5.1 Peptide vaccines: promises and pitfalls

In today's world, vaccines play an important role in keeping many of the debilitating diseases of man and animals at bay. However, we still have no vaccine for several infectious diseases and newer diseases are ever emerging as pathogens evolve in changing ecological circumstances. Much work is, therefore, being carried out on new methods to develop potential vaccines and utility of these methods is being investigated using different disease models. Of the new methods being evaluated for vaccine development, peptide based vaccination has shown a lot of promise and in the process of understanding its utility a number of limitations in this methodology have been realized. Peptide vaccines are well defined and safe as there is no infectious agent, killed or live, involved and they are chemically and biologically stable. A chosen amino acid sequence can be included or excluded in the formulation and undesirable sequences can be deleted. A major disadvantage of peptide based immunization procedure is the weak immunogenicity of peptides, and not all the facets of immune response are induced. Hence, to raise an effective immune response, peptides must be chemically conjugated to large carrier proteins. However, conjugation of peptides to the carrier is not a controlled process. Besides, peptide carrier-conjugate may require an adjuvant. Thus alternate systems for peptide presentation need to be investigated. In the present work, potential of a self-polymerizing coat protein (CP) from Johnson Grass Mosaic virus (JGMV) has been evaluated for peptide presentation using Japanese Encephalitis virus (JEV) as a model pathogen.

JGMV, causing mosaicism in monocotyledonous plants, belongs to the potyvirideae family of plant viruses. The members of this family have a flexuous, rod shaped structure, 680-900 nm long and about 11 nm wide, containing single stranded RNA as the genome. The genome encodes single large precursor polyprotein which undergoes proteolytic cleavage to yield at least eight proteins. Of these, only two proteins - VPg, which is attached at the 5'-end of the genome, and CP are present in mature virions. In vitro studies have shown that purified virion particles of potato virus Y, a member of the potyviridae family of viruses, can be dissociated into monomers by treatment with high salt concentration (0.5 M NaCl) or at a pH below 6 or above 9. The monomers can be re-associated into long flexuous rods in the absence of the viral RNA (McDonald et al., 1976). It was proposed that seven to eight monomers form a ring-like structure and several monomers stack on this ring to form a filamentous structure either in the presence or the absence of the genomic RNA (McDonald et al., 1977).
Investigations have revealed that the N- and the C-termini of potyvirus CP are exposed on the surface of the virus particle (Allison et al., 1985; Shukla et al., 1988). Mild treatment of potyvirus with trypsin or with lysyl endopeptidase removes both the N- and the C-termini without disrupting the core structure of the particle suggesting that these regions are surface exposed and are not required for particle integrity. Furthermore, it was found that particles treated with trypsin or lysyl endopeptidase could be dissociated and reassembled into potyvirus-like particles in vitro (Jagadish et al., 1991) suggesting that central core region of CP is sufficient for the assembly of virus-like particles (VLPs). The JGMV CP has been shown to express in the bacterial cells and form VLPs. The protein is thus able to take the same conformation as in the virions produced in its natural host, enabling it to form the VLPs in the absence of the viral genome.

The non-requirement of the C- and the N-termini of JGMV CP in folding and their availability on the surface of the protein that polymerizes to form VLPs led to the concept of using these termini to fuse a foreign peptide sequence by genetic engineering means and produce a chimeric protein. In this direction, an octapeptide E71 from Plasmodium falciparum, the decapeptide LHRH and a 26 kDa protein of Schistosoma japonicum were fused to the JGMV CP and VLPs obtained in E. coli (Jagadish et al., 1993). Immunization of mice with these chimeric VLPs led to the generation of anti-peptide immune response indicating to the potential of this system to present the peptides for immunization.

5.2 Over-expression of JGMV CP in E. coli

In the work described above (Jagadish et al., 1993) the level of synthesis of CP in E. coli was low precluding its use beyond experimental protocols. One of the aims of the work described in this thesis, therefore, was to substantially enhance the level of CP synthesis in E. coli. Jagadish et al. (1993) used a tac promoter based E. coli expression system using plasmid pTTQ19N:CP. In recent years, foreign gene expression in E. coli under the control of the bacteriophage T7 promoter has been shown to yield higher amounts of the expressed gene product (He et al., 2002). Thus, in order to have enhanced JGMV CP synthesis in E. coli, the CP gene was placed under the control of the bacteriophage T7 promoter in pVex 11 vector and the gene was expressed in E. coli BL-21 cells that synthesize the bacteriophage T7 RNA
polymerase. However, the amount of the CP synthesis from the T7 promoter based plasmid was found to be similar to that obtained from the tac promoter based plasmid pTTQ19N:CP.

High level of foreign gene expression in a T7 promoter based expression system is achieved by a very high level of RNA transcription from the T7 promoter in *E. coli* (Studier and Moffatt, 1986). A specific mRNA produced by T7 RNA polymerase can rapidly saturate the translational machinery of *E. coli* so that the rate of protein synthesis from such an mRNA will depend primarily on the efficiency of its translation. In fact, if the relevant mRNA is efficiently translated, the target protein could accumulate to greater than 50% of the total cell protein within a short period of time (Studier and Moffatt, 1986). However, efficiency of an mRNA translation can be affected by the mRNA secondary structure involving the translation initiation region (McPheeters *et al.*, 1986; de Smit and van Duin, 1990). In the case of phage MS2 coat protein (Schmidt *et al.*, 1987), the *E. coli* lamB gene (Hall *et al.*, 1982) and the mom gene of phage Mu (Wulczyn and Kahmann, 1991), mutation analysis showed a correlation of the inhibition of protein synthesis with the increased stability of the secondary structure in the translation initiation region. Conversely, mutations that destabilize stem loop structures by preventing base pairing (Knight *et al.*, 1987; Spanjaard and van Duin, 1989) or creating alternate AUG codon (Satchidanandam and Shivashankar, 1997) have been used to obtain enhanced expression. Secondary structure of CP mRNA was, therefore, analysed around the initiation codon. It was found that the ATG codon was part of a highly stable stem-loop structure that could potentially affect the efficiency of mRNA translation. To break this stem-loop structure nucleotide substitutions were made in the mRNA at three positions. Two of these changes (A→T and C→G) were made at the wobble base of the codon and thus they coded for the same amino acid as in the CP sequence. However, the third change, T→A, resulted in a change in the amino acid from serine to threonine. This was a conservative change considering that both the amino acids have uncharged polar side group. Such a conservative change is unlikely to drastically affect the conformation of the protein. Besides, the changed threonine constituted the second amino acid from the N-terminus of the modified CP and both the N- and the C-termini have been predicted to be exposed on the surface of the CP (Shukla *et al.*, 1988). These changes in the mRNA led to the breaking of the predicted secondary structure around the initiation codon thereby increasing the efficiency of its translation. The mutated CP gene expressed the protein in *E. coli* very efficiently and the level of CP synthesis went up by at least 16-folds. The authenticity of the synthesized protein being JGMV CP was confirmed by N-terminal sequencing of the protein and also by its western
blotting with anti-CP antibodies. The mutation did not affect the folding of the protein as it was found to auto-assemble to form VLPs that were visualized by electron microscopy.

With the expression of CP being very high, it was feared the protein might be forming inclusion bodies, as is the general trend with high level expression when the protein does not get enough time to fold properly into native structure. Inclusion bodies contain a bunch of misfolded proteins forming a heavy mass. Indeed, CP was found both in the form of the inclusion bodies as well as in the soluble fraction of the cytoplasm. No attempts, however, were made to solubilize and refold the protein from the inclusion bodies.

5.3 Japanese Encephalitis virus peptides with potential to induce virus-neutralizing antibodies

JEV infection of host cells produces 3 structural and 7 non-structural proteins. Of these, the E protein is the major envelope protein of the virion. This protein is believed to play an important role in a number of processes including viral attachment, membrane fusion and entry into the host cell. This protein is the principal target for neutralizing antibodies produced by the host against the pathogen. Kolaskar and Kale (1999) have predicted the three-dimensional structure of the E protein using the knowledge based homology approach. The 500 amino acid long protein consists of three domains: domain I, referred to as the central domain, consists of 128 residues from 1-51, 137-196 and 293-311. There are two disulphide bonds in this domain. This domain also contains the glycosylation site in addition to the epitopes with serological or biological activities. The domain II consists of 171 residues (amino acid 52-136 and 197-292) and three disulphide bonds. This domain is called the dimerization domain. It has a hydrophilic region and contains epitopes involved in neutralization and hemagglutination. The domain III is a contiguous stretch of 100 residues (amino acid 310-411) with only one disulphide bridge. This domain has been suggested to play a role in receptor binding activities (Lobigs et al., 1990).

JEV infection in a host produces both the virus neutralizing antibodies and the cytotoxic T-cells (CTLs). It has, however, been shown that protection against JEV infection is mainly antibody dependent and virus-neutralizing antibodies alone are sufficient to impart protection (Konishi et al., 1999; Pan et al., 2001). This is also evident from the fact that the formaline-inactivated JEV vaccine provides protection to vaccinees against JE without inducing
any CTLs. Studies with malaria (Molano et al., 1992) and papillomavirus (Christensen et al., 2001) have also shown that neutralizing antibodies are sufficient to clear certain pathogens from the system. Hence, it would appear that the B-cell epitopes are important determinants of protection against JEV infection.

B-cells bind to the soluble antigen and they recognize the epitopes easily accessible and exposed on the surface of the immunogen. These epitopes are hydrophilic in nature and are located on the bends of the amino acid chain, imparting a greater degree of mobility to these residues. B-cells also recognize viral proteins and glycoproteins that may be released from infected host cells prior to complete viral assembly. When the soluble antigen interacts with the surface-bound antibody on the B-cell membrane, it is internalized by the B-cell, processed and re-presented on the B-cell membrane with MHC-II molecule. The antigen specific T_{H} cell then binds to this antigen-MHC II complex, thereby secreting a number of cytokines. These cytokines trigger activation and maturation of B-cell followed by the production of antibody secreting plasma cells and memory B-cells. The secreted antibodies facilitate clearance of virus either by acting as opsonins to enhance phagocytosis or by lysis of enveloped viruses. They also play a protective role by binding to the virus particles and inhibiting them from attaching to the host cells, thereby neutralizing them. Thus specific peptides could be used to induce specific B-cell that may have virus neutralizing activity. Indeed, Seif et al. (1996) have shown that a 27 amino acid sequence derived from the C-terminal of JEV E protein (located between amino acid 373-399) fused to glutathione-S-transferase or protein A induced JEV neutralizing antibodies in mice that were immunized with the fusion protein. This sequence (peptide A) was, therefore, chosen to be presented on JGMV VLPs. Another peptide sequence that was chosen for presentation on the JGMV VLPs was a subset of the 27-amino acid sequence (peptide B); it contained residues 386 to 399 of JEV E protein. A third peptide that was chosen contained residues 151 to 163 of the E protein (peptide C). This peptide was chosen as a potential B-cell epitope based on the predicted three-dimensional structure of JEV E protein (Kolaskar and Kale, 1999). Besides, being hydrophilic and charged, this region contained a potential glycosylation site and hence a putative epitope. A 98 amino acid sequence was also chosen from the E protein (amino acid 303-400) for presentation on JGMV VLPs (peptide D) for it contained binding site for 10 anti-JEV monoclonal antibodies that neutralized virus activity in vitro. Besides, some of these antibodies passively protected mice from a fatal virus challenge and hence the peptide had the
potential to induce virus-neutralizing antibodies (Mason et al., 1989). Thus, peptides A, B and D belonged to domain III of JEV E protein while peptide C belonged to domain I.

5.4 Synthesis of JGMV CP fused with JEV peptides

Initially, attempts were made to fuse the peptide A to the N-terminus of JGMV CP by genetic engineering means. However, this did not succeed as the fusion gene did not produce detectable quantities of the fusion protein in *E. coli*. Predicted secondary structure of mRNA for the fusion gene encoding peptide A showed the formation of a stem-loop structure that included the initiation codon ATG. This perhaps attenuated the translation efficiency of the fusion mRNA. The problem was overcome by carrying out the fusion at the 3'-end of the CP gene, which would result in a fusion protein with the JEV peptide at the C-terminal of CP. The fusion was carried out using a convenient *Pst I* site at the 3'-end of CP, thereby deleting 14 amino acids from the C-terminal of CP. This construct was successful in synthesizing the fusion protein in high amounts in *E. coli*. The fusion protein auto-assembled to form VLPs. These particles had a thickness of 20-22 nm compared to 13-15 nm for just the CP containing VLPs. This demonstrated that at least the last 14 amino acids towards the C-terminus of JGMV CP were not necessary for the protein folding and assembly and these could be replaced by longer amino acid sequence, although this may have bearing on the thickness of the VLPs. Similar strategy was used for fusing peptides B and C at the C-terminus with CP. These fusions also did not affect the ability of JGMV CP to auto-assemble to form VLPs.

A free peptide in the solution, can acquire many conformations owing to the freedom it gains. The conformation of the peptide is an important determinant of its immunogenicity and it may determine if the anti-peptide antibodies would also recognize the native protein from which the peptide was derived. A peptide fused to a large protein such as JGMV CP will have constraints on its conformation imposed by the protein. Thus, to improve chances of producing anti-peptide antibodies capable of recognizing JEV E protein, a flexible linker was used to attach the peptides with the CP in the fusion protein. The flexible linker would provide the peptide freedom to take up a conformation without being affected by the conformation of the CP.

Linker sequence composition could have a significant affect on folding and stability of the fusion protein (Robinson et al., 1998). An ideal linker used to fuse two protein domains
should give them flexibility to fold independently thus retaining their individual activities. A linker with a propensity to form $\alpha$ helical or $\beta$-stranded structure is therefore not favoured. A linker containing the triple repeat of Gly-Gly-Gly-Gly-Ser residues had previously been used effectively to fuse angiogenin and a single chain antibody against human transferrin receptor without the loss of the antibody binding ability (Newton et al., 1996). Thus, $(\text{G-G-G-S})_3$ was used to fuse JEV peptides to JGMV CP that should render flexibility to the peptides to take up conformation/s independent of the CP. The DNA sequence encoding the linker was attached to the 3'-end of the CP gene, replacing the last 14 amino acids of the CP with the linker. The peptides were then fused to the CP following the linker sequence. The fusion of the linker did not affect the CP synthesis in *E. coli* and the CP fusion protein attached to JEV peptides through the linker was efficiently synthesized. The fusion protein containing JEV peptides along with the linker also auto-assembled to form VLPs as these could be purified on sucrose gradient in the same manner as the VLPs containing just the CP.

5.5 **Immunogenicity of JEV peptides**

The fusion protein VLPs, containing JEV peptides fused to JGMV CP, were purified by sucrose gradient centrifugation and these were used to immunize mice and rats without any adjuvant. Immunogenicity of the JEV peptides presented on the VLPs was studied by determining the antibody response of the animals against the cognate peptides and the JEV E protein. This was compared with the immunogenicity of JEV peptides presented using the peptide-BSA conjugates along with the complete Freund's adjuvant (CFA). Each animal received $10 \mu$g equivalent of the peptide either using the VLPs or the BSA-peptide conjugate. ELISA was carried out to study the antibody response of the immunized animals.

Anti-peptide antibodies were detectable in most of the VLP-immunized mice after the primary immunization and the level of antibodies increased significantly after the booster dose was given. In general, antibody response was low in rats compared with mice. This may simply be because of the fact that same amounts of VLPs ($10 \mu$g peptide equivalent) were used for the immunization of mice and also of rats, in spite of rats being much larger animals than mice. The anti-peptide antibody response by animals immunized with BSA-peptide conjugates was disarmingly low compared to that seen in VLP-immunized mice despite the fact that the peptide conjugates were administered with CFA, a very potent adjuvant. It may be noted that similar to
the BSA-peptide conjugates, tetanus toxoid (TT)-peptide conjugates made from JEV peptides A, B and C were also poorly immunogenic (our unpublished results). Reason for the poor immunogenicity of the peptide conjugates is not clear. BSA-peptide conjugates (and also TT-peptide conjugates) were made at a peptide density of about 15-20 peptide molecules per molecule of the carrier (Table 4.3). It is known that carrier to peptide ratio needs to be optimized for every conjugate. We have tested only one combination of peptide and BSA molecules. The peptide density of about 15 may not be optimum for immunogenicity of the peptide in the present case; this may be too low to be immunogenic or too high for making the conjugate an efficient immunogen. The high density of peptides could mask the carrier molecule making it unavailable for providing T-cell help for vigorous immune response. More surprising, though, was the observation that BSA-peptide was not very immunogenic even with CFA. Previously, it has been seen that 10 amino acid leuteinizing hormone releasing hormone (LHRH) peptide given with CFA failed to generate anti-LHRH antibodies in experimental animals (Om Singh, personal communication). Thus, these results clearly demonstrated the potential of the JGMV VLPs to efficiently present peptide antigen to the immune system and also generate vigorous anti-peptide antibody response without the use of any adjuvant. This would have implications for the development of other peptide-based vaccines as they could be administered in the form of the VLPs without using any adjuvant.

The antibody response varied for different peptides. The antibody titer was found to be very high with peptide A as compared to the other three peptides. The anti-peptide antibodies also reacted with JEV E protein and neutralized JEV activity in vitro indicating that JEV peptide containing VLPs were able to produce anti-JEV antibodies. Antibody response against JEV E protein was also the highest with peptide A containing VLPs. Interestingly, peptides A and C, presented on VLPs with the help of the linker were generally more immunogenic than when fused to the CP directly at its C-terminus. However this was not the case with peptide B. Nevertheless, it would be advisable to present the peptides on VLPs using the linker as it would provide flexibility to the peptide to take conformation/s independent of the CP.

The anti-peptide antibodies generated using the VLPs, interacted with JEV E protein and also with JEV, as seen in virus neutralization assays. Peptide A induced highest JEV neutralization activity where VLPs presenting the peptide A through the linker gave 96% virus neutralization (Table 4.4). Peptides B, C and D induced antibodies whose ability to neutralize virus in vitro was low compared to the peptide A antibodies. This is interesting considering that
peptide B sequence was a subset of the peptide A sequence; the 14 amino acid peptide B formed the C-terminus of the 27 amino acid peptide A. It could, therefore, be argued that the first 13 amino acids of the peptide A representing amino acid 373 to 385 of the JEV E protein may form the neutralization antigen of JEV. However, peptide D representing amino acids 303-400 of JEV E protein induced only low JEV neutralizing activity. It would, therefore, appear that while the sequence of the peptide would certainly be relevant, its conformation would also be important for inducing biologically relevant antibodies.

The 27 amino acid peptide A contains RGD (Arg-Gly-Asp) sequence that has been reported to be responsible for the cell-attachment-promoting activity of the extracellular glycoprotein fibronectin (Pierschbacher et al., 1984). The RGD sequence has also been shown to be involved in FMDV cell attachment and initiation of infection (Fox et al., 1989; Mason et al., 1994). The region surrounding the tripeptide RGD in Murray Valley encephalitis virus envelope protein has been suggested to be involved in the virus binding with putative receptor (Lobigs et al., 1990). It may be noted that Murray Valley encephalitis virus belongs to same subgroup of flaviviridae family of animal viruses as does JEV.

Dewasthali et al. (2001) showed the neutralizing properties of peptide 149-163 of E protein of JEV in vitro as well as in vivo using monoclonal antibodies produced with the splenocytes of mice immunized with peptide-ovalbumin conjugate. This peptide is a part of domain I, which contains the glycosylation site and epitopes with serological and biological activities. Part of this peptide, amino acid sequence 151-164 (peptide C), was used in the present studies to immunize mice as the CP fusion protein, with and without linker. The antibodies induced by peptide C containing VLPs showed a little over 30% JEV neutralization activity suggesting that sequence represented by peptide C may also be involved in the formation of the JEV neutralization antigen.

In this study, peptide A fused directly to CP produced antibodies with 88% JEV neutralizing capacity. Use of linker to fuse CP with the peptide enhanced the neutralizing ability of antibodies to 96%. Similar observation was seen in the case of peptide C although reverse was true for peptide B. Whether the linker had any role in presenting the peptide better by propping it out prominently or by giving the peptide a flexibility to take up a certain conformation resulting in enhanced neutralizing antibody titers, is debatable.
5.6 Development of a peptide based JEV vaccine using JGMV VLPs

Neutralizing antibodies alone are sufficient to impart protection to the vaccinee against JEV. Peptide based vaccine against JEV is, therefore, a distinct possibility. The success of such a vaccine would depend upon finding a suitable peptide or a combination of peptides that would generate high titers of JEV neutralizing antibodies. This study shows the generation of varying titers of JEV neutralizing antibodies by four JEV epitopes in animals that were immunized with peptides fused to JGMV CP. It is often argued that empirical rather than rational designing is critical for developing a peptide based vaccine (Regenmortel, 2000). Potential of a number of other peptide sequences derived from JEV E protein should, therefore, be examined. Another possibility lies in the use of mimotopes. These are peptide sequences that have the same conformation as a neutralization epitope in the virion. Such mimotopes could be selected by carefully screening a random peptide sequence library with JEV neutralizing monoclonal antibodies. Efforts are underway in our laboratory in this direction.

The JGMV VLPs could be used as carriers to present foreign peptides fused at the N- or C-termini of the protein. In fact, these VLPs could be used as dual vaccine by fusing immunogenic epitopes of two different virus particles or two different epitopes of the same virus particle at the C- and the N-termini. In fact, the JGMV VLP system has the potential to present several different peptides derived from the same pathogen or from different ones on the same particle by allowing the assembly of different CP molecules fused to different peptide sequences, giving rise to the possibility of a vaccine against multiple pathogens. Also, since the C- and N-termini are exposed on the surface of the coat protein, unlike other self-assembling protein systems, as big a peptide sequence as desired could be potentially attached to the protein. Generation of JEV neutralizing antibodies by VLPs presenting JEV peptides, without the use of any adjuvant, reinforces the potential of JGMV VLPs for presenting peptides for producing peptide based JEV vaccine.