CHAPTER 6
GENERAL DISCUSSION

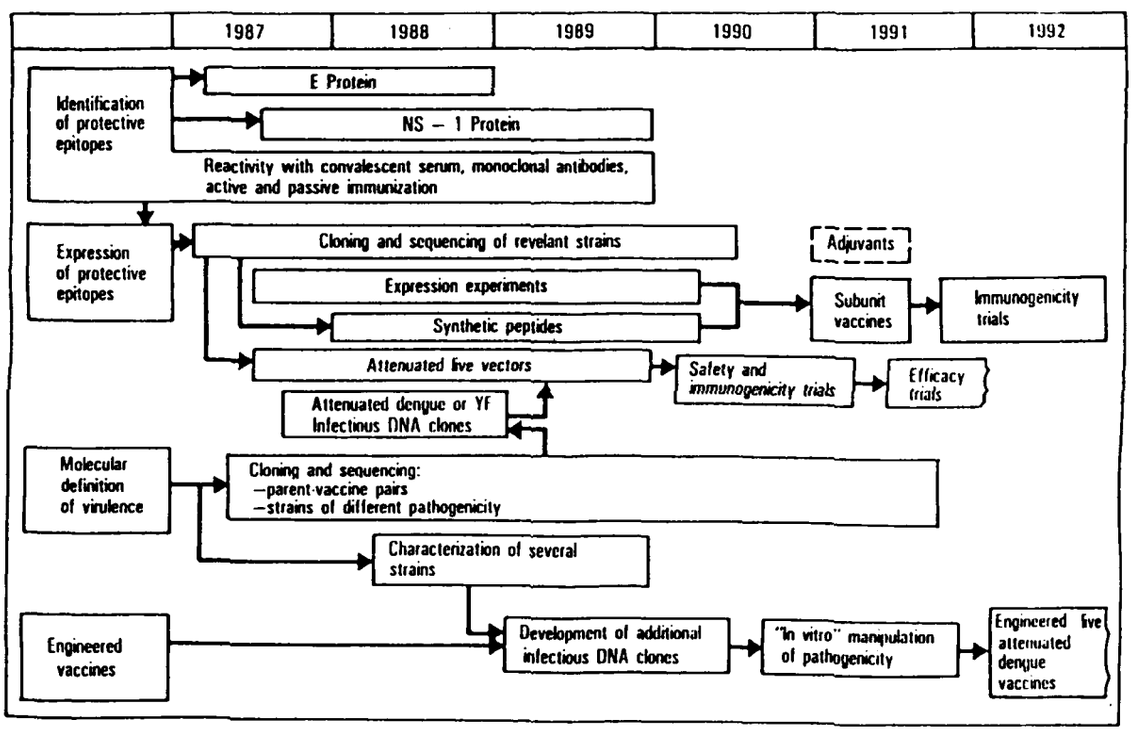
The family **Flaviviridae** consists of around 73 antigenically related viruses, 29 of which have been associated with human disease. These include the most important arthropod-borne viral afflictions of humans - DEN, JE, YF, SLE, TBE and KFD. These viruses are transmitted in nature, through the bite of an infected mosquito or tick (Porterfield, 1978). Flaviviruses produce a broad spectrum of clinical manifestations in humans, ranging from asymptomatic infection to fulminant fatal encephalitis or hemorrhagic fever. The members of this family demonstrate antigenic cross-reactivity that is strongest in the `HI' test and most specific in the `N' test (Monath, 1990).

Although a safe, cheap and efficacious live attenuated vaccine is currently available against yellow fever (17D - YF), efforts to develop similar vaccines against DEN and JE have not been as rewarding. DEN viruses, which appear to possess tropism for mononuclear phagocytes, exist in nature as four serotypes. The disease caused by these viruses is usually characterized by high fever and subsequent resolution of symptoms within a few days. However, a more severe form of dengue is manifested by hemorrhage and shock (dengue hemorrhagic fever - DHF and dengue shock syndrome - DSS). DHF/DSS has been suggested to occur as a result of secondary infection by another serotype (Halstead, 1973). Although antibodies raised against a single serotype protect against homotypic reinfection for life, these antibodies can bind to the new serotype (secondary infection), but are not able to neutralize it. The opsonization of this virus - antibody complex by Fc receptor bearing cells (macrophages) results in enhanced infection of these cells. This phenomenon has been termed antibody- dependent enhancement (ADE) and has been thought to be fundamental to the pathogenesis of most cases of DHF/DSS (Halstead, 1988).

In 1987, the Steering committee, organized a scientific meeting sponsored by WHO, to focus attention on the development of dengue vaccines (Brandt, 1988). JE, a major health problem in Asia, caused by a flavivirus serotypically and biophysically related to DEN, was also included in the discussion. As part of the deliberations, a strategic plan was proposed, as a general guide for reaching the goal of immunization against dengue and to also serve as a guide for evaluating proposals for financial support from WHO (**Fig.44**).

The envelope `E' glycoprotein of flaviviruses is the major structural protein of the virus. This protein plays an important role in a number of biological activities including virion assembly, receptor binding and membrane fusion. The E protein also contains neutralizing epitopes (Mason *et al.*, 1987,1989b,1991; McCown *et al.*, 1990; Bray *et al.*, 1989; Lee-Ron Jan *et al.*, 1993; Heinz *et al.*, 1986). However, because of the phenomenon of ADE, which is responsible for DHF/DSS, focus has been drawn towards the NS1 protein, also associated with protection (Schlesinger *et al.*, 1985, 1986, 1987). These findings have therefore initiated us to undertake the study of purifying and

FIG 44
WHO PROGRAM FOR VACCINE DEVELOPMENT : STRATEGIC PLAN FOR DENGUE



characterizing the NS1 protein of JE virus. The protective potentials of this protein have also been investigated.

(A) Purification of the NS1 protein

In order to facilitate isolation and characterization of the NS1 protein of JE virus, it was initially decided to prepare antibodies specifically reacting to this protein. Efforts were therefore made to purify the NS1 protein from JEV infected cells; devoid of other viral proteins and more importantly devoid of the `E' structural protein. Purification was attempted using JEV infected PS cells and JEV infected mouse brain suspension as the starting material. In both the cases, under native conditions NS1 protein was found to exist as a high Mw aggregate (determined by gel filtration), indicating either dimerization or oligomerization of the monomeric forms of NS1 and NS1' proteins. As the overall load of the cellular protein was very high, this strategy could not be adopted for large scale purification.

In another attempt to purify NS1, a recombinant clone expressing the NS1 protein as a trp E fusion protein was obtained from Dr.Peter Mason (Yale University, USA). Expression of the JEV sequences from the pATHNS1-2A plasmid was under the tight control of the metabolic trp E promoter of the tryptophan operon. Induction of the rNS1-2A protein was carried about by the addition of indole-acrylic acid (IAA) to an *E.coli* culture harbouring the pATHNS1-2A plasmid, under tryptophan depleted conditions. IAA mimics low levels of tryptophan and thus keeps the operon operational. Initial experiments were directed towards the standardization of the optimum time required for recombinant protein expression. Cells induced for 8 hrs with IAA expressed sufficiently high amounts of fusion protein to facilitate its purification. High level expression led to an intracellular accumulation of rNS1-2A protein as an inclusion body. The rNS1-2A protein was released from the cell after lysis and recovered as an insoluble pellet by centrifugation. This pellet was resistant to NP- 40 and 1M NaCl, hence was solubilized in SDS-PAGE sample buffer (SDS and 2-Mercaptoethanol). The rNS1-2A protein was purified on a preparative SDS-PAGE. Purification was represented by a single protein band on an analytical SDS-PAGE stained with Coomassie blue stain. Densitometric scanning confirmed the same and did not reveal minor contaminating protein bands. Around 250 ug of purified protein could be obtained from a 100 ml batch culture.

Rabbits were immunized with this preparation to prepared a NS1- 2A specific polyclonal serum. The anti NS1-2A serum specifically reacted to the NS1 and NS1' proteins of JEV in Western blot. The rabbit polyclonal serum also reacted with homologous antigen and partially purified NS1 from infected PS cells in ELISA. The IF pattern appeared diffused and cytoplasmic in fixed infected PS cells. NS1 was also found to be associated with the membrane surface of infected cells as determined by surface IF without permeabilizing the cells with acetone. The common feature in all these three tests

was that the NS1 protein was bound to the surface of a substrate, namely ELISA plate, Nitrocellulose paper - Western blot and coverslip immunofluorescence.

The ability of the NS1 polyclonal antibody to capture NS1 antigen in a solution was also investigated. Anti NS1 polyclonal antibody did not bind NS1 protein efficiently in a capture ELISA. This can be explained by the following reasons.

(i) Studies by Friguet *et al.* (1989) have indicated that binding of a protein to a substrate induces partial if not complete denaturation of the polypeptides. Thus masking epitopes normally present on the native protein and/or exposing hidden epitopes not accessible in a native state. As the polyclonal antibodies were raised against the denatured state of antigen, it is possible that the native conformation of the protein was not recognized.

(ii) The recombinant protein was expressed as a truncated moiety as it did not represent N-terminal 58 residues of the NS1 protein and also the C-terminal 106 residues of the NS2A protein. Absence of these residues, might have greatly influenced the tertiary structure of the rNS1-2A protein as compared to the native structure of authentic NS1 and NS1' protein. More importantly, the total recombinant protein contained 45% of sequences representing the trp E encoded region. This may also play an important role in the overall folding of the protein. Colman *et al.* (1987) have quoted that "the net free energy of the folded state of a protein is the sum of many stabilizing interactions which often barely counterbalance the many positive contributions to the free energy, as a result, single amino acid substitutions may suffice to destabilize the native structure".

(iii) Absence of glycosylation may also influence the tertiary structure of the rNS1-2A protein.

As the rabbit anti NS1-2A serum was not able to capture NS1 efficiently in solution, it could not be used for immunoaffinity purification studies.

Studies by Arvieux and Williams (1988), Harlow and Lane (1988), have shown that around 20% if not less, of the antibodies in a polyclonal serum are specific to the antigen. Hence, while designing an immunoaffinity column, the actual binding space available for antigen binding is far less than the theoretical space on the column. Secondly, polyclonal serum contains a large number of antibodies with varied avidities against a large number of determinants on the antigen. Hence, although the antibody will bind the antigen firmly, strong denaturants will be required to elute them from the column. Therefore it has been universally accepted that while designing an immuno-affinity column, a single MAb exhibiting intermediate binding affinity should be used (Harlow and Lane, 1988). This will enable sufficiently high binding of the protein to the column, and subsequent elution under milder conditions without denaturing the antigen.

It was for these reasons that a panel of MAbs were prepared against the NS1 protein. As the MAbs were selected on rNS1-2A and NS1 from infected cells (both purified by preparative SDS-PAGE), by ELISA, none of the other MAbs except MAb X6A10 bound NS1 efficiently in solution.

The MAb X6A10 was used for determining the titre of NS1 protein in infected mouse brain suspension and in infected PS cell culture supernatants. However, although the MAb bound the antigen, attempts to elute the antigen with various reagents, met with little success. This can be explained by the fact that there was probably high affinity antigen-antibody binding, hence maximum elution could not be achieved inspite of using extreme pH or high salt conditions. Hence this strategy could not be adopted for NS1 purification. This problem can be circumvented by preparing a larger panel of MAbs and selecting a MAb with intermediate affinity, so as to achieve elution under milder conditions.

Characterization of the NS1 protein

In order to study the kinetics of NS1 synthesis, PS cells were infected with JE virus at high MOI and viral polypeptide synthesis studied. NS1 protein synthesis was found to occur between 12 to 14 hrs post infection. Immunofluorescence and phase partitioning studies in a Triton X-114 system indicated the association of NS1 with the membrane of infected cells. Western blot studies indicated that the predominant species found in infected cells was the dimer form of NS1. The dimer was found to be resistant to SDS and 2-Mercaptoethanol indicating that neither hydrophobic bonds nor disulphide bonds were involved in dimer formation. Treatment with Urea or Guanidine-hydrochloride was also not able to break these bonds. Heating the lysates upto 55°C did not affect dimers. Temperatures above 60°C converted all dimers to monomers. The dimers were found to be resistance to alkaline pH upto 10.6, but were sensitive to a pH range between pH 2.6-6.6.

Using pulse-chase experiments, Winkler *et al.* (1988), were able to demonstrate, that the nascent NS1 of DEN was a monomer and hydrophillic. Dimerization occured between 10-20 min post-translation and the dimeric form of NS1 was found to be hydrophobic. This suggests the presence of a precursor-product relationship between the two species and also that dimerization was responsible for the protein achieving a hydrophobic state. Hydrophobicity therefore explains its association with the membrane. Glycosylation studies indicate the presence of only mannose-rich glycans on the intracellular form of NS1 and NS1' proteins.

Although the precise function of NS1 remains to be identified, Mason *et al.* (1989a) have observed that both E and NS1 protein are retained in the ER or in the early golgi compartment of infected cells in a similar manner. This suggests the involvement of NS1 in virion assembly or maturation of virus within the cellular secretory pathway.

Protection studies

The envelope (E) protein of flaviviruses is the major structural protein of the virion. This plays an important role in a number of biological activities including virion assembly, receptor binding and membrane fusion. The E glycoprotein is also the major target for

eliciting neutralizing antibodies. Analysis of the antigenic structure of flaviviruses by antibody adsorption with polyclonal or monoclonal antibodies has provided evidence that the E protein contains flavivirus group, complex and type-specific determinants. Hence major focus has been directed on this protein in terms of its importance as a candidate vaccine.

In addition to the Egp, the NS1 protein of YF, DEN-2 and TBE has also been found to confer protection (Schlesinger 1985,1986,1987; Jacobs *et al.*, 1992). The protective potentials of the rNS1-2A protein was investigated in this study.

Mice immunized with rNS1-2A alone were found to resist challenge. The AST was found to be 16.5 days compared to 5.2 with controls, where none of the mice survived. Incorporation of rNS1-2A protein along with the commercially available vaccine (Kasauli) increased the protective ability of the vaccine. The AST of these mice was also increased when compared to mice receiving only the vaccine. All these results are suggestive that NS1 does play a role in protection.

Another finding in this study is that although all mice receiving the undiluted and 1:10 dilution of Kasauli vaccine were protected, and elicited high titred antibodies against the E protein in ELISA, these antibodies exhibited low HI activity and none of these sera exhibited CF activity. All these results tend to suggest the importance of mechanisms other than those mediated via complement in protection. Also to be noted is the fact that the mice receiving only rNS1-2A protein did not elicit CF or HI antibodies and were still protected.

Mechanism of NS1 induced immunity

Inspite of the ardous efforts made by several workers to unfold the underlying mechanisms of NS1 induced immunity, the task still remains to be accomplished. Gould *et al.*(1986) have suggested several lines of thoughts to explain such a protective response.

As a consequence of infection, expression of NS1 protein occurs on the surface of infected cells. Binding of NS1 antibodies to the protein on the infected cell may prevent virus spread to neighbouring cells. Secondly, during the exvagination process, antibodies may attach to antigen containing cytoplasmic and plasma membrane fragments. This may prevent the release of virus from the cell. Thirdly, the presence of NS1 on the surface of infected cells will be an ideal target for cytolytic T cells. As a sequelae to these events, macrophage or complement may recognize the attached antibody and destroy the infected cell.

Schlesinger *et al.* (1985,86,87) have demonstrated the ability of NS1 antibodies to fix complement. Their data therefore strongly supports the involvement of CF activity in the cytolysis of virus- infected cells; although it is highly probable that other mechanisms of immune recognition of cell surface NS1 are operative as well. Studies by Henchal *et al.*(1987) support the role of antibody dependent cell cytotoxicity (ADCC) and cytolytic

T-cell induction to be involved in NS1 mediated immunity. Henchal *et al.* (1987) also suggest that if NS1 expression takes place early in the replication cycle, it is possible that as a consequence of NS1 mediated intervention, immature or less infectious virus may be released from lysed cell. This would therefore lead to an abortive or a curtailed infection. Kurane *et al.* (1990) and Rothman *et al.* (1990) have also suggested the involvement of T cells directed against the NS1 protein of the helper class in humans and mice.

NS1 based flaviviral vaccines

Halstead *et al.* (1988) have suggested that NS1 based flaviviral vaccine would have an advantage over conventional vaccines in the sense, that it would confer protection and circumvent the problem of immune enhancement (ADE). Secondly, sequence analysis of flaviviral NS1 protein indicates a considerable amount of homology at the nucleotide as well as the amino acid level (**Table 25**). The unusually high degree of homology along with the conservation in the position of cysteine residues, suggested that the overall conformation of the protein may also be conserved between the viruses of this family. A number of amino-acid substitutions and deletions that occur amongst the NS1 protein appear to be semiconservative in nature. Thus the overall hydrophilic nature of the protein is also conserved. This high level of conservation of hydrophilicity, secondary and tertiary structure strongly suggests a conservation of potential antigenic sites on NS1. This added advantage would therefore facilitate preparation of a broadly reactive flaviviral vaccine (Henchal, 1987).

However, as NS1 is a non-structural protein, a vaccine based on NS1 alone will not be able to prevent infection. Hence incorporation of NS1 protein along with other more conventional vaccines would be a better approach. Such vaccines would permit limited viral replication and induce protective immunity without the risk of vaccine associated disease (Gibson *et al.*, 1988).

TABLE 25 : SEQUENCE HOMOLOGY OF FLAVIVIRAL NS1 PROTEIN AT AMINO ACID LEVEL

	YF ASIBI	YF17D-204	WN	MVE	SLE	JE	DEN-4
YF ASIBI	-	99.5	42.1	44.7	43.1	43.3	40.9
YF 17D-204		-	41.9	44.4	43.3	43.1	41.6
WN			-	73.9	62.1	75.6	48.9
MVE				-	64.5	78.7	48.7
SLE					-	61.9	50.2
JE						-	49.4
DEN-4							-

(GIBSON 1988)

Values expressed as percentages.