CHAPTER 6

Summary

&

Conclusion
6.0 SUMMARY AND CONCLUSIONS

The objective of this work was to develop a recombinant low cost, easy to manufacture vaccine generating antibodies against LHRH which may be bioeffective in causing a decline of testosterone accompanied by atrophy of the prostate.

1) Genes pertaining to two designs of multimer LHRH units interspersed with 4 or 5 T non B peptides were sub-cloned into prokaryotic expression vectors under the control of strong promoters -T7 for design 1 (d1) and λP_L for design 2 (d2).

2) 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 expressed protein, which was mainly localized in the cytosol as insoluble inclusion bodies was purified by Ni-NTA chromatography.

3) The plasmid ICGEB-LHRH (d2) was used to express the multimeric LHRH (d2) as recombinant protein in protease deficient E.coli BL 21. The recombinant protein was abundantly expressed and it aggregated as inclusion bodies.

4) The inclusion bodies were isolated from the induced cells by mechanical disruption caused by sonication. Washing of the inclusion bodies with 1% deoxycholate in Tris EDTA buffer resulted in partial purification of the inclusion body protein. 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.

5) Instead of high concentrations of chaotropic agents, r-LHRH- d2 was solubilized in 50 mM citrate buffer at pH 3 containing 2 M urea. The protein was refolded by five fold dilution (pulsatile) with cold 10 mM citrate buffer at pH 6 in presence of 0.3 M L arginine.

6) Purification of r-LHRH-d2 was carried out by successive passages on CM Sepharose column at pH 6.0 which retained extraneous proteins and at pH 4.8 at which r- LHRH-d2 bound to the resin. The elution was carried out by using linear salt gradient (0.1-1M NaCl). The overall yield of the purified r- LHRH- d2 was 40% of the initial inclusion body proteins.

7) Characterization of the protein by SDS-PAGE, silver staining and Western Blot analysis showed the presence of a single band of purified protein, of the expected size of 16 kD, reactive with polyvalent anti LHRH antibodies.

8) 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-K-I-A-K-M-E-K-A-S-S",

9) The homogeneity and purity of the protein was confirmed by a single homogenous peak on analytical HPLC, this peak centered at 29.51 min was collected and Electron spray ionization –Mass spectroscopy was performed.
The protein mass was found to be 16604.7 which is in agreement with the theoretically calculated mass of 16.6 kDa.

10) The CD spectrum of the refolded r-LHRH- d2 showed that the multimer has considerable β sheet structure like the monomeric LHRH protein.

11) The purified and refolded r-LHRH- d2 was immunoreactive with monoclonal antibody recognizing the native molecules using goat anti-mouse-IgG HRPO as secondary antibody and also showed positive immunoreactivity with 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 second antibody.

12) Rat which serves as a homologous model for safety and efficacy studies was used for immunization experiments.

13) Recombinant LHRH protein of design1 (d1) or design (d2) was emulsified with Complete Freund’s Adjuvant (CFA) in 1:1 ratio until a stable water in oil emulsion was obtained and injected to rats subcutaneously (s.c) at multiple sites (100 μl/site). The first dose per animal was of 100 μg of the recombinant protein, two booster injections were given with 50 μg of the recombinant protein emulsified with Freund’s Incomplete Adjuvant (IFA) at 15 days interval.
14) Immunizations using Freund’s adjuvant induced anti-LHRH antibodies with both designs of the vaccine. Even though both designs of the recombinant vaccines were competent to induce bioeffective response, the design 1 (d1) LHRH vaccine generated an early response.

15) With the build-up of antibody titres in immunized animals, testosterone levels declined, which resulted in concomitant reduction of the testis size.

16) At necropsy, significant reductions in the size of reproductive organs dependant on androgen support was observed compared to those of the control group. The decline in prostate weight was highly significant.

17) Serial necropsies of animals at various stages of immunization revealed that the reduction in the prostate weight begins with the rise in antibody titres. Complete atrophy of the prostate is attained at titres of 0.2 O.D units and above.

18) Regressive changes in testicular histology were observed. In Testis tunica albuginea was thickened, seminiferous tubules were reduced in diameter to various degrees, with arrest of spermatogenesis and tubular lumen was devoid of spermatocytes. The prostate showed the most severe reaction with signs of glandular atrophy and acute necrosis, the lumina of glandular terminals and collecting tubules were collapsed and were largely free of secretory products.
19) Immunizations were also carried out with both proteinic and DNA vaccines either alone or in combination with human compatible adjuvants and immunopotentiators so as to achieve high antibody levels causing atrophy of the prostate. While such combinations were able to generate anti-LHRH antibody titres, the titres were not sustained enough so as to cause decline in testosterone to castration levels.

20) Immunization did not cause any effect on the food intake of the animals, the weight gain curves were similar in the control and immunized animals. Gross morphology did not indicate edema, abnormal organ size or appearance.

21) Pooled sera from rats undergoing drastic atrophy of the prostate was evaluated for its possible cross-reaction with human tissues. No cross reaction of the anti-LHRH antibodies, was seen with normal human tissues such as thyroid, kidney, pancreas, heart, liver, lungs, uterus and skin.

Thus the present studies amply demonstrate the proof of concept that the presently developed recombinant multimeric anti-LHRH vaccines can be used effectively to induce antibodies to inactivate LHRH, thereby reducing testosterone hormone levels to castration levels and causing atrophy of the prostate. However the application of these vaccines in humans would require an acceptable adjuvant.