CHAPTER 5

Discussion
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Immunization against LHRH by a synthetic vaccine (Talwar et al, 1992) atrophies prostate of rodents (Jayashankar et al, 1989; Rovan et al, 1992) and primates (Giri et al 1991). The vaccine inhibited the growth of androgen-sensitive rat Dunning tumours and also to some extent the androgen independent tumours, implanted in rats (Fuerst et al 1997). Given that LHRH is largely conserved in its molecular structure in mammals, rats and monkeys serve as homologous models for humans. Indeed the vaccine that atrophied the prostate in rats and monkeys was effective in humans and was safe and clinically beneficial in patients of prostate carcinoma (Talwar et al, 1999). At 400 μg each of the three doses of the vaccine given at monthly intervals, 5 out of 6 prostate carcinoma patients investigated at the All India Institute of Medical Sciences, New Delhi, were rendered either clinically stable or had improvement in symptoms. Three of these patients had reduction in prostate size and hardness and 4 had reduced levels of serum acid phosphatase. At the Dept. of Urology, General Hospital, Salzburg, Austria, two of the four D-2 stage prostate carcinoma patients showed decline in testosterone to castration levels at antibody titres above 300 pg/ml. This was accompanied by a concomitant decline of prostate specific antigen (PSA) from 600 ng/ml to less than 10 ng/ml. At the Post Graduate Institute of Medical Education and Research, Chandigarh (India), sequential nephrostograms revealed the shrinkage of prostate tissue mass accompanied by clinical improvement of an advanced stage carcinoma patient. A drawback of this vaccine was the
relatively high cost of synthesis besides loss during conjugation. There could also be difficulties of exact reproducibility during manufacture on a large scale. In this semi-synthetic vaccine, glycine at position 6 was replaced by D lysine and the linkage was made through a spacer to diptheria toxoid (DT) or tetanus toxoid (TT) as carriers (Talwar et al, 1992). While DT /TT as carriers have merit, their repeated use causes carrier induced immunosuppression in a percentage of recipients. Herzenberg and Tokuhisa (1982) were the first to report this phenomenon in mice which was confirmed for both LHRH and hCG conjugated vaccines in animals and during clinical trials (Gaur et al, 1990; Sad et al, 1991). The central objective of the present work was to develop an immunogen which may be free from the trait of the carrier induced immunosuppression induced by repeated immunizations with vaccines conjugated to tetanus toxoid (TT) or diptheria toxoid (DT) as carriers. We substituted these large carriers by small peptides which recognize T helper cells but are devoid of direct reactions with B cells. A number of promiscuous epitopes have been identified (Panina Bordignon et al, 1989; Sinigaglia et al, 1988; Vordermeir et al, 1991; Nicholas et al, 1989; Partidos et al, 1991) from many pathogens infecting humans and the reactivity of these peptides to human T cells of subjects with diverse MHC spectrum has been noted. The task was to determine whether replacement of TT or DT as carriers by such T cell helper peptides would be adequate to make a small decapeptide hormone (immunologically tolerized in both rats and humans) immunogenic to evoke antibody response against LHRH. We designed multimer anti-LHRH
vaccines with four to five oligonucleotide sequences coding for promiscuous T cell epitopes identified from circumsporozoite protein (CSP) of Plasmodium falciparum, Mycobacterium tuberculosis (MTB), tetanus toxin (TT), Respiratory synctial virus (RSV) and Measles virus. The results reported in this paper clearly demonstrate the ability of the chosen peptides to mobilise helper function for generation of antibodies to LHRH. An expected additional advantage of employing four to five T different helper peptides is to enable communication with MHC of subjects with diverse genetic background. Also the putative vaccine was aimed to be made by DNA recombinant techniques to assure consistency of the product and amenability to large scale manufacture at low cost.

Expression of recombinant proteins at high levels in E.coli is often associated with the formation of insoluble aggregates as inclusion bodies (Kane et al, 1988; Mitraki et al, 1989). Significant features of protein aggregates in inclusion bodies are the existence of native-like secondary structure of the expressed protein and reversible specific aggregation of the expressed protein (Przybycien et al,1994; Speed et al, 1996; Carrio et al,2000; Carrio et al, 2001). The formation of inclusion bodies in a way facilitates easy isolation and recovery of the expressed protein. One of the major problems associated with recovery of bioactive proteins from inclusion bodies is its aggregation during folding. In general, proteins expressed as inclusion bodies are solubilized by use of high concentrations of denaturants such as urea or guanidine hydrochloride, alongwith a reducing agent such as DTT or
β-mercaptoethanol. The solubilized proteins are then refolded by slow removal of the denaturant in the presence of an oxidizing agent. The solubilization of protein from the inclusion body by high concentration of chaotropic reagents results in the loss of its secondary structure, leading to random coil formation of the protein structure and exposure of hydrophobic surfaces (Dill et al., 1991). Loss of secondary structure during solubilization and the interactions amongst the denatured protein molecules leading to their aggregation are considered to be the main reasons for poor recovery of bioactive proteins from the inclusion bodies (Fischer et al., 1993; London et al., 1974; Rudolph et al., 1997). Since the protein in inclusion body already exists at an intermediate stage of folding pathway and thereby has considerable amount of secondary structure, it was assumed that if the proteins from the inclusion bodies could be solubilized without disturbing its native like secondary structure the overall yield of bioactive protein from inclusion body would be much higher (Patra et al., 2000a; Khan et al., 1998; Patra et al., 2000b).

Recombinant LHRH-d2 multimer from the cytoplasm in the form of inclusion body was purified with extensive washing in deoxycholate and 100 mM Tris-HCl at pH 8. Detergents are used most often for the extraction and solubilization of membrane proteins which otherwise are insoluble in aqueous solutions (Patra et al., 2000a). Sodium deoxycholate (1% w/v) as an anionic detergent added in extraction buffer facilitates the solubilization of membrane proteins. Purity and homogeneity of the r-LHRH-d2 inclusion bodies were consistent with the composition of the inclusion body proteins reported for β-
lactamase and vitreoscilla hemoglobin (Valax et al, 1993; Hart et al, 1990). As presence of contaminating proteins sometimes interfere with the optimum refolding of the solubilized protein, purified inclusion bodies with very little contaminants could be used for solubilization and refolding without multiple purification steps thus improving the overall yield. The pi of the r-LHRH-d2 multimer is 8.29. Maximum solubilization of r-LHRH-d2 inclusion bodies was obtained at pH 3 and 12. High alkaline pH may result in de-amidation of recombinant protein, thus solubilization at lower pH (pH at 3) was preferred. Solubilization of r-LHRH-d2 inclusion bodies at pH 3 in presence of 2 M urea was comparable to that achieved with 8 M urea solution at pH 8.5 and pH 6.0 and was thus employed for subsequent recovery of bioactive protein. Ionic environment at pH 3 not only helped in dissociating the inclusion body aggregates but also precipitated many E.coli host cell proteins as most of them have a pi around 3-4 (Andrews et al, 1994). Solubilization of the inclusion body proteins in acidic pH in presence of 2 M urea does not unfold the proteins into random coil conformation. This is further supported from the fluorescence spectrum of the protein samples at different pH. Such mild solubilization with help of pH, mild detergents have been reported to improve the yield of many recombinant proteins expressed as inclusion bodies in E.coli (Patra et al, 2000a; Khan et al, 1998; Puri et al, 1992; Burgess et al, 1996).

Refolding of inclusion body protein is often hindered by protein aggregation due to suboptimal renaturation process during the removal of salt and
denaturants. To avoid aggregation, protein refolding is carried out at low concentrations which reduce the propensity of intermolecular interaction. However, such process results in use of high volumes of buffer during refolding which adds to the cost of purification. Thus there is need to refold proteins at high concentration which will result in high throughput recovery of bioactive protein from the inclusion bodies. To avoid aggregation during refolding many additives and new methods have been used (De Bernardez Clarke et al, 1999). In most of the cases, these additives reduce the interaction between the partially folded protein molecules and thereby promote unimolecular protein folding. Among the additives, L-arginine has been most extensively used as an additive to reduce protein aggregation during refolding. Its structural similarity to urea is probably the reason by which it helps in reducing the protein aggregation, like urea it acts as a stabilizer at low concentration and thus helps in improved protein refolding yield (Arora and Khanna, 1996; Rudolph and Lilie, 1996). Use of L-arginine helped in reducing the protein aggregation during refolding of r-LHRH-d2 and increase in yield was observed with addition of L-arginine compared to renaturation in its absence. Solubilized r-LHRH-d2 was purified and refolded using ion exchange and gel filtration chromatography. The overall recovery of r-LHRH-d2 from the inclusion bodies was ~ 40% in comparison to 20-25% achieved in solubilizing the inclusion bodies in 8 M urea. Use of two ion exchange steps at different pH helped in maximum recovery of r-LHRH-d2 from the inclusion bodies.
Recombinant LHRH-d2 multimer was characterized for its purity, molecular mass and immunoreactivity. The purified and refolded r-LHRH-d2 multimer has β sheet types conformation and reacted with monoclonal antibody recognizing the native molecules. The CD spectra of the r-LHRH-d2 shows the presence of typical β sheets structure similar to the that of native LHRH molecules (Venamivo et al., 1996). This suggests that, in spite of being expressed as multimer, the recombinant LHRH-d2 has conformation similar to the native LHRH molecule. This suggested that with the use of mild solubilization procedures, immunoreactive proteins having native like epitopes can be produced using recombinant methods. This is simpler and easier than the normal chemical conjugation method for the large scale production of conjugated proteins with B or T cell epitopes. This protein having interspersed T cell determinants can thus be used effectively for immunization where bio-effective anti-LHRH antibody response is required.

Although the purified and refolded proteins were checked for their immunoreactivity with monoclonal antibody against LHRH, this by itself could be no guarantee that they will act as appropriate antigens to induce bioeffective response in animals. The work reported clearly indicates the competence of these recombinant proteins to cause a decline of testosterone to castration levels and to concomitantly cause atrophy of the prostate. With the build up of antibody titres, a decline in testosterone starts taking place. Serial necropsies of animals at various stages of immunization also revealed that though the reduction in the prostate weight begins with the rise in
antibody titres, it becomes prominent only at titres above 0.15 O.D units. Complete atrophy of the prostate is attained at titres of 0.2 O.D units and above, with glandular terminals showing no signs of secretory activity.

Immunization did not cause any affect on the food intake of the animals, the weight gain curves were similar in the control and the immunized animals. Gross morphology did not indicate edema, abnormal organ size or appearance. No gross morphological changes were noted in non target organs. Testis were however manifestly shrunk as would be expected from decline of testosterone. These observations are consistent with previous work carried out at New Delhi, India and at Salzburg, Austria (Jayashankar et al, 1989; Rovan et al, 1992). The testis revert to normal size in course of time with disappearance of anti-LHRH antibodies and the arrest of spermatogenesis occurring during immunization phase is reversed on decline of antibodies. The prostate weight recovers to an extent but in almost all experiments with the LHRH vaccines, the prostate size remains somewhat smaller than the original.

Though LHRH (now termed LHRH -I) was initially considered to be the main hormone made primarily by the hypothalamus from where it traveled through the portal circulation to pituitary, stimulating the release of FSH and LH. In recent years three other isoforms of LHRH (now represented by LHRH- II, III, IV) have been found to exist in various organs of higher vertebrates including humans (Sherwood et al, 1993; Powell et al, 1994; White et al, 1998). The
decapeptide in the multimer vaccines employed by us is the LHRH-I. These isoforms of LHRH have been detected not only in hypothalamus but also in organs such as prostate, kidney, bone marrow, brain, mammalian reproductive organs: testis, ovary, mammary gland, placenta and uterus (Cheiffi et al, 1991; Cheng et al, 2000; Kakkar et al, 1994; Bahk et al, 1995). Data on their preponderance and location is not adequate to draw conclusions on their role. We considered it appropriate to test the immunoreactivity of the serum, pooled from animals carrying high enough titres of anti-LHRH antibodies to cause atrophy of the prostate, with various normal human tissues. These investigations show that apart from the expected positive reactivity with hypothalamus, Leydig cells of testis also showed faint positivity. However other endocrine and somatic organs showed no reaction by immunohistochemistry (Table 5). No perceptive behaviour changes or undesired pathology was noted in rats immunized with these recombinant vaccines, which were effective in causing decline of testosterone to castration levels and bring about marked atrophy of the prostate.