CHAPTER 4

Results
4.0 RESULTS

4.1 Vaccines: Two designs of the multimeric anti-LHRH vaccines in which five oligonucleotide strands coding for LHRH were interspersed with four to five oligonucleotide sequences coding for promiscuous T cell peptides were developed. Figs. 4 and 5 show the molecular design of the multimeric LHRH peptides along with corresponding amino acid and nucleotide sequences. The constructs used for the expression of recombinant multimeric LHRH peptides linked to T cell determinants is shown in Fig. 6. The plasmid pRSET-LHRH (d1) was used to express the multimeric LHRH (d1) as recombinant protein in protease deficient E. coli BL 21(DE3) pLys S (cm$^R$). The plasmid ICGEB-LHRH (d2) was used to express the multimeric LHRH (d2) as recombinant protein in protease deficient E. coli BL 21.

4.1.1 Expression of r-LHRH-d2 and its Isolation as Pure Inclusion Bodies
The expression level of the r-LHRH-d2 was around 15% of the total cellular protein and it aggregated as inclusion bodies. The time kinetics of r-LHRH-d2 expression in shaker flask is shown in Fig 7. The level of expression plateaued around 4 hours after raising the temperature from 28°C to 42°C. The molecular weight of the expressed protein was around 16 kDa. This was close to the calculated molecular mass of the peptide constructs of r-LHRH-d2. The inclusion bodies were isolated from the induced cells by mechanical disruption. Washing of the inclusion bodies with 1% deoxycholate in Tris EDTA buffer resulted in partial purification of the inclusion body protein.
[A] Design 1 (dl) of the multimeric LHRH gene. Five units of LHRH decapptide are interspersed with nucleotide sequences encoding 4 different small peptides recognizing determinants on the T type of lymphocytes from Tetanus toxin (TT), Measles, Circumsporozoite protein (CSP) of *Plasmodium falciparum* and Respiratory syncytial virus (RSV).

[B] Amino acid sequence of multimeric LHRH peptide linked to T cell determinants.

[C] Nucleotide sequence of the gene encoding the multimeric LHRH peptide linked to T cell determinants.
Fig. 5

[A] Design 2 (d2) of the multimeric LHRH gene. Five units of LHRH decapeptide are interspersed with nucleotide sequences encoding 5 different small peptides recognizing determinants on the T type of lymphocytes from Tetanus toxin (TT), Measles, Mycobacterium tuberculosis (MTB), Circumsporozoite protein (CSP) of Plasmodium falciparum and Respiratory synctial virus (RSV).

[B] Amino acid sequence of multimeric LHRH peptide linked to T cell determinants.

[C] Nucleotide sequence of the gene encoding the multimeric LHRH peptide linked to T cell determinants.
Schematic diagrams of the constructs used for the expression of recombinant multimeric LHRH peptides linked to T cell determinants. [A] Recombinant expression vector for the expression of multimeric LHRH vaccine of design 1 under control of the bacteriophage T7 promoter. [B] Recombinant expression vector for the expression of multimeric LHRH vaccine of design 2 under control of the temperature-inducible λP1 promoter.
Fig. 7 Expression of r-LHRH-d2 multimer in *E. coli* (BL-21) at different time intervals. Lane 1, uninduced cells. Lane 2-7, induced cells at different time intervals. Lane M, molecular weight marker.
(Fig.8). At the end of the washing, the inclusion bodies contained r-LHRH-d2 multimer in the form of monomer of 16 kDa along with some higher molecular weight aggregates. Inclusion bodies thus obtained were used directly for solubilization and refolding.

4.1.2 Solubilizaton of Inclusion Bodies at Different pH

The effect of increasing urea concentration on the solubility of r-LHRH-d2 inclusion bodies at different pH is presented in Fig. 9. It was observed that only 20% of the protein could be solubilized from the inclusion bodies in the presence of 2 M urea in 100 mM Tris buffer at pH 8.5 as compared with 63% solubility in the presence of 8 M urea. Similarly only 30% of the protein could be solubilized in presence of 2M urea in 50 mM citrate buffer at pH 6.0, the solubility increased to 65% in presence of 8 M urea. Maximum solubilization of inclusion bodies was observed at pH 3 or 12. However solubilization at pH 12 resulted in degradation of the recombinant LHRH as indicated in SDS-PAGE (data not shown). Presence of 2M urea in citrate buffer at pH 3 solubilized 61% of the r-LHRH-d2 from the inclusion bodies while precipitating the E.coli contaminating proteins, the % solubility was comparable with that obtained with 8 M urea at pH 8.5 or pH 6.0

4.1.3 Refolding of Solubilized r-LHRH-d2

Purified r-LHRH-d2 inclusion bodies were solubilized in 50 mM citrate buffer pH 3.0 containing 2 M urea. The solubilized r-LHRH-d2 was diluted five folds
Fig. 8. SDS-PAGE analysis of r-LHRH-d2 inclusion bodies (IB) purification. Lane 1, induced cells. Lane 2, Purified IB, r-LHRH-d2 at 16 kDa. Lane 3, pellet remaining after solubilization of IB at pH 3.0. Lane 4, Solubilized r-LHRH-d2 in citrate buffer at pH 3.0. Lane M, Low molecular weight marker.
Fig. 9. Effect of urea concentration on the solubility of r-LHRH-d2 inclusion bodies at different pH values. Purified inclusion bodies (2 mg) were solubilized in 100 mM Tris buffer at pH values 12 and 8.5; 50 mM citrate buffer at pH values 3 and 6 in presence of increasing concentrations of urea. Solubilized protein concentration was determined by micro BCA assay and plotted as a function of urea concentration.
with 10mM citrate buffer pH 6.0/2M urea containing 0.3M L-arginine. The presence of L-arginine prevents aggregation during protein folding and dilution step (Arora and Khanna, 1996). Very little aggregation of the solubilized protein was observed during buffer exchange in the presence of 0.3M L-arginine as compared with renaturation in its absence (Fig. 10). After dilution and changing the buffer pH to 6, the protein solution was extensively dialysed to remove Arginine. Arginine free refolded protein solution was further purified using cation exchange chromatography.

4.1.4 Purification of r-LHRH-d2

Method 1- (Purification of r-LHRH-d2 by Ion Exchange followed by Gel Filtration Chromatography)

The solubilized r-LHRH-d2 protein was loaded on a CM sepharose column equilibrated with 15mM citrate buffer at pH 4.8 / 2 M urea and the bound protein was eluted in presence of salt gradient (0.1-1M NaCl). It was evident that r-LHRH-d2 which eluted between conductivity 15-20 mS/cm was homogenous but was 20% of the total protein load. r-LHRH-d2 was coeluted with higher molecular size proteins between conductivities of 25-30 mS/cm which constituted about 60% of the total protein load. High molecular weight proteins were separated by using gel filtration chromatography. This purification process served the twin purpose of buffer exchange, (so that intermolecular aggregation could be avoided) and removal of higher molecular weight proteins by size exclusion. The eluted fractions were
Fig. 10. Effect of addition of L-arginine in the refolding buffer at different pH values. Inclusion bodies were solubilized at pH 3 and diluted five folds in refolding buffer at different pH's with and without the addition of 0.3 M L-arginine.
monitored with an on-line UV detector and further analysed by SDS PAGE and silver staining. However the overall recovery of pure r-LHRH-d2 from the inclusion bodies was around 30%.

Method 2 - (Purification of r-LHRH-d2 by Twin Ion-Exchange Chromatography)

In order to overcome the loss in protein recovery associated with ion-exchange followed by gel filtration, double ion-exchange protocol was used to avoid co-elution of high molecular aggregates of r-LHRH-d2. In the first run the protein was loaded on the CM sepharose column at pH 6.0, this resulted in binding of high molecular weight proteins whereas most of r-LHRH-d2 did not bind to the column and was collected as flow through. The r-LHRH-d2 obtained as flow through was again reloaded on CM-Sepharose column at pH 4.8 and pure r-LHRH-d2 was eluted at a conductivity of 25mS/cm. The purified r-LHRH-d2 gave a single band in SDS-PAGE and on silver staining. The recovery of r-LHRH-d2 from ion-exchange matrix was around 80% of the protein load. The overall yield of the purified r-LHRH-d2 from the inclusion bodies of E.coli was ~ 40%. The overall yield of pure refolded r-LHRH-d2 from inclusion bodies using both the methods are printed in Table 1. In both cases, highly pure r-LHRH-d2 was obtained (Fig. 11). As the second method gave higher yield of the purified r-LHRH-d2, it was followed for large scale recovery of r-LHRH-d2 multimer.
<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Protein (mg)</th>
<th>Step yield (%)</th>
<th>Overall Yield (%)</th>
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<tr>
<td>Cell lysate</td>
<td>256</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Inclusion body</td>
<td>65</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Solubilization (50 mM citrate buffer pH 3/2 M urea)</td>
<td>40</td>
<td>61</td>
<td>61</td>
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<tr>
<td>Refolding and dialysis</td>
<td>32</td>
<td>80</td>
<td>49</td>
</tr>
<tr>
<td>Method-1 Ion-exchange at (pH 4.8) and Gel-filtration chromatography</td>
<td>20</td>
<td>62</td>
<td>30</td>
</tr>
<tr>
<td>Method-2 Ion-exchange chromatography at pH 6.0 and at pH 4.8</td>
<td>26</td>
<td>81</td>
<td>40</td>
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</table>
4.1.5 Characterization of Refolded r-LHRH-d2

Protein purified and refolded by the above method was characterized using CD, fluorescence, ELISA, SDS-PAGE. The fluorescence spectra in both the cases showed max at 360 nm, indicating the presence of tryptophan residues in the protein (Fig. 12B). The intensity of fluorescence spectra reduced as the pH lowered from 10 to 4 indicating the presence of compact protein structure. At pH 2 the shift in the fluorescence spectra by 10 mm indicates the unfolding of the protein at very low pH (< 2). Estimation of protein by both BCA assay and ELISA were equivalent within experimental error of 5-10%.

Characterization of the protein by SDS-PAGE and Western Blot analysis showed the presence of purified protein of the expected size. Authenticity of the purified protein was further confirmed from the N-terminal amino acid sequencing (Edman degradation) of r-LHRH-d2 which confirmed the sequence "M-D-I-E-K-I-A-K-M-E-K-A-S-S", presence of r-LHRH-d2 as a single band in silver staining and western blot analysis. The HPLC analysis of the purified r-LHRH-d2 showed a single peak at 29.51 min indicating the high purity of r-LHRH-d2 (Fig. 12A). Finally the authenticity of the purified protein was verified using mass spectroscopic analysis (Fig. 12C). Protein mass was found to be 16604.7 which is in agreement with the theoretically calculated mass of 16.6 kDa. The CD spectrum of the refolded r-LHRH multimer showed the presence of β-sheet with single negative band around 215-218 nm (Fig. 12D) at two different pH. The endotoxin levels of the protein batches were less than 0.5 EU/mL.
Fig. 11. SDS-PAGE analysis of purified r-LHRH-d2. Lyophilized protein (2 mg) from method 1 and method 2 was dissolved each in 500μl loading dye and 5 μl and 15μl of aliquots were loaded on to 12% SDS-PAGE. Lanes 1, 4 pure r-LHRH-d2 eluted from S-100 gel-filtration column (Method-1). Lanes 2, 3 pure r-LHRH-d2 eluted from CM Sepharose column pH 4.8 (Method-2).
Fig. 12. (A) HPLC analysis of purified r-LHRH-d2 on a Shimadzu LC10AD HPLC system at 280 nm showed a single peak at 29.51 min.
Fig. 12. (B) Fluorescence emission spectra of purified r-LHRH-d2 at different pH. Lines 1-5 indicate emission spectra at different pH. Line 1, pH 10; line 2, pH 8; line 3, pH 6; line 4, pH 4; line 5, pH 2.
Fig. 12. (C) Mass spectroscopic analysis of r-LHRH-d2 in agreement with the theoretically calculated mass of 16.6 kDa.
Fig. 12. (D) The CD spectrum of the refolded r-LHRH-d2 showed the presence of β-sheet with single negative band around 215-218 nm at pH 6 and pH 8.
4.1.6 Immunoreactivity of Refolded r-LHRH-d2

The purified rLHRH-d2 was immunoreactive against anti LHRH ascites (1:100 dilution) in ELISA using goat anti-mouse-IgG HRPO as secondary antibody (Fig. 13A) and also showed positive immunoreactivity against anti-LHRH serum (pooled sera of hyperimmune male animal in which immunization against LHRH in earlier experiments with LHRH-DT vaccine had caused a decline of testosterone and atrophy of the prostate) using goat anti-rat-IgG HRPO (1:25,000 dilution) as a secondary antibody (Fig. 13B).

4.2 Immunization with proteinic vaccine

The recombinant proteins expressed in E.coli (purified and refolded) were examined for their ability to induce bio-effective antibodies in rats, a species in which LHRH moiety is identical to humans.

4.2.1 Immunization with w/o emulsion of r-LHRH protein and Freund's adjuvant

Hyperimmunization was carried out with the presently developed recombinant multimer vaccines employing Complete Freund's adjuvant so as to evoke maximal potential toxicity of anti-LHRH antibodies induced by these vaccines. Figure 14 gives the results of the antibody titres induced in rats by design 1 (d1) vaccine (100μg). Each rat generated anti-LHRH antibodies. The antibodies were detectable on day 15 after the first injection and titres increased further with the two boosters given at 15 day intervals.
Fig. 13A. Immunoreactivity of native LHRH and r-LHRH-d2 with mAb specific to native LHRH (anti LHRH ascites 1:100 dilution).
Fig. 13B. Immunoreactivity of native LHRH and r-LHRH-d2 with polyvalent antibodies (pooled sera of hyper immunized male rats having undergone prostatic atrophy after immunization with synthetic LHRH vaccine). Antibody titres were expressed as absorbance values obtained at 1:100 serum dilution by the ELISA.
consonance with the increase in the anti-LHRH antibodies, serum testosterone declined in each animal and reached below 1ng/ml by the 40th day. Rats immunized with the design 2 (d2) recombinant vaccine (100μg) also generated anti-LHRH antibodies but the titres went beyond the bioefficacy levels after the first (primary) and two booster injections of the vaccine (Fig. 15). The testosterone levels started declining after day 40 to reach the castration levels in course of time in tune with the building up of antibody titres. Even though both designs of the recombinant vaccines were competent to induce bioeffective response, the design 1 (d1) LHRH vaccine generated early response. All the eight animals immunized with one or the other vaccine showed decline of testosterone concomitant with reduction of the testis size. In order to determine whether immunization with one or the other vaccine caused atrophy of the prostate, necropsy was performed and the prostate as well as other accessory reproductive organs were taken out and weighed. Table 2 gives the size of various organs in control (adjuvant alone) and immunized animals. It will be noted that the decline in prostate weight was highly significant. A reduction in weight of seminal vesicles, epididymis and testis, all dependant on androgen support was also noted. Other organs of the animals like heart, liver, kidney and lungs had no significant change in weight.

From previous work (Jayashankar et al., 1989; Giri et al., 1991; Rovan et al., 1992; Fuerst et al., 1997 and Talwar et al., 1999), it was expected that with the decline of testosterone, atrophy of the prostate would occur. It was
Fig. 14 (A). Antibody response to recombinant design 1 (d1) LHRH vaccine in rats. Arrows indicate the time points at which primary immunization and boosters were given. (B). Blood testosterone levels in each of the immunized rats represented by different symbols.
Fig. 15. Antibody response and testosterone levels in rats immunized with recombinant design 2 (d2) LHRH vaccine.
**Table 2.** Wet weights* of prostate, testis, accessory reproductive organs and some other organs normalized on the basis of 100 g body weight of rats immunized with either d1 or d2 LHRH vaccine.

<table>
<thead>
<tr>
<th>Group</th>
<th>Prostate</th>
<th>Testis</th>
<th>Seminal vesicles</th>
<th>Epididymis</th>
<th>Heart</th>
<th>Liver</th>
<th>Lungs</th>
<th>Spleen</th>
<th>Kidney</th>
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<tr>
<td></td>
<td>L (g)</td>
<td>R (g)</td>
<td>L (g)</td>
<td>R (g)</td>
<td>L (g)</td>
<td>R (g)</td>
<td>L (g)</td>
<td>R (g)</td>
<td>L (g)</td>
</tr>
<tr>
<td>d-1 LHRH</td>
<td>0.010 ± 0.007</td>
<td>0.045 ± 0.002</td>
<td>0.043 ± 0.002</td>
<td>0.022 ± 0.002</td>
<td>0.023 ± 0.003</td>
<td>0.028 ± 0.001</td>
<td>0.026 ± 0.002</td>
<td>0.344 ± 0.056</td>
<td>2.44 ± 0.214</td>
</tr>
<tr>
<td>d-2 LHRH</td>
<td>0.011 ± 0.001</td>
<td>0.042 ± 0.001</td>
<td>0.042 ± 0.001</td>
<td>0.023 ± 0.001</td>
<td>0.024 ± 0.002</td>
<td>0.028 ± 0.002</td>
<td>0.028 ± 0.002</td>
<td>0.316 ± 0.047</td>
<td>2.59 ± 0.419</td>
</tr>
<tr>
<td>Control (Ctl)</td>
<td>0.223 ± 0.001</td>
<td>0.332 ± 0.002</td>
<td>0.333 ± 0.001</td>
<td>0.323 ± 0.001</td>
<td>0.324 ± 0.001</td>
<td>0.146 ± 0.001</td>
<td>0.145 ± 0.001</td>
<td>0.339 ± 0.018</td>
<td>2.52 ± 0.338</td>
</tr>
<tr>
<td>Ical d1 vs Ctl</td>
<td>224.0</td>
<td>202.0</td>
<td>204.4</td>
<td>223.37</td>
<td>212.2</td>
<td>103.98</td>
<td>72.95</td>
<td>0.167</td>
<td>0.402</td>
</tr>
<tr>
<td>Ical d2 vs Ctl</td>
<td>199.27</td>
<td>204.4</td>
<td>205.1</td>
<td>234.99</td>
<td>248.8</td>
<td>110.91</td>
<td>74.98</td>
<td>0.900</td>
<td>0.251</td>
</tr>
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</table>

*Values are mean ± S.D of four rats in each group. The four rats in control group received only the adjuvant but no antigen.

The tabulated 't' for (n1 - n2 - 2) i.e 6 degrees of freedom at 95% confidence level (t' tab 2.47) is more than the calculated value 't' cal for heart, liver, lungs and spleen so there is no statistically significant difference in the weights of these organs whereas 't' cal is more than 't' tab 2.47 for testis, seminal vesicles, epididymis and prostate i.e there is statistically significant difference in the organ weights of the reproductive organs between treated (d1 or d2) and the control group.
however of interest to enquire whether the reduction in prostate starts early and is manifest at antibody titres below 0.15 O.D units, a threshold which was reported previously (Diwan et al, 1998) for achieving bio-efficacy. In a new experiment eight rats were immunized with d1 and an equal number with d2 LHRH vaccines and one animal from each group necropsied at various titres of antibodies. Figure 16 shows that the reduction of prostate size is prominent at 0.12 O.D units of antibody titres, but the maximal atrophy is attained at titres of 0.2 O.D units and above.

Animals were individually examined for any local effect at the site of injection i.e erythema, granuloma, pain on touch. Injections of small amounts given subcutaneously at multiple sites ensured that none of the immunized animals developed any severe reaction (lesions, ulcerative granulomas) at the site of injection. Behaviourally animals were found normal. Immunizations did not have any effect on food intake and normal growth of the animal as seen by gain in body weight (Fig.17).

4.2.2 Immunization regimes with human compatible adjuvants and immunopotentiators

Having established that the proteinic vaccine given with Freund's adjuvant caused prostatic atrophy in experimental animals, experiments were continued to explore immunization regimes with human compatible adjuvants and immunopotentiators to achieve high antibody levels causing atrophy of the prostate.
Fig. 16. Prostate weights normalized on 100 g body weight basis at different circulating titres of anti-LHRH antibodies. (A) Rats immunized with (d1) vaccine. (B) Rats immunized with (d2) vaccine.
Fig. 17. Weight chart of control (-----o-----) animals, d1 (....Δ....) and d2 (—#—) immunized rats. The lines represent the arithmetic mean of each group of four rats.
Both proteinic and DNA vaccines were investigated, either alone or in combination to evoke high enough anti-LHRH response to reduce the testosterone to castration levels thereby causing atrophy of the prostate.

4.2.2.1 Immunization with r-LHRH protein adsorbed on alum

Three groups of animals (n=3) were immunized with different doses (50µg, 100µg and 200µg) of proteinic vaccines. The protein was adsorbed on 2% alhydrogel and administered in male rats along with SPLPS (200 µg /rat, in the first injection only) followed by two boosters at 15 day intervals. It however did not generate high titres of antibodies to elicit bioeffective response (Fig 18-19). While pilot experiments with proteinic vaccine given with Freund's adjuvant caused prostatic atrophy in experiment animals, proteinic vaccine adsorbed on alhydrogel failed to cause the same.

4.2.2.2 Immunization with r-LHRH protein and MF-59 emulsion

Figure 20' shows the plot of geometric mean of anti-LHRH antibody titres obtained with LHRH(d1) vaccine (100µg) mixed with the adjuvant MF-59 emulsion and injected to rats intramuscularly. Following the first injection on day 0 the antibodies quickly reached 0.06 O.D units by day 5. A second injection on day 13 increased the titres to 0.19 O.D units by day 15. The titres declined by day 30 to 0.08 O.D units but a third injection on day 33 increased the titres to 0.13 O.D units. A fourth injection on day 50 boosted the titres (which had decreased to 0.07 O.D units by day 45) to 0.15 O.D units.
Fig. 18. Immune response obtained in male rats immunized with different doses of proteinic vaccine (LHRH-d1) adsorbed on alum.

* n = 3 for each dose
* doses (50μg, 100μg and 200μg)
* i.m. injections, given on d=0, d=15 and d=30.
Fig. 19. Immune response obtained in male rats immunized with different doses of proteinic vaccine (LHRH-d2) adsorbed on alum.

- n = 3 for each dose
- doses (50μg, 100μg and 200μg)
- 3 immunizations given on d=0, d=15 and d=30.
Fig. 21. Immune response obtained in male rats after i.m immunization with LHRH (d1) vaccine (100µg) mixed with MF-59 emulsion.

* n = 4
* i.m injection given on d=0, d=13, d=33, d=50.
Fig. 20. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m. administration of LHRH (d1) vaccine (100μg) mixed with MF-59 emulsion. Data for individual animals are given as scatter, while the geometric mean is represented by -•- joined by a continuous line.

* n = 4
* i.m. injection given on d=0, d=13, d=33, d=50.
Fig. 21. Immune response obtained in male rats after i.m immunization with LHRH (d1) vaccine (100μg) given 1 hr after i.m administration of MF-59 emulsion.

* n = 2.
* i.m injection given on d=0, d=13, d=33, d=50.
Fig. 21'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m. administration of LHRH (d1) vaccine (100μg) given 1 hr after i.m. administration. MF-59 emulsion. Data for individual animals are given as scatter, while the geometric mean is represented by -*-, joined by a continuous line.

* n = 2
* i.m. injection given on d=0, d=13, d=33, d=50.
However the titres did not go beyond 0.15 O.D units and decreased to 0.08 O.D units by day 75.

Figure 21 shows the plot of geometric mean of anti-LHRH antibody titres obtained with LHRH (d1) vaccine (100μg) when injected 1 hour after the administration of the adjuvant MF-59 emulsion. Following the first injection on day 0 the antibodies quickly reached 0.11 O.D units by day 5. A second injection on day 13 increased the titres to 0.17 O.D units by day 15. The titres declined by day 30 to 0.08 O.D units but a third injection on day 33 increased the titres to 0.18 O.D units. A fourth injection on day 50 boosted the titres (which had decreased to 0.08 O.D units by day 45) to 0.18 O.D units. However the titres were not sustained and decreased to 0.07 O.D units by day 75. It was observed that all animals turned sero-positive when MF-59 emulsion was used as an adjuvant, either mixed with proteinic vaccine or given prior to immunization. While the adjuvant induced a quicker response the titres declined quickly and were not sustained so as to elicit a bioeffective response.

4.2.2.3 Immunization with r-LHRH protein admixed with Immuvac

Figure 22 shows the plot of geometric mean of anti-LHRH antibody titres obtained on intramuscular co-administration of LHRH (d1) vaccine (100μg) with the adjuvant Immuvac (0.5 x 10^9 heat killed M.w.). The antibody titres emerged soon after the first injection and were detectable on day 5 as 0.09 O.D units, which declined to 0.08 O.D units by day 10. A second injection on
day 13 increased the titres to 0.11 O.D units by day 15. The rise seen in the antibody titres just after the injection showed a decline again, remaining at 0.07 O.D units till day 30. A third injection on day 33 increased the titres to 0.19 O.D units by day 35, the titres declined by day 45 to 0.09 O.D units. A fourth injection on day 50 did boost the titres to reach 0.19 O.D units but even this booster dose was not able to sustain the titres, the decline of the titres started again and reached 0.08 O.D units by day 75.

Figure 23' shows the plot of geometric mean of anti-LHRH antibody titres obtained on intradermal co-administration of LHRH (d1) vaccine (100μg) with the adjuvant Immuvac (0.5 x10^9 heat killed M.w.). The antibody titres emerged soon after the first injection and were detectable on day 5 as 0.09 O.D units, which declined to 0.08 O.D units by day 10. A second injection on day 13 increased the titres to 0.13 O.D units by day 15. The rise seen in the antibody titres just after the injection showed a decline again, remaining at 0.06 O.D units till day 30. A third injection on day 33 increased the titres to 0.23 O.D units by day 35, the titres declined by day 40 to 0.12 O.D units. A fourth injection on day 50 did boost the titres to reach 0.2 O.D units but even this booster dose was not able to sustain the titres, the decline of the titres started again and reached 0.08 O.D units by day 75. It was observed that Immuvac as an adjuvant with LHRH (d1) vaccine (admixture given either intramuscularly or intradermally) induced a quicker response, the titres increased immediately after the injections but declined soon after and then
Fig. 22. Immune response obtained in male rats after i.m immunization with LHRH (d1) vaccine (100μg) mixed with adjuvant Immunvac (0.5 x 10^9 heat killed M.w).

* n = 2
* i.m injection given on d=0, d=13, d=33, d=50.
Fig. 22. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m. immunization with LHRH (d1) vaccine (100µg) mixed with adjuvant Immuve (0.5 x 10^9 heat killed M. w). Data for individual animals are given as scatter, while the geometric mean is represented by -*-* joined by a continuous line.

* n=2
* i.m injection given on d=0, d=13, d=33, d=50.
Fig. 23. Immune response obtained in male rats after i.d immunization with LHRH (d1) vaccine (100μg) mixed with adjuvant Immuvac (0.5 x 10⁹ heat killed M.w).

* n = 2
* i.d injection given on d=0, d=13, d=33, d=50.
Fig. 23'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.d immunization with LHRH (d1) vaccine (100μg) mixed with adjuvant Immuvac (0.5 x 10⁹ heat killed M. vac). Data for individual animals are given as scatter, while the geometric mean is represented by *- joined by a continuous line.

* n=2
* i.d injection given on d=0, d=13, d=33, d=50.
increased only when another booster dose was given. Thus the titres were not sustained so as to elicit a bioeffective response.

4.3 Immunization with DNA vaccine

Preliminary immunization studies with anti LHRH (d1) DNA vaccine (using expression vector VR1012 was carried out in male rats. Anti-LHRH (d1) DNA (VR1012) vaccine was injected intra muscularly into male rats 24 hours after pretreatment of muscle with 0.5% bupivacaine. Different doses of anti-LHRH DNA (VR1012) vaccine varying from 25-200 μg per injection were used in order to determine the optimum concentration which would elicit immune response sufficient to decrease testosterone concentration and prostate weight. It was observed that all doses of anti-LHRH (d1) DNA (VR1012) were immunogenic i.e all experimental animals turned sero-positive for anti-LHRH antibodies. Figure 24 shows the effect on testosterone levels of male rats with anti-LHRH immune response due to anti-LHRH (d1) DNA (VR1012) vaccine, n=4 for 25, 50 and 200 μg dose and n=8 for 100 μg dose. Even though testosterone declined with the emergence of antibodies, the castration levels of testosterone were not achieved. Moreover the antibodies generated only after the 2nd injection and multiple injections were required to sustain the titres. Figure 25 gives the linear regression for fall in testosterone levels with increase in anti-LHRH antibody titres after DNA vaccine immunizations with different doses. Figure 26 shows the plot of geometric mean of anti-LHRH antibody titres obtained with different doses of DNA vaccine. The data interpreted statistically (Table 3-4) shows that there is no statistical significant
Fig. 24. Effect on testosterone levels of male rats with anti-LHRH immune response after administration of anti-LHRH(d1)DNA(VR1012) vaccine. The vaccine was given i.m. after pretreatment of muscle 24 hr earlier with 0.5% bupivacaine.

# doses (25µg, 50µg, 100µg and 200µg)
* i.m injection given on d=0, d=45, d=91, d=162
Anti LHRH antibody (OD 492 nm)

Testosterone levels ng/ml

Days post immunization

Animal no. 6 (Group 1)

Testosterone levels ng/ml

Days post immunization

Animal no. 6 (Group 1)

Testosterone levels ng/ml

Days post immunization

Animal no. 6 (Group 1)

Testosterone levels ng/ml

Days post immunization

Animal no. 6 (Group 1)
Anti LHRH antibody (OD 492 nm)

Testosterone levels ng/ml

Days post immunization

Anti LHRH antibody (OD 492 nm)

Testosterone levels ng/ml

Days post immunization

Anti LHRH antibody (OD 492 nm)

Testosterone levels ng/ml

Days post immunization

Anti LHRH antibody (OD 492 nm)

Testosterone levels ng/ml

Days post immunization
Fig. 25. Linear regression for fall in testosterone levels with increase in anti-LHRH antibody titres after immunization of male rats with different doses of anti-LHRH (d1) DNA vaccine.
Fig. 26. Geometric mean of anti-LHRH antibody titres obtained in male rats against different doses of anti-LHRH(d1)DNA(VR1012) vaccine. Each point is indicative of geometric mean of titres obtained in animals after administration of a particular dose.

* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=45, d=91, d=162
Table 3: Comparison of anti-LHRH antibody titres generated in male rats after immunization with different doses of anti-LHRH (d1) DNA (VR1012) vaccine.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares (S. S)</th>
<th>Degree of freedom (d.f)</th>
<th>Mean sum of squares (M.S.S)</th>
<th>F cal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between the sample</td>
<td>S.S.B = 0.009</td>
<td>K-1 = 3</td>
<td>S.S.B/ K-1 = 0.003</td>
<td>1.933</td>
</tr>
<tr>
<td>Within the sample</td>
<td>S.S.W = 0.239</td>
<td>N-K = 41</td>
<td>S.S.W/ N-K = 0.0058</td>
<td></td>
</tr>
</tbody>
</table>

The calculated value of F (F cal 1.93) is less than the tabulated value (F tab 2.84) at 5% level of significance. Hence there is no statistical significant difference in the mean anti-LHRH antibody titres generated by different doses of anti-LHRH (d1) DNA (VR1012) vaccine.

K = no. of samples = (4 different doses i.e., 25μg, 50μg, 100μg and 200μg)

N = total no. of observations (44) = mean anti-LHRH antibody titres taken at 11 different time points.
Table 4: Comparison of anti-LHRH antibody titres generated in male rats due to anti-LHRH (d1) DNA(VR1012) vaccine after each booster injection

<table>
<thead>
<tr>
<th>Dose DNA (µg)</th>
<th>Mean Titres</th>
<th>Arithmetic Mean</th>
<th>Std. Deviation</th>
<th>Variance</th>
<th>t-test 95% - 6°</th>
<th>Statistical significance</th>
</tr>
</thead>
</table>
|               | After 2
|               | in J (for 21 days) | 2
| in J | 2
| in J | No significant difference between titers after 2
|       | 25 | 0.129 | 0.140 | ± 0.027 | 1.06<9.28 | 2.32<2.447 | between titers after 2
|       | 50 | 0.122 |                  |           |               |                          | nd & 3
|       | 100 | 0.182 |                  |           |               |                          | rd injection |
|               | After 3
|               | Injection (for 45 days) | 2
| in J | 2
| in J | Significant difference between titers after 2
|       | 25 | 0.224 | 0.186 | ± 0.028 | 1.32<9.28 | 6.66<2.447 | between titers after 2
|       | 50 | 0.156 |                  |           |               |                          | nd & 4
|       | 100 | 0.189 |                  |           |               |                          | th injection |
|               | After 4
|               | Injection (for 41 days) | 3
| in J | 2
| in J | Significant difference between titers after 3
|       | 25 | 0.296 | 0.262 | ± 0.024 | 1.40<9.28 | 4.12<2.447 | between titers after 3
|       | 50 | 0.262 |                  |           |               |                          | rd & 4
|       | 100 | 0.247 |                  |           |               |                          | th injection |
|               | 200 | 0.243 |                  |           |               |                          |
difference at 5% level of significance (F cal. by ANOVA) between the antibody titres generated by different doses of DNA. While there is no statistical significant difference (t-test) between antibody titres generated after 2\textsuperscript{nd} and 3\textsuperscript{rd} injections there is significant difference between 2\textsuperscript{nd} and 4\textsuperscript{th} injections i.e, it was only the fourth injection that boosted the titres.

Different strategies were thus employed to enhance the immune response with the idea of having a quicker response and of simplifying the treatment.

4.3.1 DNA vaccine co-administered with alum

Immunization with anti-LHRH (d1) DNA (VR1012) vaccine alone in PBS required at least two injections for the antibodies to appear. The first injection did not cause the generation of antibodies. Experiments were carried out to see whether the DNA vaccine given along with alum improved in anyway its immunogenicity. Anti-LHRH (d1) DNA (VR1012) vaccine dose 100 μg + 25% of dose admixed with alhydrogel (1:1 admixture of DNA and alum was given i.m at multiple sites) and 200 μg SPLPS (SPLPS given in first injection only) was given i.m to male rats (n=3), 24 hrs after muscle pretreatment with 0.5% bupivacaine. Booster injections of the same dose were given on day 34, day 96 and day 150. Figure 27 shows the plot of geometric mean of anti-LHRH antibody titres obtained with DNA vaccine when co-administered along with alum. It was observed that inclusion of alum elicited early response i.e Ab titres emerged within one month of 1\textsuperscript{st} injection which was boosted by 2\textsuperscript{nd} injection after a month as against DNA vaccine when given without alum.
Fig. 27. Immune response obtained in male rats after i.m administration of anti-LHRH(d1)DNA(VR1012) vaccine given partially along with alum.

* n = 3
* Anti-LHRH (d1) DNA (VR1012) vaccine dose 100 µg + 25% of dose admixed with alhydrogel (1:1 admixture of DNA and alum was given i.m at multiple sites) and 200 µg SPLPS (SPLPS given in first injection only)
* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m. injection given on d=0, d=34, d=96 and d=150.
Fig. 27. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m administration of anti-LHRH(d1)DNA(VR1012) vaccine given partially along with alum. Data for individual animals are given as scatter, while the geometric mean is represented by -⁎- joined by a continuous line.

♦ n = 3
♦ Anti-LHRH (d1) DNA (VR1012) vaccine dose 100 μg + 25% of dose admixed with alhydrogel (1:1 admixture of DNA and alum was given i.m at multiple sites) and 200 μg SPLPS (SPLPS given in first injection only)
♦ site of DNA injection was pretreated with 0.5% bupivacaine
♦ i.m injection given on d=0, d=34, d=96 and d=150.
(where the titres appeared only after the 2nd injection. A third injection on day 96 increased the titres to 0.28 O.D units by day 126, however the titres were not sustained and declined to 0.14 O. D units by day 145, the decline in titres continued till another booster injection was given. A fourth injection on day 150 again boosted the titres, which reached peak levels of 0.24 O.D units by day 187. The antibody titres after the fourth injection could not be sustained and declined by day 210 to 0.1 O.D units. It was evident that while the presence of alum decreased the time taken for the onset of action, it had little effect on the duration of response i.e a fourth injection was desirable.

4.3.2 DNA vaccine administered simultaneously with alum adsorbed protein

In another series of experiments, immunization was carried out with both DNA and proteinic vaccine given simultaneously so as to determine whether such a co-administration would enhance the immune response. Anti-LHRH (d1) DNA (VR1012) vaccine dose 100 µg (1µg/2µl) was given i.m (100µl /TA muscle on both legs) to male rats (n=5), 24 hrs after muscle pretreatment with 0.5% bupivacaine. The animals were also given 100µg of proteinic vaccine LHRH (d1) adsorbed on 2% alhydrogel along with 200µg SPLPS (SPLPS given in first injection only) intramuscularly at multiple sites. Booster injections of the same dose of DNA vaccine and alum adsorbed proteinic vaccine were given on day 35, day 101 and day 145. Figure 28' shows the plot of geometric mean of anti-LHRH antibody titres obtained with DNA vaccine when co-administered along with proteinic vaccine adsorbed on alum. It was observed
Fig. 28. Immune response obtained in male rats after simultaneous i.m administration of anti-LHRH(dll)DNA(VR1012) vaccine (100µg) and proteinic vaccine (100µg), proteinic vaccine given as adsorbed on alhydrogel along with 200µg SPLPS (SPLPS given in first injection only).

* n = 5
* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=35, d=101, d=145
Fig. 28. Geometric mean of anti-LHRH antibody titres obtained in male rats after simultaneous i.m administration of anti-LHRH(d1)DNA(VR1012) vaccine (100μg) and proteinic vaccine (100μg), proteinic vaccine given as adsorbed on alhydrogel along with 200μg SPLPS (SPLPS given in first injection only). Data for individual animals are given as scatter, while the geometric mean is represented by -*-* joined by a continuous line.

* n = 5
* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=35, d=101, d=145
that co-administration of alum adsorbed protein along with DNA vaccine elicited early response i.e Ab titres emerged within one month of 1\textsuperscript{st} injection as against DNA vaccine when given alone (where the titres appeared only after the 2\textsuperscript{nd} injection). Antibody titres were 0.13 O.D units on day 20, after the second injection on day 35 the titres went beyond 0.15 O.D units and reached 0.20 O.D units by day 95 but showed a decline soon after by day 100. A third injection on day 101 did not increase the titres, which remained at 0.15 O.D units and then declined by day 145. A fourth injection given on day 145 increased the titres to 0.2 O.D units. However the titres were not sustained and declined by day 175 and reached 0.13 O.D units by day 190, the decline of the titres further continued and reached 0.07 O.D units by day 200.

4.3.3 DNA inoculations along with cytokine genes

Cytokines have been shown to modulate immune responses in DNA immunization studies. In this study the adjuvant effect of the cytokine genes was manifested by codelivery of DNA with granulocyte macrophage colony stimulating factor (GM-CSF) gene so as to improve vaccine efficacy. Anti-LHRH (d1) DNA (VR1012) vaccine (1\(\mu\)g/\(\mu\)l) was admixed with VR1012 (GM-CSF) DNA (1\(\mu\)g/\(\mu\)l) and 200 \(\mu\)l of this admixture was administered to male rats (n=6) i.m on both legs, 24 hours after pretreatment of the TA muscle with 0.5% bupivacaine (as per section 3.7.2). 25 \(\mu\)g of the DNA vaccine admixture was also mixed with alum (1:1) along with 200\(\mu\)g SPLPS (SPLPS given in first injection only) and administered i.m at multiple sites. Booster injections of the
same dose were given on day 30, day 62 and day 106. Figure 29 shows the plot of geometric mean of anti-LHRH antibody titres obtained after immunization with anti-LHRH (d1) DNA (VR1012) vaccine admixed with VR1012 (GM-CSF) DNA. Each rat generated anti-LHRH antibodies which were detectable after first injection and even though four injections were given, the booster doses were unable to sustain the titres above the bioefficacy levels so as to bring about a decline in testosterone levels to castration levels.

4.3.4 DNA immunization by cationic lipid mediated gene delivery method

The lipid DNA complex having anti-LHRH (d1) DNA (VR1012) concentration of 1μg /2μl was directly injected i.m to quadriceps of male rats (n=6) at a dose of 100μg DNA per rat. Figure 30 shows the plot of geometric mean of anti-LHRH antibody titres obtained with DNA vaccine given by cationic lipid mediated gene delivery method. Following the injection on day 0 there was a delay in generation of antibody response, the antibodies emerged after 60 days, reaching 0.13 O.D units by day 75. The titres decreased to 0.12 O.D units by day 109 and finally declined by day 124. Even though there was a delay in the onset of action, all animals turned sero-positive but the titres did not go beyond 0.15 O.D units.
Fig. 29. Immune response obtained in male rats after i.m immunization with 100μg of anti-LHRH (d1)DNA (VR1012) vaccine admixed with 100μg of VR1012 (GM-CSF) DNA, 25 μg of the DNA vaccine admixture was also mixed with alum (1:1) along with 200μg SPLPS (SPLPS given in first injection only) and administered i.m at multiple sites.

* n=6  
* site of DNA injection was pretreated with 0.5% bupivacaine  
* i.m injection given on d=0, d=30, d=62, d=106.
Fig. 29'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m immunization with 100μg of anti-LHRH (d1)DNA (VR1012) vaccine admixed with 100μg of VR1012 (GM-CSF) DNA, 25 μg of the DNA vaccine admixture was also mixed with alum (1:1) along with 200μg SPLPS (SPLPS given in first injection only) and administered i.m at multiple sites. Data for individual animals are given as scatter, while the geometric mean is represented by -⁎- joined by a continuous line.

♦ 1=6
♦ site of DNA injection was pretreated with 0.5% bupivacaine
♦ i.m injection given on d=0, d=30, d=62, d=106.
Fig. 30. Immune response obtained in male rats after i.m administration of 100μg of anti-LHRH(d1)DNA (VR1012) vaccine given as lipid DNA complex.

* n=6
* i.m injection
Fig. 30'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m immunization with 100μg of anti-LHRH(d1)DNA (VR1012) vaccine given as lipid DNA complex. Data for individual animals is given as scatter, while the geometric mean is represented by -∗- joined by a continuous line.

♦ 11 = i.m injection

* n=6
* i.m injection
4.3.5 Immunization with DNA vaccine entrapped in microspheres

In another experiment, animals (n=3) were immunized with anti-LHRH (d1) DNA (VR1012) vaccine entrapped in microspheres. 50 mg of P(DL) Lactide microspheres containing 25µg of DNA was suspended in 400µl PBS pH 7.4 (containing 50µl of alum). 200 µl of this mixture was directly injected i.m to male rats on both legs. Figure 31’ shows the plot of geometric mean of anti-LHRH antibody titres obtained after immunization with DNA vaccine entrapped in microspheres. Following the injection on day 0, the antibodies emerged on day 38, reaching 0.12 O.D units by day 62 and then declined soon after by day 75 to 0.09 O.D units. There was no increase in titres thereafter. Even though all animals turned sero-positive for anti-LHRH antibody, the titres did not go beyond the bio-efficacy levels of 0.15 O.D units.

4.3.6 DNA immunization with different expression vectors

While the DNA immunizations with VR1012-LHRH(d1) showed 100% positivity i.e all animals turned sero-positive for anti-LHRH antibodies, the titres were not high enough to cause prostatic atrophy hence immunization was carried out with different expression vectors, VR1020 (DJ) and VR1020 (KJ). Two groups of animals (n=4) were taken and immunized with either anti-LHRH (d1) DNA (VR1020-DJ) vaccine or anti-LHRH (d1) DNA (VR1020-KJ) vaccine, dose 100 µg (1µg/2µl) + 25% of dose admixed with alhydrogel (1:1 admixture of DNA and alum was given i.m at multiple sites) and 200 µg SPLPS (SPLPS given in first injection only) was given i.m to male rats, 24 hrs after muscle pretreatment with 0.5% bupivacaine (as per section 3.7.2).
Fig. 31. Immune response obtained in male rats after i.m administration of 25μg of anti-LHRH (d1) DNA (VR1012) vaccine, given entrapped in P (DL) LA microspheres.

* n=3
* i.m injection
Fig. 31'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m administration of 25μg of anti-LHRH(d1) DNA (VR1012) vaccine, given entrapped in P(DL)LA microspheres. Data for individual animals is given as scatter, while the geometric mean is represented by -*-, joined by a continuous line.

* n=3
# i.m injection
Booster injections of the same dose were given on day 33, day 64 and day 99.

Figure 32' shows the plot of geometric mean of anti-LHRH antibody titres obtained with anti-LHRH (d1) DNA (VR1020-DJ) vaccine. Antibodies were detectable on day 15 after the first injection. On being given a second injection on day 33 the titres reached 0.15 O.D units by day 55. The titres reached 0.17 O.D units by day 84 after being boosted on day 64 and then declined below 0.15 O.D units by day 96. On day 99 the animals were given a fourth injection which gradually increased the titres to 0.2 O.D units by day 134. However the titres declined soon after by day 148. Figure 33' shows the plot of geometric mean of anti-LHRH antibody titres obtained with anti-LHRH (d1) DNA (VR1020-KJ) vaccine. Antibodies were detectable on day 15 after the first injection. After the second injection on day 33 the titres reached 0.15 O.D units by day 55. The titres reached 0.19 O.D units by day 84 after being boosted on day 64 and then declined to 0.11 O.D units by day 96. On day 99 the animals were given a fourth injection which increased the titres to 0.18 O.D units by day 108 and by day 134 the titres increased to 0.2 O.D units. However the titres declined below 0.1 O.D units by day 148. While both the DNA vaccines showed 100% positivity i.e all rats generated anti-LHRH antibodies which were detectable after first injection, the booster doses were unable to sustain the titres above the bio-efficacy levels so as to bring about a decline in testosterone levels to castration levels.
Fig. 32. Immune response obtained in male rats after i.m administration of anti-LHRH(d1)DNA(VR1020-DJ) vaccine given partially along with alum.

* n = 4.
* Anti-LHRH (d1) DNA (VR1012) vaccine dose 100 μg + 25% of dose admixed with alhydrogel (1:1 admixture of DNA and alum was given i.m at multiple sites) and 200 μg SPLPS (SPLPS given in first injection only)
* Site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=33, d=64 and d=99.
Fig. 32'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m immunization with anti-LHRH (d1)DNA (VR1020-DJ) vaccine given partially along with alum. Data for individual animals is given as scatter, while the geometric mean is represented by -**- joined by a continuous line.

* n = 4
* Anti-LHRH (d1) DNA (VR1012) vaccine dose 100 µg + 25% of dose admixed with alhydrogel (1:1 admixture of DNA and alum was given i.m at multiple sites) and 200 µg SPLPS (SPLPS given in first injection only)
* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=33, d=64 and d=99.
Fig. 33. Immune response obtained in male rats after i.m administration of anti-LHRH (d1)DNA (VR1020-KJ) vaccine given partially along with alum.

* n = 4
* Anti-LHRH (d1) DNA (VR1012) vaccine dose 100 µg + 25% of dose admixed with alhydrogel (1:1 admixture of DNA and alum was given i.m at multiple sites) and 200 µg SPLPS (SPLPS given in first injection only)
* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=33, d=64 and d=99.
Fig. 33’. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m immunization with anti-LHRH (d1)DNA (VR1020-KJ) vaccine given partially along with alum. Data for individual animals is given as scatter, while the geometric mean is represented by a continuous line.

* n = 4
* Anti-LHRH (d1) DNA (VR1012) vaccine dose 100 µg + 25% of dose admixed with alhydrogel (1:1 admixture of DNA and alum was given i.m at multiple sites) and 200 µg SPLPS (SPLPS given in first injection only)
* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=33, d=64 and d=99.
In another set of experiment, anti-LHRH (d1) DNA (VR1020-DJ) vaccine (1μg/μl) was admixed with VR1012 (GM-CSF) DNA (1μg/μl) and 200 μl of this admixture was administered to male rats (n=3) i.m on both legs, 24 hours after pretreatment of the TA muscle with 0.5% bupivacaine (as per section 3.7.2). 25 μg of the DNA vaccine admixture was also mixed with alum (1:1) along with 200μg SPLPS (SPLPS given in first injection only) and administered i.m at multiple sites. Booster injections of the same dose were given on day 40, day 84 and day 126. Figure 34 shows the plot of geometric mean of anti-LHRH antibody titres obtained after immunization with anti-LHRH (d1) DNA (VR1020-DJ) vaccine admixed with VR1012 (GM-CSF) DNA. Antibodies were detectable on day 17 after the first injection. After the second injection on day 40 the titres reached 0.2 O.D units by day 69 and the started declining by day 81. The third injection given on day 84 boosted the titres which again reached 0.2 O.D units followed by a gradual decline to 0.18 O.D units by day 119. The fourth injection given on day 126 boosted the titres which reached 0.2 O.D units by day 155 and were sustained till a short period till day 167. The titres declined to 0.1 O.D units by day 175 and the decline continued further from day 190 onwards. It was observed that on immunization with anti-LHRH (d1) DNA (VR1020-DJ) vaccine and VR1012 (GM-CSF) DNA, all animals turned sero-positive and the titres increased on giving booster injections but declined quickly till further injection was given. The fourth injection was able to sustain the titres for some time but they also declined soon after.
Fig. 34. Immune response obtained in male rats after i.m immunization with 100μg of anti-LHRH (d1)DNA (VR1020-DJ) vaccine admixed with 100μg of VR1012 (GM-CSF) DNA, 25 μg of the DNA vaccine admixture was also mixed with alum (1:1) along with 200μg SPLPS (SPLPS given in first injection only) and administered i.m at multiple sites.

* n=3
* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=40, d=84, d=126.
Fig. 34'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m immunization with 100µg of anti-LHRH (d1)DNA (VR1020-DJ) vaccine admixed with 100µg of VR1012 (GM-CSF) DNA, 25 µg of the DNA vaccine admixture was also mixed with alum (1:1) along with 200µg SPLPS (SPLPS given in first injection only) and administered i.m at multiple sites. Data for individual animals are given as scatter, while the geometric mean is represented by -•- joined by a continuous line.

* n=3
* site of DNA injection was pretreated with 0.5% bupivacaine
* i.m injection given on d=0, d=40, d=84, d=126.
4.3.7 DNA immunizations using *Immuvac*

100μg of anti-LHRH (d1) DNA (VR1020-DJ) vaccine (1μg/2μl) was mixed with 0.1 ml (0.5x 10⁸ heat killed Mw) suspension of *Immuvac* and administered to male rats either intramuscularly on both legs (150μl/site) or given by intradermal route at multiple sites (100 μl/site). The animals were boosted thrice with 100μg of anti-LHRH (d1) DNA (VR1020-DJ) vaccine and 0.5x 10⁹ heat killed Mw at the indicated time intervals. Figure 35’ shows the plot of geometric mean of anti-LHRH antibody titres obtained with anti-LHRH (d1) DNA (VR1020-DJ) vaccine and *Immuvac* given intramuscularly to male rats. Booster injections of the same dose were given on day 38, day 69 and day 97. Co-administration of Immuvac with DNA vaccine resulted in a quicker response i.e antibodies emerged in the first month itself, which reached 0.13 O.D units by day 32. A second injection was given on day 38 followed by a third injection on day 69, which increased the titres to 0.15 O.D units by day 75. The titres were not sustained and declined by d=93. A fourth injection was given on day 97 which finally boosted the titres, the titres reached 0.19 O.D units by day 105 and remained on 0.19 O.D units till day 115. The titres decreased by day 134 to 0.12 O.D units and then the decline continued thereafter.

Figure 36’ shows the plot of geometric mean of anti-LHRH antibody titres obtained on intradermal administration of anti-LHRH (d1) DNA (VR1020-DJ) vaccine admixed with *Immuvac*. Even though four injection of the same dose
Fig. 35. Immune response obtained in male rats after i.m immunization with anti-LHRH (d1)DNA (VR1020-DJ) vaccine (100μg) mixed with adjuvant Immuvac (0.5 x 10⁶ heat killed M. w).

★ n=2
★ i.m injection given on d=0, d=38, d=69, d=97.
Fig. 35'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.m immunization with anti-LHRH (d1)DNA (VR1020-DJ) vaccine (100μg) mixed with adjuvant Immuvac (0.5 x 10⁹ heat killed M.w). Data for individual animals are given as scatter, while the geometric mean is represented by - Asterisk- joined by a continuous line.

* n=2
* i.m injection given on d=0, d=38, d=69, d=97.
Fig. 36. Immune response obtained in male rats after i.d immunization with anti-LHRH (d1)DNA (VR1020-DJ) vaccine (100μg) mixed with adjuvant *Immuvac* (0.5 x 10^9 heat killed M.w).

* n=2
* i.d injection given on d=0, d=38, d=69, d=97.
Fig. 36'. Geometric mean of anti-LHRH antibody titres obtained in male rats after i.d immunization with anti-LHRH (dl)DNA (VR1020-DJ) vaccine (100μg) mixed with adjuvant Immuvac (0.5 x 10⁹ heat killed M.w). Data for individual animals are given as scatter, while the geometric mean is represented by -•- joined by a continuous line.

* n=2
* i.d injection given on d=0, d=38, d=69, d=97.
were given on day 0, day 38, day 69 and day 97 the titres did not go beyond 0.10 D units.

4.4 Histology of Reproductive Organs

Though there was no remarkable difference of body weights between controls and animals immunized with Freund's adjuvant during the treatment period, however at necropsy significant reductions in the size of reproductive organs were observed compared to those of the control group. While the control group which received the same adjuvants had normal morphology, the experimental group animals showed regressive changes in the histology of reproductive organs. In Testis (Fig. 37) tunica albuginea was thickened, seminiferous tubules were reduced in diameter to various degrees, with arrest of spermatogenesis and tubular lumen was devoid of spermatocytes. In general regressive changes in testicular histology were observed. The prostate showed the most severe reaction (Fig. 38), there was glandular atrophy with acute necrosis and denudation of the epithelium. Prostate acini were regressed. The epithelium of glandular terminals was transformed to a necrotic cluster of cells. The lumina of glandular terminals and collecting tubules were collapsed and were largely free of secretory products or filled with remnants of former secretion. Lymphoid cellular infiltration was observed with increase in mast cells. The normal interstitial tissue was transformed to a collagenous fibrotic network. Atrophic transformations were also found in the seminal vesicles and epididymis, accompanied by complete arrest of functional activities and interstitial fibrosis.
Fig. 37. Photomicrograph of control rat testis (A,C) showing cross-section of seminiferous tubules with spermatogenic cells at various stages of development. A, × 20; C, × 40. (B,D) Immunized animals have diminished size of seminiferous tubules and thickened tunica albuginea. B, × 20. Arrest of spermatogenesis indicated by absence of spermatozoa (D, × 40).
In these studies, hyper-immunization was carried out with the presently developed recombinant multimer vaccines employing a strong adjuvant so as to evoke maximal potential toxicity of anti-LHRH antibodies induced by these vaccines. All animals were examined for gross and microscopic pathology. Necropsy did not show any evidence of edema, abnormal organ size or appearance in non target organs.

4.5 Immunoreactivity of Antibodies with Normal Human Tissues

In order to gauge potential immunopathology of the circulating antibodies, cross-reactivity of pooled sera from animals whose prostate was significantly atrophied was investigated with normal human tissues. The antibodies did not react with normal human tissues (fixed, paraffin embedded) such as thyroid, kidney, pancreas, heart, liver, lungs, uterus and skin, but showed positive reactivity with hypothalamus and Leydig cells of the testis as determined by immunohistochemistry (Table 5).
Table 5: Immunoreactivity of Anti-LHRH antibody with fixed Human Tissues as determined by Immunohistochemistry

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid</td>
<td>-</td>
</tr>
<tr>
<td>Kidney (left)</td>
<td>-</td>
</tr>
<tr>
<td>Kidney (right)</td>
<td>-</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
</tr>
<tr>
<td>Lungs (left)</td>
<td>-</td>
</tr>
<tr>
<td>Lungs (right)</td>
<td>-</td>
</tr>
<tr>
<td>Uterus</td>
<td>-</td>
</tr>
<tr>
<td>Skin</td>
<td>-</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>+</td>
</tr>
<tr>
<td>Testis</td>
<td>+*</td>
</tr>
</tbody>
</table>

* Leydig cells were faintly positive.