WORLD HEALTH ORGANIZATION

ORGANISATION MONDIALE DE LA SANTE

WHO VACCINES AND BIOLOGICALS **VAB/IVR/VIR/2003.01**
Initiative for Vaccine Research

and

PEDIATRIC DENGUE VACCINE INITIATIVE (PDVI)

Minutes of the Informal Consultation on Antibody Protection against Dengue Infection: 1. Viral Entry: Structure, Function 1 April 2003

Co-sponsored by: Ellison Medical Foundation, Rockefeller Foundation, Henry M. Jackson Foundation

Welcome and Introduction

Dr Yuri Pervikov: Welcomed the attendees and reviewed the objectives of the meeting.

1. Evaluate role of antibodies in protection against dengue infection and modulation of immune responses.
2. Discuss research studies for further activity in the area of anti-dengue antibody.

Dr Gubler: Described the new entity, Pediatric Dengue Vaccine Initiative (PDVI) that was started one year ago. PDVI is an alliance of stakeholders formed to facilitate the introduction of safe pediatric dengue vaccines. The Gates Foundation funds PDVI. There are three main components of this initiative. The development of dual use field stations for the purpose of conducting dengue vaccine efficacy trials (sites), and to support the conduct of these trials by funding research in both developing and developed areas (capacity building), and to develop research and vaccine safety. To this end, Dr Halstead has organized a series of three meetings, the first of which was held in June 2002 in Bethesda, MD on "Antibody Protection against Viral Infections with Special Reference to Dengue Virus". This meeting is the second and the next meeting will take place in Vienna and will cover Antibodies and Viral Entry. This latter meeting will be organized with Dr Franz Heinz and is scheduled for June 2003. It will be a more comprehensive 3-day science meeting. Goals for this meeting today include setting the research agenda for PDVI and proposing issues to be addressed in Vienna.

Topic: Dengue Vaccines under Development-Overview

Speaker**:** Dr Wellington Sun

Goals: Review dengue vaccines currently in clinical trials and more promising candidates under development. The target population for dengue vaccines is largely a pediatric population (~ one billion children in endemic area) with military and travelers only

comprising a small fraction of the potential vaccine recipients. Obstacles to vaccine development include the presence of four serotypes capable of producing disease (DF and DHF), lack of animal models of disease, neutralizing antibody as a surrogate marker of protection which needs validation and standardization, and the incomplete understanding of determinants of DHF in immunologically primed individuals.

Strategies: Ten vaccine candidates were reviewed:

• LAV, PDK	10 dengue genes	Phase 2 trials (Mahidol/AvP)
• LAV, FRhL	10 dengue genes	Phase 2 trials (WRAIR/GSK)
• 3'deletion + chimera	2+8? dengue genes	Phase 1/2 DEN-4 (NIAID)
• YF Chimera	2 dengue genes +8 YF	Phase 1/2 (Acambis/AvP)
DNA	2+ dengue genes	Protects monkeys (NMRC,WRAIR, JHU, Cytopulse, Powderject, Maxygen)
PIV	3 dengue genes	Protects monkeys (WRAIR, NMRC)
3' mutation	10 dengue genes	Preclinical (FDA/WRAIR)
rDN-2 Env	<1	Protects monkeys (HBG, NMRC, IP)
LAV, PDK Chimera,	3+7 dengue genes	Immunogenic in mice (CDC)
Adenovirus	1 dengue gene	Immunogenic in mice (GenPhar)

Progress:

Results from the LAV trial in Thailand:

- The most common reactions after the first dose were fever, headache, myalgias and rash
- Symptoms decreased after revaccination but increased after the booster.
- The Nab response against all 4 serotypes 28d after second booster of either formulation (F3212 or 3313) was between 80-100%.

Results from LAV, FRhL vaccine from WRAIR, GSK:

- Reactogenicity decreases with revaccination
- Multiple dosing required for tetravalent antibody response, after 2 doses seroconversion rates ranged from 70-100% against all 4 serotypes.
- Recommend that the dosing interval be greater than 3 months.
- No evidence for vaccine virus causing disease enhancement in humans.

NIH approach using attenuated DEN viruses or chimeric antigenic viruses looked at immunogenicity in a dose finding study (n=68), included 8 placebos/controls:

- Doses were log 2, 3 and 5 pfu with viremia in 11/19, 7/20 and 14/20 respectively.
- Rash developed in about 60% but were mostly minimal
- Many developed a rash (16/19,11/20, or 10/20) respectively.
- Very few developed a fever (1/19, 0/20, 1/20)
- 95-100% seroconversion with GMT Nabs ranging from 165-399 at Day 42.

The Acambis YF chimera vaccine approach was reviewed:

- Tetravalent vaccine (mix of individual serotype-specific chimeras) elicits Nabs against all 4 serotypes in 100% of monkeys.
- The Phase 1/2 trial of ChimeriVax-DEN 2 monovalent vaccine I was completed and showed it was well-tolerated with serocoversion rates of 93-100%.
- Phase 1/2 trial of ChimeraVax-DEN tetravalent vaccine planned for 2003.

DNA vaccines form NMRC, WRAIR:

- Monkey studies delivered dengue-2 prM/E DNA (2 x 1 μg) by gene gun induced Nabs and short term protection against live virus challenge.
- NS1-NS3 genes increased immuonogenicity of DEN-1 prM/E gene DNA vaccine in monkeys
- Phase I study of DEN-1 DNA vaccine 2003 planned.
- Shuffled tetravalent DNA vaccine constructs produced anti-dengue Abs by ELISA all 4 serotypes in primates.

Purified Inactivated Vaccine (PIV) WRAIR

- Immunogenicity and protective efficacy of DEN-2 PIV shown in mice and primates.
- cGMP lots of DEN-2 PIV manufactured at the WRAIR.
- Formulation with novel adjuvants enhanced immunogenicity in monkeys.
- DEN-2 PIV IND submitted with Phase I planned for 2003.
- Relatively frequent boosting may be required with PIV vaccine as Nabs may wane.

Dengue "Mutant F" candidate vaccines, FDA:

- Monkey study compared DEN-I WP wt virus with MutF.
- Fewer days of viremia (RT-PCR) 4.6 vs 1.1 and lower peak titer (1.5 vs. 0.2) in MutF.
- Similar PRNT50 320 vs. 240 (average for 9 monkeys per group).
- Challenge with DEN-1 wt showed no viremia (0/5) in MutF group vs 5/6 in controls.

Topic: Epidemiologic evidence of ADE

Speaker: Dr Maria Guzman

Goals: Use the Cuban experience as a unique model and clean epidemiologic setting to study the role of secondary infection as a risk factor for DHF/DSS.

Strategies:

- Analysis of four distinct Dengue epidemics in Cuba over a 25-year period.
- 1977 DEN-1: entire country affected with 400,000 cases; 44.5% IH Ab+ (prior to 1977 only 2.6% population had dengue Abs). No DHF/DSS cases were reported.
- 1981 DEN-2: 344,000 cases, 10,000 severe cases and 158 deaths (100 children, 58 adults). No cases in 1 and 2 year olds as they were not born in 1977 during previous DEN epidemic.
- 1997 DEN-2: No DHF/DSS observed in children experiencing primary DEN-2 infection. DHF/DSS observed only in adults with 205 cases, 12 deaths. Secondary infection shown in 98% DHF/DSS cases and in 92% of fatalities.
- 2001-02 DEN-3: No DHF/DSS observed in children experiencing their primary DEN-3 infection, and again DHF/DSS observed only in adults. Preliminary results show that secondary infection was a risk factor for DHF/DSS.

Progress:

- Important aspects of secondary infections were identified.
- Age-risk of severe disease s higher in children than adults and the outcome is worse in children with a fall in death rate seen with increasing age.
- Interval between epidemics: marked increase in severity with longer intervals between epidemics (4 vs. 20 years).
- Sequence: Den-1 then Den-2 or Den-1 then Den-3 are capable of inducing DHF/DSS.

Questions and discussion:
Using a cut-off in PRNT of 80% (vs. 50%) may lessen cross -reactivity. Also, likely to be virus strain differences that play a role in disease severity as the DEN-1 then DEN-2 sequence (with similar circulating virus strains) has been encountered in others areas before with much less severe disease. Genetic background of the population may also support the development of severe disease in Cuba.

Topic: in vitro ADE
Speaker: Dr Guzman (for Dr Halstead)

Goals: Review the data for ADE

Strategies/Progress:
- Initial observation (1964 Hawkes) in the Murray Valley encephalitis virus system
- many influences on in vitro ADE assay: cell system, level of activation and age of cells, MOI, temperature, incubation period, and maturation
- In vivo ADE occurs in undiluted sera and Nabs, if present, will block ADE
- only one relevant study in the literature (Kliks 1989) which prospectively correlated in vitro ADE with disease severity in vivo
- ADE observed after sequential infections is restricted to dengue subgroups, not to other flaviviruses with dengue infections
- ADE thought to occur via the usual viral receptor as antibody attracts the virus to the cell surface and enters via its normal receptor
- Much more needs to be done to identify cells supporting dengue infections in vivo
- biologically relevant systems then needed to study these receptor systems on FcR-bearing cells
- Identification of cellular receptors, viral entry mechanisms, and understanding of ADE at the molecular level will help improve assays for neutralization tests and the assessment of polyvalent dengue vaccine candidates.

Questions and discussion:
In a recent clinical trial with ChimeriVax-DEN 2, they found distinctly different viremic patterns on the basis of prior YF immunity. There was a higher, longer, and later viremia in YF immunes than YF naives-maybe the first demonstration in vivo of ADE that would involve another flavivrus with dengue. These vaccine trials afford a unique opportunity to study ADE in vivo. We need to thoughtfully take advantage of the vaccine clinical setting as an opportunity to understand ADE (including immunity to other flaviviruses outside the dengue subgroup). Kinetics of viremia, but not kinetics of antibody responses, were studied. These samples could be available for further study.

Topic: The relationship of pre-existing dengue virus neutralizing antibody levels to acute dengue viremia and disease severity
Speaker**:** Dr Daniel Libraty

Goals: prospectively examine pre-existing antibody levels and disease severity in secondary infections

Strategies:
- Selection of a well-defined cohort for analysis using an active surveillance system for school absences in Thailand (n~2000/yr)
- Each January sample pre-existing/pre-illness Ab levels

- June, Aug and November survey for illness and school absences
- Acute and 2 week convalescence samples (DV, JEV EIA and HI, DV PCR) with diagnosis based on serologies, viral isolation and identification
- 90% of cohort had secondary infections using previously established criteria
- Disease severity classified from most severe to least: DHF (hospitalized), hDF (hospitalized DF), and nhDF (mild DF)

Progress:

- In secondary DEN-3 infections (n=45), the $PRNT_{50}$>100 was associated with milder disease (DF vs. DHF p=0.02) and higher titers of pre-existing Nabs associated with a lower peak viremia
- In secondary DEN-2 infections (n=37) there was no relationship between pre-existing Nabs levels and disease severity nor was there any correlation between Nabs and peak viremia
- Hypothesis is that in the presence of high cross reactive Abs ($PRNT_{50}$100-1000) to DV3 there would be in vivo neutralizing capacity, limited viral replication, reduced disease severity
- But with lower levels of serotype cross reactive Abs ($PRNT_{50}$GMT 25-45) to DV2 there was no in vivo neutralizing capacity.

Questions and discussion:
Discussion followed regarding the potential differences between the reference strain and DEN-3 isolates. The viruses were matched for each individual (diagnostics included viral isolation). What happens early after infection in these primed individuals? This study in children only allowed two timepoints to obtain blood: one acute and one convalescent draw. Were any of these infections tertiary? They were anemnestic by definition (i.e. previous flavivirus infections). The suggestion was made to analyze the data using $PRNT_{90}$. What is the correlation between in vitro neutralization and in vivo neutralization? How can we best study this in vaccinees? Dengue infections seem to be different than other viral infections. For example, an in vitro Nab titer of 1:50 is pretty convincing and would be protective in other viral infections (measles) but it is not in dengue infection. Part of this meeting will address ways in which the neutralization assay can be made more sensitive. Varying the dose of the virus versus varying the dose of the antibody could give very different results. Some suggested using cells with and without Fc receptors. Other raised the idea of a log neutralizing index (LNI). Also look at dynamics of antibody response (check multiple timepoints).

Topic: Antibody neutralization of virus
Speaker: Dr Klasse

Semantics:

- Neutralization is the reduction of viral infectivity by ligand (Ab) binding to virion
- Demarcation: blocks step before major biosynthetic event in replicative cycle of virus
- Other inhibitory effects of antibodies on viral replication occur, e.g. block of viral release

Hypotheses and evidence:

- Block of virus attachment is a major mechanism of neutralization: HIV as example
- Exceptions for particular Abs and target cells
- Two major theories of virus neutralization compete: “special-effect” and “occupancy” theory
- Special-effect theory: single-hit; conformational change; crucial epitopes; post-attachment or even post-entry mechanisms; enhancing and neutralizing Abs always distinct
- Occupancy theory: multi-hit; binding is enough; minimum occupancy; block of attachment or entry; enhancement of infectivity possible at low occupancies
- Current evidence generally favors occupancy theory, which may be differentiated:
- Critical occupancy on functional viral attachment or entry-mediating protein->neutralization

- Ab coat on virion (whether on viral or cellular antigens)->neutralization

Questions and discussion:
Functional affinity (including avidity effects) predicts occupancy and neutralization well for Fab and IgG. Differential steric effects of Fab and of bivalently or monovalently binding whole IgG were discussed. Persistent non-neutralizing fraction still incompletely understood: but maximal extent of neutralization, as a complement to titers of neutralizing Abs, may predict efficiency *in vivo*.

Topic: Cryo-EM Dengue virus structure
Speaker: Dr R. J. Kuhn

Strategies:
- Using cryo-electron microscopy and image reconstruction techniques and the known atomic structure of the homologous E protein of TBEV
- A homology-modeled Dengue E protein dimer was fitted into the outer layer of density of the Cryo-EM reconstruction (model sequences)

Progress:
- Dengue virus structure was presented
- High resolution structures presented ranging from 24 - 9.5A
- At higher resolution, the particle appears more convoluted (see"bumps")
- Icosahedral scaffold has 90 dimers with 3 monomers in the icosohedral asymmetric unit
- Well organized outer protein shell complex covers (a minimal) lipid bilayer membrane
- Lipid bilayer is polygonal, the dengue particle causes the membrane to bend
- The bumps are asparagine sites (2-3kD of extra density)
- Discussed physical differences between the immature dengue particle (prM/E heterodimer) and the mature particle (E-E homodimer) determined to 16A
- Mature particle is smooth, ~ 500 A; immature particle is spiky, ~ 600 A
- Conversion of the immature dengue to the mature virus involves major rearrangement of E glycoprotein
- Each spike consists of 3 prM:E heterodimers; E is the envelope glycoprotein and prM is the precursor to the membrane protein M
- Conclusions – ordered arrangement of E protein with 3 molecules in an asymmetric unit
- The majority of the prM in the immature particle sits on top of the E protein as a "capped" spike-like structures.

Questions and answers:
Low pH rearrangements-these are different experiments-there is a lot of aggregation at low pH. In most virions, are M proteins on the surface? Cannot answer that as we only see 18-20 residues of M (it runs under the E protein); it comes close to the holes but does it come through? In the mature reconstruction, do you know the location of the TM sequences? The cleavage of prM proteins results in movement of the TM-in fact there are dramatic rearrangements. We need better resolution to look at some of these questions (i.e. <11A). There are neutralizing Abs to prM and NS1 Nabs effect neutralization, there may be other epitopes besides E involved in protection. Low pH mature particle experiments are in progress.

Topic: Domain III of the flavivirus envelope protein is the cell receptor-binding domain
Speaker: Dr A.D.T. Barrett

Strategies:

- Uses recombinant envelope protein domain III (E-D3) for various flaviviruses
- Domain III is conserved between different flaviviruses
- ED3 is 10kD protein approximately 100 amino acids (residues 300-400), depending on the virus, with a single disulfide bridge
- ED3 was expressed as fusion proteins with GST, MBP or HIS-tag
- ED3 is recognized by polyclonal and monoclonal antibodies
- Experimental question: do the recombinant Domain III clones from other flaviviriuses bind to VERO cells with a measurable affinity? And is there competition?
- Domain III is monovalent whereas the virus is a multivalent ligand (affinity vs. avidity)
- Therefore at low MOIs there is easy competition but at high MOIs there is no competition
- D3 neutralization is complex specific
- E-D3 binding measurements were presented using immunofluorescence, biotin labeled E-D3, iodinated E-D3 and ELISAs
- All of these results obtained using multiple techniques point to E-D3 specifically binding to cells with Kd's~ μM or less, depending on the flavivirus

Progress:

- E-D3 is antigenic, immunogenic, highly stable
- Experimental evidence suggests that E-D3 is the cell receptor binding domain
- DIII binding fits a single site model (this explains the lowish affinity when compared to the virus itself which is multivalent)
- Mutations in DIII do account for some differences in virulence but do not account for large differences in neuroinvasive phenotypes
- Mutations in single amino acid (E332) in WNV E-D3 had large effects on recognition of epitopes and neutralization of virions.

Questions and answers:
Could you use these antibodies to look at interference? Yes, not done yet though.

Topic: interaction between neutralizing antibody and domain 3 of the dengue virus envelope protein
Speaker**:** Dr H. Bedouelle

Strategies: cloning cDNAs, sequencing techniques, recombinant expression systems, and fluorochrome/chemistry.

- Cloned a Fab4E11 fragment
- Fab4E11 and Mab4E11 neutralized all 4 serotypes of DV (PRNT)-which suggests that the bivalence and Fc fragment are not absolutely necessary for neutralization (although the concentration of mAb giving PRNT 50% is at least a log lower).
- Efficiency of neutralization was DEN-1> DEN-2~DEN-3>DEN-4.
- MAb4E11 (0.4 mg) protected mice from 100 LD_{50} DEN-1 challenge (currently testing other serotypes).

Progress:

- Expressed mAb4E11 and it's antigen in E. coli.
- Mapped the epitope (contains residues 306-314 of protein E) and paratope of the Ab (residues mapped in L-CDR3 and H-CDR3).

- Characterized its neutralization mechanism (via proposed binding to heparan sulfates).
- MAb4E11 binds P'1-peptide and this peptide inhibits cellular infection with Den-1 virus (50% inhibition at 0.5 mg/ml).
- Design rule strategy (target residue must: belong to the hypervariable loops of the Ab, be adjacent to a functionally important residue of the mAb, not be functionally important for the interaction) was successfully used to create a fluorophore containing mAb.
- When the Ag binds to the modified mAb there is a detectable change in fluorescence.
- Converted mAb4E11 into a reagentless fluorescent immunosensor able to measure nanomolar amounts of Ag in sera.
- This technology can be applied generally to any antibody and has multiple potential uses (in solution, or as antibody chips, or applied to the tip of optical micro/nanofibers, or for detection and quantification of proteins in complex mixtures).
- Currently attempting to transform mAb4E11 into a therapeutic molecule against DHF/DSS.

Questions and answers:
Discussion ensued regarding comparing the Kd of the recombinant to either naturally acquired Fab or the Kd of virus binding to recombinant Ag Does the peptide induce antibodies in animals? Yes, we saw Abs to domain III but they were not neutralizing Abs. Remember though, that this is an incomplete peptide that does not cover the full epitope. MAb4E11 neutralizes all 4 serotypes but with variable efficacy: DEN-1 10 fold easier to neutralize. If there is cross-reactivity, what about aligning the residues in all 4 serotypes? We are trying to work out exactly which residues are involved.

Topic: Membrane fusion proteins
Speaker: Dr F. Rey

Goals**:** structural studies using recombinant subviral particles.

Strategies: performs structural studies using:
Class II enveloped viruses-Semliki Forest Virus (prototype), alphaviruses, and flaviviurses, with a protein shell and lipid envelope.
- Mature virions contain 3 proteins: C (capsid), E (envelope), and M (membrane).
- Flavivirus E protein is the major constituent of the virus surface and has dual functions: Binding cellular receptors and mediating low pH triggered membrane fusion.
- In contrast to alpha-viruses which have glycoproteins with split functions (E1-binding, E2-membrane fusion); E1 fusion protein, a soluble ectodomain is monomeric in solution, if the soluble monomer is put in the presence of liposomes at low pH this catalyzes the trimerization reaction; there are specific lipid requirements for this reaction (cholesterol and sphingolipids)

For flaviviruses:
- prM has a chaperone function.
- Maturation includes cleavage of prM and rearrangement of E into homodimer.
- Used this trimer to understand how it inserts into the membrane.
- A ring of 5-6 trimers to make the pore.
- EM and cryo-EM shows this ring is a cone shaped molecule.
- Post fusion conformation shows 70A projections (conical shaped).
- The E1 trimer spans the whole thickness of the lipid layer.
- The length of the trimer matched the length of the monomer.
- Plausible model to study other possible class II viruses via reconstruction on liposomes.

Comparison of viral membrane fusion proteins:

Class I (irregular shape)- Influenza, HIV examples

- Intravesicular budding, trimeric; mature protein results from proteolytic cleavage of a precursor (HA0-> HA1 + HA2 in influenza virus and gp160->gp120 + gp41).
- Fusion peptide is in general at or near the N-terminus.
- Fusogenic conformational change leads to a trimeric coiled-coil, with both N- and C-terminus at the same end of a stable protein rod.

Class II (regular)- Alphavirus and Flaviviurs examples, helical, icosahedral,

- Extravesicular budding, forms protein network at the viral surface, folds as a heterodimer with a chaperone protein (prE2/E1 in alphaviruses, prM/E in flaviviruses).
- Cleavage occurs on companion protein (pE2->E2 in alphaviruses, prM-> M in flaviviruses).
- Fusion peptide is internal.
- Fusogenic conformational change leads to a very stable homotrimer.

Other possible class II enveloped viruses:
Flaviviridiae-pestiviruses, hepatitis C virus.
Togaviridae-rubella virus.
Bunyaviruses?

Questions and answers:
Is the flexing in the E protein at the junction between domain I and II or I and III? No, we do think there is need for flexing-just need a strong interaction between the fusion protein and the membrane particle. Have you looked at the electrostatic rearrangement of the amino acids? Other discussion ensued regarding the various ultrastrucutral distances and opening of the lipid leaflet lipid to assess the porin.

Topic: The entry machinery of flaviviruses
Speaker: Dr F Heinz

Goals: Elucidation of the molecular mechanisms controlling and driving flavivirus membrane fusion.

Strategies: Combining structural information, in vitro mutagenesis, and functional analyses (including in vitro liposome fusion experiments) using the tick-borne encephalitis (TBE) virus model

Background:

- Cell entry by enveloped viruses requires fusion of the viral membrane with a cellular membrane
- Entry is triggered by receptor binding and/or low pH and mediated by viral fusion proteins (specific viral envelope glycoproteins)
- There are 2 structurally defined classes of viral fusion proteins thus far: Class I and II
- Class I proteins (prototype influenza virus hemaglutinin) are oriented perpendicular to the membrane (spike) with N-terminal fusion peptides
- Class II proteins (flavi and alphaviruses) are oriented parallel to the membrane and have an internal fusion peptide
- Both classes of viral fusions proteins exist in mature virions in a metastable conformation that is converted into an energetically more stable conformation during fusion.

Progress:

- Using in vitro liposome fusion experiments, TBE virus was shown to have the fastest fusion mechanism of all known enveloped viruses analyzed to date (fusion at 37C-and even fusion at 15C-occurs within seconds and without measurable lag phase)
- This model was used to investigate the molecular characterisitics of a class II viral fusion machinery
- The trigger for fusion activation is mildly acidic pH (pH threshold is 6.4)
- Low pH triggers structural changes required for fusion; the E proteins on the surface of the virus undergo dramatic irreversible structural changes and exhibit an oligomeric switch from dimers to trimers
- Target membrane binding requires the dissociation of the E dimer into monomers (if the dimer is covalently cross-linked no binding occurs)
- The structural changes in E required for fusion are at least a two step process consisting of its dissociation (dimer to monomer) followed by irreversible trimerization.
- Structural elements involved in membrane fusion: the highly conserved cd loop at the tip of domain II in the E protein serves as the fusion peptide.
- Low-pH induced dissociation of E dimers makes this region accessible for target membrane interactions
- Interactions of the class II viral fusion proteins of both flaviviruses and alphaviruses with target membranes involve the recognition of cholesterol and its hydroxyl group at carbon 3.
- The overall fusion process of flaviviruses, however, is relatively independent of cholesterol in the target membrane. This is in contrast to alphavirus fusion which is absolutely dependent on the presence of cholesterol.

Questions and answers:
Can these assays be used to dissect the roles of the trimer and the monomer in neutralization? Yes, that is on the list. One specific Ab, which is a good fusion inhibitor in the in vitro fusion assay (an Ab directed at the fusion peptide) does not react with native virus. It could act only internally at the level of the endosome as the epitope only becomes accessible after low pH-induced dissociation of E. Therefore, it is unlikely to be important in viral neutralization. However, we do have a whole panel of monoclonal Abs that do bind to the virus and inhibit fusion to a lesser extent. Have you done mapping studies of the trimer as it sits in the liposome (ie using proteases) to see which regions might be inserted into the membrane? No, not yet. We have done comparative studies of the full-length trimer and the C-terminally truncated form of the trimer (lipid induced). We have seen similarities but one of the differences we see is that the specific mAb that is a good fusion inhibitor recognizes its epitope only in the case of the truncated form but not in the full length trimer.

Topic: Interactions of human dendritic cells (DC) and dengue virus
Speaker: Dr M. Marovich

Goals: Review work with human DC and dengue virus regarding infection of DC and consequences of infection. Also, review data regarding human DC and antibody dependent enhancement and the identification of a DC specific molecule involved in dengue virus entry.

Strategies:

- Using blood derived myeloid (primary) human dendritic cells
- THP-1 cells (wild type cell line) or THP-1 transfected with DC-SIGN/L-SIGN for in vitro experiments;
- In vivo data from skin biopsies in a LAV recipient

Progress:

- Myeloid DC, in particular at the immature DC stage, were infected with Den 1-4
- Both lab adapted (highly passaged) and low passage isolates infected these cells
- Dengue infection of immature DC led to TNF-alpha mediated maturation (in bulk assays) although some dengue infected cells did not undergo maturation
- DC do not undergo antibody dependent enhancement using a flow based assay adaptation of the standardized K562 ADE assay, in spite of similar levels of Fc receptor expression
- DC-SIGN (CD209), dendritic cell specific ICAM-3 grabbing non-integrin, a c-type lectin, mediated DEN 1-4 virus entry into human DC
- THP-1 cells become susceptible to dengue infection only after transfection with DC-SIGN or L-SIGN (a homologous molecule expressed in liver sinusoidal endothelial cells and lymph nodes and placenta)
- Anti-DC-SIGN monoclonal Abs blocked DV infection of DC (and THP-DC-SIGN cells)
- Dengue infection of DC or THP-DC-SIGN cells results in a productive infection with the release of infectious virions into the culture supernatant
- This supernatant was capable of transmitting infection to susceptible cells and the infection was significantly inhibited in the presence of the anti DC-SIGN mAbs
- It is not clear if standard targets for dengue infection (C6/36 or VERO cells) express a functionally equivalent molecule or use another distinct pathway for infection

Questions and answers:

A reference was made to previous published primate studies which showed that skin was one of the few sites (skin and lymph nodes) from which virus could be cultured in both early and later stages of dengue infection. Regarding the lack of ADE in these DC that express Fc receptors, why not? Perhaps there is a qualitative (versus quantitative) difference in the Fc receptors. DC Fc receptors may contain inhibitory motifs (ITIMs) and do not allow "enhanced" infection. Or maybe the presence of DC-SIGN on these cells is overbearing and "masks" any increase in infection that might be offered by enhancement through Fc receptors. One way to address that question is by looking at K562 cells transfected with DC-SIGN. We now have that cell type in house and can answer the question directly. Are the DC/LC in skin the only infected cells there? It seems so. Some of the darker (pigmented) cells shown in the figure were actually melanocytes at the dermal-epidermal junction. This staining is difficult, at best, and we were only successful using the frozen sections. Did you notice any qualitative differences in the types of infections between the immature and the mature DC? Yes. The immature cells were more readily infected (30-40%) than their mature counterparts (4-5%), but there was about the same amount of virus produced in the culture supernatants suggesting that the mature DC were somehow much more efficient at virus production. This may reflect differences in the intracellular environment (i.e. pH levels) that favor virus replication.

Topic: **mAbs as a tool to evaluate specificity of dengue antibody**

Speaker: Dr J. Aaskov

Goals: characterize anti-dengue antibodies present at the start of a secondary dengue in terms of their ability to cross react and neutralize or enhance infection and to understand what in vitro neutralization tests tell us.

Strategies:

- Review variations of in vitro neutralization assays
- Propose using monoclonal antibody competition ELISAs to determine the role of cross reactive Abs in dengue virus infection.

Progress:

- Multiple dengue virus receptors exist
- Receptors expressed on cell lines are different than on susceptible cells in vivo
- Heparin sulfate receptor is the first point of contact between the virus and the cell
- Uptake and fusion into endosomal vesicles requires a 22kD oligomeric protein (under investigation)
- Neutralization occurs when Ab blocks attachment of virus to either receptor
- dengue virus receptor blocking assay described using cell lysates and nitrocellulose blotting techniques indicate that some in vitro Nabs block binding to blotted cellular proteins while others do not
- Conclusion is that there are diverse dengue virus populations and the "isolate" is a neutralizable subpopulation of that which caused disease
- Presented data showing in vivo variation of E protein gene within populations of dengue-3 in a patients serum
- Epitopes seen by cross reactive Abs are different from those recognized by serotype specific Abs
- Not all hosts produce Abs against the same dengue virus epitopes
- Collections of mAbs against the 4 serotypes of dengue were compared for neutralizing capability
- Few monoclonal Abs directed against Den-1 (7-20%) or Den-3 (6.5%) neutralize virus, but approximately 50% of the Den-2 mAbs and 30% of Den-4 mAbs neutralize virus
- MHC background (mouse strain) also influences the % of mAbs that neutralize virus
- Perhaps humans produce large amounts of cross-reactive but non-neutralizing Abs in Den-1 and Den-3 infections which could explain the tendency towards more severe disease in secondary infections ending with Den-2
- Designed a competitive blocking assay to look at the competition between polyclonal sera from patients that better assessed neutralization
- Hypothesis is that some hosts are unable to produce Abs against epitopes critical to the infection or neutralization process in vivo.

Questions and answers:

Have you seen this in vivo? We are working backwards here, first looking for cross-reactive sera, then we'll do blocking experiments. Are you doing dilutions with the competition binding assays? Yes, with human sera at 1:10 you'll see competition.

Have you ever seen enhanced binding of mAbs? Using mAbs, we usually see huge enhancement, but we do not see enhancement with polyclonal human sera.

Topic: **Dengue virus prM as a target of neutralization/infection enhancement**

Speaker: Dr I. Kurane (presented proposal for Dr. Keelapang)

Goals:

- Determine whether Abs to prM neutralize DV or enhance infection
- If so, characterize (map) the neutralization or enhancing epitopes on dengue prM proteins
- This project has been funded since August 2002 as a one-year project.
- If successful, then the subcommittee will consider an extension.

Strategies:

- This lab recently generated 5 anti-prM monoclonal antibodies: 4C1,1G1,1H10, A8, 2E11
- All monoclonals were purified from ascites and quantitated

Progress:

- All mAbs were generated after dengue 2 virus immunization, except 2E11 which was generated after DNA vaccination and were characterized by PRNT 50%
- ADE assays: use human monocytic cell lines, U937 cells, exposed to virus (lab adapted and low-passage strains) that has reacted with purified, serially diluted mAbs
- Epitope mapping: react mAbs against N-terminally truncated or point mutated prM proteins generated in vitro using a mammalian expression system; read out will be immunofluorescence (IF) staining in a human embryonic kidney cell line
- Preliminary results showed that the antigen was expressed after transfection using IF
- Checking the role of prM on protection or ADE
- Knowledge on the function and spatial distribution of these determinants may help identify important targets.

Questions and answers**:**
It is good to revisit the prM story. There good titers induced after prM exposure but is there actual protection after prM? We did not find protection after prM, although 2H2 did protect. prM is always a contaminant in in vitro Ab generation (due to contamination of virus). prM could be released as part of cytolytic effect. "M" Abs do not neutralize. There can be some low level prM expression (without spike formation) in a native looking particle, but in raw micrographs we can easily identifiy a very smooth particle from a not completely smooth one. Currently, it is unclear whether prM cleavage from mature virus is the trigger for rearrangement or if the low pH (as it moves through the trans-golgi network) itself causes the rearrangement.

Topic: **Studies on Dengue Antibody Avidity and Human Dengue Challenge Model**
Speaker: Dr W. Sun

Goals: Identification of more specific markers of protective immunity

- development of an optimized and validated assay as a marker for protective immunity following natural infection or after vaccination.

Strategies:

- Using available sera from tetravalent dengue vaccine recipients after challenge with DEN-1 or DEN-3.
- Detailed analysis of antibody responses and comparisons between individuals who were or were not protected against challenge.
- Antibody avidity will be measured using BIA-Core1000 technology (proposed).

Progress:

- Total of 20 volunteers have been challenged
- Challenge virus criteria: GMP produced, 90% induction of mild-moderate DF, viremia, seroconversion within 30-60 days by PRNT, no DHF.
- Protection is defined as prevention of clinical dengue or prevention of viremia.
- DEN-1 45AZ5 (n=6) and DEN-3 CH53489 (n=7) used most frequently.
- Assessment after challenge includes tourniquet test, CBC with differential, liver function studies, PT/PTT, chest X-ray and abdominal ultrasound.
- Reactogenicity index (RI) used to grade the severity of symptoms (fever/chills, myalgias, nausea/vomiting/abdominal pain, headache).
- Naïve DEN-1 challenge (n=6): dose 10^3 or 10^4 pfu, all developed fever, HA and most (5/6) had myalgia, rash and viremia. All developed Nabs (range 155-2992).

- Naïve DEN-2 challenge (n=3): 10^5 pfu, no fever in any, and very low RI. Nabs ranged from 28-255.
- Naïve DEN-3 challenge (n=7): 10^4 or 10^5 pfu, all had fever, headache, myalgias/arthralgias, rash, and viremia. Nab ranged from 235-5100.
- Naïve DEN-4 challenge (n=4): 10^4,10^5,or 10^6 pfu, only 1/4 with fever, all with headache, 2/4 with myalgia/arthralgia and viremia, and only one with a rash. Only 3/4 developed Nab (range 32-789).
- Challenge after tetravalent dengue vaccine (TDV): 3 groups, challenge or vaccine virus assigned, subjects and clinical investigators blinded.
- DEN-1 45AZ5 challenge (n=7; 5 post-vaccination and 2 naives) at $2x10^3$ pfu .
- DEN-3 CH53489 challenge (n=7; 5 post-vaccination and 2 naives) at 10^5 pfu.
- Inclusion criteria: 18-45 year old healthy with known flavivirus serostatus (Hep B,C HIV negative); range of time post-vaccination = 12-42 months.
- No viremia in the DEN-1 challenge group who were previously vaccinated, naives were viremic
- DEN-3 challenge 4/5 post-vaccinated volunteers were viremic (10^2-10^3 pfu) and both naives were viremic (10^2-10^4 pfu).
- TDV recipients were protected form DEN-1 challenge but not DEN-3 challenge.
- No evidence of enhanced illness or viremia in TDV recipients in DEN-3 challenge.
- Level of Nab may be important in DEN-3 challenge.
- Future plans involve the comparison of antibody responses in protected versus non-protected recipients.
- Planning to test the avidity of Ab after primary and revaccination with monovalent and tetravalent dengue vaccination, also characterize Ab isotypes, subtypes, after tetravalent vaccination and finally examine IgG subtype avidity and protection.
- These studies would be performed using BIA (Biomolecular Interaction Analysis) Surface Plasmon Resonance (SPR) technology with a gold dextran surface chip.
- Many different types of biomolecules can be detected including antibodies and viruses or viral proteins.
- Plans to monitor avidity during the maturation of the immune response and hopefully can use the information from this data to develop more specific assay to assess protection.

Questions and answers:
What do the structural people say about addressing avidity? Avidity is due to conformational changes. How do you plan to immobilize serum versus virus (i.e. capture piece of the assay). BioCore technology planned to be used in this study normally uses monoclonal antibodies, not polyclonal sera. It may be necessary to bind virus, or E protein on the chip. We plan to try anti-Fc antibodies or anti-IgG Abs to coat the surface, then overlay with volunteer's serum (capture aspect) then use either whole virus or purified envelope. This is an area that needs development. It may be difficult to evaluate the surface. It was noted that the NIH just sent out an announcement that funds are available for human immunity and technology transfer to get assays up and running that may be relevant.

Topic: **Evaluation of a live attenuated tetravalent vaccine in a non-human primate model**
Speaker: Dr K. Stittelaar

Goals: Design a cynomolgus macaque model to evaluate dengue vaccines.

Strategies:
- Reviewed prior uses of the cynomolgus model in disease systems
- Infection /vaccination /challenge protocol reviewed

- 6 animals/group, 4 groups, each group is infected with one of the four dengue serotypes
- 6-9 months later all vaccinated with a tetravalent LAV (including 6 control animals)
- 6-9 months later animals receive same dengue virus challenge (2 challenge controls)
- Initial challenge with low does virus ($5x10^5$pfu) with daily "sampling" x 10 days
- Ability to titrate up on challenge virus, if necessary
- Extensive immunomonitoring post-intervention with plasma/plasmapheresis for IgG , IgM, IgE, IgA, virus neutralization, ADE studies and development of serotype-specific assays, passive immunization experiments
- PBMC for T cell responses, RT-PCR for inflammatory markers, dengue specific RT-PCR
- Isolation of lymph nodes
- Vaccination objective is for safety determination of the tetravalent vaccine
- Challenge objective for efficacy of the tetravalent vaccine in immune-and non-immune animals
- Timing of vaccination and challenge will be determined based on Ab levels and consultation with collaborators
- Daily sampling x 10 days for serology and T cell responses, telemetry temperature probe.

Progress:

- Development of dengue virus serotype specific RT-PCR
- Development of mRNA detection system for inflammatory markers
- Generation of recombinant vaccinia viruses for immunomonitoring (Env, NS2a)
- Growing dengue challenge viruses: Den-140541 Brazil 1990, Den-2 Jamaica 1982,Den-3 PaH881 Thailand 1988, Den-4 28128 Tahiti 1987.
- Selection/pre-screening of animals
- Approval by local animal ethics committee

Questions and answers**:**
Questions centered around the design and sequence of infection, then vaccinating before the challenge; why not vaccinate first (before infection) then challenge with all 4 serotypes? Clarification regarding the vaccinia recombinants-they would be used for preparing targets cells for immune assays. One suggestion one to focus on the serology and protection and ADE issues, and to save the T cell work for another study.

Note from Dr Scott. B. Halstead: The increased viremia in YF antibody-positive compared with flavivirus-naïve individuals is interesting and is reminiscent of the enhanced seroconversions observed in YF vaccinated versus non-vaccinated individuals who were given the S-1 dengue-2 vaccine, now known to be an over-attenuated American genotype dengue-2 virus. (Bancroft et al. JID

1984; 149: 1005-1010).

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