

Immune Complex Deposition in Adult Male Sprague–Dawley Rats Chronically Immunized with GnRH

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Introduction

The pursuit of a long lasting, yet reversible, male contraceptive has been a focus of research for many years. One approach is to inhibit sperm production for a defined length of time, thus rendering the male temporarily infertile. Because spermatogenesis is initiated through a hormonal cascade that culminates in the production of testosterone (T), one method of stopping sperm production is to disrupt this pathway, thus preventing T from being produced. Without T, sperm production is abolished.

Problem

This study was undertaken to evaluate whether the anti-GnRH antibodies and immune complexes (IC) generated by immunization with GnRH-TT cause cellular damage within the animal.

Method of study

Chronic immunization of rats with GnRH-TT injected i.m. was followed by tissue/organ analysis for immune complex deposition by immunofluorescence microscopy. Two groups were studied: (1) those immunized throughout the experiment until their ultimate demise, and (2) those given a chance to recover from the effects of chronic immunization before final analysis.

Results

GnRH-TT was effective in stopping spermatogenesis, which resumed after withdrawal of the immunogen. Most tissues from chronically immunized animals were not significantly different than controls, however the kidneys of treated animals exhibited a higher accumulation of IC. Despite increased IC deposition, pathologic effects were not detected at the cellular level.

Conclusions

GnRH-TT is an effective immunocontraceptive although the accumulation of glomerular IC represents a potential deleterious side effect.

The hormonal pathway to sperm production begins in the hypothalamus where the GnRH is secreted. GnRH stimulates the pituitary to secrete the gonadotropins (LH and FSH), which in turn act upon the testes to initiate and sustain spermatogenesis. The major player at this point is the gonadotropin LH that stimulates the Leydig cells of the testis to produce T. The resulting high concentration of T in the testes is what directly initiates and maintains spermatogenesis.¹ Therefore, inhibiting T production would cause spermatogenesis to shut down. In this study, we stopped spermatogenesis by interrupting the

hormone cascade leading to T production by blocking the effect of GnRH on the pituitary using anti-GnRH antibodies.

In 1973, just after GnRH was identified, porcine GnRH was purified, bound to a carrier protein, and injected into rabbits to generate antibodies against GnRH. Anti-GnRH antibodies were needed to develop assays for hormone quantification, function, and mechanism. During this antibody generation process, an altered phenotype was noted in the immunized male rabbits. The testes of the immunized rabbits reduced in size, and, when they were examined histologically, they were found to be devoid of sperm.² What was not known at the time of these experiments was that GnRH has been highly conserved through evolution and that rabbit and porcine GnRH are 100% identical in structure. Therefore, the anti-porcine GnRH antibodies generated by the rabbit reacted with the rabbits' intrinsic GnRH and neutralized the stimulatory effect of GnRH on the pituitary, which shut down the hormonal cascade that culminates in sperm production. With spermatogenesis halted, the testes in the rabbits shrank to near their pre-pubertal size.²

GnRH is a hapten and thus by itself not immunogenic because it lacks a T-cell-stimulating epitope. For GnRH to be immunogenic it must be bound to a carrier protein that can supply the missing T-cell stimulation.²⁻⁵ This hapten-carrier immunogen stimulates antibody production to both the hapten and the carrier portions of the chimera. Many carrier proteins have been used to generate anti-GnRH antibodies. The carrier we selected for this study was tetanus toxoid (TT). We have shown that if GnRH is covalently bound to TT it can generate a strong immune response in rats.⁶ We have also shown that this immunogen produces bioreactive anti-GnRH antibodies that neutralize the effect of native GnRH on the pituitary, thus halting LH and FSH production and the subsequent production of T and spermatogenesis.⁷

The fact that GnRH alone is not immunogenic is the feature that has made it an attractive candidate for a reversible immunocontraceptive. Many immunocontraceptives are theoretically possible, but if they cannot be reversed then no significant improvement is made over vasectomy. Furthermore, using a self-antigen as an immunogen harbors the potential to induce an autoimmune disease state because once the self-antigen has been stimulated to produce an immune response it could become self-sustaining in

its immune-priming effect. This risk, however, is eliminated using GnRH because it is a hapten and thus incapable of mounting an immune response unless it is coupled to a carrier protein. Thus, once the GnRH-TT immunogen is depleted from the body, anti-GnRH antibody titers will drop. As the anti-GnRH antibody titers diminish, the normal hormonal cascade is reestablished and spermatogenesis resumes.⁷ This illustrates the mechanism for reversal of contraception. Reinstatement of the contraceptive condition is accomplished simply by re-boosting with more of the GnRH-carrier immunogen.

We, and others, have shown this to be the case with GnRH bound to a variety of carriers.^{2,8-24} We have also shown that after three GnRH-TT immunizations, given 1 month apart, circulating T levels in the rat are suppressed to minute or undetectable values.⁶ However, if no more boosts of GnRH-TT are given to the rats, T levels will return to normal approximately 14 weeks after the last immunization, and spermatogenesis will resume.

Besides the autoimmune considerations, other potential problem areas must be examined when using an immunological approach to contraceptive development. For example, immune complexes have the potential to cause tissue damage. Some immune complexes stimulate complement binding that can result in cell lysis. In this study, we wanted to evaluate whether immune complexes generated after GnRH-TT immunization caused tissue damage. To this end, we immunized rats with GnRH-TT for various prolonged lengths of time to fully explore any problems generated by the immunogen and its complexes over time. At the end of experiments, the animals were terminated and their tissues examined for immunocomplex deposition as well as cellular damage. We also studied animals that underwent GnRH-TT immunization followed by a non-immunization rest phase. These animals were used to determine if any tissue or cellular injury seen from immunizations would be permanent or transient. The results from these experiments will be used to assess the safety and feasibility of developing GnRH-TT into an immunocontraceptive for men.

Materials and methods

Animals

The experiments were initiated on 12-week old, male Sprague-Dawley rats (Charles River Laborat-

ories, Wilmington, MA, USA). The animals were housed following conventional housing and caging protocols at The Rockefeller University Laboratory Animals Research Care facility. Animals were fed a standard diet (Purina Rat Chow), and both food and water were available *ad libitum*. Adult, male Brown Norway (BN) rats (Charles River Laboratories) were also housed and maintained as above (BN rats were induced to produce auto anti-kidney-glomeruli antibodies and their kidney sections used as positive controls in IF studies). All protocols were approved by the institutional IACUC and all procedures performed followed animal safety and welfare guidelines put out by the National Research Council.²⁵

Buffers and Chemicals

All chemicals used were reagent grade and obtained from Sigma[®] Chemical Company (St. Louis, MO, USA) unless otherwise noted. Phosphate Buffered Saline (PBS): 0.01 M phosphate, 0.15 M NaCl; Adjuvant Pluronic-Tween 80-PBS (PT-PBS): 2.5% Pluronic L121, 0.2% Tween 80 in PBS.

Immunogens and the Immunization Schedule

Generation of anti-renal-glomerular antibodies for positive controls

A 2% mercuric chloride stock solution was prepared in water. For immunizations, the stock solution was diluted 1:10 in PBS and filter-sterilized through a

0.2 µm filter. Male BN rats (~200 g) were injected i.p. with 100 µl three times per week for 2 weeks. Control rats were injected with filter-sterilized PBS. At week 3 the rats were killed and their kidneys removed for cryosectioning.

All animals in groups 1–10 were immunized starting on day 1, and received booster immunizations every 4 weeks thereafter for the length of time shown in Fig. 1. All immunizations were performed in the mornings, at approximately the same time each day, while the animal was anesthetized for blood drawing.

Immunization with TT

Tetanus toxoid by itself (TT; Mass Biological Laboratory, Boston, MA, USA) was injected in groups 6–10 via i.m. in the hind thigh with 100 µg TT/500 µL of adjuvant PT-PBS.

Immunization with GnRH-TT

The GnRH-TT immunogen (made in-house; TT covalently conjugated to the first amino acid of GnRH) injected i.m. as above 100 µg GnRH-TT in 500 µL of adjuvant PT-PBS.

Blood Collection and Processing

Every fourth week each animal was anesthetized by inhaling a metaphane/air mixture, bled via their tail vein, and ~1.5 mL of blood collected. At termination, the animals' bloods were taken by cardiac puncture. The bloods were incubated at RT (RT) for

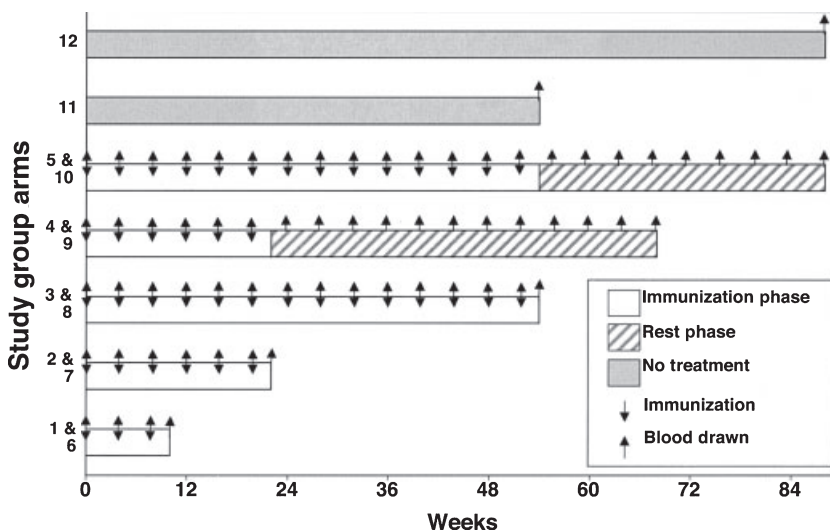


Fig. 1 Diagram of experimental study design. The various groups of animals in the study arms are represented by the bars with their length spanning the X-axis denoting the weeks of study for each group. White bars indicate the immunization phase of the study (groups 1–10), while the hatched bars indicate the rest phase of the study (groups 4, 5, 9, and 10). Gray bars represent the non-immunized, aged-matched control animals. Upward pointing arrows indicate when bloods were drawn, while downward pointing arrows represent immunizations events.

1–2 hr, spun at ($\sim 10\,000\ g_{av}$) for 10 min, and the serum removed and stored at -20°C .

Tissue/organ removal, Processing, and Cryosectioning

Animals were killed by CO_2 inhalation. The brain was removed first, placed in a coronal slicing mold (Zivic Laboratories, Inc., Portersville, PA, USA), and cut at the coronal planes framing the median eminence (ME). This brain section was placed in a sectioning mold, surrounded by OCT, and fresh-frozen with powdered dry ice. The pituitary, cervical lymph nodes, heart, lungs, spleen, liver, kidneys, testes, epididymides, and prostate were also removed and likewise processed. Tissues were serially cryosectioned to generate 5- to $7\text{-}\mu\text{m}$ -thick sections on a Zeiss Microm HM500M cryostat, placed on Superfrost[®] Plus slides and stored at -80°C .

Histochemical Staining

Hematoxylin and eosin

Tissue slides were incubated in 10% formalin at RT for 15 min, rinsed in water, incubated in hematoxylin and eosin according to the manufacturer's instructions (Richard Allan, Kalamazoo, MI, USA). Slides were air dried then sealed with Cytoseal[™] 60 mounting medium and a glass cover slip and stored in an opaque box at 4°C .

Cresyl violet

The brain tissue slides were incubated in decreasing concentrations of ethanol for 5 min each (100, 95, and 70%), then water rinsed. Slides were incubated with cresyl violet acetate staining solution (2.5 g cresyl violet acetate, 300 mL water, 30 mL of 1 M sodium acetate, and 170 mL of 1 M acetic acid, stirred 7 days at RT then filtered through a $0.45\ \mu\text{m}$ nylon filter) for 1 hr, rinsed in water, and air dried, mounted, and stored as above.

Immunofluorescence Assay

Tissue slides were brought to RT and incubated in PBS supplemented with 10% horse serum for 30 min. The tissue sections were covered with a 1:50 dilution of FITC-labeled, goat anti-rat IgG Fab₂ according to the manufacturer's recommendation, and incubated in a dark 37°C humidified incubator for 30 min. The tissue slides were washed for three

10 min washes in PBS. Slides were mounted and stored as above.

Microscopic Examination and Photography

Tissue samples were examined using an Olympus BX60 epifluorescence (NY/NJ Scientific, Somerset, NJ, USA), short arc mercury lamp microscope and pictures were taken with either: T_{max} 1000 b/w film or Ectachome 400 slide film using an Olympus 35 mm camera or obtained digitally using an Olympus QImaging Micropublisher 3.3 megapixel, Peltier-cooled, CCD color digital camera run using QCapture 2.68. 6 software (Quantitative Image Corporation) and imported into Adobe[®] Photoshop[®] 7.01 for analysis. All 35 mm slides were scanned at 1200 dpi on an Epson Expression 1680 scanner using an Epson Scan plug-in through Photoshop[®] 7.0. An identical adjustment to tonal range was made to all images using the levels settings in Photoshop[®].

Radioimmunoassays

Testosterone

The Coat-a-Count testosterone kit (Diagnostic Products Corp., Los Angeles, CA, USA), consisting of anti-testosterone antibody-coated tubes, was used to assay testosterone levels in serum following the manufacturer's protocol. Briefly, a standard curve was established (in triplicate) consisting of 0, 0.035, 0.175, 0.7, 1.4, and 2.75 pmol testosterone. Three quality control tubes supplied by the manufacturer were also run (in triplicate) to ensure the assay itself was within normal operating parameters. All unknown samples were run in duplicate. ^{125}I -labeled testosterone was added to each tube, and the tubes were vortexed and incubated for 3 hr at 37°C . The liquid was aspirated, and the tubes were counted for 1 min in a gamma counter. Total counts and non-specific binding controls were run. The results were calculated using a modification of the Rodbard-NIH RIA program (Bethesda, MD, USA).

GnRH antibody titers

All samples were run in duplicate. 100 μL of 1% BSA in 10 mM PBS was placed into 12×75 mm plastic test tubes. Serum samples were diluted 1:100 in 1% BSA/PBS and 100 μL of these samples were added to the test tubes. Positive controls were generated by adding 100 μL of anti-GnRH-TT pooled rabbit sera diluted 1:100 (triplicate) and 1:400 (triplicate).

Negative, non-specific-binding control tubes received an additional 100 μL of 1% BSA/PBS. Next, 100 μL of iodinated GnRH (New England Nuclear), with a specific activity of 2200 curies/nmol and diluted in 1% BSA/PBS to $\sim 15,000$ cpm/100 μL , was added to each tube and incubated 18–20 hr at RT. Each tube then received 200 μL bovine gamma globulin (5 mg/mL PBS) followed by 500 μL of 25% polyethylene glycol 8000 in PBS. The tubes were vortexed then centrifuged at 2600 g (Sorval H6000A rotor) for 25 min at 4°C to pellet the anti-GnRH antibodies bound to labeled GnRH. The pellets were counted for 3 min in a Micromedic 4/600 plus gamma counter and the antibody titers expressed in nmol of GnRH bound per liter of serum.

BUN Micro-Spectrophotometric Assay

This assay was a modification of a standardized urea nitrogen colorimetric assay protocol (Sigma®). 20 μL of buffered urease was placed in each well of a 96-well, flat-bottom plate. Serum samples were diluted 1:10 with Milli-Q^{uf}Plus-filtered water (Millipore, Billerica, MA, USA), and 4.0 μL of the diluted samples was added per well, in duplicate. For a positive control, 4.0 μL of a 1:10 dilution of the 30 mg/dL urea nitrogen standard solution in Milli-Q^{uf}Plus-filtered water was added per well, in triplicate. Negative controls received 4.0 μL of Milli-Q^{uf}Plus-filtered water. The plate was gently shaken and incubated at RT for 15 min to hydrolyze the urea into NH_3 and CO_2 . To each well, we added in order the following: 40 μL phenol nitroprusside, 40 μL alkaline hypochlorite, and 200 μL Milli-Q^{uf}Plus-filtered water. Incubated the plate 20 min at RT to allow the NH_3 to produce indophenol, which was then detected using a Dynex MRX plate reader (Chantilly, VA, USA) set at 570 nm. The BUN value for each sample was obtained by either mapping the value directly to the urea nitrogen standard curve or by determining it using the following equation:

$$\text{Serum BUN (mg/dL)} = (\text{OD}_{\text{test serum}}) / (\text{OD}_{\text{standard}}) \times 30 \text{ mg/dL.}$$

Creatinine Micro-Spectrophotometric Assay

The microassay for creatinine was a modification of Sigma®'s creatinine kit. 30 μL of serum from each sample was added to duplicate wells of a 96-well, flat-bottom plate. Blanks were generated by placing

30 μL of Milli-Q^{uf}Plus-filtered water into each of three wells. Three positive control standards (30 μL of 3 mg/dL creatinine) were included in each assay for internal reference. To each well, 300 μL of alkaline picrate solution (made by mixing five volumes of creatinine color reagent, with 1 volume of 1.0 N sodium hydroxide solution) was added with a multichannel pipettor, mixed, and incubated at RT for 8 min. The plate was then read at 490 nm in a Dynex MRX plate reader zeroed to the blanks. The OD readings of the serum test samples and the standard samples were recorded as Initial A_{test} and Initial A_{standard} values. To each well of the plate, 10 μL of acid reagent was added using a multichannel pipettor, mixed, and incubated at RT for 5 min. The plate was once again blanked and read at 490 nm. The OD readings of the serum test samples and the standard samples were recorded this time as Final A_{test} and Final A_{standard} values. The creatinine concentration per sample as calculated as:

$$\text{Creatinine (mg/dL)} = \frac{(\text{initial } A_{\text{test}} - \text{final } A_{\text{test}})}{(\text{initial } A_{\text{standard}} - \text{final } A_{\text{standard}})} \times 3 \text{ mg/dL}$$

and the values averaged. A standard curve from 0 to 10 mg/dL showed linearity of the plate reader spectrophotometer at 490 nm with an $R^2 = \sim 1.0$. If a test sample's value fell outside of this linearity range, the sample was diluted and the serum rerun and the resulting creatinine concentration multiplied by the dilution factor.

Statistical tests

Descriptive statistics were performed using Microsoft® Excel for Mac® statistical package and the Mann-Whitney test was performed using GraphPad's InStat® 3 for Macintosh. Fisher's Exact test was performed using the web-based program at <http://www.matforsk.no/ola/fisher.htm>. The analysis of mean (ANOM) was performed according to Schilling and Ott.²⁶

Results

Experimental Design

The study consisted of 12 groups of male rats with 10–12 animals in each group at the start of the study (Fig. 1). Two immunogens were used in the study:

groups 1–5 were the experimental animal groups immunized with 100 µg/injection of gonadotrophin releasing hormone attached to the carrier protein tetanus toxoid (GnRH-TT). Groups 6–10 were the corresponding carrier protein control animal groups that were immunized with 100 µg/injection of TT only. (Over 99% of the mass of the GnRH-TT immunogen is from the TT component – MW of GnRH $\sim 1.2 \times 10^3$, MW of TT $\sim 1.5 \times 10^5$. Because of this, the animals immunized with just the carrier protein TT were immunizing with 100 µg of TT/injection and considered equivalent in antigen load to the GnRH-TT-immunized animals.) The remaining two animal groups, 11 and 12, were control rats that were never immunized and yet age-matched to the animals in groups 3 and 8, and groups 5 and 10, respectively.

The immunizations given to groups 1–10 were done every fourth week for the times specified for each arm of the study (Fig. 1). Blood was drawn every 4 weeks from each animal in groups 1–10 until each arm of the study was terminated (Fig. 1). The blood draws were processed for serum. Blood was drawn from each animal in all the groups (groups 1–12) at the conclusion of each study arm when the animals were killed. Groups 1, 2, and 3 (GnRH-TT-immunized) and groups 6, 7, and 8 (TT-immunized) were terminated 2 weeks after their last immunization. Terminating the animals at this time point was done to ensure that the animals would have high concentrations of circulating antibodies at their death. Animals in groups 4 and 5 (GnRH-TT-immunized) and groups 9 and 10 (TT-immunized), however, were kept alive for several months after their last immunization. This provided those animals with a resting phase, in which they were no longer challenged with immunogen, so circulating antibody titers could drop. This strategy was designed so we could compare tissues from animals with high circulating levels of immune complexes (IC) to tissues from animals with lowered levels of circulating IC. Any damage or alterations seen to tissues when IC levels were high could then be re-examined in animals after the resting phase to determine if the damage was permanent or transitory. Monthly blood samples, however, were still drawn from the animals during the rest phase to evaluate antibody titers and testosterone levels.

When each group reached the end of its study arm, the animals were killed and the following organs were removed and fresh-frozen on dry ice: brain, pituitary, testes, epididymides, prostate,

spleen, liver, heart, lungs, and cervical lymph nodes. The organs were cryosectioned and used in both histological and immunofluorescent assays to assess tissue architecture and immune complex deposition, respectively. If, at any time during the experiment, any of the animals in the 12 groups became moribund, the animal was killed and its organs removed.

Animal Deaths During the Experiment

The experiment began with 140 animals, 19 of which died before the end of the study. None of the deaths occurred in the age-matched, no treatment controls, while 12 animals died in the TT-immunized controls, and seven animals died in the GnRH-TT-immunized animals. Six animals died while under anesthesia during the study (four from the TT-immunized controls and two from the GnRH-TT-immunized animals). Five animals died from unknown causes and their tissues were not recovered (three from the TT-immunized groups and two from the GnRH-TT-immunized groups). These animals were found dead in their cages by the animal care facility staff and had no prior indication of ill health. It is significant to note that all of these animals died within 1 or 2 days after having been anesthetized and immunized or bled, suggesting the cause of death was likely due to an unobserved stress experienced by the animal because of the procedure. Eight animals died or were put down after becoming moribund during the study (five animals were from the TT-immunized group and three animals were from the GnRH-TT-immunized group). Their tissues were recovered and examined, and a cause of death determined if possible. However, necropsy of only one of the animals gave a conclusive reason for its moribund state with the discovery of a lymphosarcoma (the animal had been immunized with GnRH-TT). Although necropsies of the remaining seven animals were inconclusive for their cause of death, each of these deaths occurred near the end of the 82-week long study when the animals were ~ 100 weeks old, an age corresponding to the end of the normal average lifespan for these rats. Any general, non-specific deterioration seen at this time was thus attributed to old age.

Efficacy of GnRH-TT Immunizations

We examined several parameters to confirm the biological efficacy of the GnRH-TT immunogen. First, to

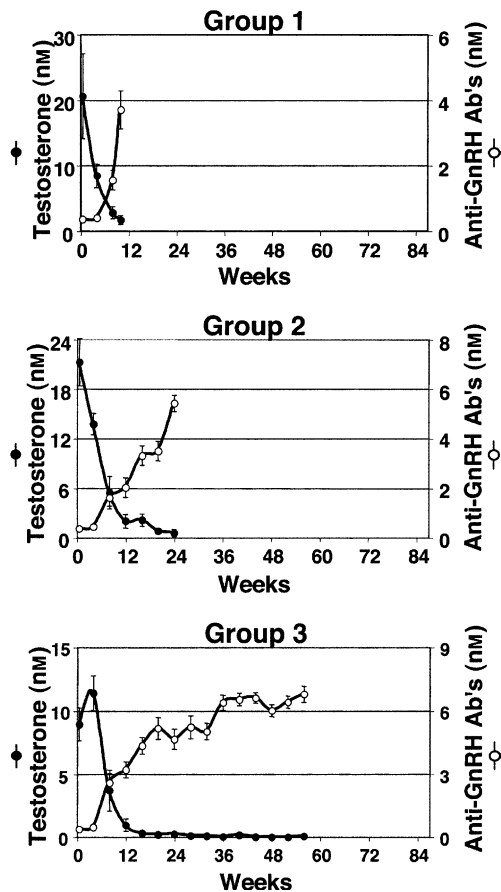


Fig. 2 Efficacy of GnRH-TT immunization. Groups 1–3 show the levels of serum testosterone and antibody titers measured in the GnRH-TT-immunized animals for the length of each study. Open circles show the average group molarity of anti-GnRH antibodies in the serum for each blood drawing; dark circles show the average group serum testosterone molarity at each bleed. Error bars are given as SEM.

show GnRH-TT was immunogenic in each animal, we analyzed every animal's serum for anti-GnRH antibodies. Figs 2 and 4 confirm the immunogenicity of GnRH-TT as seen by the increase in anti-GnRH antibody titers of groups 1–3 and groups 4–5.

We next determined if the antibodies produced by the immunization were biologically effective in disrupting the hormonal cascade stimulated by GnRH. The biological effectiveness of the anti-GnRH antibodies is demonstrated by the decrease in levels of circulating testosterone (T), which are plotted in Figs 2 and 4. Serum T levels dropped as anti-GnRH antibody titers rose. Furthermore, as levels of T declined and remained low, the size of the animals' testes and prostate also diminished (data not shown),

as was expected from other studies showing efficacy of anti-GnRH antibodies.^{6,7}

Shrinkage of the testes and prostate suggests that T stimulation of these organs has diminished or stopped. The decrease in the size of the testis is most likely the result of the cessation of spermatogenesis. To evaluate this hypothesis, we microscopically examined cryosections of the testes and the epididymides for the presence of developing and mature spermatozoa, respectively. Fig. 3A,B show representative cryosections of a testis and epididymis from a TT-immunized control animal. Normal spermatogenesis is observed in the testis and the lumen of the epididymis is seen filled with spermatozoa. In contrast, Fig. 3C,D show representative cryosections of testis and epididymis from a GnRH-TT-immunized animal showing that spermatogenesis has abated. The lumens of the seminiferous tubules and the epididymides were void of sperm and without the stimulation of T had dramatically contracted in size. This is especially evident in comparing Fig. 3D with the epididymal cross-sections shown in Fig. 3B as the photograph was taken of a control epididymis at the same magnification and similar epididymal region. The lumens of the contracted seminiferous tubules appeared to be filled with a webbing of Sertoli cells, while the germ cells could be seen on the periphery of the tubules in a condensed state (Fig. 3C). Not all of the animals had the same severity of hypogonadism or azoospermia. Some of the animals in groups 1 and 2 were oligozoospermic, and a few of the animals in these groups had normal-appearing levels of sperm within the lumen of the seminiferous tubules and epididymides. Table I summarizes and quantifies the results obtained for all the groups within this study.

In group 1, 70% of the animals at termination had testes void of sperm or below normal concentrations. This value rose to 75% for group 2, and by the termination of group 3, all animals were azoospermic. When the epididymides were examined, 70% of the animals in group 1 and 50% of the animals in group 2 had sperm in the lumen. The sperm, however, were not always distributed evenly throughout the epididymis; some animals only showed sperm in the most distal region of the epididymis (the cauda). This indicates that although spermatogenesis had stopped, the sperm continued to be moved through the epididymis until expulsion. In group 3, no sperm were found in the epididymides of any of the animals.

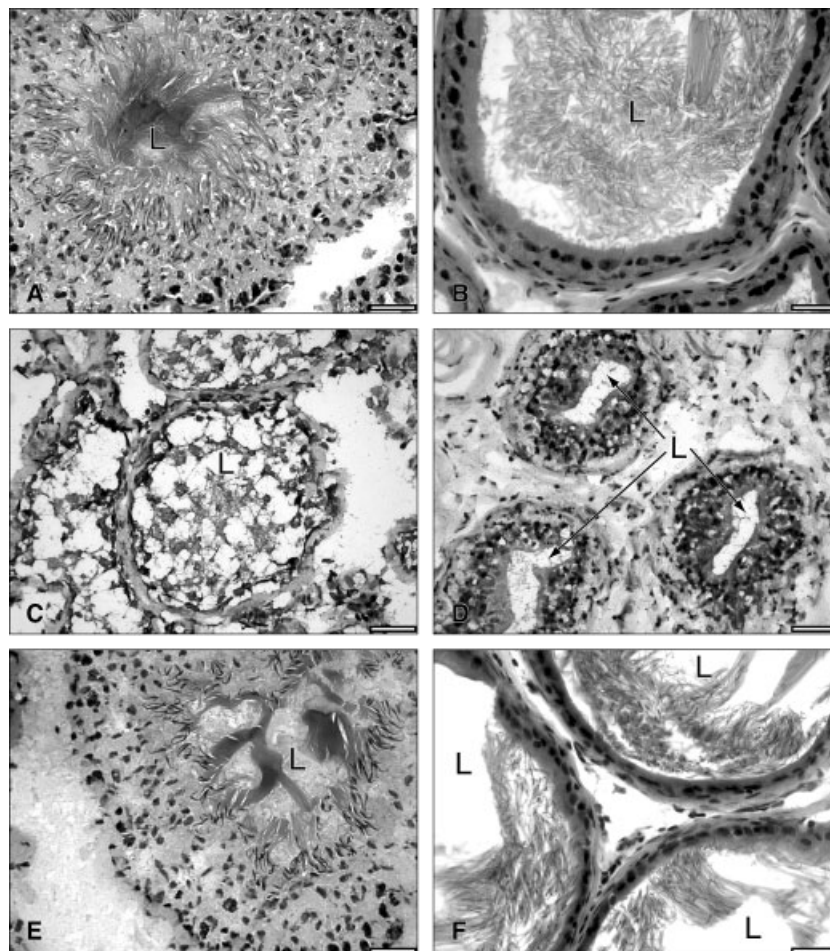


Fig. 3 Testes and epididymides from GnRH-TT- and TT-immunized animals. A, C, and E are fresh-frozen, unfixed cryosections of seminiferous tubules. B, D, and F are cryosections of epididymides. A and B (group 8) are representative of groups 6–12 showing normal spermatogenesis. C and D (group 3) are representative of GnRH-TT immunizations showing loss of spermatogenesis and cohesive tubule structure, luminal contraction and thickening of epididymal interstitial tissue. E and F (group 4) are representatives showing recovery of spermatogenesis in some animals from groups 4 and 5. L designates lumens. Scale bars = 50 μ m.

Recovery of Spermatogenesis

Groups 4 and 5 were immunized with GnRH-TT every 4 weeks for one-half and one-full year, respectively. Then, instead of being killed at this point, the animals were maintained without further boosting for an extended period referred to as the resting phase (Fig. 1). During the resting phase, we continued to collect blood samples every four weeks until the end of the study to monitor levels of anti-GnRH-TT antibodies and T. The data to the left of the dotted line in Fig. 4 show the antibody titers and T levels from blood collected during the immunizing phase of the experiment. This shows the efficacy of the antibodies. The data points to the right of the dotted line represent samples collected during the resting phase. During the resting phase, group 4 showed a slow increase in the level of T with a concomitant decreased titer in anti-GnRH antibodies

(Fig. 4A). In group 5, however, circulating T levels remained essentially undetectable, although a decrease in anti-GnRH antibody titer was beginning (Fig. 4B). The slow-to-non-existent recovery of T levels was different from what had been seen in previous studies of animals injected with GnRH-TT. In those earlier studies, animals had recovered T production approximately 8–16 weeks after their last injection (data not shown). In the earlier studies, however, the animals had only been immunized monthly for 3 months. This is a significant contrast to the hyperimmunization schedule used in the current study. The slower recovery seen in this study may reflect the extra time needed for the increased antigen load to be eliminated from the animals.

Several of the testes from animals in groups 4 and 5 appeared normal in size and weight upon necropsy. We cryosectioned and examined the testes and epididymides from these two groups to see if

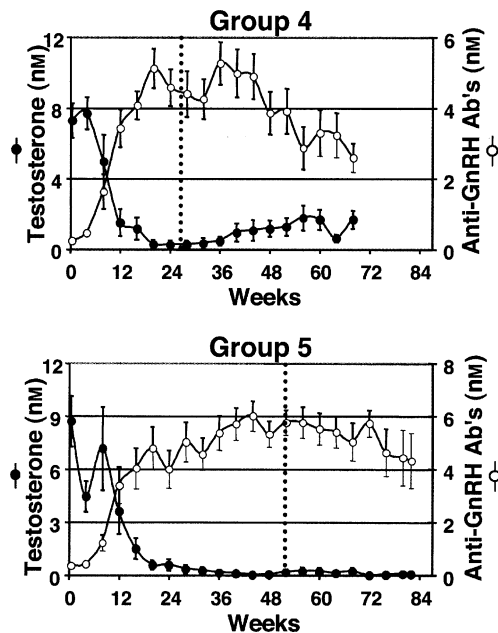


Fig. 4 Levels of serum testosterone and antibody titers from animals immunized with GnRH-TT followed with a non-immunizing resting phase. Animals in groups 4 and 5 were immunized with GnRH-TT monthly until they entered the start of their rest phase (no immunizations) as denoted by the dotted line. Open circles show the average group molarity of anti-GnRH antibodies in the serum for each blood drawing; dark circles show the average group serum testosterone molarity from each bleed. Error bars are given as SEM.

spermatogenesis had reinitiated in any of the animals. Fig. 3E,F show representative micrographs from these normal-appearing GnRH-TT-immunized testis and epididymis after the resting phase. Sperm can be seen in the lumens of both tissues, showing that spermatogenesis had resumed. In group 4, 67% of the animals had resumed spermatogenesis, while 33% remained azoospermic (Table I). In group 5, however, only 25% of the animals had recovered spermatogenesis by the conclusion of the study (Table I). This difference in recovery could result from both the increased antigen load as well as the shorter resting phase given to the animals in group 5 as compared with group 4 (30- versus 48-weeks of resting).

Circulating testosterone concentrations in the animals of groups 4 and 5 where spermatogenesis had returned were ≤ 4.0 nM (≤ 1.2 ng/mL) with an average concentration of 1.8 nM (0.52 ng/mL). This average testosterone concentration value is slightly lower than the normal lower limit of rat serum T. The normal range of serum T in rats has been reported from 2 to 20 nM or 0.6–6.0 ng/mL.²⁷

Table I Levels of Sperm within the Testis and Epididymis at the End of the Study

Group	Animals with testicular sperm (%)	Animals with epididymal sperm (%)
1	50 (two of 10 animals with normal levels (three of 10 animals oligospermic))	70 (four of 10 animals had sperm throughout the epididymis (three of 10 animals had sperm only in the distal epididymis))
2	50 (three of 12 animals with normal levels (three of 12 animals oligospermic))	50 (three of 12 animals had sperm throughout the epididymis (three of 12 animals had sperm only in the distal epididymis))
3	0	0
4	67	67
5	25	25
6	100	100
7	100	100
8	100	100
9	100	100
10	100	100
11	100	100
12	100	100

Organ Analysis for Immune Complex Depositions

Having shown that GnRH-TT is an effective immunogen, we examined several organs to determine if there were changes in the tissues that could be attributed to the GnRH-TT immunization. Such changes could include signs of inflammation, disrupted cellular architecture, or the deposition of immune complexes. Immune complexes (IC) can be deposited on cells and in some instances stimulate the complement cascade. If this happens, cell death and tissue damage result. Because immunization with GnRH-TT produces antibodies that cross-react with the animal's native GnRH, we examined whether these autoantibodies bound complement and damaged various organs directly or indirectly through the formation and subsequent deposition of immune complexes.

We examined the brain, pituitary, kidney, liver, spleen, lung, heart, cervical lymph nodes, prostate, testis, and epididymis. The organs were removed, fresh-frozen, and cryostat-sectioned. They were analyzed using histochemical stains to monitor overall

tissue architecture and look for signs of inflammation, such as macrophage and lymphocyte infiltration. We also inspected the tissue for the presence of IC using immunofluorescence methods to detect the autoantibodies. For each tissue section examined using histochemical stains, a serial tissue section was also inspected immunofluorescently for anti-rat immunoglobulin that would indicate immune complex deposits.

We saw no differences in the liver, spleen, lung, heart, or cervical lymph nodes of the GnRH-TT groups when compared with the TT-immunized animals or to non-immunized, age-matched controls (age-matched controls were done for group 3 only) (data not shown).

As we have reported above, GnRH-TT immunized animals did show an altered cellular architecture in the testis and epididymis as seen by the contraction of seminiferous tubules and epididymal lumen as well as the elimination of spermatogenesis. Likewise, the prostate was found to have contracted in volume without testosterone stimulation, and the anterior lobe of the pituitary appeared to diminish in size, which was expected with the loss of gonadotroph stimulation and secretion. However, none of these tissues showed signs of inflammation nor did we observe any IC deposits using immunofluorescence (data not shown).

Brain Examination

GnRH is produced within the hypothalamic region of the brain, and thus that area is a key site potentially affected by the anti-GnRH antibodies. The brain, however, protects itself from immunologic injury through the blood-brain barrier, which consists of a series of tight junctions. However, not all of the brain is protected by the blood-brain barrier. We focused our examination of the brain on the hypothalamus, but more specifically on the ME. The ME is where hypothalamic neurons containing GnRH project through the blood-brain barrier and are exposed to the circulatory system. Here GnRH is secreted into the portal blood supply, which carries the hormone to the pituitary. Because the neurons in this region are not protected by the blood-brain barrier, we speculated that they would be the most vulnerable to attack by anti-GnRH antibodies.

The rat ME spans a distance in the brain of about 1.5 mm in a rostral to caudal direction. We coronally cryosectioned each rat brain in a rostral to caudal

direction through the expanse of the brain containing the ME. We then placed the brain sections into three sequential groupings based upon their rostral to caudal location. Thus, the first grouping contained ~0.5 mm of the most rostral region of ME, followed by ~0.5 mm of the middle region of the ME, and concluding with ~0.5 mm of the most caudal region of the ME. These three regions were labeled anterior, medial, and posterior ME, respectively. Serial sections from each region were stained with Cresyl violet to assess the cellular architecture of the ME and labeled with FITC-labeled anti-rat Fab immunoglobulins to detect the presence of any immune complexes. We saw sporadic labeling of the ME in several brain sections from rats in all arms of the study (rats immunized with GnRH-TT or with TT with or without an accompanying rest period and in the aged-matched, non-immunized control rats) making the interpretation of our results difficult. In an effort to increase specificity of our assay over the surrounding background noise, we established the following parameters: Any rat that showed labeling of the ME in two consecutive serial sections from either the anterior, medial, or the posterior regions of the ME was scored as positive for ME labeling. We also did a second ME analysis using a more stringent discrimination of the data. Rat brains that showed positive labeling (defined above) of the ME in all three ME regions (anterior, medial, and posterior) were scored as strongly positive. We hypothesized that non-specific binding should show a random distribution pattern of labeling over the tissue sections, while IC deposition in the ME would show a more organized and specific labeling pattern. Fig. 5 shows quantification of these findings, comparing specific control and experimental groups along with the standard error of their mean. The Fisher's exact test was used to analyze the intra-group pairings. In all cases, even though label at the ME could be seen in some animals, no statistical difference was found between the groups even when placing $P = 0.1$ (data not shown). These results were also confirmed using an ANOMs comparison between the groupings. Furthermore, none of the brains showed signs of tissue inflammation or damage within the ME (data not shown).

Kidney Examination

The renal glomeruli are known to be sites of IC deposition because one of their functions is to filter

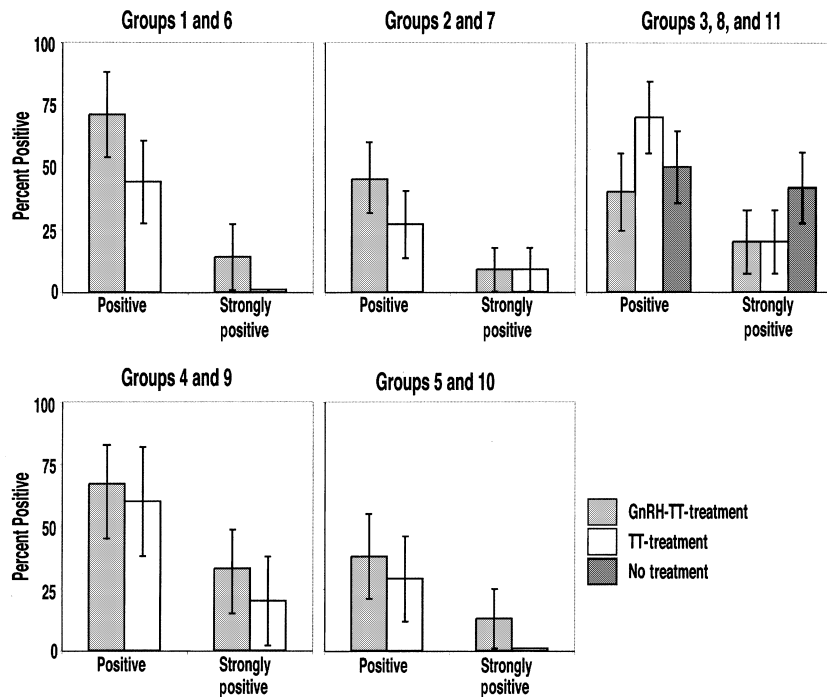


Fig. 5 Quantification of labeling of the median eminence. A *positive* labeling of the median eminence (ME) was given if adjacent serial sections of brain labeled. A *strongly positive* ME labeling was given when adjacent serial sections from the rostral, medial, and caudal regions of the brain were each scored positive for ME labeling. Light gray bars represent data from GnRH-immunized animals; white bars represent the data from TT-immunized animals; and dark gray bars are the data from no-treatment control animals. Error bars given as SEM.

out circulating ICs. However, persistent IC deposition along with complement binding can damage the glomeruli. No systematic examination of the kidneys has been undertaken for immunocontraceptives so we wanted to determine the effects of GnRH-TT IC deposition and examine the kidneys. To achieve this, we cryosectioned the cortical regions of the kidneys and, using immunofluorescence, examined them for IC depositions. Fluorescence intensity in the glomeruli was quantified using a qualitatively scale that ranged from no detectable fluorescence (0) to intense fluorescence seen in positive controls (4). The positive controls were BN mice that had been induced into an autoimmune state to produce anti-glomerular antibodies. Representative photographs of kidney sections from GnRH-TT-immunized showing both strong positive and negative findings for deposition of ICs within the glomeruli can be seen in Fig. 6. We detected ICs in many of the kidneys of animals immunized with either GnRH-TT or TT. Few ICs were found in the non-immunized, age-matched control animals of group 11, and those that were found had minimal levels of immunofluorescence (Fig. 7). Group 12 animals were not analyzed.

There were statistically significant differences between both the GnRH-TT-immunized animals and the TT-immunized animals when compared with the

No Treatment control animals of group 11 using Mann-Whitney's non-parametric analysis. The immunized animals groups consistently showed a higher labeling of the renal glomeruli over the non-immunized animals (Table II). When the GnRH-TT-immunized animal groups were compared with the TT-immunized animal group counterparts, a significant difference was also seen in all the comparisons except for groups 1 versus 6 and groups 5 versus 10. In each case of significant difference, the direction of difference showed the GnRH-TT-immunized animals to have higher levels of IC deposition within the glomeruli over the TT-immunized animals (Table II). An intra-group analysis was also performed between the groups that the same length of immunizations but with or without a following rest phase to see if a diminishment in the levels of IC deposition would occur. In both the GnRH-TT- and the TT-immunized animals, the rest phase did not show any significant decrease in levels of ICs within the glomeruli (Table II) as we had hypothesized and may reflect the high antigen load from the chronic immunizations. The significant increase in GnRH-TT ICs over the TT-immunized animals might indicate that the GnRH-TT animals were suffering from an onset of kidney damage. The kidneys were thus evaluated further for potential damage.

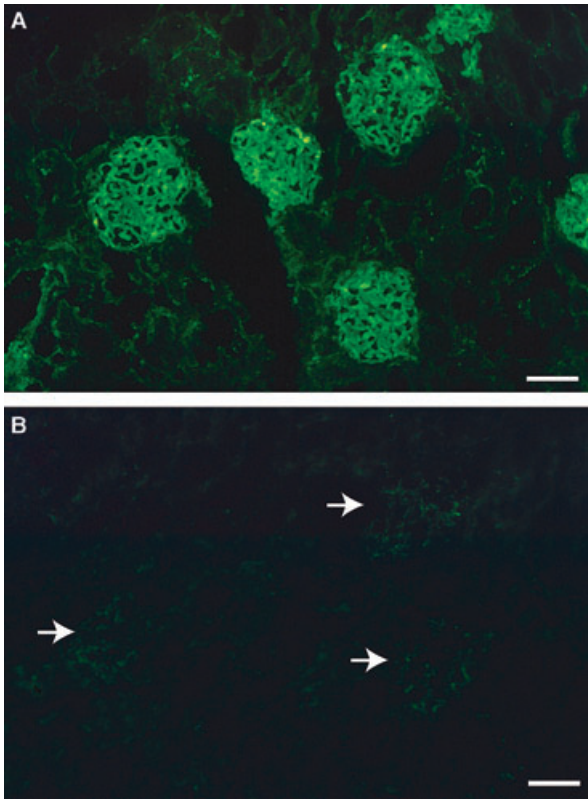


Fig. 6 Immunofluorescence of rat immunoglobulin in tissue sections of kidney from immunized animals. Fresh-frozen, unfixed cryosections from the kidney cortex labeled using FITC-labeled anti-rat Fab fragments for immune complex detection. A spectrum of labeling intensity was seen in the sections from the various animals. Representative micrographs showing what was considered intense labeling of glomeruli (A) and extremely weak staining of the glomeruli (B) are presented. Arrows indicate three glomeruli in the weakly staining micrograph. Scale bars = 50 μ m.

The presence of ICs in the glomeruli does not imply kidney damage has taken place because filtering out IC is a normal function.²⁸ However, it does increase the potential for tissue damage, because complement might bind to the ICs and cause the underlying cells to be lysed. We stained the kidneys with H&E and looked for architectural anomalies such as thickening of the glomeruli in the region of their basement membranes, which is often seen if the glomerulus's is damaged.²⁹ No thickening or other pathology was detected surrounding the glomeruli in kidneys from either the GnRH-TT-immunized, TT-immunized, or non-immunized animals (data not shown). We then examined serial kidney sections from those animals that had been strongly immunopositive for glomerular ICs, checking for the presence of complement C3b via immunofluorescence microscopy. No complement was detected in any of the samples, again suggesting that the kidneys were not damaged (data not shown).

We next examined markers for kidney function: serum blood urea nitrogen (BUN) and creatinine. If the kidneys were not functioning properly, these substances would not have been removed from the blood very efficiently, and thus their levels in serum would be elevated. To test for these markers, we developed a high throughput microassay requiring only microliters of serum per test. This allowed us to examine in triplicate a large number of serum samples collected. Sera from 135 untreated male Sprague-Dawley rats were used to determine the normal range for these markers in the microassay. The upper end of the normal range was chosen to be two standard deviations above the average from the

Fig. 7 Quantification of immune complex deposition within kidney glomeruli. Fluorescence intensity was determined using a subjective scale of 0 (background fluorescence) to 4 (intense fluorescence comparable with the positive controls) with 0.5 unit increments. Two evaluators did independent and blinded scoring and their observations averaged making 0.25 unit increments possible. GnRH-TT-immunized animal results are shown with triangles, TT-immunized animal results are shown with circles, while the No-Treatment control animal results are presented as diamonds.

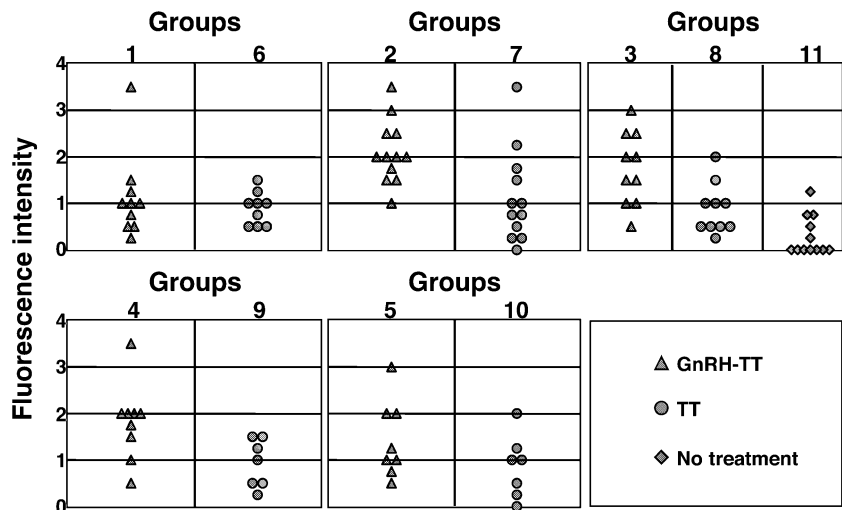


Table II Mann–Whitney Analyses of Inter and Intra Treatment Groups for Immune Complex Deposition within the Renal Golmeruli via Immunofluorescence Data

Inter-group comparisons				Intra-group comparisons					
GnRH-TT-immunized groups versus TT-immunized groups		GnRH-TT-immunized groups versus no treatment control group		TT-immunized groups versus no treatment control groups		GnRH-TT-immunized groups		TT-immunized groups	
Group comparisons	Significance (P-value)	Group comparisons	Significance (P-value)	Group comparisons	Significance (P-value)	Group comparisons	Significance (P-value)	Group comparisons	Significance (P-value)
1 versus 6	NS*	1 versus 11	<0.01	6 versus 11	<0.01	2 versus 4	NS	7 versus 9	NS
2 versus 7	0.01	2 versus 11	<0.01	7 versus 11	0.01	3 versus 5	NS	8 versus 10	NS
3 versus 8	0.02	3 versus 11	<0.01	8 versus 11	0.01				
4 versus 9	0.02	4 versus 11	<0.01	9 versus 11	0.02				
5 versus 10	NS	5 versus 11	<0.01	10 versus 11	0.06				
1–5 versus 6–10	<0.01	1–5 versus 11	<0.01	6–10 versus 11	<0.01				

*Not significant.

135 animals assayed. Thus in our assays, creatinine values above 1.92 mg/dL and BUN values above 29.4 mg/dL were considered abnormally high. Figs 8 and 9 show representative BUN and creatinine values, respectively, taken at various stages throughout the course of the experiment from each group of animals. Occasionally, a transiently high creatinine or BUN value was recorded from an animal from one of the monthly bleeds. If such a value was recorded for the animal only once, this was not considered significant, since such fluctuations do occur normally. However, there were two exceptions to this rule: (1) If the high value occurred at the time of death it was considered important because no further bleedings could be done to determine if the value was only transiently high; and (2) If the transiently high BUN had a corresponding transiently high creatinine value during the same bleeding, even if the elevated values were short-lived, they were considered significant. Only two animals fell into the second exception category, and both of those animals had repeatedly high BUN values. Therefore, to avoid double-counting these animals, they each were accounted for within the multiple BUN only category (Table III). The percentage of animals in each of the arms of the study with elevated BUN or creatinine was essentially no different between the treatments, indicating that kidney damage was unlikely to result from GnRH-TT immunization.

Discussion

This study was undertaken to evaluate whether the anti-GnRH antibodies and immune complexes generated by immunization with GnRH-TT cause cellular damage within the animal. To examine this, the animals were chronically immunized to exacerbate any potentially damaging effects the anti-GnRH antibodies or the IC might have on the animal. We divided the study into two parts: (1) animals immunized until the time of their demise, which therefore occurred at or near the height of their anti-GnRH antibody titers, and (2) animals given a rest period, so the immune complexes could ebb within the animal by the time of their death. Comparing these two arms of the study could reveal if any damage resulting from the immunizations was permanent or just transient.

In this study, as in other studies where animals were immunized with GnRH (albeit those studies did not involve chronically immunize animals), the animals showed no ill side effects after immunization.³⁰ The majority of the tissues examined showed no sign of change whether or not the animals experienced a post-immunization rest phase. The expected size shrinkage in the testes, epididymides, prostate, and anterior pituitary were observed, but no immune complex deposition was detected in any of these tissues. Because the tissues were fresh-frozen for immunohistochemical analy-

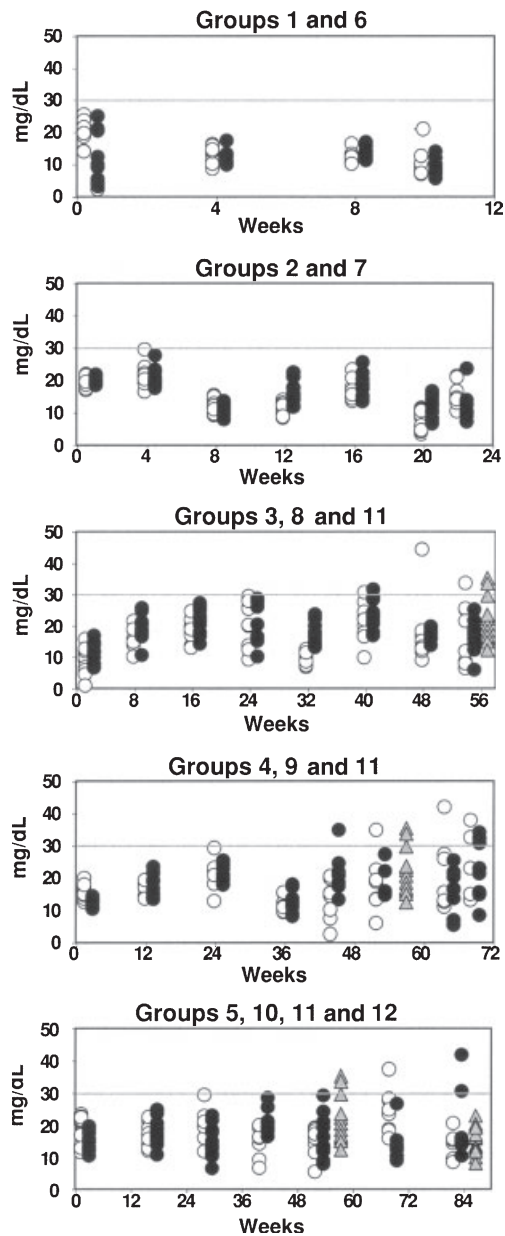


Fig. 8. Serum BUN analysis of the groups at various weeks over the course of the study. Serum levels of BUN were determined using a micro ELISA protocol (see M&M). Values are expressed in mg/dL. The dashed line represents the upper limit value of normal for rats in this assay. Results are given for GnRH-TT-immunized animals (open circles), TT-immunized animals (closed circles), and the no-treatment control animals (gray triangles).

sis, the cellular architecture of the tissues lacked the cohesion fixed tissues would have retained. This was especially pronounced in the testes of the GnRH-TT-immunized animals with the contracted

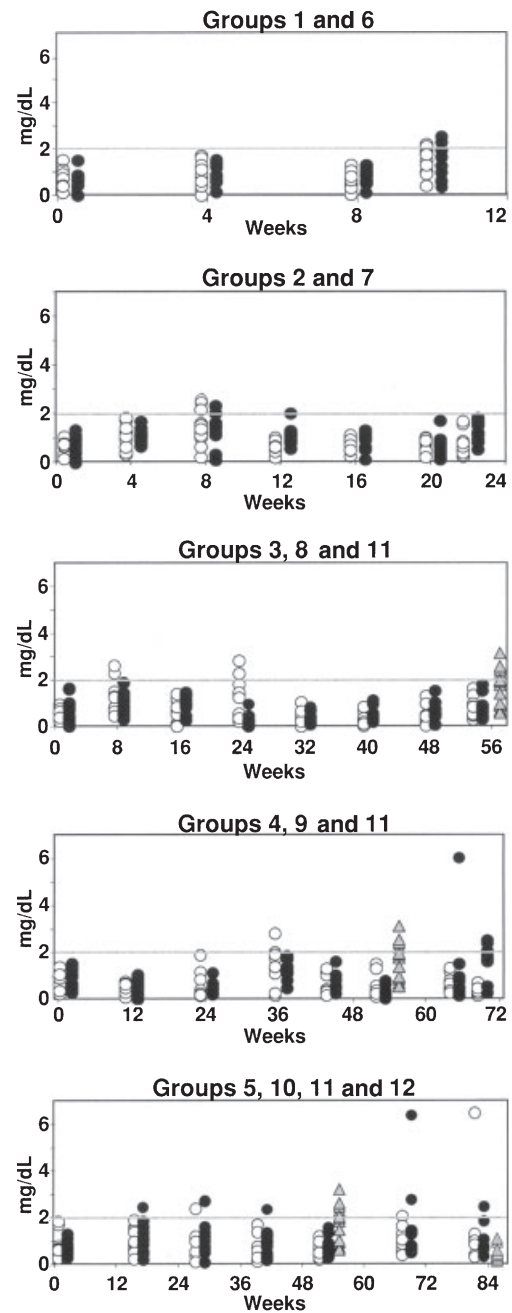


Fig. 9. Serum creatinine analysis of the groups at various weeks over the course of the study. Serum levels of creatinine were determined using a micro ELISA protocol (see M&M). Values are expressed in mg/dL. The dashed line represents the upper limit value of normal for rats in this assay. Results are given for GnRH-TT-immunized animals (open circles), TT-immunized animals (closed circles), and the no-treatment control animals (gray triangles).

Table III Animals with Abnormally High Serum BUN or Creatinine Values

Treatment	Rats with multiple high BUN values or a high BUN at death	Rats with multiple high creatinine values or a high creatinine at death	Rats with non-transient high BUN or creatinine values over the total number of rats examined
TT-immunized animals	10 animals (three from group 8; six from group 9; one from group 10)	5 animals (3 from group 6; one from group 9; one from group 10)	13/48 (27.1%)
GnRH-TT-immunized animals	5 animals (three rats from group 3; one rat from group 4; one rat from group 5)	7 animals (three from group 1; two from group 4; two from group 5)	10/50 (20%)
No Treatment, age-matched control animals	2 animal (from group 11)	4 animals (from group 11)	4/24 (16.7%)

seminiferous tubules (Fig. 3). During the atrophic spermatogenic stage of the GnRH-immunized animals, the lumens of seminiferous tubules were filled with interwoven Sertoli cells. Germ cells appeared quiescent and sitting along the basement membrane of the lumen wall. However, the presence or absence of spermatogenesis was always clear. Additionally, the tissue sections of testes from the rested animals undergoing recovery of gonadal function appeared to have a less densely packed cellular arrangement than the TT-immunized control animals. We speculate that this cellular sparsity may be explained by our observation that, although spermatogenesis was returning in these animals, the majority of the animals still had low levels of serum testosterone. This situation might be further exacerbated by the age of the animals. We also cannot rule out from this study that chronic immunization with GnRH might permanently affect testicular architecture adversely resulting in only partial recovery.

The ME of the brain and the glomeruli of the kidneys did show fluorescent labeling in some of the animals for immune complexes. The labeling in the ME of the brain was not specific to the GnRH-TT-immunized animals but was seen also in the TT-immunized and the non-immunized controls at similar frequencies and statistically showed no difference between these study arms. We do not understand why the ME labeled in many of the animals regardless of whether they were immunized with GnRH or not. It could be that immunoglobulins transiently adhere to the ME as a normally occurring phenomenon in the animal or the assay inherently has a high level of background reporting. In an effort

to circumvent this later possibility we applied a more stringent test to the samples for them to be considered positively labeled and reported this as *strongly positive* in Fig. 5. As with the less stringent method of analysis, these data also showed no significant difference between the three study arms. Cresyl violet staining of the brain sections revealed normal appearing ME as well with no signs of inflammation. Our data suggests that immunization with GnRH-TT in an adult animal does not have an adverse effect on the ME. On pre-pubescent animals, however, an effect of the ME has been detected after immunization with GnRH.

In a study done on pre-pubescent male boars immunized with GnRH-KLH, the investigators found damage to the ME of the brain using histological examination.²¹ In their study, the brains showed various degrees of inflammation at the ME after immunization. These disruptions consisted of edema, collapsed capillaries, fibrosis, fibroblast infiltration, and accumulation of neurosecretum within the ME. However, these were not things we detected in the immunized adult male rats. Some of these elements may not have been detected in our system because the design of our experiments focused on IC deposition. For example, the tissues for this study were fresh frozen but purposely not fixed to prevent any loss of IC detection that the fixation might cause. The discrepancy between our data and that of the boar study might be the result of species differences, but it may also reflect the fact that the boars used in the Molenaar study were immunized pre-pubertally. The rats immunized in our study were adults. It is possible that the plasticity of the hypothalamus during puberty makes the ME more susceptible to anti-

GnRH antibody damage. This hypothesis is supported by studies by Brown *et al.*,²² where ewes and rams were actively immunized against GnRH both pre-pubertally and peripubertally and then followed over the course of 2 years to determine the long-term effects of such immunizations. Some of the immunized females never developed normal gonadal function and sexual behavior during the study even when the anti-GnRH antibody titers had fallen. Injection with GnRH at 90 weeks of age elicited little or no LH release in both the males and females again even though anti-GnRH antibody titers were no longer detected. These investigators suggested that deprivation of GnRH in early life might result in partial permanent impairment of the hypothalamic and/or pituitary function.

Unlike immunizations for illnesses, which are used to protect individuals from life-threatening contagions, an immunocontraceptive would be used to protect healthy individuals from unwanted pregnancy. It would therefore be required that any risk from immunization be very low for it to have general acceptance. In assessing the risk of an immunocontraceptive, one needs to take into account that antibody levels will need to be sustained at high circulating concentrations for long periods of time – the amount of time contraception desired. For diseases, the goal of the immunization is to establish a line of memory B-cells that act as sentinels in case the body is challenged with the contagion in the future. Thus circulating antibody titers need not be high continuously; instead, they simply need to reach high titers upon exposure to the pathogen. For an immunocontraceptive to be efficacious, boosting immunizations would need to be given to maintain high levels of circulating antibody. This sustained (potentially for years) high level of circulating antibody titer might have detrimental effects that only manifest themselves after an extended period of time. These antibodies would bind to their antigens and produce a continuous supply of immune complexes that could result in tissue damage.

Molenarr *et al.* reported seeing lesions in the ME of pre-pubertal pigs immunized against GnRH²¹ although we did not observe this in the adult rats. Another potential site of injury are the renal glomeruli.^{29,31,32} However, a systematic examination of the kidneys of animals after long-term, chronic exposure to an immunocontraceptive has not been examined. Therefore, an aim of this study was to determine if the GnRH-TT immunocontraceptive

immunogen places the kidneys at risk of damage because of sustained deposition of the IC within the glomeruli. In this study, the kidneys of the rats did show anti-immunoglobulin labeling within the renal glomeruli of both the GnRH-TT- and control TT-immunized animals. Because a function of the kidney is to filter out immune complexes, finding them was expected. However, because IC can stimulate the complement cascade, tissue damage is a potential side effect of their presence. A subjective evaluation of the concentration of IC deposition within the renal glomeruli, based on the immunofluorescence intensity, showed the GnRH-TT-immunized animals to have a significantly higher concentration of IC than the TT-immunized animals. However, we suggest that these deposits are not harmful to the rats based on the following observations: (1) Histologically, the glomeruli appeared the same in the all three study branches (GnRH-TT-immunized, TT-immunized, and non-immunized, age-matched controls). No signs of inflammation, neither thickening of the basement membrane nor cellular necrosis, were seen in any of these three study arms. (2) Cryosections from animals showing the highest concentrations of IC from each study arm were analyzed for the presence of complement component C3 and yet no C3 was detected in any of the samples (data not shown). (3) Serum BUN and creatinine levels were measured in all the animals yet no significant difference was detected in the values among the study groups. (4) There was no increase in the number of animal deaths in the GnRH-TT-immunized animals over the TT-immunized animals. Furthermore, the animals in groups 4, 5, 9, and 10 (the groups where a rest phase was given) were followed to the end of their normal lifespan, and showed no downward shift in the life expectancy of the GnRH-immunized animals. There was a significant increase in IC deposition in three of the five groups GnRH-TT-immunized animals when compared with the TT-immunized control animals and might be because of ICs being made not only with the GnRH-TT immunogen but with native GnRH as well. However, this increase in IC deposition did not appear to cause kidney dysfunction in the animals and thus our results suggest that deposition of GnRH-TT or TT ICs within the renal glomeruli do not appear to damage the kidneys in immunized animals as seen by several measurements of kidney function.

We had hypothesized that the animals given the resting phase would exhibit a reduction of antibody

titers so significant that little or no IC labeling would be evident in the glomeruli of these animals. This, however, was not observed, which likely reflects that the antigen load from the chronic hyperimmunizations was simply too sizeable to be overcome within the time frame of the study. This is supported by the >2 and >4 nM of circulating anti-GnRH antibodies at the end of the study for groups 4 and 5, respectively, along with the low levels of circulating testosterone in these groups (Fig. 4), but the persistent presence of ICs in renal glomeruli of the rested animals does raise a note of concern.

Although some gonadal restoration was beginning to occur among the immunized animals given the resting phase, we did not see recovery of testosterone in the immunized animals at our anticipated recovery rate. In earlier studies, we found that serum testosterone returned to normal levels approximately 14–16 weeks after cessation of the immunizations. Histological examination of testes from those testosterone-recovered animals showed that they had regained spermatogenesis (unpublished results). In those studies, however, the animals were given only three injections of GnRH-TT at 4-week intervals, which was sufficient to drop the serum testosterone levels to an undetectable level. For this study, a much longer length of time was necessary for testosterone to begin its recovery in the majority of the animals (groups 4 and 5) and for spermatogenesis to return (Fig. 3, Table I). In most of the animals from those groups, testosterone levels only returned to values associated with hypogonadism (<2 nM). Most of testes from the animals in group 4 (67%) and some from the animals in group 5 (25%) had returned to a normal size at the end of the study. Spermatogenesis had also resumed in each of these recovered animals. The concentration and quality of these sperm, however, is unknown. Resumption of fertility would be expected because of the previous results by Kumar et al.,³⁰ which showed that GnRH-TT-immunized rats recovered fertility with time after withdrawal of the immunogen. We cannot be certain that all of the animals in our study would have recovered spermatogenesis given more time. In this study, because the majority of the animals in group 4 were recovering after hyperimmunization, we suspect that the animals showing no signs of spermatogenesis were simply delayed in their recovery. Likely this is because an insufficient drop in anti-GnRH antibody titers had occurred by the end of the study for certain animals.

Conclusion

Our results indicate that GnRH-TT immunization would be an effective, reversible, and potentially safe procedure in the development of a male immunocontraceptive. Although we did not detect IC deposition in multiple tissues examined, we did detect IC deposition in the kidneys of chronically immunized animals, but we did not detect concomitant kidney failure. However, as new targets for immunocontraception continue to be identified,³³ future studies of candidates for long-acting, reversible immunocontraceptives should include monitoring of renal IC deposition in laboratory animals or of renal function in human and non-human primate subjects.

Acknowledgments

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