Dengue

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- Disease burden for dengue (Establishing the base line data required for designing a study)
- Evaluation of DENV- NS1 Ag ELISA for early diagnosis of the disease
- Evolution of dengue viruses in the country
- The role of host/viral factors in dengue immunopathogenesis
- Interactions of DENV with cellular organelles during viral morphogenesis
- Studies on the effect of dengue viruses on *in-vitro* hematopoiesis and demo static physiology of vasenlar endotrelial cells
- siRNAs an antiviral tool against dengue virus
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  Generation of HIV-1 subtype C based DNA vaccine candidate & assessment of prime boost immunization strategy in mouse model

Disease burden for dengue (Establishing the base line data required for designing a study on disease burden)

PS Shah, Anand Singh, Rupali Bachal, Asha Bhagat, Cecilia D.

Dengue is endemic to Pune and several other cities in Maharashtra. It is of major relevance to disease management that the cost of the disease to public health is established. Before designing a study on disease burden we have addressed the following questions to generate base line data for dengue in Pune. Do the intensity of outbreaks, the demography, the severity of the disease, the dominating virus serotype and the seasonal pattern change from year to year? We have taken up the study in collaboration with two major hospitals, KEM, Pune, YCMH, Pimpri and five clinics spread out in the city and will be covering a period of five years from 2005 to 2009. The data that has been generated each year has been reported in the annual reports and will be collated at the end of the five year period.

Objectives

- Determine the proportion of clinically suspected dengue cases with confirmed DENV aetiology.
- Categorize the confirmed dengue cases according to the disease severity in Pune.
- Determine the seasonal incidence of dengue
- Determine the serotype of the circulating dengue virus

Work done

A study of the febrile cases referred by the clinicians in and around Pune revealed that the incidence of dengue this year was very high compared to 2006. A total of 882 samples were tested from suspected dengue cases in Pune during Jan 07-Dec 07. Of these, 332 (37%) sera were positive for dengue-specific IgM antibodies. **Fig 1** shows the categorization of the confirmed dengue cases in 2007. DF cases (81%) were predominant with 29% cases showing thrombocytopenia. Only 19% of the cases were DHF with 6% belonging to DHFI, 12% to DHFII and 1% to DHFIII, there were no DHFIV cases observed in the cases we investigated.

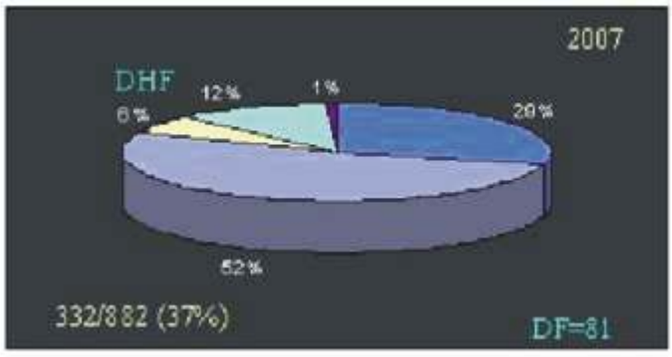


Fig. 1. The confirmed dengue cases were categorized according to WHO classification

The largest group that was affected was the 20-30 yr age group. The males were better represented than females. The largest number of cases was observed in September. Three seroytpes DENV-1, 2 and 3 were found to be circulating.

We also test samples from different hospitals and PHCs across the state of Maharashtra. Mumbai had 546 suspected cases of which 48% were confirmed. The other cities with 70-100 cases with a positivity ranging between 20-30% were Nashik, Solapur, Sangli, Washim and Nagpur. Altogether Maharashtra had 1336 suspected

cases of which 439 were DEN-IgM positive (32.9%) from Jan-November 2007. A rural outbreak in a village of Bhor (Pune district) was investigated in June 2007. Thirty one cases were investigated of which 13 were IgM positive and 15 were PCR positive resulting in a 74% confirmed cases. DENV-1 was the predominant serotype and all cases were DF. In March 2008, another rural outbreak in Gojubavi, a village in Baramati was investigated. Of 78 suspected cases, 48 were IgM positive and four were PCR positive, thus resulting in 66% positivity. The predominant serotype was once again DENV-1 and like Bhor all cases were DF. Therefore DENV-1 seems predominant in rural Pune causing less severe disease.

Future Plans

Similar studies will be carried out in the next year and more aggressive investigations will spearheaded in periurban areas. At the end of five years the data will be collated and analyzed to achieve the objectives mentioned above.

Evaluation of DENV- NS1 Ag ELISA for early diagnosis of the disease

PS Shah, Anand Singh, Cecilia D

Detection of dengue cases before seroconversion, which is important for patient management and determination of disease incidence, remains a challenge. We have been carrying out RT-PCR for detection of viral RNA in the early phase of infection. Another option for the early diagnosis of DENV-infection is the detection of NS1 Ag in patient sera. Commercially available Platelia Dengue NS1 Ag assay kit was evaluated for its ability to detect early cases in comparison to RT-PCR.

Objective

- Assess the use of Platelia Dengue NS1 Antigen Capture ELISA for early detection of dengue cases.

Work done

Initially 41 dengue samples, known to be positive by Real time RT-PCR were used to evaluate the NS1 ELISA in comparison with multiplex RT-PCR. NS1 antigen was detected in 30 of 41(73%) samples while dengue RNA could be detected by RT-PCR in 27(66%) samples. The NS1 ELISA was then tested with 39 sera from suspected dengue cases, which showed IgM in 14 (36%) samples. Out of 39 samples, 13 (33%) were positive by NS1 ELISA and 21 (54%) turned out to be positive by RT-PCR **(Table 1)**.

Table 1. Comparison of NS1 Ag ELISA with RT PCR in the detection of dengue cases

Cases tested	No. pos for NS-1 Ag (%)	No. pos by RT-PCR (%)
Known cases (Real time PCR pos), n=41	30 (73)	27 (66)
Suspected cases, n=39	13 (33.3)	21 (53.8)
Total n=80	46 (57.5)	48 (60)

The RT-PCR assay detected more cases (29) than NS/ELISA (23) before seroconversion (n=46) while the NS1 Ag ELISA detected more cases (23) than RT-PCR (15) after seroconversion. Considering the ease of the NS1 Ag ELISA and the smaller volume of sample required, the test could be used for early diagnosis of the disease.

Future plans

The NS1 antigen based ELISA will be applied to our investigations in rural areas.

Evolution of dengue viruses in the country

JA Pawar, Cecilia D

Phylogenetic analysis of the DENV circulating in India is being pursued since 2003 to understand the evolution of dengue viruses in the country. We had earlier reported the genotypes of the Indian strains of DENV-1, 2, 3 and 4 circulating in the country during the last fifty years. To further understand the evolution and dispersion of the virus in the country, full genome sequencing was undertaken.

Objectives

- Sequencing of the full genome of DENV strains of all four serotypes.
- Analysis of the sequence- data based on the genetic diversity of the different genes, selection pressures and possible recombination sites.

Work Done

Virus strains representing each decade were selected. The full genome of 15 strains of the four serotypes has been sequenced. The sequences are being analyzed for phylogeny based on whole genome, genetic diversity of the different genes, the selection pressures and possible recombination sites.

Seven genes C, prM, E, NS1, NS2a, NS2b and NS3 of two strains of DEN-4 which were 17 years apart were compared and it was found that the prM, NS2A, NS2B, NS3 genes were strongly conserved Capsid, E gene and NS1 were less conserved.

Future plans

Analysis of the whole genome sequences generated will be completed and a few more strains of DENV-3 will be sequenced.

The role of host/viral factors in dengue immunopathogenesis

Debargh Dutta, Cecilia D

Both host and virus factors are important in the development of severe dengue disease. So far we had shown that both arms of the immune response, the cytokine and antibody response were differentially regulated in DF and DHF cases. This time we used the C57BL/6 mice to study the immunoregulation by infected DCs in mice.

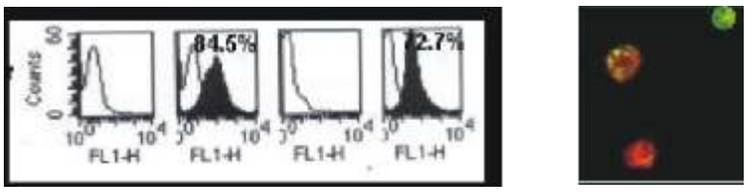
Objectives

- Assess the susceptibility of DCs myeloid (DC1) and plasmacytoid (DC2) to DENV infection.
- Study the effect of DENV infection on the two populations of DCs.
- Determine the effect of dengue-infected DC1 and DC2 on T cells

Work done

The two populations of DCs DC1 and DC2, derived from the bone marrow of C57BL/6 mice were found to be

susceptible to DENV infection. However, the virus was found to regulate the two populations differently. There was increased expression of co-stimulatory and regulatory molecules on DENV infected DC1 as compared to DENV infected DC2. Inoculation of mice with infected DC2 showed generation of regulatory T cells (Treg cells) in the spleen, whereas, inoculation with infected DC1 induced a strong proliferative response in the spleen. $CD4^+$ T cell from DENV immunized mice upon secondary stimulation with infected DC1 showed marked decrease in their proliferation suggesting activation-induced cell death. No difference in T cell proliferation was noticed when cells were restimulated with infected DC2.



A B

Fig.2. Purified populations of DC1 and DC2 infected with DENV-2. A) Shows the flowcytometric analysis and B) shows the presence of virus antigen in DCs (green) by IFA

Future plans

The differential effect of dengue virus on the two populations of DCs and their immunostimulatory potential in mice was an interesting finding and it will be interesting to see whether the same phenomenon is observed with human DCs.

Interactions of DENV with cellular organelles during viral morphogenesis

Samatha Sripada, Cecilia D.

Majority of viruses use cytoskeletal components of the host cells to gain entry into cells, replicate and spread to adjacent uninfected cells. Our earlier studies on dengue morphogenesis demonstrated that all three cytoskeletal elements of fibroblast cells are modified upon virus infection. This year the detailed interaction of DENV with microfilaments was investigated. During entry DENV was found to induce the *de novo* formation of filopodia facilitating entry of virus particles. In normal cells de novo actin polymerization is regulated by the Arp2/3 complex. We therefore investigated the possible interaction of DENV with Arp2/3 complex during virus entry.

Objectives

- Analysis of changes induced in microfilament structure during entry, maturation and egress of DENV-2.
- Investigating the role of Arp2/3 complex in nucleation of actin microfilaments during entry.

Work Done

PS (porcine kidney fibroblast cells) were infected with DENV, fixed at various time points and stained for actin and DENV with fluorescent probes. For analyzing the early events of virus entry, surface staining of cells for DENV was carried out without fixing or permeabilization. The images were acquired on Zeiss LSM 510 confocal microscope. Virus was labeled with secondary antibody-TRITC (red), actin and Arp2/3 were labeled with probe-FITC (green). During the process of infection, within 15-30 min of virus addition to cells, Arp2/3, which showed cytoplasmic distribution in normal cells, was found to relocate to the cell periphery associated with virus **(Fig.3A)**. High magnification rendering of infected cells showed close association of DENV on the cell surface (concluded because cells were stained for virus without permeabilization) with Arp2/3 **(Fig.3B)**. This suggested that DENV-2

recruited Arp2/3 as the nucleating complex for inducing actin rich filopodia.

Ours is the first report to show the selective recruitment of Arp2/3 complex by a virus to cell surface thereby facilitating entry.

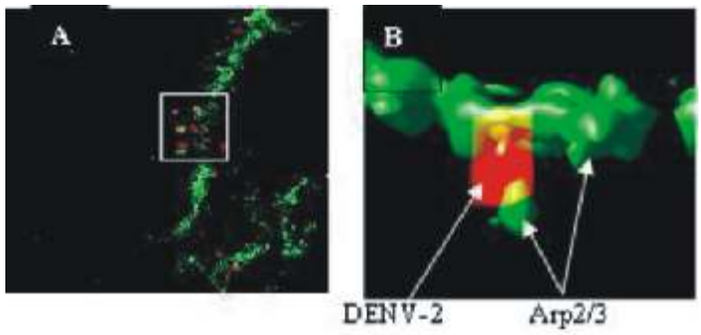


Fig. 3: Association of DENV-2 with Arp2/3 complex. Deconvolved images of DENV-2 infected cells showing the distribution of Arp2/3 complex (green) and viral antigen (red). (B is the area magnified from A)

Double labeling of DENV and actin at 15 min showed virus aligned along the filopodia (**Fig.4A)**. At 60 min p.i. there was thinning of the actin cortex **(Fig.4B)** which is characteristic of endocytic hotspots. Thus on binding to the cells DENV induced formation of filopodia and thinning of the cortex to facilitate entry.

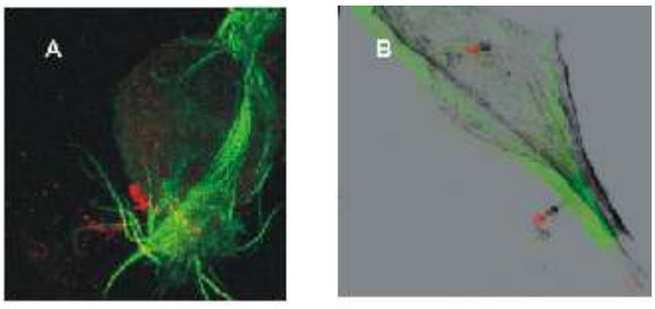


Fig.4: A) shows association of virus aggregates with filopodial extensions at 15 min p.i. ; B) Shows localization of DENV at the surface of the cell at 60 min p.i. DENV Infected cells were labeled for actin (green) and DENV (red).

Maturation phase of DENV was characterized by intact MF, which may be required for virus protein translation and assembly. However, there was no direct association between viral antigen and MF. **(Fig.5).**

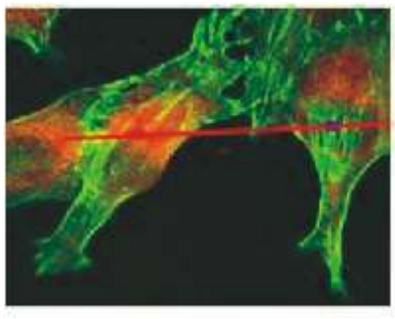


Fig. 5; Actin reorganization during DENV maturation. Infected cells were fixed at 48 h p.i. and double labeled for actin (green) and virus (red).

During egress, the virus induced the formation of thick peripheral bundles possibly a mechanism to propel mature virions out of the cell **(Fig.6)**. Inhibition of actin polymerization by treatment of cells with cytochalasin D at different time points pre and post infection resulted in decrease in virus yield as measured by PFU and real time PCR assays. The microfilament structure was thus required for all stages of virus replication.

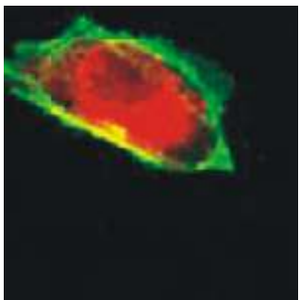


Fig.6; Actin reorganization during DENV egress. DENV-2 infected cells were fixed at 72 h p.i. and double labeled for actin (green) and virus (red).

Future plans

The role of the other cytoskeletal components in DENV replication will be investigated.

Studies on the effect of dengue viruses on in-vitro hematopoiesis and hemostatic physiology of vascular endothelial cells

A Basu, P Jain, SV Gaongodkar

This program was initiated in 2005 in collaboration with the National Institute of Immunohematology (ICMR) Mumbai and is extramurally funded by the Dept of Biotechnology Govt of India. The major objectives are to characterize at a molecular and cellular level, the mechanisms by which dengue viruses cause hematological dysfunctions-specially thrombocytopenia and related platelet disorders.

In continuation with the previous years work, the effect of dengue 2 virus on the in-vitro differentiation of hematpoietic progenitor stem cells was carried out to include multilineage differentiation including thrombopoiesis. The dengue 2 virus was shown to selectively inhibit human megakaryocyte growth and differentiation and induce apoptosis in early CD34+CD61+ megakaryocytic progenitors. [(1)] (Basu A et al 2008) Importantly, erythropoiesis remains unaffected and detailed studies on granulopoiesis are ongoing.

The major achievement in the second year of the project was the standardization of components of vascular biology. Human endothelial cells from HUVEC and primary lung microvascular beds were grown and characterized with an objective to study the effects of dengue virus induced alterations in vascular physiology and hemostatic properties. The complete envelope glycoprotein (E) and NS1 from dengue 2, dengue 3 virus and JEV have also been cloned into pcDNA3.1 for transfection studies in these cells. The optimization of efficient transfection of the endothelial cells with these constructs are currently undergoing.

siRNAs an antiviral tool against dengue virus

PS Shah, Guru Kumar KR and Cecilia D

Partial inhibition of virus replication in vitro by treatment of cells with chemically synthesized siRNAs as well as cloned siRNA sequences (shRNA) targeted against the 3'UTR of DENV genome was reported previously. To achieve complete inhibition of virus replication, we targeted two additional regions, the junction of core and prM and the NS3 gene. For sustained delivery of siRNA, plasmid based vector pSilencer H1 neo (Ambion) was used, which had an antibiotic resistance marker (neoR), useful in generating cell lines that stably express siRNAs.

Objective

- Cloning of the oligonucleotides representing the target siRNA sequences into pSilencer H1 neo vector.
- Testing the antiviral effect of 3'UTR, C-prM and NS3 shRNAs against DENV.

Work done

To select the potential siRNA targets, sequences of 11 Indian strains of DENV1, 2, 3 and DENV 4 serotypes of dengue viruses were aligned and analyzed. No continuous stretch of 21 nucletotides conserved in all four serotypes was found. Therefore, two sets of oligonucleotides representing C-prM conserved in DENV-1, 2 & 3 and NS-3 conserved in DENV-1, 3 & 4 were synthesized.

The oligonucleotides were cloned into the pSilencer-H1 Neo vector and two clones targeting CprM (pSil-CprM) and NS3 (pSilNS3) were generated. The tests to assess the antiviral effect have been standardized.

Future plans

The inhibitory effect of pSil-UTR, pSil- NS3, and pSil-CprM against different serotypes of DENV will be assessed. Stably transfected cells expressing siRNA will also be generated.

Additional Studies

Generation of HIV-1 subtype C based DNA vaccine candidate & assessment of prime boost immunization strategy in mouse model

(Extramural project RS 72, 06,494)

Srikant Tripathi, SM Mehendale, Madhuri Thakar, Smita Kulkarni, PS Shah, Cecilia D

Introduction

The development of a candidate vaccine for HIV-1 Clade C using the DNA prime protein boost vaccine approach was undertaken in collaboration with NARI. The aim was to generate recombinant pVax expressing the envelope, codon optimized envelope and gag genes and test their immunogenicity with and without immunomodulators in mice.

Objectives

- Evaluate the immunogenicity of HIV-1 Clade C envelope and gag genes with or without immunomodulators in mice.

Work Done

Three week old mice were immunized using 6 different constructs:

Native envelope (gp150), Codon optimized envelope, Codon optimized envelope with immuno-modulators, Native envelope (gp150) with immuno-modulators, Gag, Gag with immuno-modulators and control pVAX-1.

The sera samples were collected on 0, 3, 5 and 7 weeks post immunization and were assessed for antibodies against gp120 by ELISA. The best antibody response was seen with codon-optimized gp150 gene. The response was slightly improved with the addition of immunodulators.

Future plans

Further experiments are ongoing with improved vectors and schedules.

Publications

- Basu A, Jain P, Gangodkar SV, Shetty S, Ghosh K. Dengue 2 virus inhibits in-vitro megakaryocytic colony formation and induce apoptosis in thrombopoietin-inducible megakaryocytic differentiation from cord blood CD34+ cells **FEMS Immunology & Medical Microbiology** 2008: 53: 46-51

Presentations in the Conferences:

- Priyadarshini D and Cecilia.D. The levels of cytokines and neutralizing antibodies in dengue cases in India and its relevance to DHF. Oral presentation at the 13th International Congress of Immunology, Rio de Janeiro, Brazil, August 21-25, 2007.
- PS Shah, Harshad Patil and Cecilia.D siRNAs an antiviral tool against dengue. Oral presentation at Third Asian Regional Dengue Research Network Meeting Taipei, Taiwan, August 22- 24, 2007.
- Jayashri Pawar and Cecilia D. Molecular evolution of Dengue 1 in India. Third Asian Regional Dengue Research Network Meeting Taipei, Taiwan, August 22- 24, 2007.
- Samatha Sripada and Cecilia D. Significance of cytoskeleton in dengue virus replication. Frontiers in Cellular, Molecular and Developmental Biology, Dresden, Germany, September 1- 4, 2007.
- Anand Singh, PS Shah and Cecilia D Early detection of Dengue cases using NS1 antigen based ELISA. International Conference of Emerging and Reemerging Viral Diseases of Tropics and Sub-tropics, New Delhi, December 11-14, 2007.
- Guru Kumar KR., Priyadarshini D, JA Pawar, Asha Bhagat and Cecilia D. Detection and quantitation of Dengue viruses by Real Time PCR Method. International Conference of Emerging and Reemerging Viral Diseases of Tropics and Sub-tropics, New Delhi, December 11-14, 2007.

Workshops / Conferences / Seminar / Meetings attended

Cecilia D.

- Meeting of Health Minister of the States to review Chikungunya fever situation held at Vigyan Bhawan. DGHS, Ministry of Health and Family Welfare on June 21st, 2007.
- Invited to attend the Asia-Pacific Dengue Prevention Board meeting at Colombo, Sri Lanka, June 2007.
- International Workshop on Molecular Epidemiology and Immunology of Malaria and Other Vector Borne diseases at Jabalpur, 16-19 October 2007.
- Dr. Ananthanarayan Symposium at PGI, Chandigarh on 29 November 2007.
- 1st Meeting in March 2007 with Health Ministry of Kerala regarding the establishment of a Field Unit of NIV at Alappuzha, Kerala.