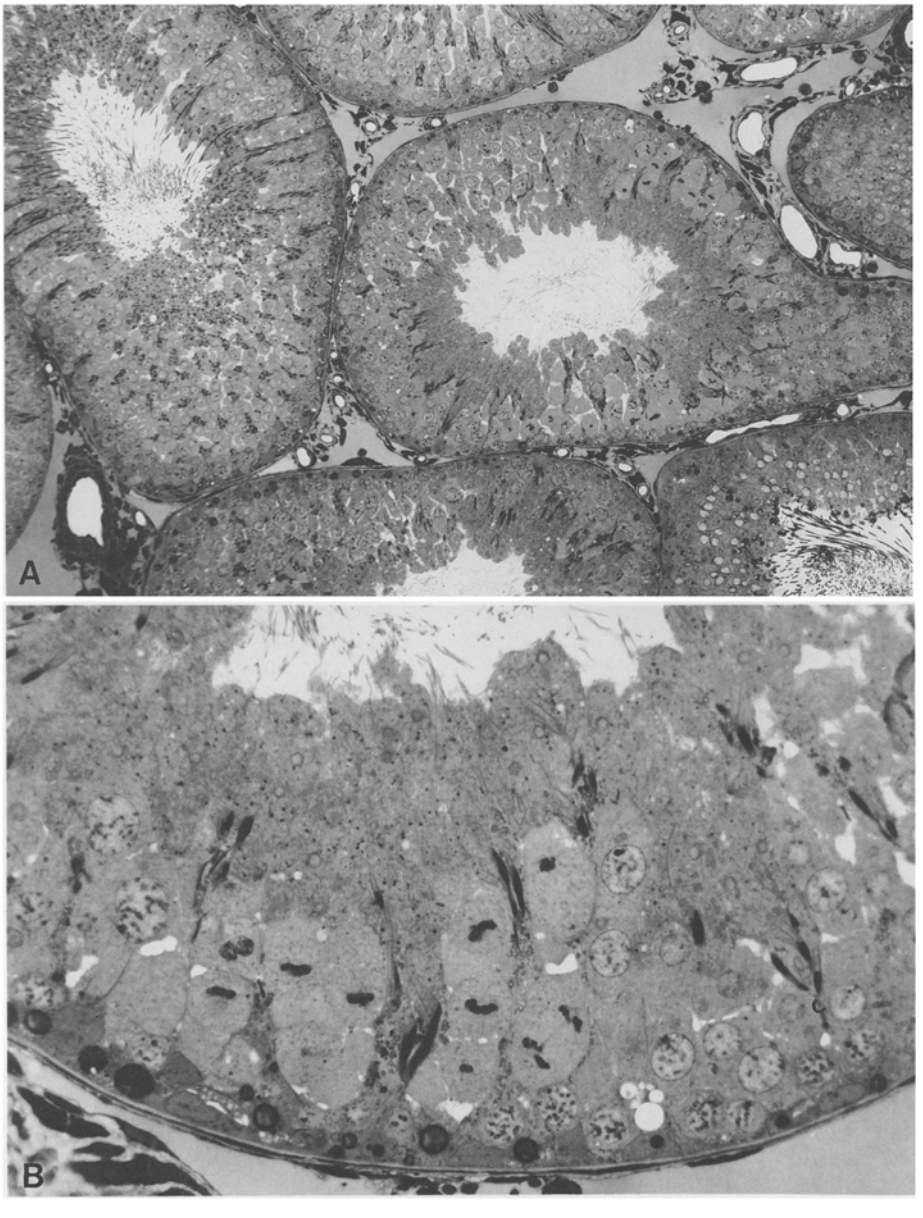


Fig. 18. A Regeneration of tubular and interstitial compartments on day 154: note the copmpleted spermatogenic cycle, the meiotic division patterns and the spermatozoa within the lumen. ×650. B Detail of meiotic division patterns at the stage of spermatocytes. ×2600

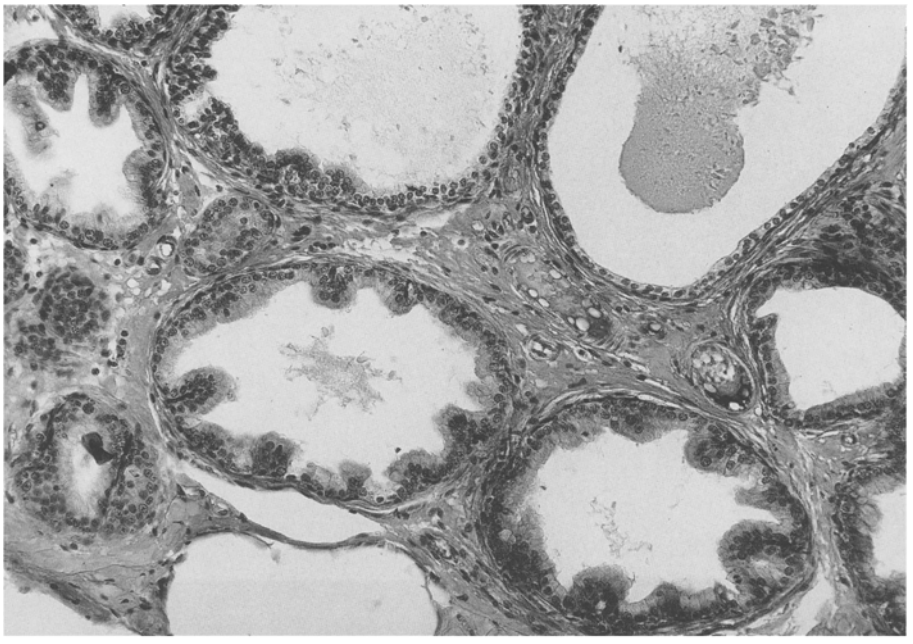

**Fig. 19.** Regeneration of secretory epithelia of the ventral prostate on day 154. ×1040

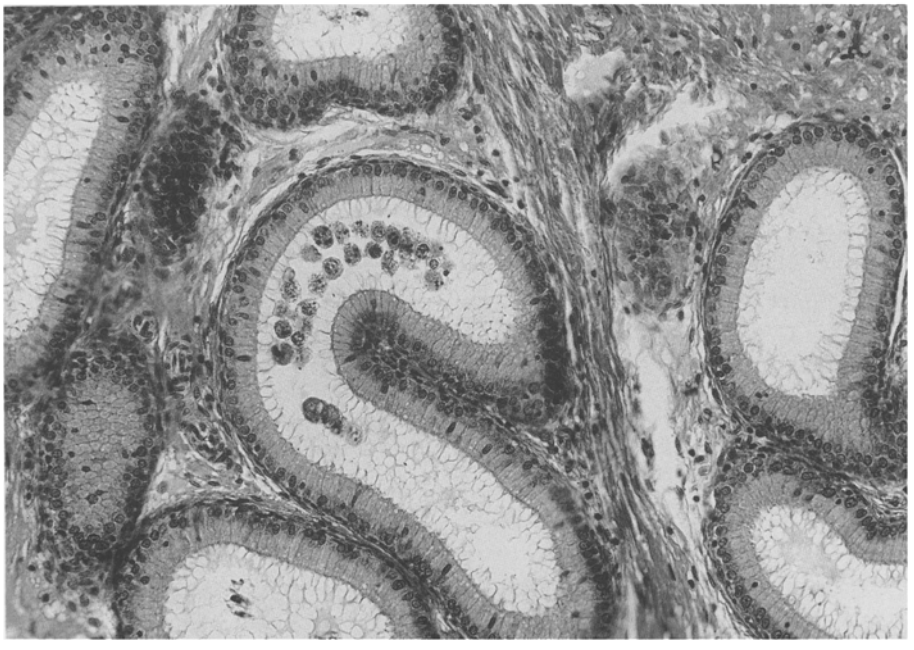

**Fig. 20.** Histoarchitecture of the caput epididymis on day 154: note the presence of few spermatozoa and exfoliated germinal cells within the ductus lumen. ×1040

testosterone biosynthesis. Since LHRH has been reported to have a direct effect on rat testis [8], this axis may be an additional factor for the atrophic effect of LHRH vaccine on spermatogenesis. Whatever the mechanism of inhibition of spermatogenesis by LHRH vaccine, it is clear that the dose schedule and route applied in this study prevent spermatogenesis and fertility. Restoration of complete fertility by day 300 indicates total reversibility of the vaccination. Since this vaccination schedule for regulation of male fertility would require supplementation with androgen for the maintenence of libido [12], LHRH vaccine, supplemented with a long-acting androgen at a particular time in the dose schedule, and an adequate delivery system can be effectively used in the management of male fertility.

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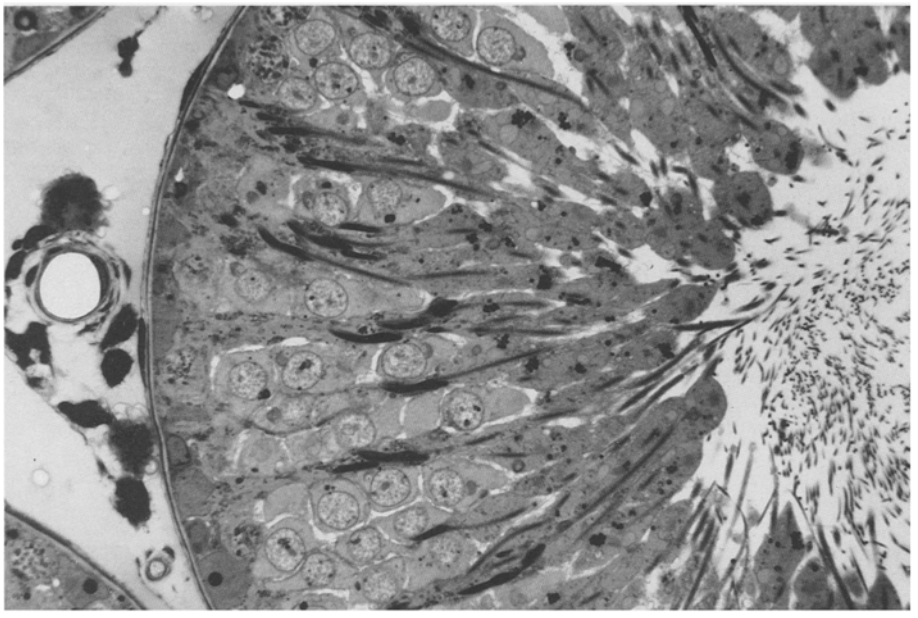

**Fig. 21.** Histology of testis on day 300. ×2600

**Fig. 22.** Histology of epididymis on day 300. ×650

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Prof. Dr. J. Frick
Urologische Abteilung
Landeskrankenanstalten
A-5020 Salzburg
Austria