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Washington D.C., USA
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Abbreviations and acronyms

ALT	Acute Liver Toxicity
CDC	Centers for Disease Control and Prevention
cGMP	Current Good Manufacturing Practices
CMI	Cell Mediated Immune Response
DEN	Dengue
ELISA	Enzyme Linked ImmunoSorbent Assay
FACS	Fluorescence Activated Cell Sorter
FDA	Food and Drug Administration
GMT	Geometric Mean Titre
GSK	GlaxoSmithKline
HLA	Human Leukocyte Antigen
IFN	Interferon
IND	Investigational New Drug
IL	Interleukin
IVR	Initiative for Vaccine Research
JE	Japanese Encephalitis
LAV	Live Attenuated Vaccine
NS	Non Structural
PBL	Peripheral Blood Leucocyte
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PDK	Primary Dog Kidney
PDVI	Pediatric Dengue Vaccine Initiative
PFU	Plaque Forming Unit
PIV	Purified Inactivated Vaccine
prME	Pre-Membrane Envelope
PRNT	Plaque Reduction Neutralization Test

RNA	Ribonucleic Acid
SLE	Saint Louis encephalitis
SOP	Standard Operating Procedure
TCID	Tissue Culture Infective Dose
TDR	Special Programme for Research and Training in Tropical Diseases
Th	T Helper
TNFα	Tumor Necrosis Factor Alpha
US NIH	United States National Institutes of Health
UTR	Untranslated Region
VDV	Vaccine Derived Virus
WN	West Nile
WRAIR	Walter Reed Army Institute of Medical Research
YF	Yellow Fever

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Preface

Introduction

The fifth meeting of the WHO Task Force on Clinical Trials of Dengue Vaccines was held on 11 December 2005 in conjunction with a workshop on neutralization assays organized by the Pediatric Dengue Vaccine Initiative (PDVI) and the 54th Annual Meeting of the American Society of Tropical Medicine and Hygiene.

The general objectives and expected outcomes of the WHO task force were to:

Objectives

1) Critically evaluate observational data on immune responses linked to protection against dengue disease;
2) Assess immune response quality and quantity in relation to protection;
3) Assess correlates as a proxy for protection;
4) Review readouts and methods.

Outcomes

1) Review of dengue vaccine pipeline (new clinical data were presented);
2) Recommendations to WHO to facilitate vaccine development and evaluation;
3) Discussion and recommendations on prime-boost immunization strategies for dengue vaccines;
4) Recommendations on immunological readouts and assay standardizations;
5) Meeting report.

The meeting was attended by representatives from private, academic, and government organizations with active dengue vaccine development programmes at or close to clinical stages. The plenary meeting was followed by a closed session, attended by WHO temporary advisers and participants from non-commercial entities, to identify a list of recommendations to WHO, which are attached to this report.

1. Progress with existing vaccine candidates in late preclinical or clinical development

Dr. M. Mammen presented an update for the live-attenuated Den vaccine in Thai infants that has been developed by GSK and the Walter Reed Army Institute of Medical research (WRAIR). In an initial pediatric study in Thailand involving 7 flavivirus antibody-negative 6-9 year old children who received 2 doses of formulation 17 containing DEN-1 (based on strain 45AZ5, Western Pacific), DEN-2 (S16803, Thailand), DEN-3 (CH53489, Thailand), and DEN-4 (341750, Colombia) vaccine viruses, all vaccinees in the "according to protocol" group (N=6) developed PRNT antibodies against DEN 1-4 viruses when measured 1 month following administration of the second dose. The target age for the first dengue vaccine dose is after 12 months of age, as maternal antibody may persist and reduce vaccine take in children up to 12 months of age. In a phase 1/2 randomized, controlled, double-blinded trial in 12-15 month old infants, 51 flavivirus-naïve individuals were enrolled in 3 cohorts, in compliance with FDA recommendations for an escalating cohort size. Cohort A (n=4+2) received 10% of the full tetravalent dose, while cohorts B (n=10+5) and C (n=20+10) received the full dose. The full dose vaccine was well tolerated, and no serious vaccine-related adverse events occurred. Cohort A, receiving 10% of the full dose, did not present a more advantageous safety profile. Mild to moderate redness and swelling, usually lasting one day, occasionally up to 3 days, were more frequent in vaccinees than in the control group. General solicited symptoms in vaccinees were comparable to those of the control group. At 1 month after the first dose, all 3 cohorts developed $PRNT_{50}$ titers in approximately 25% (cohort A) and 33-40% (cohorts A and B) of the vaccinees against DEN-2 and DEN-4 viruses. An anti-DEN-1 response occurred in about 5% of the infants in cohort C. At 1 month after secondary immunization, which was administered 6 months after the first dose, cohort B developed neutralizing antibodies against all 4 serotypes in 89-100% of recipients. Cohort C developed antibodies against DEN-1, -2, -3, and -4 viruses in 40%, 100%, 80%, and 94% of the recipients, respectively. Cohort A responded against DEN-1, -2, and -4 viruses in about 25%, 100%, and 75% of recipients, respectively. In cohort A, the 10% tetravalent dose was poorly immunogenic, particularly against DEN-3. Cohorts B and C showed a tetravalent response in 53.6% of recipients at 1 month after the second vaccine dose.

Discussion: The observed cohort effect (B > C) was not believed to be due to vaccine potency issues in this pilot study. Although the proprietary potency could not be provided here, the vaccine was considered stable, and the potency was what was intended. Blinded sera were sent to WRAIR for PRNT testing. Pre- and post-immunization sera were not all tested at the same time, and cohort B and C sera were tested at different times. Cohorts B and C were separated according to FDA-recommended safety recommendation to incrementally escalate cohort size.

Statistically, there remains a 3% likelihood that the different immunogenicity observed in the two cohorts was due to chance. Low-level antibody responses to DEN serotypes following tetravalent immunization might represent heterotypic immunity. In the future, it will be important to look at T-cell responses to various DEN strains. If titers following tetravalent immunization are lower than the corresponding serotype-specific titers following monovalent immunization, then interference is probably occurring. Even in the absence of demonstrable antibody following primary immunization, successful priming may result in an efficient response following secondary vaccination. Most children in this study were not viremic after vaccination. Of 34 recipients, 6 (4 after dose 1, 2 after dose 2) were viremic at a single time point (bled when febrile). The booster immunization was given at 6 months. A general concern was expressed that primary immunization which induces low levels of immunity, coincident with a lack of breadth of response, may place children at risk for wild-type DEN infection during a long interval between primary and secondary immunizations. In relation to vaccine safety and quality, it was mentioned that the vaccine seeds have been regenerated by extracting RNA from the previous seeds and transfecting vaccine-certified cells. There was some question as to whether a DEN-4 outbreak in 2004-2005 could have influenced some results in this study, particularly regarding the polyvalent antibody response following the second vaccine dose, but there were no unexpected rises in antibody or other indications of sub clinical infection. JE vaccination, which is recommended at 18 months for children, was delayed 1-2 months, so that the DEN vaccinations could be completed prior to JE immunization. It was recommended that more and earlier time points be examined for viremia. The weak immunogenicity of the 10% vaccine dose in cohort A indicated that this formulation may be close to the threshold vaccine dose. Perhaps the full-dose formulation used in the B and C cohorts were also close to the threshold dose, contributing to the immunogenic differences observed in these two cohorts. The manufacturer is working on new formulations of the vaccine.

Dr B. Guy of Sanofi Pasteur presented analyses of cellular immunity after immunization of human volunteers with VDV3, a clonal derivative of LAV3, produced in vaccine-certified Vero cells. The LAV3 candidate vaccine had been extensively tested in volunteers, and had shown significant reactogenicity in some individuals across different clinical trials. Preclinical studies with VDV3 had shown commonly accepted markers of attenuation, including smaller plaque phenotype than wild-type DEN-3 16562 virus, temperature sensitivity, reduced growth and lack of transmission in mosquitoes, a stable genotype with conserved loci associated with the original Mahidol LAV3 vaccine virus, low viremia in monkeys (peak viremia < 100 PFU/ml, viremia of < 3 days duration), dramatically reduced TNFα, IL6, and IL12 production in human dendritic cells, and higher IFN type 1 secretion, compared to the parental DEN-3 16562 virus. In summary, the preclinical studies suggested a good attenuation profile, while maintained a strong capacity to trigger innate immune responses. Fifteen flavivirus-naïve adults received 100 $TCID_{50}$ of the VDV3 vaccine. Vaccinees exhibited high reactogenicity (fever, malaise, decreased white cell counts, elevation of liver enzymes) and high viremia levels. Levels of serum cytokines were assessed during the first 2 weeks after vaccination, and interferon titres coincided with viraemia and fever. No increase of IL10 and TNFα was observed. Viruses isolated from vaccinees' sera showed no reversion at the putative VDV-3 attenuation loci. Cellular responses were analyzed, using PBMCs collected from vaccinees at days 0 and 28. Lymphoproliferation, Th1/Th2 cytokines

in the supernatant, and IFNγ/TNFα were quantitated. PBLs were stimulated with live or inactivated vaccine and wild-type virus, NS3 peptide pools (15-mers, overlapping by 11 amino acids), and individual NS3 peptides. In monkeys, immunization with a low VDV3 dose (100 PFU, same dose used in the human trial) produced viremias of higher peak titer (> 200 PFU/ml) and longer duration (> 7 days, up to day 14) than immunization with a high dose (10^4 PFU) of VDV3. The reactogenicity observed in vaccinees appears to be due to the virus itself, but the CMI results indicate that reactogenicity may have been worsened by different factors, including HLAs and insufficiently potent initial innate responses at the low dose administered. The VDV3 candidate vaccine was clearly too reactogenic, and is no longer a viable candidate. The predictive value of preclinical endpoints may be mostly negative, allowing the elimination of obviously virulent candidates. The subsequent clinical safety of candidates that are advanced cannot be predicted with certainty. For VDV candidates, it appears that even low, short-lasting viremia in monkeys is not acceptable. Other VDVs have been shown to induce no viremia in monkeys, and show good tolerance in humans. Vaccinations should be performed in sequential cohorts comprised of a few volunteers, and relatively high doses of vaccines should be used. Vaccinated populations should be more heterogeneous to be more representative.

Discussion: The original Mahidol live-attenuated DEN-3 candidate (LAV3), produced in fetal Rhesus lung cells, and from which the VDV3 candidate was derived in Vero cells, was attenuated in Thai volunteers. Changes in plaque size have been noted following replication of the LAV3 vaccine virus in Vero cells. It was mentioned that requiring an absence of viremia in monkeys as a necessary correlate of attenuation for vaccine candidates is probably expecting too much. Such a stringent safety requirement may be necessary only for certain vaccine candidates, such as the VDV3 virus. The desire was expressed to have a gene array expression pattern that predicts the attenuation profile of a candidate virus.

Dr. N. Kanesa-thasan presented a randomized, double-blind, placebo-controlled Phase 1 study of the safety and immunogenicity of the tetravalent live-attenuated ChimeriVax-DEN vaccine and the YF-Vax vaccine in adult volunteers (Study H-050-002). These two vaccines were administered subcutaneously in two groups of 33 volunteers, and a 33-volunteer placebo group was included as a control. The tetra-ChimeriVax-DEN vaccine contained $10^{4.1}$, $10^{3.3}$, $10^{4.0}$, and $10^{3.4}$ $TCID_{50}$ of the component DEN-1, -2, -3, and -4 viruses, respectively (target dose of 10^4 $TCID_{50}$ per serotype). All 99 volunteers in all three experimental groups received tetra-ChimeriVax-DEN at 6 months following primary immunization. One or two doses of tetra-ChimeriVax-DEN presented a safety profile that was intermediate between YF-Vax and the placebo (saline) recipients. Transient viremia was more common in tetra-ChimeriVax-DEN recipients than in the YF-Vax group. The tetra-ChimeriVax-DEN vaccine produced a biphasic viremic profile at day 7 (viremia detected in 45% of recipients, maximum titer of about 140 PFU/ml) and day 15 (approximately 13% of recipients, 85 PFU/ml). Viremia was comprised predominantly of the DEN-3 and DEN-4 components of the tetravalent vaccine. Following primary immunization with YF-Vax, a single viremic peak (maximum titer of about 80 PFU/ml) occurred at day 5 in approximately 11% of the recipients. All 3 experimental groups received the tetra-ChimeriVax-DEN vaccine at approximately 6 months after the primary or placebo immunization.

A biphasic viremia, comprised predominantly of DEN-3 and DEN-4 components, was again noted. For the placebo group, about 36% of the volunteers were viremic (maximum titer of about 115 PFU/ml) at day 7 after the second immunization, and about 4% were viremic at day 13 (30 PFU/ml). In the YF-Vax group, about 14% of volunteers were viremic (about 250 PFU/ml maximum) at day 7, and about 15% were viremic (about 50 PFU/ml) at day 13. Neutralizing antibody results were presented for sera obtained at day 30 after primary immunization. Volunteer sera were tested against the homologous DEN strain and two other strains of each serotype in PRNTs. In the tetra-ChimeriVax-DEN group, 67.7%, 64.5%, 93.55, and 80.6% of the recipients seroconverted ($PRNT_{50} \geq 1:10$) to DEN-1, DEN-2, DEN-3, or DEN-4 virus, respectively, at day 30 after primary immunization. Seroconversion to at least one, two, three, or all four DEN serotypes occurred in 96.8%, 87.1%, 67.7%, and 32.3% of these volunteers, respectively. In the YF-Vax group, 100%, 96.1%, 92.3%, and 69.2% of the recipients seroconverted to at least one, two, three, or all four DEN serotypes, respectively. In summary, the tetra-ChimeriVax-DEN vaccine elicited seroconversion against at least one DEN serotype in 96.8% of the volunteers, and at least trivalent seroconversion occurred in two-thirds of the subjects after the first dose of vaccine. Rather than being inhibitory to the response to the tetra-ChimeriVax-DEN vaccine, prior YF immunity may have increased the response. Immunogenicity data from the second dose of tetra-ChimeriVax-DEN vaccine were not yet available for presentation.

Discussion: Immunofocus assay was used to identify the DEN serotypes involved in viremias. The individual ChimeriVax-DEN viruses served as the homologous DEN strains in the PRNT tests. It was commented that geometric mean titers should reflect seronegatives, not just seropositive titers above a certain threshold. In terms of the presence of pre-existing YF immunity in volunteers prior to this study, two individuals in each of the three groups had previous flaviviral immunity to JE, YF, or DEN – this has not been broken out yet. Each of the component viruses in the tetra-ChimeriVax-DEN vaccine exhibited different replication traits. All of the monovalent vaccines produced viremia in monkeys. It was pointed out that quantitative virology is important to determine which dose and viremic levels are well tolerated. It might be useful to test larger doses, perhaps increasing the targeted 4444 $\log_{10}$ PFU dose regimen used in this study to 5555 $\log_{10}$ PFU, to determine if the lower dose regimen represents threshold doses that permit expression of viral interference. The $10^{3.3}$ and $10^{3.4}$ PFU doses used for the DEN-2 and DEN-4 virus-specific components here were quite low. Variable virus-specific responses might be due to the characteristics of each component virus, rather than necessarily evidence of interference among the viruses. No evidence of rash was observed in any of the vaccinees, even though requesting such information was part of the SOP. It was pointed out that mild rashes might not be noticed without direct observation of the trunk area. There was some question as to whether occurrence of rash with YF vaccination is unusual. It was pointed out that the presence of a rash indicates a complete DEN cycle of infection, where central monocytes migrate to the skin. YF-based chimeric vaccines might exhibit different tissue tropisms, which might account for different post-vaccination clinical observations, relative to vaccinees receiving a DEN vaccine or a DEN-based chimeric virus vaccine.

Dr. A. Durbin provided an update on the clinical evaluation of live-attenuated rDEN vaccine candidates in humans. This was a collaboration between US NIH and Johns Hopkins University. The candidate DEN vaccine viruses developed by NIH involve two live-attenuated approaches based on (1) incorporating into DEN-1, DEN-2 and DEN-3 viruses the previously described Δ30 deletion in the 3'-untranslated region of DEN-4 virus (rDEN4Δ30), resulting in rDEN1Δ30, rDEN2Δ30, and rDEN3Δ30 viruses, and (2) chimeric DEN-4/DEN-1, -2, -3 viruses expressing the prM/E gene region of DEN-1, -2, or -3 virus in the attenuated genetic background of the rDEN4Δ30 virus. [rDEN4Δ30]: Four cohorts, 20 healthy volunteers each, received a single subcutaneous dose of 10^1 ,10^2, 10^3, or 10^5 PFU of the rDEN4Δ30 virus. None of the volunteers developed systemic illness. Low viremias (mean peak titer maximum of 16 PFU/ml in viremic individuals) were detected in 7-14 recipients of each group. Fever was either mild or most often absent, neutropenia occurred in 3-5 individuals in each group, and increased ALT occurred in 5 individuals of the 10^5 dose group and 1 recipient in the 10^3 dose group. A rash, usually asymptomatic and not generally noticed by the volunteer until it was identified upon examination, was observable in 10-16 volunteers per group. A mild pruritis occurred in 3 volunteers at the 10^2 dose. Mean reciprocal neutralizing antibody titers for the four dose groups ranged from 139–567 at 28 days and 129-399 at 42 days post-immunization. A 100% seroconversion rate and a robust antibody response were observed in each group, even at the 10^1 PFU dose. [rDEN1Δ30]: Based on the rDEN4Δ30 dose titration in volunteers, 20 volunteers received a single 10^3 PFU dose of the rDEN1Δ30 vaccine subcutaneously. No fever, ALT elevation, or systemic illness was observed. Nine recipients developed low viremias of <10 PFU/ml average titer. Neutropenia occurred in 8 volunteers. A macular papular rash (not a typical DEN rash) occurred only over the trunk area, with onset at 10-14 days post-immunization, in 8 volunteers. PBMCs were collected every other day. Increased concentration of monocytes at about 10 days was predictive of who would develop a rash. The rashes were less prominent than following immunization with rDEN4Δ30. A seroconversion rate of 95%, and robust mean neutralization titers of 919 (range of <10 – 7778), 768 (<10 – 5923), and 303 (11-1043) were measured at days 28, 42, and 180 days after immunization, respectively. This compared with mean titers of 139 (5-2365) and 129 (12-1222) at days 28 and 42 for the previous rDEN4Δ30 study. Skin biopsies were taken from consenting volunteers. [rDEN2/4Δ30]: Twenty volunteers received a single 10^3 dose of this vaccine. Neither systemic illness nor fever was evident in the recipients. Viremias were detected in 9 volunteers (mean titer < 10 PFU/ml), increased ALT occurred in 3, neutropenia occurred in 7, and asymptomatic rash occurred in 7 volunteers. Rashes were similar to those following the rDEN1Δ30 vaccination, and occurred on the trunk at 10-14 days. Increases in circulating monocytes were again observed for these individuals. Seroconversion was evident in 100% of the 18 volunteers analyzed to date, with mean neutralizing titers of 309 (<10 – 1669) and 467 (42-3799) at days 28 and 42, respectively. Skin biopsies were taken from consenting volunteers around peak of rash. Future plans include the development a second generation of rDEN4Δ30 viruses with engineered mutations in NS5, expected to reduce ALT. Clinical trials of two of these candidates are planned for early 2006. The rDEN3/4Δ30 will be characterized. The investigators will proceed with the tetravalent vaccine formulation, likely to include rDEN1Δ30 + rDEN2/4Δ30 + rDEN3/4Δ30 + rDEN4Δ30. Two dose studies are planned to define the best interval for immunization.

Discussion: No decreases in platelets were observed. African American individuals (more than 50% of the volunteers in the trial) are more likely to become neutropenic. Pruritic itching, observed in earlier DEN vaccine trials, was only observed in three volunteers in this study. Skin biopsies were taken to look for virus in the skin. Such biopsies are important, as we are still in the dark as to where wild-type DEN viruses replicate. It was suggested that probing for viral replication should be done, not just antigenic engulfment by cells. It was considered that small, transient elevations of ALT may be permissible in a DEN vaccine. In the reported trial, all volunteers were seronegative to YF, WN, SLE, and JE flaviviruses. Because a tetravalent DEN vaccine will be used in people primed with DEN, it will be important to test vaccine candidates in volunteers with prior DEN flaviviral immunity.

Dr R. Kinney provided a summary of the preclinical development of the CDC/Mahidol chimeric DEN-2(PDK-53)/DEN-1, -3, and -4 candidates expressing the prM/E genes of wild-type DEN-1 16007, DEN-3 16562, and DEN-4 1036 viruses in the attenuated genetic backgrounds of the PDK53-E and PDK53-V variants of the Mahidol candidate DEN-2 PDK-53 vaccine virus. Both PDK-53 variants contain experimentally identified attenuation loci at nucleotide 57 of the 5' noncoding region (5'NCR-57) and at amino acid residue NS1-53. The PDK53-V variant possesses an additional experimentally identified attenuation locus at NS3-250. The chimeric viruses retain the *in vitro* phenotypic markers (small plaque size, temperature sensitivity, poor replication in C6/36 mosquito cells) and attenuation of neurovirulence for newborn mice that characterize the attenuated DEN-2 PDK-53 viral vector. In collaboration with Sanofi Pasteur, the chimeric PDK53-E-based viruses have been tested in cynomolgus monkeys in monomeric and tetravalent formulations. The chimeric PDK53-V-based viruses have been tested in tetravalent formulation only. The monomeric-E candidates elicited high neutralizing antibody titers. The lowest geometric mean titers (1:48 after primary immunization, 1:196 after boost) were in response to the chimeric DEN-2/4-E candidate. The chimeric tetravalent-E and -V formulations elicited neutralizing antibodies against all four DEN serotypes, although titers versus DEN-3 and DEN-4 viruses were low, indicating that interference was probably occurring. In the monkey studies, viremias ranged from undetectable (< 5 PFU/ml) to 215 PFU/ml, except for one monovalent DEN-2 PDK53-E recipient which developed viremia of 1500 and 900 PFU/ml at days 8 and 9 post-immunization, respectively, and one monovalent DEN-2 PDK53-V recipient which had a 580 PFU/ml titer at day 5 post-immunization. Viremias were generally lower following immunization with the tetravalent-E formulation than with the tetravalent-V formulation. To date, no variable-dose tetravalent formulations have been tested. The chimeric viruses constructed in the PDK53-V genetic background have been selected as the most viable set of candidates to move forward, based on (a) the presence of all three well-characterized DEN-2 PDK-53 virus-specific genetic markers of attenuation in each viral component of the candidate tetravalent-V vaccine, (b) the additional contribution of the NS3-250-Val locus to the attenuated viral phenotypes of small plaque-size, temperature-sensitivity, and attenuation of neurovirulence for newborn mice, and (c) the increased genetic stability of the 5'UTR-57-T locus of attenuation in the chimeric-V viruses, relative to the chimeric-E viruses, following ten serial passages in Vero cell culture. The chimeric viral phenotypic markers of attenuation result predominantly from their shared DEN-2 PDK-53 genetic background containing attenuation loci in nonstructural regions of the genome, and partially from the chimeric constructions per se, as shown by direct comparison with chimeras constructed in the wild-type DEN-2 16681 genetic background. The chimeric viruses replicate to

$10^{6.7}$ – $10^{7.3}$ PFU/ml in Vero cell culture. The preclinical results appear to warrant further development and testing, and a commercial partner is being sought to advance these vaccine candidates. Discussions are currently being pursued with InViragen of Fort Collins, Colorado.

Discussion: Although the DEN-2 PDK-53 prM-29 Asp-to-Val mutation, which has been eliminated in chimeric viruses expressing heterologous prM/E proteins, has not been shown to have a significant attenuating effect in vitro or in mice, this locus may be important in humans. The PDK53-E and PDK53-V nomenclature tends to cause some confusion.

Dr. B-A Coller, representing Hawaii Biotech, discussed preclinical results for Hawaii Biotech's recombinant subunit DEN vaccine developed in the Drosophila S2 expression system. This system is capable of producing commercially viable quantities of native-like protein. Preclinical results of a subunit vaccine containing 80%E in combination with NS1 (abbreviated as E/NS1 in this summary) were presented. The E protein is associated with virus neutralization, cell fusion, and binding to virus receptors. NS1 is implicated in cell-mediated immune responses and antibody-mediated protective responses. The E/NS1 vaccine is expected to induce a higher level of protective immunity than either 80%E or NS1 subunit vaccine alone. Hawaii Biotech has determined that immunoaffinity purification of the proteins is critical to maintaining their antigenicity. Suckling mice vaccinated with DEN-2 E/NS1, containing 5 µg of each viral protein, showed 100% survival against DEN-2 viral challenge, versus 10% survival for unimmunized mice. Immunization with 80%E alone resulted in 80% protection. After two vaccinations in nonhuman primates, E/NS1 elicited a greater percentage of $CD3^{+}IFN\gamma^{+}$ cells than did two immunizations with 80%E alone. In a pilot study involving four experimental monkey groups, 2 Rhesus macaques per group, 4 doses of four formulations of the tetravalent-E/monovalent(DEN2)-NS1 vaccine (Group 1: 3 µg[of each DEN 1-4]/0.3 µg[NS1] in 10 µg each of adjuvants A and B; Group 2: 3 µg/0.3 µg in 50 µg each of adjuvants A and B; Group 3: 1 µg/0.1 µg in 60 µg of adjuvant D; Group 4: 5 µg/0.5 µg in 60 µg of adjuvant D) were administered over the course of 102 days. The adjuvants, which were well tolerated, were novel compounds not yet registered for human use. The safety profiles of all 4 formulations were good. Groups 2 and 3 showed the most consistent robust neutralization responses. One animal in each group was challenged with either DEN-2 S16803 or DEN-4 341750-Carib virus after the fourth tetra-E/NS1 vaccine dose. A weak anamnestic increase in neutralizing antibody response occurred in groups 2 and 3, possibly indicating protective immunity, whereas stronger secondary responses occurred in Groups 1 and 4. In study 1, also involving Rhesus macaques, the most promising tetra-E/NS1 formulation (1µg/0.1 µg in 60 µg of adjuvant D) from the pilot study was compared to immunization with tetra-80%E alone (1 µg each of DEN 1-4) in 60 µg of adjuvant D. Animals were vaccinated at days 0, 60, and 120, and challenged 6 months after the last immunization. *In vitro* analyses were ongoing. No significant toxicity or reactogenicity occurred with the vaccine, adjuvant, or combination of the two. Study 2, planned to start in early January 2006, has been designed to test tetra-E/NS1 (1 µg/0.1 µg) in formulation with either 0.5 mg or 2.0 mg of a different adjuvant, designated adjuvant E. The immunization and challenge schedule have been designed as in study 1. Currently, cGMP manufacture of the vaccine antigens is being performed at Cobra Biomanufacturing in the United Kingdom, with fill and finish of the vaccine and adjuvant scheduled for the end of 2006. Hawaii Biotech plans to file for an IND in February 2007, and is planning to initiate Phase 1/2 clinical trial for March 2007.

Discussion: Hawaii Biotech is switching to WHO standard reagents for all of its PRNT assays. While not being able to share details on adjuvant E, Dr Coller re-stated the excellent safety profile the product has shown to date. In terms of manufacturing, Cobra Biomanufacturing, which is experienced in monoclonal antibody work, is investigating reproducibility of cell growth for scale up for manufacturing. For the Phase 1 trial, a 2-vial approach to investigate escalation of antigen and adjuvant is planned. The goal is to develop a single-vial vaccine containing the DEN 1-4 80%E and DEN-2 NS1 antigens. A third dose of vaccine may increase the duration, if not the titer, of the immune response, but the assessment of the durability of the response will require further studies.

Dr. R .Putnak of WRAIR presented results of a candidate dengue-2 purified inactivated virus (PIV) vaccine which might serve as an effective backup to a live-attenuated vaccine or comprise a component of a prime-boost vaccination strategy. DEN 1-4 cGMP vaccine lots have been produced in certified Vero cells in serum-free medium at WRAIR. A DEN-2 PIV has been successfully tested in a nonhuman primate model (data published). Virus was concentrated by ultrafiltration of infectious medium, purified on sucrose density gradients, inactivated with 0.05% formalin for 10 days at 22°C, combined with adjuvant, and bottled. Protein concentrations of about 12 µg/ml were consistent from lot-to-lot preparations of DNE-2 PIV. Adjuvants were produced and vaccines were formulated at GSK. Evaluation of the PIV vaccine in monkeys was performed in collaboration with GSK and Hawaii Biotech. Rhesus macaques received 5 µg of DEN-2 PIV in formulation with one of five adjuvants (Alum, AS04-OH, AS04-PO4, AS05, AS08) by intramuscular injection at days 0 and 90. Animals were challenged at day 160. All vaccines were non-reactogenic and stimulated neutralizing antibodies after one or two doses. Antibodies persisted to the day of challenge. PIV in adjuvants AS05 and AS08 elicited the highest $PRNT_{50}$ titers (approximately 1:5012 after the booster and before challenge), but the most stable neutralizing titers (peak of about 1:630) were elicited by the live-attenuated DEN-2 PDK-50 virus immunization control group. Post-challenge viremia was not detected in sera of monkeys immunized with PIV/AS05, PIV/AS08, or PDK-50. However, there was no correlation between higher $PRNT_{50}$ titers and levels of viremia assayed by Taqman. Only the PDK-50 vaccine appeared to induce sterile immunity, as indicated by the lack of measurable anti-NS3 antibodies, in all three monkeys of the group. In each of the PIV groups, one monkey in each group responded to virus challenge by developing measurable anti-NS3 antibody. Future plans include trying to improve PIV yields by using alternative cell lines for cGMP production, produce and test tetravalent PIV formulations, determine safety profiles of newer, more immuno-stimulatory adjuvants to replace Alum, determine the significance of Taqman-detectable viremia, and investigate the significance of the less stable neutralizing antibody titers following immunization with PIV versus the live-attenuated DEN vaccine.

Discussion: The differences in viremia levels determined by conventional plaque assay versus rapid genetic Taqman assay may be due to neutralized virions not being cleared from the circulation as rapidly. Sterile immunity should not be a standard of achievement for DEN vaccines, because such level of immunity is usually extremely difficult to achieve, and even live-attenuated vaccines generally do not elicit sterile immunity. In terms of protection of a previously immunized individual against infection with wild-type DEN virus, how much reliance should be placed on long lasting levels of circulating neutralizing antibodies, versus development of a secondary

response in the presence of waning antibody levels? This important question currently remains unanswered, but the very short time periods between the bite of a DEN virus-infected mosquito and viremia production and clinical response was highlighted. The potential use of dengue PIV vaccines in a prime boost regimen was highlighted, leading to the subsequent agenda item.

Dr. S. Thomas of WRAIR presented heterologous prime boost vaccination strategies for dengue. Repeat immunization with an antigen presented in the same vehicle may be effective for boosting the humoral response, but may be inefficient in inducing CMI responses due to primary immunity impairment of antigen presentation and inflammatory signals. A heterologous prime boost (He P/B) strategy, where the boosting antigen is delivered in a different vehicle, may generate higher levels of T cell memory and improved immunogenicity after primary vaccination. In addition, antibodies of higher avidity antibody might be induced in the absence of clonal expansion to the primary vehicle antigen. The Naval Medical Research Center conducted three different He P/B strategies for a tetravalent DEN vaccine in Rhesus macaques (4 per group). The vaccines included: [1] tetravalent DNA vaccine (prM/E, C-terminally modified with LAMP, administered by biojector) at day (-30), followed by TDNA at day 0 and then tetravalent live attenuated PDK-passaged virus (10^5 PFU per serotype, subcutaneously) at day 60; [2] tetravalent purified inactivated vaccine (1 µg per serotype) at day 0, followed by TLAV at day 60; and [3] TLAV at day 0 followed by TLAV at day 60. Monkeys were challenged at 6 months after the day-60 booster. Animals developed high $PRNT_{50}$ GMT titers at day 90 (30 days after the booster immunization) in all three groups, with approximate reciprocal anti-DEN-1/2/3/4 titers of TDNA_TDNA_TLAV: 820/2510/450/1240, TPIV_TLAV: 750/2250/320/2260, and TLAV_TLAV: 600/2420/250/1600. Titers declined over time. By day 240 (180 days after booster), reciprocal anti-DEN-2 titers had fallen to approximately 310-670, while the anti-DEN-1, -3, -4 titers fell to < 250. The highest PRNT titers (GMT DEN-2 titer =DEN-4, >DEN-1, >DEN-3 in all three groups) occurred 30 days after the TLAV booster. The day 240 titers were somewhat better for the TPIV_TLAV group. Except for the anti-DEN-2 response, which was > 250 at day 30 in the TPIV_TLAV group and at days 30 and 60 in the TLAV_TLAV group, the anti-DEN $PRNT_{50}$ responses were low at days 30 and 60 in all three groups. The He P/B strategy has been encouraging in non-DEN animal and initial human studies. The DEN monkey data reported here are encouraging. Protective efficacy data are pending for the post-challenge results. Future plans include Phase 1 human monovalent testing of the He P/B strategy, using DNA and PIV vaccines.

Discussion: Without the TLAV boost, little neutralizing antibody was seen following DNA vaccination, although a good boost response occurred after primary administration of TDNA vaccine, followed by secondary vaccination with the TLAV vaccine. Post-challenge results were not presented – viremia determinations are in progress. A TPIV_TLAV HE P/B immunization strategy might permit a greater degree of TLAV replication with a more robust boost in neutralizing antibody titer, relative to the TLAV_TLAV strategy where replication of the secondary TLAV dose may be inhibited.

2. Development and standardization of PRNT assays

Dr. A. Barrett , chair of the WHO steering committee on flavivirus vaccines, opened this session by highlighting the importance of highly reproducible, validated assays to determine levels of neutralizing antibodies for late stage clinical trials. Surrogate markers for protection against DEN viral infection are also needed. Unique neutralization assays are used by each laboratory and manufacturer, hereby rendering the comparison of results between laboratories very difficult. Therefore, methods and reagents should be harmonized to facilitate inter-laboratory comparison of data and regulatory interactions. This might include the provision and use of standard reagents, such as cells and virus seeds, as well as guidelines for techniques, reagents, and methods of analysis. Vaccine developers were solicited to give their views on the perceived needs and avenues towards harmonization of assays, as well as on the role the public sector could take in facilitating this task.

Dr. Coller stressed the need for highly reproducible, validated assays to support late stage clinical trials and efforts to establish a surrogate marker for protection. While each manufacturer/ laboratory may have unique assay and standardization of approaches, general methods, and whenever possible reagents (e.g. WHO cell and virus seeds) will facilitate comparison of data and regulatory interactions (recognized test method). A "points to consider" type document could provide guidance on key technical aspects (such as methods of production/QC of viral working seeds, cell banks, methods of analysis, etc) to harmonize as much as possible/assure quality of data. She also mentioned opportunities for collaboration in development of standardized high throughput assay as work was still in relatively early stages.

Dr. Bruce Innis (GSK) summarized the manufacturers need to correlate test results with clinical protection. He mentioned that GSK is working on a micro neutralization test, that would permit the high throughput needed for large scale clinical trials. That test should correlate well with the conventional PRNT, while having better reproducibility and accuracy. He stressed that companies would develop their own tests, but such methods should employ standard cells and virus strains, and protocols and their validation status should be in the public domain. In his view, WHO can play important roles by providing written guidelines, building data-driven consensus, and identifying and providing reference sera of low and medium potency for assay validation. Vaccine developers, on their side, could contribute by developing and validating tests that use appropriate viruses, cells, and methods of data reduction, and by supporting the relevance of protocols with clinical data.

Dr. Denis Crevat (Sanofi Pasteur) underlined that standardization of neutralization assays is needed for data comparisons and development of immunological correlates of protection. More efficient second generation assays must be developed, because the conventional PRNT is too labor intensive, time consuming, and serum sample consuming for routine testing in large scale Phase 3 trials. There is need for assays to detect homotypic and heterotypic neutralization responses, so as to be able to assess the serotype-specific immune response and the specific infecting DEN serotype. Efforts should continue to harmonize test results obtained in different laboratories. Use of nonessential reagents, such as complement, should be avoided. Other considerations include viral plaque staining versus vital dye staining of cells, possibly providing efficiencies of automated plaque counting, recommended method for titer calculation, use of appropriate standardized controls for run and results validation, automation of microtiter methods with 96/384 well plate formats, and development of new tests based on FACS, focus neutralization, ELISA, and PCR.

Discussion: There was general consensus that there is a great need for a validated assay for DEN neutralizing antibodies. It was noted that the conventional PRNT has never been validated. As important as assay validation was the need for harmonization. While assay validation is the responsibility of the vaccine developer, the public sector and the research community can greatly contribute to this task, and at the same time provide guidance for harmonization of methods. Based on previous experiences from a WHO collaborative study to identify candidate international reference reagents, it was suggested that a two pronged approach should be adopted: (1) the provision of serum samples suitable for assay validation, and (2) the development of a guidance document for the gold standard assay PRNT. In relation to (1), adequate volumes of quality human reference sera are needed to serve as reference reagents. Different potential sources of sera were discussed. While sera from vaccinees could be acceptable and represent a convenient source, sera resulting from wild-type DEN infection should be the gold standard. In particular clonal vaccines may produce clonal antibody responses not suitable for reference purposes. A large volume of serum can be obtained from an individual by plasmaphoresis. A validation panel should contain different natural antibody titres, from low to high, and suitable control panels with sera for WN, SLE, YF and JE should be included. In relation to (2), the guidance on PRNT, a document should be assembled that discusses the critical variables of the assay, including procedures, reagents and interpretation of results . There was no consensus for the development of a uniform standard protocol, however. A small working group should be set up to move forward the issues of identifying and obtaining reference reagents and harmonization of test methods. PDVI should partner with WHO to launch and conduct this process. The development of calibrated reference sera should be pursued once assay validation and guidance document be on track.

3. Recommendations

1. The task force meeting was considered a very successful venue for interaction among vaccine developers. Participants recommended the establishment of additional communication platforms including IVR, PDVI, TDR (Special Program for Research and Training in Tropical Diseases) and vaccine manufacturers.
2. Consideration should be given to improve information by means of continuing electronic commentaries. A diagnostic DEN working group could be formed and use a web portal to post neutralization test information and protocols.
3. Panels of sera should be established suitable for dengue neutralization test validation. Requirements for these sera should be developed in close consultation with vaccine developers. Synergies with TDR/PDVI efforts to validate acute dengue tests should be sought.
4. In close collaboration with vaccine developers, and guidance document on dengue PRNT should be produced.
5. For the purpose of these planned activities, an ad hoc committee should be constituted with strong representation of vaccine developers.
6. The development of calibrated international reference reagents remains a priority and should be pursued together with the efforts on assay validation and method harmonization.
7. Vaccine developers and academic groups should support and interact with that group, particular in relation to the identification of suitable serum samples.
8. While no formal collaboration on innovative assays, such as high throughput assays has been recommended, regular review of progress in this field should be conducted.
9. The discussion of the prime boost strategies was considered informative, but no specific recommendations could be made at that time. Progress in that area should be reviewed regularly.

Annex 1:
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The World Health Organization has managed cooperation with its Member States and provided technical support in the field of vaccine-preventable diseases since 1975. In 2003, the office carrying out this function was renamed the WHO Department of Immunization, Vaccines and Biologicals.

The Department's goal is the achievement of a world in which all people at risk are protected against vaccine-preventable diseases. Work towards this goal can be visualized as occurring along a continuum. The range of activities spans from research, development and evaluation of vaccines to implementation and evaluation of immunization programmes in countries.

WHO facilitates and coordinates research and development on new vaccines and immunization-related technologies for viral, bacterial and parasitic diseases. Existing life-saving vaccines are further improved and new vaccines targeted at public health crises, such as HIV/AIDS and SARS, are discovered and tested *(Initiative for Vaccine Research).*

The quality and safety of vaccines and other biological medicines is ensured through the development and establishment of global norms and standards *(Quality Assurance and Safety of Biologicals).*

The evaluation of the impact of vaccine-preventable diseases informs decisions to introduce new vaccines. Optimal strategies and activities for reducing morbidity and mortality through the use of vaccines are implemented *(Vaccine Assessment and Monitoring).*

Efforts are directed towards reducing financial and technical barriers to the introduction of new and established vaccines and immunization-related technologies *(Access to Technologies).*

Under the guidance of its Member States, WHO, in conjunction with outside world experts, develops and promotes policies and strategies to maximize the use and delivery of vaccines of public health importance. Countries are supported so that they acquire the technical and managerial skills, competence and infrastructure needed to achieve disease control and/or elimination and eradication objectives *(Expanded Programme on Immunization).*

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