

Journal of Andrology, Vol. 22, No. 6, November/December 2001
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Immunobiology of a Synthetic Luteinizing Hormone Receptor Peptide 21–41

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ABSTRACT: Immunization of adult male rabbits with a synthetic luteinizing hormone–receptor peptide (LH-RP; representing amino-acids 21–41 of the extracellular domain of the rat LH receptor) resulted in production of high-titer antibodies capable of interacting with particulate and cell-based LH receptors. The antibody produced was able to inhibit binding of ¹²⁵I-labeled human chorionic gonadotropin (hCG) to a particulate sheep luteal LH receptor preparation by 40%–50%. Maximal inhibitory activity was correlated with high antibody titer. Immunocytometry revealed that the antibody could directly bind to cells having LH receptors, such as rat granulosa and Leydig cells. The antibodies recognized a 77-kilodalton membrane protein in Western blots of mouse testicular extracts. Interaction of endogenous Leydig cell LH receptor with the LH-RP antibody resulted in both hormone agonist and antagonistic activi-

ties. The hormone-mimicking activity (increase in serum testosterone over control) was confined only to the early phase of immunization when the antibody titer was low. Blockade of LH receptor during the later part of immunization resulted in a significant reduction in serum testosterone over controls and inhibition of spermatogenesis. DNA flow cytometry showed that a specific and significant inhibition of meiosis (transformation of primary spermatocytes to round and elongated spermatids $P < .01$) and spermiogenesis (transformation of round spermatids to elongated spermatids $P < .0001$) occurred following blockade of LH function.

Key words: Testicular function, spermatogenesis, receptor antibodies.

J Androl 2001;22:992–998

Spermatogenesis in mammals is mainly regulated by 2 glycoprotein hormones; namely, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Immunization of adult male bonnet monkeys (*Macaca radiata*) with ovine FSH (Aravindan et al, 1993) as well as a recombinant FSH receptor protein has been shown to result in impairment of spermatogenesis (Moudgal et al, 1997). Immunization of male rabbits (Jeyakumar et al, 1995) and monkeys (Suresh et al, 1995) with ovine LH also results in blockade of testosterone production and spermatogenesis. Immunization of rabbits with a holo LH receptor (LH-R) preparation isolated from sheep corpora lutea led to the production of receptor-specific antibodies, but not to testicular dysfunction (Jeyakumar and Moudgal, 1996). This was shown to be due to the presence of antibodies expressing both hormone-mimicking and antagonistic activities throughout the period of immunization. During the early period of immunization, when only hormone-mim-

icking antibodies were present, there was a sustained (lasting over several weeks) and significant (fourfold to sevenfold) increase in serum testosterone levels. The return of serum testosterone to normal levels during later periods of immunization could be correlated to the appearance of receptor antagonistic (blocking) antibodies. The occurrence of both types of antibodies in the serum could be demonstrated by *in vitro* tests (Jeyakumar and Moudgal, 1996). The presence of hormone-mimicking antibodies of sufficiently high titer throughout immunization was held responsible for the lack of inhibition of serum testosterone over control and maintenance of normal testicular function. This study suggested that the epitopic regions of LH-R capable of generating hormone-mimicking and hormone antagonistic antibodies could be different.

The amino acid sequence of the LH receptor of a number of species has been enumerated, and peptide regions of the extracellular domain, which could be involved in specific binding to LH/human chorionic gonadotropin (hCG), have been recognized (Roche et al, 1992). Two peptides corresponding to amino acid residues 21–38 and 21–41 of the extracellular domain of the rat LH/hCG receptor exhibited significant inhibitory activity in the *in vitro* ¹²⁵I hCG-receptor binding assay, and region 21–38

Supported by Jawaharlal Nehru Center for Advanced Scientific Research, Bangalore and Canadian Institutes for Health Research.

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Received for publication December 8, 2000; accepted for publication May 10, 2001.

has been suggested to be an LH/hCG binding site (Roche et al, 1992). Because significant inhibition could be obtained with the synthetic peptide using only molar concentrations (IC_{50} 9.67 to 15.4×10^{-6} M), it was believed that obtaining corroborating evidence by other means would be useful to ensure that these regions do indeed represent the ligand binding site of the receptor. In the current study, an attempt has been made to investigate whether the antibody raised to the synthetic peptide, 21–41, of the rat LH-R, is capable of blocking endogenous LH activity in the male rabbit. It was believed that availability of one or more such synthetic antigens whose antibody could specifically block the responsiveness of gonadal cells to LH could be useful in generating newer therapeutic agents.

Materials and Methods

The Antigen

The rat LH-RP_{21–41}, corresponding to amino acid sequence Arg-Cys-Pro-Gly-Pro-Arg-Ala-Gly-Leu-Ala-Arg-Leu-Ser-Leu-Thr-Tyr-Leu-Pro-Val-Lys-Val, was synthesized by the solid-phase method, using a semiautomated LKB Biolynx 4175 peptide synthesizer employing fluorenylmethoxy carbonyl (Fmoc) chemistry (Atherton and Sheppard, 1989). Amino acids were coupled as 2-(1H benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTu) esters in the presence of an equivalent amount of 1-hydroxybenzotriazole (HOBt) using diisopropylethylamine as a catalyst. The peptide was cleaved from the Nova Syn KA resin by treatment for 5 hours with a mixture of trifluoroacetic acid, thioanisole, metacresol, and phenol (10:0.1:0.05 v/v). The resulting 21–41 synthetic peptide (LH-RP) was checked for purity by fast-performance liquid chromatography and amino acid analysis using an LKB 4151 Alpha Plus Amino Acid Analyzer. The LH-RP concentration of the final solution used as the antigen was 0.44 mg/mL.

Immunization and Titration of Antibodies

Three adult male healthy rabbits bred in the laboratory (New Zealand white) were immunized with the LH-RP conjugated with Keyhole Limpet Hemocyanin (KLH) in the proportion of 1:500 (Briand et al, 1985). The immunization schedule consisted of subcutaneous injections of 500 μ g LH-RP equivalent of the conjugate emulsified in Freund's complete adjuvant on day 1. The antigen was injected at several sites on the back of rabbits. This was followed by injecting a 500 and 300 μ g LH-RP equivalent of the conjugate in Freund's incomplete adjuvant on days 15 and 40, respectively. Booster injections (300 μ g) in saline were given on days 80 and 130.

Production of antibodies to LH-RP was monitored by a standard enzyme-linked immunosorbent assay (ELISA) procedure (Suter, 1982). To obtain a proper assessment of the specific LH-RP antibody titer, the wells of the ELISA plate were coated with LH-RP (100 ng/well) conjugated to a protein carrier other than KLH. Control wells coated with the above protein carrier alone were used to determine nonspecific antibody binding. The plates

were washed 4 times with phosphate-buffered saline (PBS) containing Tween 20 (PBST) before the sites were blocked with 5% dry milk (250 μ L) for 1 hour. After washing 3 times with PBST, antiserum of different dilutions was added to the wells and incubated for 2 hours at room temperature. Following further washing with PBST, 200 μ L of antibody (1:600) to rabbit immunoglobulin (Ig) G conjugated with horseradish peroxidase (HRP) was added to the wells and incubated for 1 hour at room temperature. The color that developed following the addition of orthophenylenediamine (0.03% in citric acid buffer pH 5.5) and incubation for 10 minutes was quantitated by measuring the optical density at 490 nm in an ELISA reader.

The ability of the LH-RP antibodies to block the binding of 125 I-labeled hCG to particulate sheep luteal LH receptor preparation was tested primarily according to the procedure described in detail by Jeyakumar and Moudgal (1996). In brief, this consisted of incubating sheep luteal receptor preparation (1 mg/tube) with 125 I hCG (1.5 – 2.0×10^5 cpm/tube) in the presence of 100 μ L of normal rabbit serum (control) or antiserum to LH-RP for 2 hours (total volume, 300 μ L) at room temperature. To facilitate precipitation of receptor and processing, 5% polyethylene glycol (PEG) was added, the receptor pellet collected by centrifugation was washed with 1 mL radioreceptor assay (RRA) buffer, and radioactivity was monitored. Total and nonspecific binding values determined in the absence and presence of excess unlabeled hCG were in the order of 30%–40% and 2%–6%, respectively, of added radioactivity.

Western Blotting of LH Receptor

Adult mouse testis was extracted using a lysis buffer as described (Babu et al, 2000) for examining the solubilized LH-Rs. Skeletal muscle was also extracted to serve as a control. Equivalent amounts of solubilized protein (25 μ g) was run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Polyscreen, NENTM Life Science Products, Boston, Mass) for reaction with antibodies. Following reaction with a 1:10000 dilution of each rabbit antibody, detection was performed by chemiluminescence using commercial kits (ECL+plus, Amersham, Buckinghamshire, United Kingdom). Molecular weight markers were used to compute the mass of the detected bands. After tests with LH-R antibodies, each blot was stripped for reprobing with an actin antibody (Sigma Chemical Company, St Louis, Mo) to test for equivalent loading and transfer.

Flow Cytometric Analysis: Preparation of Single Cell Suspension From Testicular Biopsy

The procedure used for preparing single-cell suspensions from a testicular biopsy was essentially that as described earlier (Suresh et al, 1992). In brief, testicular cells were released from the seminiferous tubules in calcium- and magnesium-free PBS by mincing the tissue obtained at biopsy with fine, curved scissors. The tissue mince was gently aspirated to disperse the cells, washed with PBS, and centrifuged for 10 minutes at $800 \times g$. The pellet was resuspended in 1 mL PBS; filtered through 100- μ m nylon mesh; and the cells were fixed in chilled, 70% ethanol and stored at 4°C until further analysis (Suresh et al, 1992).

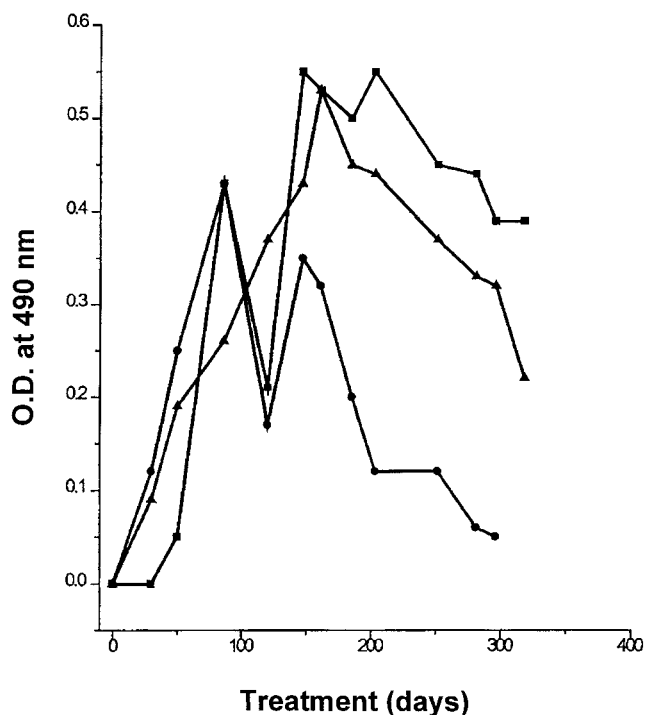


Figure 1. Effect of LH-RP immunization of rabbits on its antibody titer. The rabbit numbers 123, 126, and 127 are represented by symbols ■, ●, and ▲, respectively. The optical density (O.D.) at 490 nm of each antiserum tested at 1:800 dilution is shown. For details of methodology used in determining antibody titer see text.

Propidium Iodide Staining of Testicular Cells

The ethanol-fixed cells were stained with propidium iodide (PI) as described earlier (Suresh et al, 1992). Briefly, an aliquot of 1–2 million cells/mL was washed twice with PBS and incubated in 300 μ L of 0.5% pepsin solution (prepared in 0.9% saline pH 2) for 10 minutes at 37°C. After centrifugation the cells were stained with PI staining solution (25 μ g/mL PI, 40 μ g/mL RNase, and 0.3% Nonidet P-40 in PBS) for 20 minutes at room temperature. The PI-stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif) equipped with a 15-mw argon-ion laser at an excitation wave length of 488 nm. The fluorescence signal of PI was collected after being passed through a 620-bandpass filter (605–635 nm).

Preparation of Leydig Cells and Granulosa Cells for Determining LH-RP Binding Sites

A Leydig cell-rich fraction from the testes of adult Wistar rats was prepared according to the procedure of Van Damme et al (1974). Granulosa cells were isolated from equine chorionic gonadotropin (eCG)-primed immature female rats according to the procedure described elsewhere (Selvaraj and Moudgal, 1994). The cells were fixed in 1% buffered-paraformaldehyde for 30 minutes at 4°C, washed with 1 mL PBS containing glycine (GB-PBS; 10 mM glycine and 1% BSA) 3 times, and incubated with 50 μ g IgG equivalent of respective antiserum or normal rabbit serum (NRS; for nonspecific) for 60 minutes at room temperature. Cells were washed 3 times using 1 mL GB-PBS each time

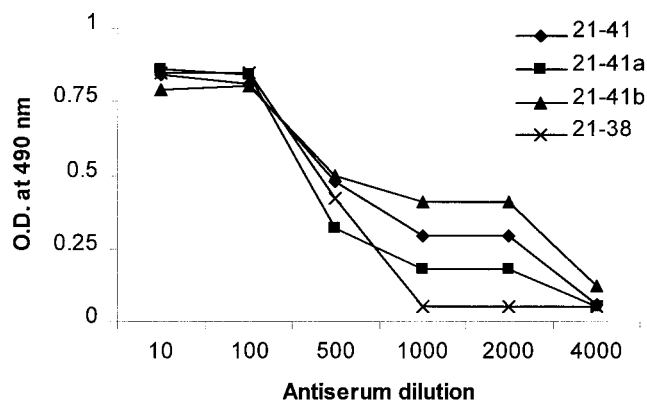


Figure 2. Demonstrating the ability of the LH-RP antibody to bind LH-RP 21-41 (◆), used in current study, and LH-RP 21-38 (×), LH-RP21-41a (■), and b (▲), which were procured from other sources.

following incubation with anti-rabbit IgG coupled with fluorescein isothiocyanate (FITC; Sigma) for 30 minutes at room temperature. While the washed granulosa and Leydig cells were re-suspended in GB-PBS, the green (FITC) fluorescence was measured using a FACScan flow cytometer equipped with a 15-mw argon-ion laser at an excitation wave length of 488 nm. The fluorescence signal for FITC was collected after a 525 band pass (505–545 nm).

Serum Testosterone Assay

Testosterone in ether-extracted sera was assayed using a well-standardized and routinely used radioimmunoassay procedure (Mukku et al, 1981). The sensitivity of the assay was 10 pg/tube, and the interassay and intraassay coefficients of variation were 9.3% and 8.3%, respectively.

Data Analysis

The statistical significance was assessed by one-way ANOVA and the Mann-Whitney *t*-test. The level of significance was set at 5%.

Results

Antibody Titration and Characterization

All 3 rabbits responded to immunization by producing antibodies that bound the LH-RP (Figure 1). They responded to the booster injection by producing relatively high antibody titers that were measurable for more than 100 days. The antibody to our synthetic LH-RP₂₁₋₄₁ preparation showed complete cross-reaction with LH-RP₂₁₋₄₁ preparations obtained from different sources. The antibody further cross-reacted with a synthetic LH-RP₂₁₋₃₈ preparation and the ED₅₀ of all 4 synthetic preparations ranged between 1:1000 and 1:1800 (Figure 2).

Western blot analysis was used to demonstrate the interaction of the peptide antibodies with mouse testicular LH receptor. All 3 rabbits reacted with a major component of the mouse testis (Figure 3) with an estimated mo-

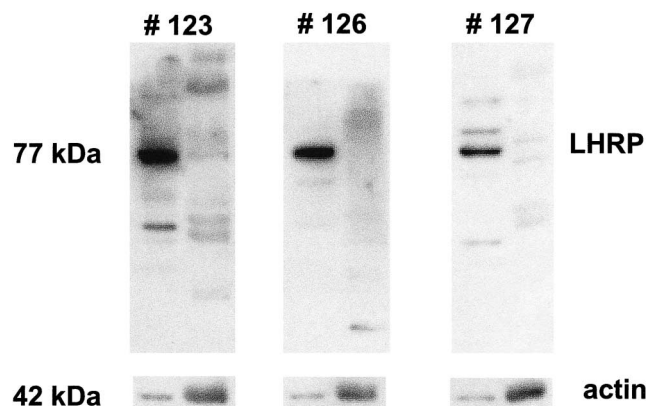


Figure 3. Western blot analysis of mouse testicular extract using the 3 anti-LH receptor antisera. The numbers on top indicate the respective antisera shown in Figures 1 and 2. In each panel, the lane on the left is the detergent solubilized testicular extract, and the right shows skeletal muscle protein. The M_r deduced in comparison to molecular weight markers is shown on the extreme left. The bottom panel shows the same blots reprobbed with an antibody actin following stripping of the LH receptor antibody. Note that actin intensity is stronger in skeletal muscle.

molecular weight of 77 kilodaltons (kd), equivalent to the mass of the full-length glycosylated LH receptor predicted by complementary DNA cloning (Loosfelt et al, 1989). The intensity of the band revealed by each antibody for the same testicular extract was, however, different, revealing differences in the titer of the antibodies. Some smaller bands were also apparent, but these were not consistent for all 3 rabbit antisera. Extracts of skeletal muscle used as a control showed no reaction at the 77-kd region. Other lighter bands observed are attributable to some minor, nonspecific reactions. The bottom panel of each blot shows equivalent protein loading as revealed by the actin antibody. As expected, the intensity of actin band in skeletal muscle is much stronger than in testis.

The ability of the antibody to recognize cell-associated LH receptor was demonstrated using flow cytometry. The antibody specifically bound rat granulosa and Leydig cells, both of which are known to have LH receptors (Figure 4A and B). The histogram shows that it is possible to clearly distinguish between the specific binding of the LH-RP antibody and the nonspecific binding of preimmune IgG. The antibody also specifically bound to monkey Leydig cells (Figure 4C).

The specificity of the LH-RP antibody was further checked by determining its ability to inhibit binding of ^{125}I hCG to a particulate sheep luteal LH receptor preparation. Antiserum of all 3 rabbits exhibited inhibitory activity, maximal inhibition in binding of 40%–50% being observed between days 125–150 of immunization (Figure 5), the time period when the antibody titer was also high ($R = 0.93$). Nonspecific inhibition observed with an equal volume of preimmune sera was less than 5%.

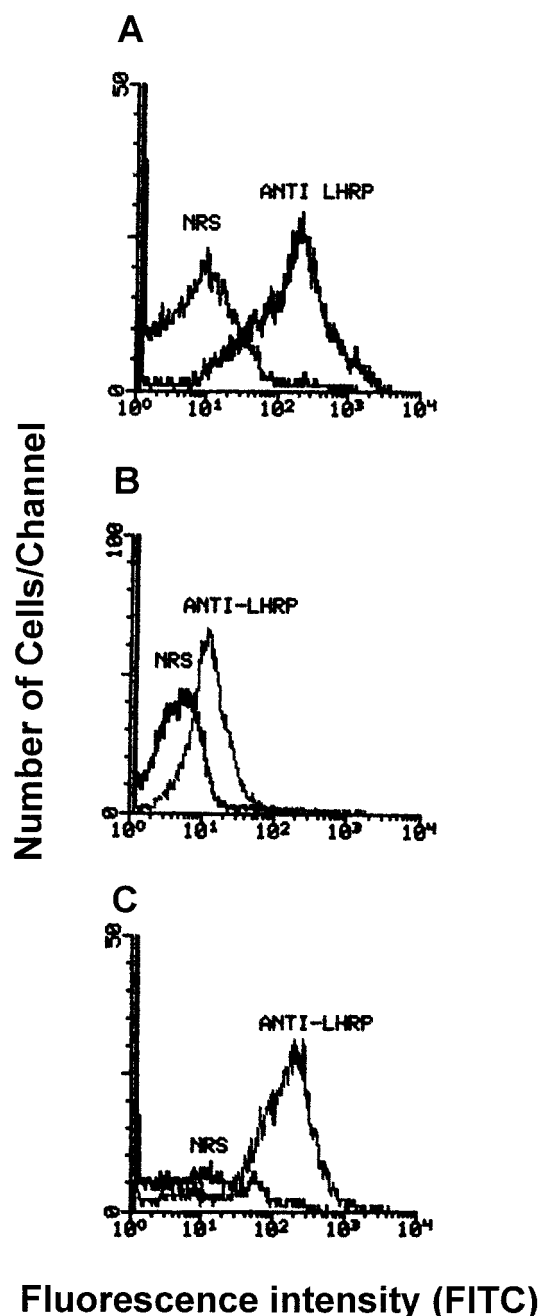


Figure 4. The representative overlay-histograms of normal rabbit serum (NRS) and anti-LH-RP bound to (A) rat granulosa cells, (B) rat Leydig cells, and (C) monkey Leydig cells as determined by immunocytometry. The x-axis shows the green fluorescence intensity of FITC on log scale and the y-axis indicates the number of cells per channel (see text for details).

Effect of Immunization with LH-RP₂₁₋₄₁ on Testicular Function

During the first 100 days of immunization the serum testosterone levels (Figure 6) showed a twofold to threefold increase over the control values (control, 7.8 ± 0.47 ng/mL vs immunized, 17.38 ± 1.2 ng/mL; $P < .0001$). This

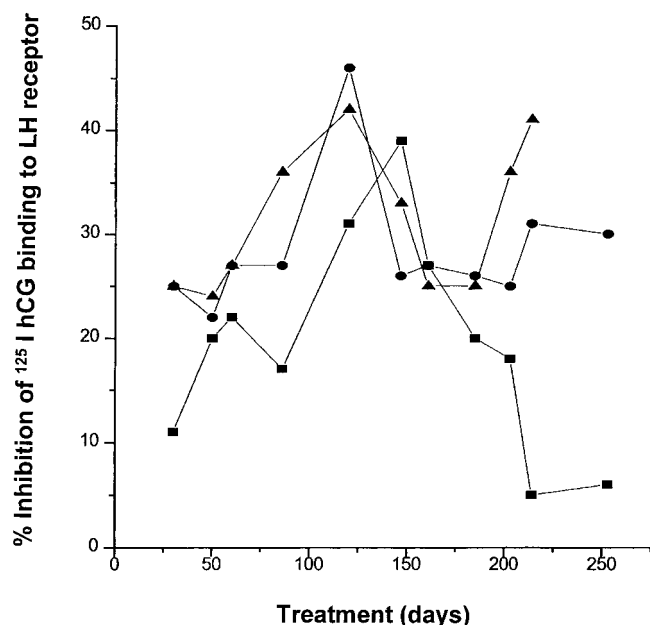


Figure 5. Ability of the LH-RP antibody at different times of immunization to inhibit binding of ^{125}I hCG to a sheep luteal LH receptor preparation (see text for details). The rabbit numbers 123, 126, and 127 are represented by symbols ■, ●, and ▲, respectively. Note that an equivalent amount of nonimmune sera obtained from control rabbits inhibited binding of ^{125}I hCG to the sheep luteal LH receptor preparation to a maximal extent of 5%.

was reminiscent of the hormone agonist activity, observed when rabbits were immunized with the natural holo sheep LH receptor protein (Jeyakumar and Moudgal, 1996). During later periods of immunization (more than 100 days), however, the serum testosterone levels generally showed marked reduction (by 80%) in its level over control (immunized, 1.76 ± 0.3 ng/mL vs control, 7.80 ± 0.47 ng/mL; $P < .0001$). Because blood samples for assay were always taken around the same time of the day, the

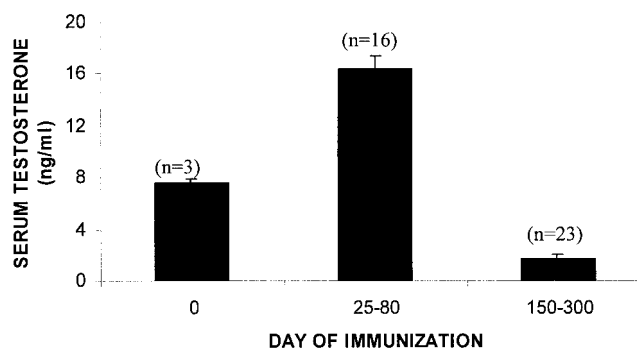


Figure 6. Effect of LH-RP immunization on serum testosterone levels. Three rabbits were used in each case. The n in parentheses refers to the total number of blood samples analyzed from the 3 rabbits during a particular time interval.

reduction in testosterone levels cannot be ascribed to any diurnal variations in serum testosterone levels. Besides, we had earlier shown that the species of rabbit used in the current study does not exhibit diurnal rhythms or seasonality (Jeyakumar et al, 1995). We also observed earlier that immunization of rabbits with a nonspecific protein-like mouse IgG does not lead to any change in testicular function (Jeyakumar et al, 1995). The reduction in testosterone level was observed to negatively correlate with the increase in hCG receptor binding inhibition activity.

Blockade of LH function with an antibody to LH-RP may lead to arrest in spermatogenesis. The effect of active immunization with LH-RP on testicular germ cell transformation was monitored by flow cytometry using a single-cell preparation obtained from testicular biopsy material taken following 0, 120, and 300 days of immunization. The percentage of different germ cell types present was quantified according to a procedure standardized earlier (Suresh et al, 1992). Overall spermatogenesis monitored by quantifying transformation of spermatogonia

The effect of immunization of rabbits on the distribution pattern of testicular germ cell composition and transformation ratios as determined by flow cytometry

Day of Immunization	Percentage of Germ Cells (mean \pm SEM)				
	HC	1C	2C	S-ph	4C
0	26.2 \pm 2.9	52.4 \pm 3.6	10.6 \pm 0.6	2.45 \pm 0.4	4.93 \pm 0.2
120	9.3 \pm 4.7*	37.7 \pm 18.9	39.2 \pm 5.6†	5.39 \pm 1.0*	8.35 \pm 1.4
300	11.4 \pm 3.8*	46.93 \pm 7.5	17.86 \pm 4.7	3.9 \pm 0.9	19.8 \pm 5.5*
Transformation Ratios (mean \pm SEM)					
	HC:2C	1C:2C	1C:4C	HC:4C	HC:1C
0	2.76 \pm 0.1	4.96 \pm 0.7	10.56 \pm 0.8	5.66 \pm 0.7	0.57 \pm 0.008
120	0.69 \pm 0.3†	2.82 \pm 1.5	3.93 \pm 2.0*	0.94 \pm 0.5‡	0.16 \pm 0.008‡
300	0.73 \pm 0.4†	3.32 \pm 1.4	2.86 \pm 0.9†	0.79 \pm 0.5‡	0.24 \pm 0.007‡

Results of analysis of testicular cell preparations obtained from testicular biopsy samples of the 3 immunized rabbits are provided. At each time point, 10,000 cells/sample were analyzed by flow cytometry. Each data point is the mean \pm SEM of 3 rabbits.

HC indicates elongated spermatids; 1C, round spermatids; 2C, spermatogonia and non-germ cells; S-ph, spermatogonial cells synthesizing DNA; 4C, primary spermatocytes.

* = $p < .05$, † = $p < .01$ and ‡ = $p < .0001$.

(2C) to elongate (HC) spermatids showed the HC:2C ratio to be significantly inhibited (75%; $P < .01$) in all 3 rabbits at both 120 and 300 days of immunization (Table). This effect appeared to be due to a block in meiosis as seen by significant inhibition in the transformation of primary spermatocytes (4C) to round (1C; $P < .05$) and elongated (HC; $P < .0001$) spermatids (Table). The transformation of round to elongate spermatids (HC:1C) was also significantly ($P < .0001$) inhibited (Table). Normally, the cells having a 2C and 4C amount of DNA do not exceed 15%–20% of total germ cells analyzed. However, if meiosis is blocked, the percentage of cells having 2C and 4C amounts of DNA could show a relative but substantial increase (41%–52%), as in the present case.

Discussion

A primary objective of the current study was to determine whether we could obtain antibodies capable of interacting with a natural LH receptor using as the immunogen a synthetic peptide representing an apparent active site (Roche et al, 1992) of the LH receptor. High antibody titer was maintained between 100–250 days of immunization and its ability to specifically interact with the LH receptor was established using both in vitro and in vivo tests. The LH-RP antibody bound 3 preparations of synthetic LH-RP_{21–41} procured from different sources as well as an LH-RP_{21–38} preparation with equal avidity. In Western blot analysis using mouse testicular extract as the protein source, the antibody of all 3 rabbits showed binding to a 77-kd protein, a molecular species similar to the mouse LH receptor. In addition, the antibody of all 3 rabbits significantly inhibited the binding of ¹²⁵I-hCG to a particulate sheep luteal LH receptor preparation.

The LH-RP antibody and not the preimmune rabbit serum bound granulosa and Leydig cells. The significant inhibition (40%–50%) in binding of ¹²⁵I hCG to sheep luteal receptor obtained with LH-RP antibody is suggestive of the antibody interacting with at least one of the hCG binding sites in the receptor. In our earlier study, use of hCG monoclonal antibodies in the binding inhibition assay had shown that individually, no one agent could inhibit binding to the receptor by more than 45%. However, 2 or more monoclonal antibodies added together resulted in close to 80% inhibition, indicating that hCG is interacting with its receptor through more than one site (Jeyakumar et al, 1997).

Depending on the period of immunization (early, less than 100 days vs late, more than 100 days of immunization), the LH-RP antibody generated by the rabbits appeared to simulate as well as block endogenous LH activity. This was reflected by a marked change in serum testosterone levels, an increase over control levels before

100 days (120% increase; $P < .0001$), and in a significant decrease in testosterone levels below the control beyond 100 days (80% decrease; $P < .0001$). This decrease in serum testosterone correlated, interestingly, with an increase in the ability of the antibody to block in vitro ¹²⁵I hCG binding to the receptor.

There seems to be a distinct difference in the way rabbits respond to the holo sheep LH receptor protein and the rat LH-RP_{21–41} peptide. In the earlier study on the holo receptor, serum testosterone level was never suppressed below control even after prolonged immunization and consequently, no inhibition of spermatogenesis was observed. This was indicative of the coexistence of LH mimicking and antagonistic antibodies through the period of immunization (Jeyakumar and Moudgal, 1996). In the current study with rat LH-RP_{21–41} peptide, however, the serum testosterone concentration as well as spermatogenesis were significantly inhibited following prolonged immunization. Such effects are expected to follow the blockade of LH function. We have shown earlier that immunization of male rabbits with the hormone ovine LH results in significant reduction in serum testosterone levels, leading to blockade of meiosis and consequently, spermatogenesis (Jeyakumar et al, 1995). A similar observation was also made using a different approach altogether, such as the use of norethisterone as a feedback inhibitor of LH secretion (Suresh et al, 1995; Shetty et al, 1997).

In conclusion, it appears from the foregoing that the synthetic LH-RP peptide used in the current study represents a functional receptor binding site and can supplant the holo LH receptor as an effective immunogen in any future vaccine study. By blocking even one of several receptor binding sites of hCG with the LH-RP antibody we were able to obtain substantial suppression of in vivo LH function. Whether it is possible to completely eliminate hormone-mimicking activity by synthesizing analogues of this peptide remains to be determined, as they may prove to be useful therapeutic agents.

Acknowledgments

We thank Dr Medhamurthy of the Indian Institute of Science, Bangalore, for providing the LHRP peptides. We appreciate the help of Odile Royer in manuscript preparation.

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