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The effect of active immunization against gonadotropin-releasing hormone on the ultrastructure of the rat ventral prostate

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Abstract To evaluate the effects of active immunization against gonadotropin-releasing hormone (GnRH) on the ultrastructure of the rat ventral prostate, male Sprague-Dawley rats received three consecutive intramuscular injections of 10 µg/100 g body weight (D-Lys^6)-GnRH-diphtheria toxoid conjugate (GnRH-DT vaccine). Following immunization, test animals developed sufficiently high antibody titres to block the pituitary gonadal axis. Consequently testosterone values dropped to the levels in castrates. This therapy leads to atrophy of the prostate. Following immunization a strong immunological response, indicating the presence of considerable amounts of a GnRH-like peptide, was observed in the ventral prostates as early as 14 days after the first injection of GnRH-DT. Immunoneutralisation of GnRH-like activity may contribute to the effects observed.

Key words GnRH-DT vaccine · Testosterone · Ultrastructure · Rat · Prostate

Introduction

Blocking of gonadal testosterone synthesis has been effectively used in the management of advanced prostate carcinoma. This can be achieved by desensitization or down-regulation of the anterior pituitary with gonadotropin-releasing hormone (GnRH) analogues, currently the substances used most commonly for this purpose [6, 11, 14, 16].

Another approach to blocking the pituitary gonadal axis is based on the idea of active immunization against GnRH. Since native GnRH has a very short biological half-life and – as a decapeptide – low immunogenicity, synthetic GnRH analogs conjugated to carriers such as albumin fractions, IgG or diphtheria toxoid are used as vaccines. Together with suitable adjuvants, i.e. "Freund's complete adjuvant", "MDP" or "SPLPS from *Salmonella enteritidis*", these vaccines induce an immune response as a result of which native GnRH is neutralized [19]. The anterior pituitary is consequently understimulated and therefore testosterone synthesis in the testis is blocked. This should lead to regression of testosterone-dependent organs such as the tubular compartment of the testis, the epididymis, seminal vesicles and, most important, the prostate [1–2, 5, 17]. Using an appropriate application protocol active immunization against GnRH could be a promising approach for the management of prostate cancer and, together with adequate testosterone substitution, for fertility control [10].

In this paper we report the effect of active immunization against GnRH with a GnRH-diphtheria toxoid conjugate (GnRH-DT) on the pituitary gonadal axis by examining serum testosterone levels and antibody titres as well as the ultrastructure of the rat ventral prostate.

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Materials and methods

Experimental animals and immunization schedule

Male Sprague-Dawley rats with an initial body weight of 191 ± 12 g were kept under controlled temperature, light and diet conditions. The anti-GnRH-DT vaccine was provided by Prof. G.P. Talwar of the National Institute of Immunology, New Delhi, India. Test animals were immunized with three consecutive intramuscular injections (every 28 days) of 20 µg GnRH-DT in 200 µl physiological saline solution substituted with 5% squalene and 0.2% Tween 80. The first injection contained SPLPS from *S. enteritidis* as an adjuvant; the other two injections were adsorbed to alums. Control animals received equal amounts of the substituted physiological saline solution at the same times.

A group of six animals was killed every 2 weeks after initiation of the vaccination protocol. Blood was drawn by heart puncture, the serum separated and stored at –20°C until further use. Ventral

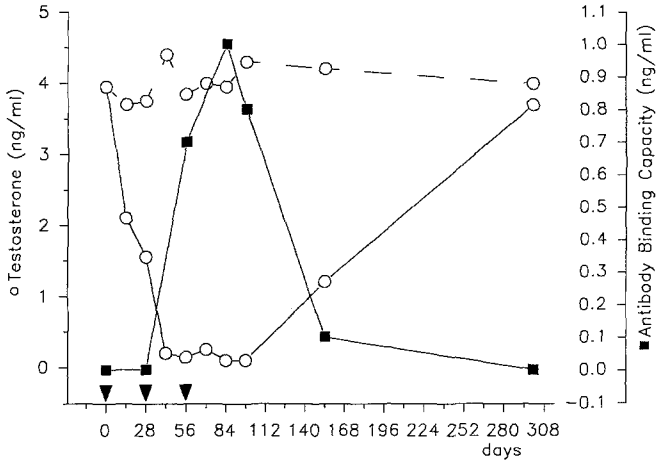


Fig. 1 With rising antibody titres, testosterone drops to the level in castrates. O– – –O Control; O——O vaccine; ▼ application of vaccine

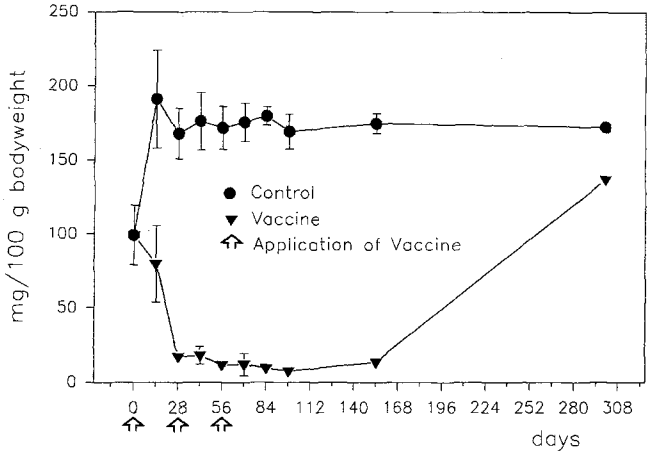


Fig. 2 Following vaccination, prostate weights decrease 5% that of controls

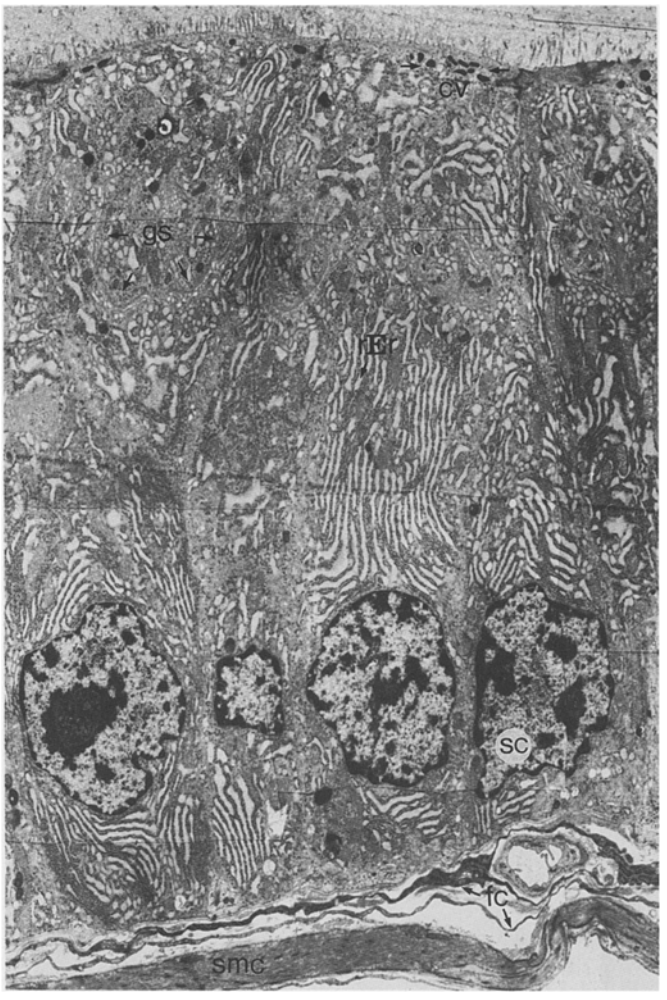


Fig. 3 the day 42: the first atrophic events within secretory cells (*sc*). Rough endoplasmic reticulum (*rER*) is partially dilated and appears "empty". Condensed secretory vesicles (*cv*) come from the final cycles of vesicle formation. Fibrocyte (*fc*), Golgi system (*gs*), smooth muscle cell (*smc*). ×5600

prostates were removed, weighed and prepared for light and electron microscopy.

Testosterone assay

Serum levels of testosterone were estimated by radioimmunoassay (RIA) using the Serono testosterone kit with ^{125}I-testosterone as tracer. Sera of orchiectomized animals served as controls to estimate the detection limit of the assay, which is about 0.2 ng/ml testosterone.

GnRH-antibody titre

GnRH-antibody titres were measured at the National Institute of Immunology, New Delhi, India, using a modified RIA method. In brief, the assay protocol consisted of 50 µl each of normal horse serum (diluted 2.5-fold in assay buffer), diluted antiserum, phosphate buffer (50 mM, pH 7.4) and ^{125}I-GnRH. After incubation for 18–20 h at 4°C the antibody-bound ^{125}I-GnRH fraction was separated and measured.

Electron microscopy

Organs were perfused with 2.5%–4.0% glutardialdehyde buffered with 175 mM cacodylate (pH 7.3) and subsequently postfixed in 2.0% aqueous OsO_4. After dehydration in a graded series of alcohol, organs were embedded in Epon 812. Semithin sections were stained with Azur II and observed under a Reichert Univar microscope. Ultrathin sections were contrasted with lead citrate and uranyl acetate and observed under a Phillips EM 300 transmission electron microscope.

Results

All rats from the immunized group developed antibodies against GnRH. Although antibody titres were highest after the third injection of GnRH-DT, a high titre was maintained until day 98 of the protocol. With rising antibody titres, testosterone dropped to castrate levels (Fig. 1).

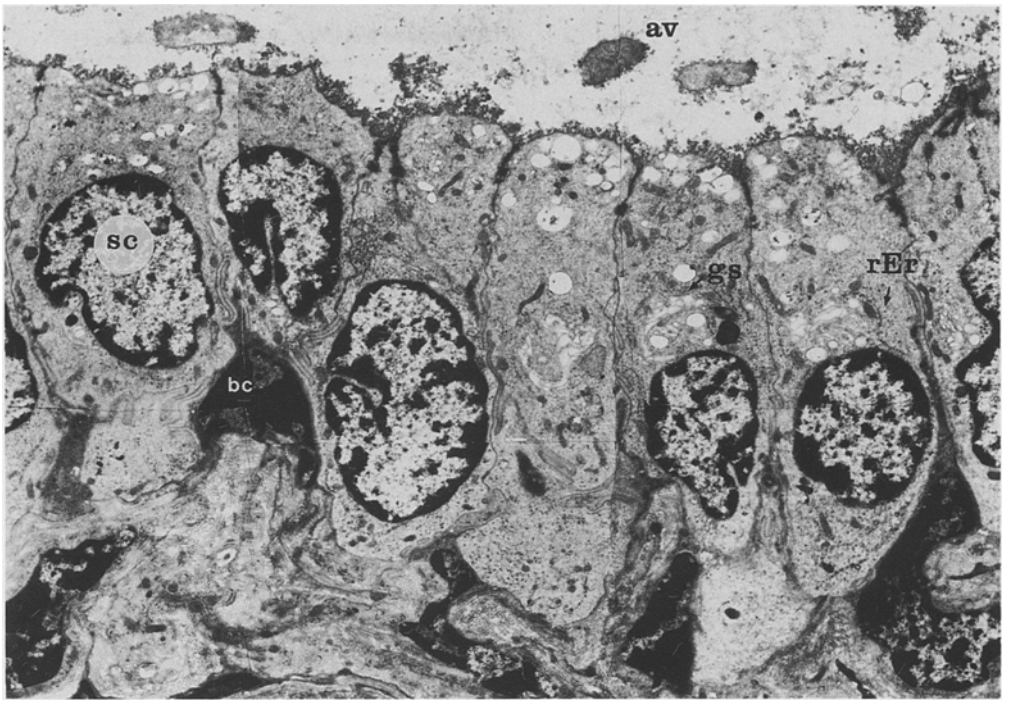
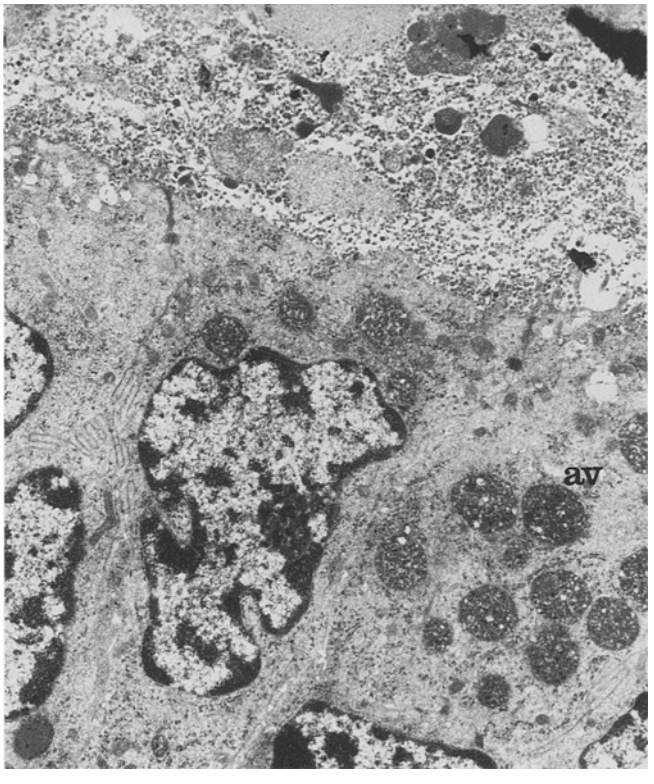


Fig. 4 Day 70: virtually the entire rough endoplasmic reticulum (*rER*) and Golgi system (*gs*) of secretory cells (*sc*) have regressed. Note the expelled autophagic vacuoles (*av*) within the lumen of acini. ×5600



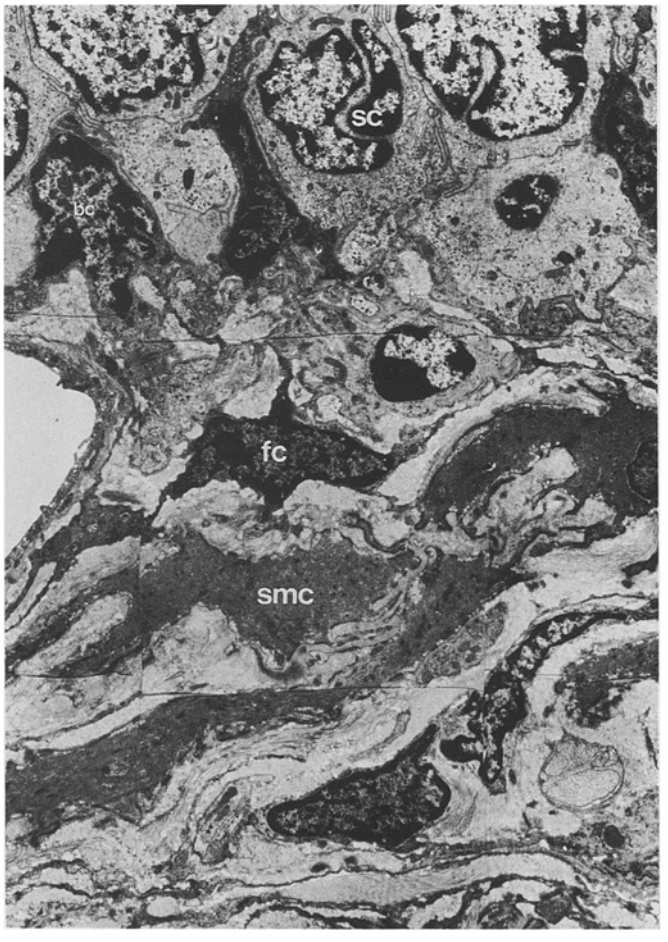


Fig. 5 Day 70: autophagic vacuoles (*av*) within secretory cells (*sc*). ×5600

Fig. 6 Day 70: due to necrosis of secretory cells (*sc*), basal cells (*bc*) with their characteristic, condensed nucleus can be observed more frequently. The acinar wall becomes fibrotic; layers of fibrocytes (*fc*) and smooth muscle cells (*smc*) are increased. Due to collagenous fibrosis, smooth muscle cells lose contact with each other. ×5600

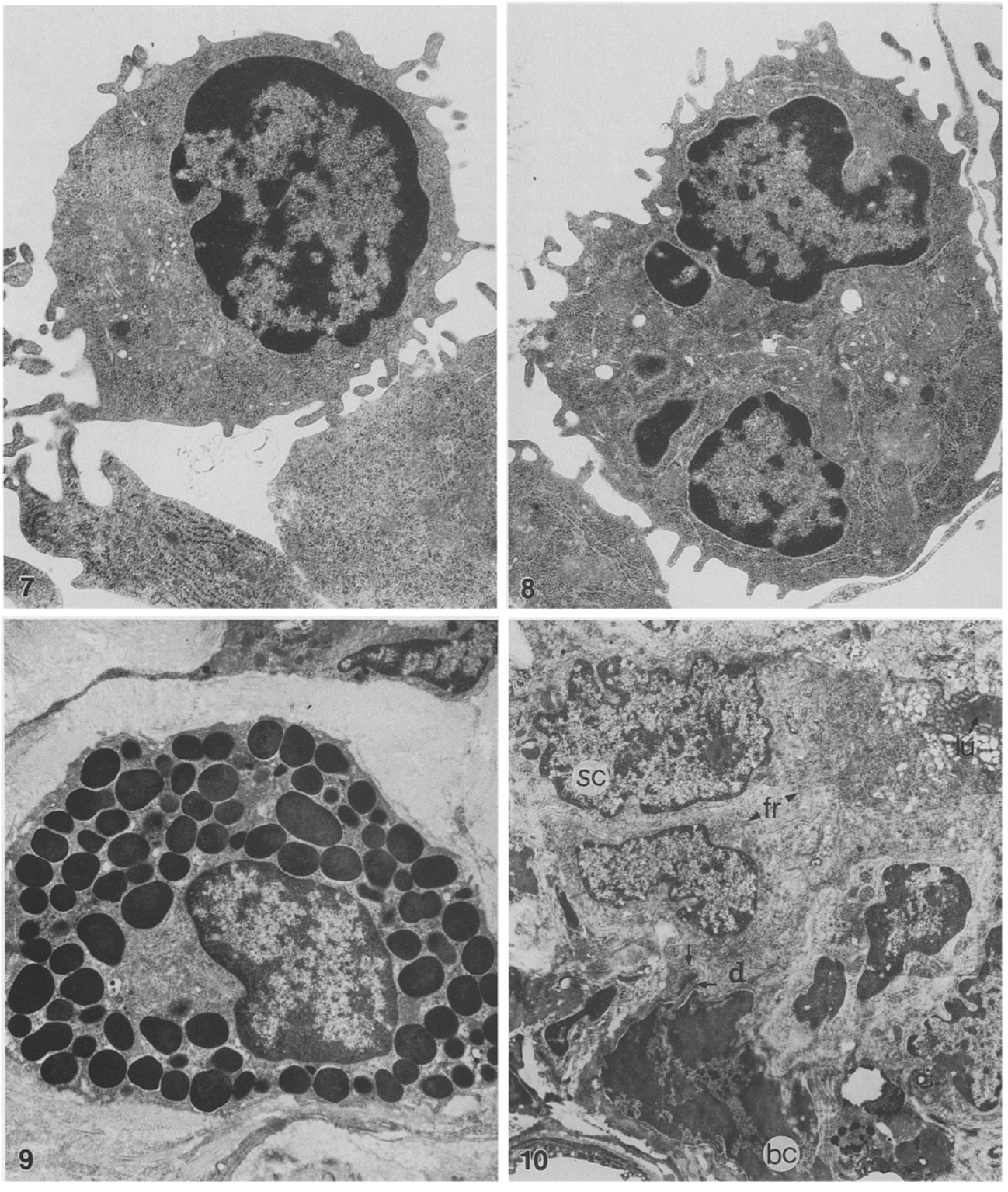


Fig. 7 Perivascular prolymphocyte. ×14500. **Fig. 8** Perivascular monocyte. ×14500. **Fig. 9** Mast cell; the loose reticular connecting tissue is transformed into a collagenous fibrotic network. ×7800. **Fig. 10** Day 98: acini have regressed to small clusters of secretory cells (*sc*) without any signs of secretory activity. Note the high number of free ribosomes (*fr*). The lumen of acini has almost disappeared. Basal cell (*bc*), desmosome (*d*), lumen (*lu*). ×6000

Due to the low testosterone values prostate weights decreased to 5% of the control weights at day 98 (Fig. 2).

Using electron microscopy, the first signs of cellular atrophy were observed at day 42 as a partially dilated and "empty" rough endoplasmic reticulum. Since the Golgi system appeared unaltered, remaining secretory vesicles came from the final cycles of vesicle formation. The

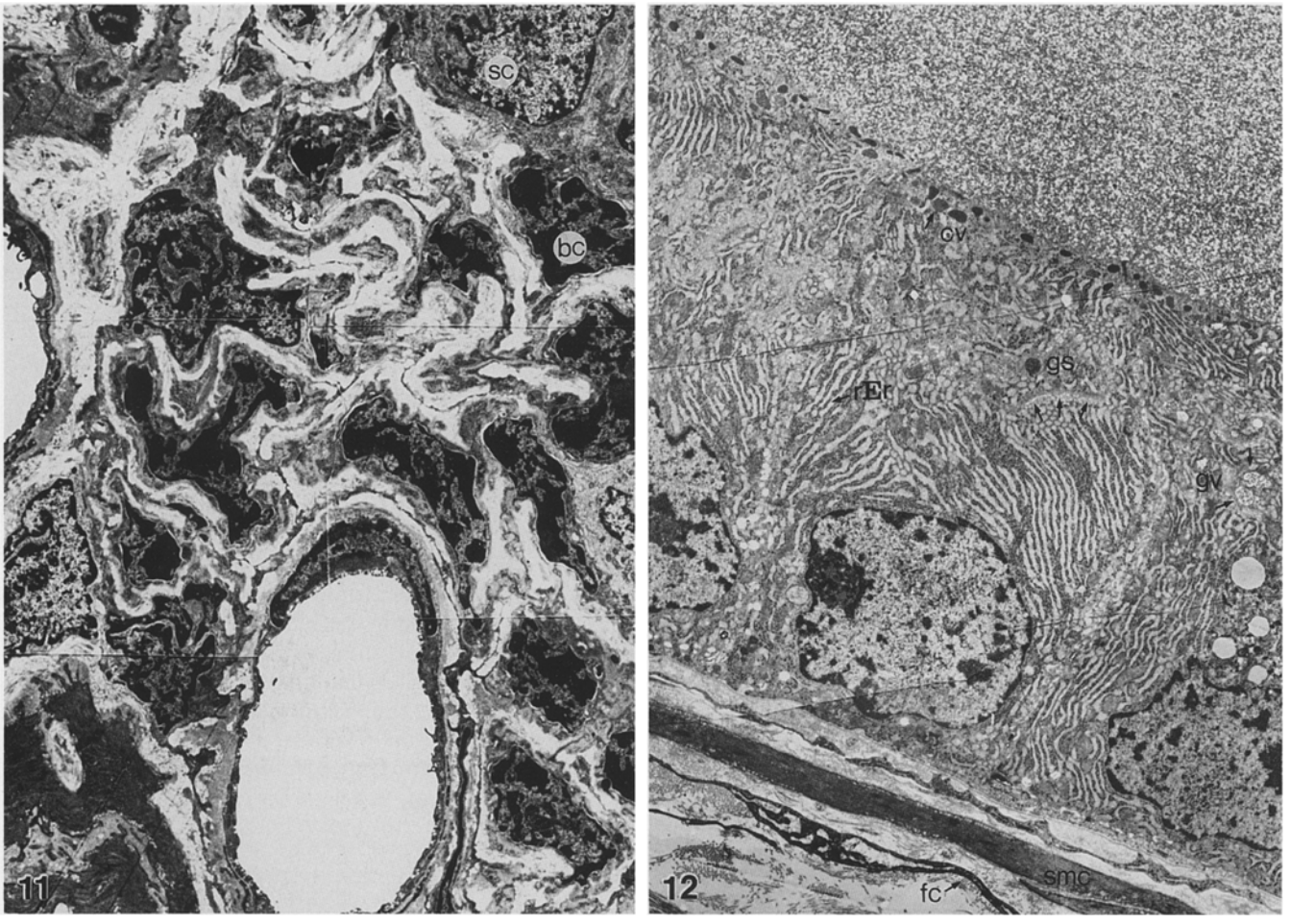


Fig. 11 Day 98: due to necrosis of secretory cells (*sc*), basal cells (*bc*) are increased in number and build several layers beneath the remaining "silent" secretory cells. ×5600. **Fig. 12** Day 300: both the stroma and the parenchyma appear normal. Note the normal distribution of rough endoplasmic reticulum (*rER*), Golgi system (*gs*) and secretory vesicles [granular (*gv*) and condensed (*cv*)]. No differences from control prostates can be observed in the acinar wall. Smooth muscle cells (*smc*), fibrocytes (*fc*). ×5600

acinar, consisting of one to two layers of fibrocytes and smooth muscle cells, as well as the loose reticular connective tissue appeared unaltered (Fig. 3).

Seventy days after the first injection of GnRH-DT virtually the entire rough endoplasmic reticulum and the Golgi system had regressed. Parts of them were found in autophagic vacuoles within what were formerly secretory cells or the lumen of the acini (Figs. 4, 5). As the number of secretory cells decreased, basal cells – identified by their nuclear configuration and the location of desmosomes to secretory cells – as well as the layers of the acinar wall appeared to be increased in number. The wall of the acini became fibrotic. As a consequence of collagenous fibrosis, smooth muscle cells lost contact with each other (Fig. 6). The loose reticular connecting tissue was transformed into a collagenous fibrotic network with massive accumulations of lympho-, granulo- and monocytes; mastocytosis was observed (Figs. 7–9). This immune response started at day 14, when no atrophic effects could be observed, and became more and more apparent until day 98.

Atrophic events progressed. Most of what were formerly secretory cells showed severe damage and become phagocytosed by macrophages (day 98). Therefore "basal cells" built several layers beneath the remaining silent secretory cells (Figs. 10, 11). Surviving secretory cells contained mainly free ribosomes and very few cisternae of rough endoplasmic reticulum. About 98 days after the first injection of GnRH-DT, antibody titres started to decline and testosterone levels to rise, indicating recovery of the pituitary gonadal axis (Fig. 1). With rising testosterone levels, ventral prostates gained weight (Fig. 2). Light and electron microscopic examination showed a recovery of the secretory epithelium as well as of the stroma. Reorganization of the secretory epithelium seemed to emanate from basal cells. The first secretory vesicles appeared in adluminal parts of secretory cells, indicating the beginning of new cycles of secretory vesicle formation. By day 300 testosterone levels (Fig. 1), prostate weights (Fig. 2) and prostate ultrastructure both of the parenchyma and the stroma (Fig. 12) appeared normal.

Discussion

Since it is well established that the structural and functional integrity as well as the growth of accessory sex organs are controlled by androgens [3], most attempts at managing growth disorders of the prostate, i.e. benign prostatic hyperplasia or prostate cancer, are based on the possibility of interrupting the action of testosterone on target organs such as the prostate. Atrophy or involution of these testosterone-dependent organs can be induced by the administration of testosterone antagonists and GnRH analogues [13, 15, 16]. Unfortunately prostate cancer acquires testosterone independence after some time, making therapy with GnRH analogues and testosterone antagonists ineffective.

Active immunization against GnRH leads to testosterone down-regulation and induction of an immune response within the prostate. The immune response seems to be due to the presence of intraprostatic GnRH, since it was observed as early as 14 days after the first injection of GnRH-DT. At this time no atrophic effects were observed, excluding the possibility that inflammation occurred as a result of necrosis of secretory cells in the prostate. It is known from the testis that GnRH is synthesized locally and plays an important role as a paracrine modulator of Leydig-cell function [4]. In the testis, too, an immune response occurs before atrophy of the prostate begins, strengthening the theory that GnRH is present in the ventral prostate of the rat. The existence of GnRH receptors has already been demonstrated in the prostate of man and rat [9, 18]. Although there is increasing evidence that GnRH might be a paracrine or autocrine modulator of prostate function (i.e. regulation of hormone receptors), the physiological role of GnRH in normal and transformed prostates remains uncertain [9, 12, 14, 17, 18]. Only recently the synthesis of GnRH was demonstrated in the human cancer cell line LNCaP-FGC [12]. Whether GnRH is synthesized in the prostate of rodents is unknown; thus we cannot exclude the possibility that the observed immune response is due to accumulation of native GnRH and/or GnRH-DT within the prostate. Hence, there might be a new circuit in the regulation of the prostate which can be utilized for the management of prostate cancer, even if testosterone-independent.

There have been a number of very detailed reviews of the effects of castration on the rat ventral prostate [3, 7, 8]. In brief, castration leads to atrophy of the prostate within 3 weeks, accompanied by a marked decrease of prostatic weight. Using electron microscopy, the first changes in the rough endoplasmic reticulum can be observed within 2 days after castration. This process includes the formation of so-called whorls of the rough endoplasmic reticulum, which then become entrapped and are subjected to autophagy. Autophagic vacuoles reach their numerical peak 3 days after castration and are then replaced by dense bodies. Apical cisternae of rough endoplasmic reticulum become dilated, and secretory activity is enhanced prior to cessation of synthesis. Regression of the Golgi system starts between days 3 and 5. Both the size and appearance of the cisternae are affected. Changes reach there endpoint about 3 weeks post-castration. Secretory cells appear reduced in height and contain few cisternae of the rough endoplasmic reticulum and Golgi system as well as frequent lipid droplets. The lumen of the acini is narrowed and contains no secretion.

Following vaccination comparable events can be observed. Major differences can be seen at the time at which these alterations occur. Orchiectomy results in a rapid and substantial decrease of circulating androgens; therefore, histological alterations appear very soon and are much more pronounced than after vaccination. In animals immunized against GnRH, testosterone deprivation develops relatively slow, so that secretory cells of the prostate can adapt to the declining serum testosterone levels in terms of secretory activity and abundance of intracellular organelles such as the rough endoplasmic reticulum or the Golgi system. Formation of "whorls" was rarely observed. Below a critical concentration of testosterone, synthesis of secretory products is terminated (about day 42) and the organelles involved in synthesis begin to regress. Once again the mode of action is autophagy. The result is that "secretory cells" contain mainly free ribosomes and only a few cisternae of the rough endoplasmic reticulum and the Golgi system. In terms of cellular atrophy, vaccination can therefore be designated as effective as castration.

At the first sight, Fig. 1 shows a discrepancy between declining testosterone levels, rising antibody titres and weight reduction of the prostate. Testosterone levels as well as prostate weights decline before GnRH antibody titres become measurable (days 0–28). This can be explained by the detection limit of the assay used to determine serum concentrations of GnRH antibodies. Since vaccination against GnRH leads to an intraprostatic and intratesticular immune response (perivascular infiltrates of lympho- and monocytes) until day 14 of the immunization protocol, the presence of GnRH antibodies seems likely. Hypothalamic GnRH is secreted cyclically and in very low amounts, so that hardly detectable concentrations of antibodies directed against GnRH can lead to the onset of down-regulation of luteinizing hormone (LH) within the pituitary.

Prostate weight is mainly determined by the amount of secretion present in the lumen of the acini. With declining testosterone levels, the synthesis rate of the prostatic epithelium and therefore prostatic weight declines, before atrophic events can be observed at the cellular level.

In previous studies dealing with active immunization against GnRH, GnRH conjugated to human or bovine serum albumin together with Freund's complete adjuvant was used as vaccine. However, these conjugates proved not to be as effective in inducing high antibody titres as the GnRH-DT conjugate used in this study [1, 2, 10, 20]. Since Freund's complete adjuvant is not recommended for use in humans, SPLPS from *S. enteritidis* was used to make this vaccine suitable for clinical application.

Figure 1 shows that high antibody titres can be maintained with monthly boosters of GnRH-DT. Except for

the expected loss of libido, we were able to demonstrate that active immunization against GnRH has no adverse effects on other endocrine organs such as the thyroid, the adrenal gland and the pituitary (except for a loss of LH immunoreactivity). Test animals were healthy with normal food intake and weight gain, so there seems to be no reason why therapy with a GnRH-DT vaccine should not be prolonged. Testicular effects, i.e. arrest of spermatogenesis and fibrosis of the tubular wall, seem to be dose- and time-dependent, so that for use as fertility control, adequate testosterone substitution to compensate for loss of libido and the tendency for a Sertoli-cell-only syndrome to develop should be employed to ensure recovery of testicular function [10, 17]. Using three consecutive injections of GnRH-DT, lack of testosterone does not seem to be critical as regards recovery, since by day 300 after the first injection of GnRH-DT, test animals were as fertile as controls, confirming the complete recovery of prostate tissue and testis as seen using light and electron microscopy.

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