2003 REPORT OF THE STEERING COMMITTEE ON DENGUE AND OTHER FLAVIVIRUSES VACCINES
Including

MINUTES OF THE SC MEETING

WHO, Geneva, 2-3 April 2003

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1. CONTEXT OF THE STEERING COMMITTEE MEETING

Dr M.P. Kieny introduced the Steering Committee (SC) meeting by stating that the objectives for the meeting are to evaluate progress in the following areas: (1) dengue vaccines that are in early stages of development and pre-clinical evaluation, (2) research related to the assessment of efficacy and immunogenicity of JE vaccines and (3) vaccines and vaccination against other flaviviruses. The anticipated outcomes are to define research priority for further WHO activity, and recommend new research projects for support in 2003-4.

No progress in clinical trials of dengue vaccines was included in the agenda of the 2003 SC meeting. It is based on the fact that WHO/IVR has established the Task Force for clinical trials of dengue vaccine and this Group is responsible for scientific advice on the next steps of clinical trials paying special attention to the vaccine safety. The previous TF meeting was held in November 2002 in Denver. The next one will be convened in December 2003 probably in conjunction with the annual meeting of the ASTMH.

WHO decided to extend the area of the SC research by including some other flaviviruses vaccines of high priority for developing countries. The SC will consider yellow fever, West Nile and tick-borne encephalitis vaccines as potential components of the future SC activity.

2. PLAN OF ACTIVITY

2.1 Strategic Plan

**DENGUE**

A. Overall objective

The development of safe, effective and inexpensive vaccines for dengue (DEN) with the prospect for integration in the national immunization programmes (EPIs)

B. Research Priorities

- Use of infectious clone technology to study viral pathogenesis and immunity and to construct genetically well defined mutant viruses as potential vaccines and challenge viruses.
- Investigation of alternative biotechnological approaches (e.g. nucleic acid based vaccines, recombinant – expressed proteins) for the development of DEN candidate vaccines.
- Research aimed at evaluating, comparing and then optimizing the protective effects of candidate vaccines in current or newly developed animal model systems with the special attention to the monkey model.
- Facilitate the development of clinical and laboratory based field sites for the study of dengue infection and dengue vaccine safety and efficacy.
- Research on the clinical evaluation of safety, immunogenicity and efficacy of candidate vaccines.
- Research on the role of humoral and cellular immunity in protection of dengue virus infection.
JAPANESE ENCEPHALITIS

A. Overall objective:

- The development of a second generation Japanese encephalitis vaccine that is safer, requires fewer doses, and is more amenable to integration into the EPI in developing countries.

B. Research priorities:

- Evaluation of efficacy of JE vaccines against JE virus strains circulating in different geographic regions.
- Standardization of neutralizing assays for evaluation of JE vaccines in field trials.

WEST NILE

- Studies to evaluate the need of West Nile vaccine in developing countries including monitoring of WN epidemics.
- Evaluation of the role of antibody and T cell immune systems in protection against West Nile virus infection.

YELLOW FEVER

- Studies on safety of yellow fever vaccines, including potential of neurotropism and viscerotropism and possible human genetic elements that may predispose vaccinees to adverse events.

TICK BORNE ENCEPHALITIS

- Assessment of efficacy of currently available vaccines against three existing subtypes of TBE virus.

2.2 What has been achieved in the last two years?

Dengue

Ongoing or completed projects

- Development of standardized neutralization test for dengue virus. Dr R. Putnak (USA), Dr M. Ferguson (UK), Dr I Kurane, (USA).
- Preparation of anti-dengue reference human serum. Dr M Ferguson (UK), Dr S. Yoksan (Thailand).
- Development of standardized protocol for evaluation of dengue vaccines in monkeys including repository of reference and working materials. A. Osterhaus (The Netherlands).
- Characterization of protective neutralizing antibodies induced by vaccination with tetravalent dengue vaccine. Dr W. Sun (USA).
- Dengue virus PrM as target of neutralization/infection enhancement. Dr Keelapang Poonsook (Thailand).
- Monoclonal antibody competitive ELISA to determine the role of cross-reactive antibodies in dengue virus infection. Dr J Aaskov (Australia).

**Outcomes**

- Evaluation of four live attenuated tetravalent vaccines in phases I and II clinical trials.
- Considerable progress in definition of T cell epitopes on prM, E, NS1 and NS3 proteins of dengue viruses.
- Increased understanding of mechanisms of a) stimulation of T cell immune responses and b) attenuation of dengue viruses.

*Japanese encephalitis*

**Ongoing or completed projects**

- Specificity of human T cell responses to Japanese encephalitis viruses. Dr S. Green (USA).
- Contribution of genotypic variation to the degree of protection conferred by JEV vaccine-induced immunity. Dr A.Barrett (USA).
- Improving clinical endpoint for phases I and II clinical trials of JE candidate vaccines. Dr S. Yoksan (Thailand).
- Development of JE standard reagents. Dr M. Ferguson (UK).

**Outcomes**

- Three Vero cell-derived inactivated vaccines and YF/JE chimeric vaccine are at phases II and III clinical trials.
- Repository of well characterized JEV strains established.
- Important information on specificity of human T cell immune responses to JE virus.
- Neutralizing activity of sera from people immunized with different JE vaccines against different genotypes of JE virus identified.

2.3 **Future plan of action for 2003**

*Dengue*

Studies recommended being initiated/carried out in 2004-2005:

- Assess the role of humoral and cellular immunity in protection against dengue virus infection.
- Evaluate the role of the first flavivirus infection on manifestation of the secondary flavivirus infection.
- Develop infectious clone-derived dengue viruses for challenge studies.
- Initiate study for standardization of the methods for evaluating humoral and cellular immune response and the quantitation of flavivirus RNA.
- Encourage active participation of arbovirus collaborating and reference laboratories in
flavivirus vaccine research and development programme.
- Support the preservation and distribution of clinical samples (sera, PBMC) for evaluation of the effect of T and B cell memories on dengue viral challenge on behalf of WHO.
- Finalise the WHO Guidelines on production and control of tetravalent live attenuated and chimeric dengue vaccine.

**Japanese encephalitis**

**Activity recommended for 2004-2005**

- Finalize project on the development of standard reagents for evaluation of JE vaccines.
- Continue to evaluate progress in clinical trials of JE vaccines.
- Strengthen collaboration with SEAR-WPR Working Group on JE infection with special attention to JE vaccines and vaccination.
- Organise a training course on immunological methods for evaluation of JE vaccines and JE diagnosis, August 2003, Mahidol University, Bangkok.

3. **DENGUE (DEN) VACCINE DEVELOPMENT**

3.1. **Review on the development of subunit dengue vaccines.**

**Presenter:** Dr. T. Humphreys, Hawaii Biotech, Inc., Aiea, Hawaii, USA

**Goals:** To develop tetravalent, recombinant subunit vaccines for dengue, with the rationale that non-replicating vaccines have some advantages over live vaccines, e.g., by their capability to induce a more balanced antibody response against each virus serotype.

**Strategies:** Focus on an expression system that is capable of producing sufficient quantities of correctly conformed recombinant dengue subunit antigens, and on identifying safe and effective adjuvants, which when formulated with antigen are capable of eliciting a good immune response and protection.

**Progress and Future Plans:** Recombinant N-terminal 80% envelope (E) antigens for all 4 dengue virus serotypes and full-length recombinant NS1 antigen for dengue-2 were produced in a drosophila cell expression system. Expression levels of 20-50 ug of antigen per ml of culture fluid were achieved, which represented a significant fraction of the total secreted protein. The antigens were demonstrated to react with conformation-sensitive monoclonal antibodies. Purification was achieved using monoclonal antibody affinity chromatography; however, conventional absorption chromatography could also be used. Antigens were formulated with several different adjuvants, including alum, and tested in animal models (mice and non-human primates, NHP). Primarily endpoints were the measurement of neutralizing antibodies (in a virus plaque reduction neutralization test, PRNT50) and ELISA antibodies. The highest antibody titers were seen against dengue-1 followed by dengue-2 and 3, while the lowest titers were against dengue-4. Virus challenge experiments were also performed. Protection against dengue-2 virus challenge was observed in vaccinated mice and NHP (rhesus macaques). The typical mouse immunization schedule was 5 ug of antigen, 2
doses, 4 weeks apart, with antibodies measured 2 weeks after the second dose. Primates received 3 doses of 5-100 ug per dose. Interestingly, in some cases lower antigen doses were more protective than higher doses; this is possibly because higher antigen doses may lead to decreased Th1 and increased Th2 responses. The addition of dengue-2 NS1 to the vaccine is expected to increase the level of protection. The tetravalent vaccine formulation proposed for pre-clinical evaluation in NHP and testing in Phase 1 clinical trials will consist of 3 ug each of dengue-1, 2, 3, and 4 E antigen and 0.3 ug of dengue-2 NS1 antigen, formulated with an adjuvant(s) to be determined in an upcoming study in NHP.

Questions, Discussion, and Recommendations: A question was asked regarding the methods used for antigen purification. Monoclonal antibody affinity chromatography is the principal method used, although purification to 95% or greater can also be achieved using classical chromatographic methods. A question was asked whether the recombinant 80% E antigen exists as a dimer or a monomer. Although all of the physicochemical characterizations have not been completed, the current evidence is that 80% E is probably a monomer. The strategy for increasing the antibody responses to dengue-4 was addressed; this will be to increase the antigen dose of that component. The issue of adjuvants was raised. Freund’s adjuvant did not appear to be highly effective even though it was predicted that it should induce a good Th1 type response. The fact that virus-neutralizing antibodies were sometimes seen in NHP without good protection was another important issue that was raised. This suggests that cell mediated immunity (CMI) as well as antibody may be important; the addition of dengue-2 NS1 antigen to the vaccine may result in the stimulation of a Th1 response. A question was raised about prM-E expression vs. 80% E expression. Since prM was not found to be secreted from the drosophila cells (i.e., in VLPs), 80% E may be just as effective. The importance of demonstrating protection against challenge with each of the four dengue virus serotypes in an NHP model before proceeding to the human challenge model was strongly advised in order to reduce any potential risk to the volunteers for DHF.

3.2 Review on the development of chimeric dengue vaccine at CDC, Fort Collins.

**Presenter:** Dr. J. Roehrig, CDC, Fort Collins, CO, USA

**Goals:** Develop an intratypic chimeric, tetravalent dengue vaccine.

**Strategies:** Use the dengue-2 live-attenuated vaccine candidate (S16803, PDK-53) co-developed at Mahidol University, Thailand and Aventis, France, as a carrier for the structural prM-E genes of the other dengue serotypes for making a chimeric, tetravalent vaccine. The rationale is that the dengue-2 vaccine is immunogenic and highly attenuated and the attenuating mutations are located outside of the structural gene region, in the nonstructural genes and the 5'-noncoding region (5'-NCR).

**Progress and Future Plans:** The chimeric viruses (i.e., dengue-2/1, dengue-2/3, and dengue-2/4) were successfully produced and demonstrated to replicate to high titers, i.e., 6-7 log10 pfu/ml. Assessment of the attenuated phenotypes of the chimeras is being carried out along with studies to determine immunogenicity. The in vitro neutralization profiles of the chimeras with monoclonal and polyclonal antibodies were found to be type-specific and similar to those seen using wild-type viruses. Mice were immunized with the chimeric vaccines. A dose of 5 log10 pfu was administered ip. Sera from vaccinated animals were assayed for virus neutralizing antibodies using a PRNT assay with a 70% plaque reduction endpoint (i.e., PRNT70). It was found that the dengue-2/1 chimera elicited the highest
antibody titers followed by the dengue-2/3 chimera, while the dengue-2/4 chimera elicited the lowest neutralizing antibody titer. Challenge experiments were performed using ‘interferon knockout’ mice (strain AG 129), which are susceptible to a peripheral dengue virus challenge. Animals vaccinated with the dengue-2/1 chimera exhibited good protection against virus challenge, with some anamnestic antibody responses following challenge. Immunization of NHP with the backbone dengue-2 virus and the chimeras resulted in the highest titered antibodies (measured in a PRNT50 assay) being produced against dengue-2, followed by dengue-1 and 3, with the lowest titers against dengue-4. Therefore, the strategy will be to increase the dose of the dengue-2/4 chimera. The future aim is to identify an effective tetravalent vaccine formulation by testing in NHP, followed by Phase 1 clinical trials.

Questions, Discussion, and Recommendations: There was discussion of dosing schedules, and the need to improve the antibody titers against dengue-4 by increasing the dose of the dengue-2/4 virus. A one or two-dose scheduled is proposed. The genetic stability of the attenuating mutations in the dengue-2 backbone was addressed; there appears to be no evidence for reversion to wild-type in any of the chimeras. Although the mosquito competence of the chimeras has not yet been assessed, they demonstrated reduced replication in $C6/36$ *Aedes albopictus* cells, which may be an important safety consideration when doing clinical trials.

3.3. DNA vaccines for dengue. Presenter: Dr. K. Porter, NMRC, Silver Spring, MD, USA

Goals: To develop nucleic acid (DNA) vaccines capable of immunizing and protecting against dengue.

Strategies: The strategy is to use DNA vaccination to mimic the immune response following natural infection, with the induction of virus neutralizing antibodies and MHC class 1 and 2 restricted cell-mediated immune (CMI) responses. The potential advantages of DNA vaccines are that infectious viruses do not need to be used, it is easy to produce, and relatively stable. Recently, a novel approach is being evaluated for producing a tetravalent dengue DNA vaccine using a gene shuffling technique to produce a plasmid that expresses epitopes of all 4 virus serotypes.

Progress and Future Plans: The prM-E gene region from each of the 4 virus serotypes was cloned in a standard expression plasmid in order to make a tetravalent vaccine. In addition to prM-E, several other gene constructs were evaluated, e.g., 80% and 92% of the E gene. It was found that expression of prM and 100% E (prME) induced the most long-lived antibody responses. Expression of these genes in transfected cells resulted in the secretion from the cells of E protein-containing virus-like particles (VLP), which sedimented like viral SHA on sucrose gradients and could be visualized by EM. The prME constructs were capable of inducing virus neutralizing antibodies in mice against each of the 4 virus serotypes. In experiments in NHP, 50 to 80% protection was observed. Most work has been done with dengue-1. Strategies for optimizing the DNA vaccines included the testing of different delivery devices, e.g., the Biojector device, which may be a good way to deliver DNA, and accessory factors, e.g., GM-CSF and immuno-stimulatory sequences, which may augment the immune responses to DNA vaccines. Various immunization schedules were also evaluated. The vaccination schedule used for Aotus monkeys was 0, 1, and 5 months, with virus challenge at 6 or 11 months. In these experiments, the co-administration of GM-CSF with the
DNA vaccine did not increase virus-neutralizing antibody titers over those achieved with dengue DNA alone. With DNA vaccines, the presence of virus neutralizing antibody at the time of virus challenge was found to correlate with protection against viremia, however, some vaccinated animals without antibody were also protected. Animals vaccinated with the Biojector device tended to maintain higher antibody levels than those vaccinated with needle and syringe. The gene gun was also evaluated in rhesus macaques for the delivery of smaller doses of DNA (1-2 ug), resulting in the induction of low-titered neutralizing antibodies and complete to partial protection. Recently, a gene shuffling (Molecular Breeding™) approach developed by MaxyGen, Inc., is being explored under a collaborative development agreement for producing a tetravalent dengue DNA vaccine using a single plasmid construct. For this work, the prM-E genes of all 4 types were genetically shuffled, and individual clones were tested in mice for their ability to induce a tetravalent neutralizing antibody response. Three clones that induced tetravalent antibody responses are being evaluated in NHP (rhesus macaques). The monkeys were vaccinated at 0, 1, and 3 months; neutralizing antibodies were measured at month 4, and the animals will be challenged at month 8 with dengue-1 or dengue-2 virus. Three of 6 animals that received a mixture of the 4 monotypic constructs made tetravalent neutralizing antibodies, as did 4 of 6 animals that received the shuffled genes (chimera #13). However, chimera #11, which lacks the prM gene, failed to induce dengue-4 neutralizing antibody. Tetravalent DNA vaccine candidates including the gene-shuffled constructs are undergoing further testing in rhesus macaques. The most promising candidates will be developed and evaluated in Phase 1 clinical trials.

Questions, Discussion, and Recommendations: Several questions centered on the utility of the gene shuffling approach for making a tetravalent dengue DNA vaccine. It was suggested that the antigens produced from the shuffled genes might lack important epitopes or might be conformationally altered compared with native flavivirus antigens. It was suggested that some of these questions might be resolved by more extensive epitope mapping with important monoclonal antibodies, comparison with the native antigens, e.g., by gel analysis and perhaps 3-dimentional structure analysis. The shuffled antigen was secreted from cells (presumably as a VLP) and reacted with dengue monoclonal antibody 2H2 and polyclonal antisera against all 4 serotypes. Questions were asked about the doses of DNA used; these were 100 ug for mice and 5 mg for NHP. It was suggested that low antibody titers following 3 inoculations might indicate an important role for T-cell immunity in protection with the DNA vaccines. This idea appears tenable since protection was sometimes observed in the absence of measurable levels of neutralizing antibody titers at the time of virus challenge, and some positive results have already been obtained in T-cell proliferation assays. Additional assays to assess CMI responses are planned, as well as the exploration of a prime-boost strategy for increasing antibody titers. Better immune responses might also be achieved by delivering DNA more efficiently to skin dendritic cells, e.g., by using the Biojector device. A question was asked about pre-clinical safety studies, specifically DNA integration assays. There is a plan to perform these assays using the dengue-1 DNA construct.

3.4. Information on Task Force on clinical trials of dengue vaccines
November 2002. Denver. Presenter: Dr. F. Ennis, University of Massachusetts, Worcester, MA, USA

Goals: This meeting of the Task Force on Clinical Trials of Dengue Vaccines was part of the Initiative for Vaccine Research (IVR) and was co-sponsored by the Pediatric Dengue Vaccine Initiative (PDVI). The focus of the meeting was on dengue vaccines currently in clinical
trials. Among the roles of the Task Force are to provide input and guidance in the areas of vaccine development and testing and to make recommendations.

Strategies: To provide guidance to vaccine developers, make recommendations, and help to find support in order to facilitate the development of dengue vaccines.

Progress and Future Plans: Several recommendations were made by the Task Force: (1) establish banks for vaccine testing reagents; (2) support development of better correlates of protection, animal models (e.g., primate testing), and the human challenge model; (3) more studies are needed in the area of immune correlates of protection, e.g., T-cell responses and antibody responses after vaccination and natural infection; (4) provide standard reagents (sera and viruses) for measuring antibodies against dengue and JE, and RNA standards for measuring flavivirus burden by PCR methods; and (5) establish standards for tetravalent dengue vaccines and set up vaccine working groups. It was agreed that the PVI and the Steering Committee should consider supporting some of these recommendations.

Questions, Discussion, and Recommendations: Several additional suggestions were made; these included the writing of human use protocols to allow for the sharing of clinical specimens, the development of antibody standards for neutralization assays, and the establishment of a repository for flavivirus reagents such as viruses, antisera, cDNA clones, and cell lines. Some flavivirus reagents should already be available from various repositories: e.g., the extensive collection of flavivirus isolates at Galveston, TX (POC: Dr A. Barrett), numerous virus isolates and monoclonal antibodies at Queensland, Australia (POC: Dr. J. Aaskov), and over 4,000 flavivirus isolates, including many important historical isolates and low passage viruses available at the CDC (POC: Dr. D. Gubler). Dr Pervikov indicated that the NIBSC (POC: Dr M. Ferguson) is being considered as a reference repository for monoclonal antibodies, and banks of qualified Vero cells, viruses and serum standards for neutralization assays have recently been established there. There are also 26 Collaborative Centers for Arbovirus Research, which receive a small amount of yearly funding, publish annual reports, and jointly manage many flavivirus reference reagents, including the large collection formerly maintained at YARU. It was suggested that the available reference reagents and related information could be published on an Internet site, which could be updated frequently. It was suggested that such a site could be managed or coordinated by the WHO. There is an existing website managed by the Collaborative Centers, which lists available viruses, reagents, diagnostic tests, and perhaps reports. This site is currently being updated and the Internet address will be made available soon (POC: Dr Y. Pervikov). Laboratories outside the Collaborating Centers may also post information on the website. Some impediments to the distribution of reference reagents were mentioned, these include shipping costs, which may have to be borne by the requestor, the need for permits, e.g., import/export permits and special permits for Select Agents, BL-3 agents, etc., and the need to possibly restrict publication of and access to some level-2 and Select Agents. Finally, it was agreed that the IVR and PDVI need additional funding in order to continue to be effective in advancing flavivirus vaccine development and testing.

3.5. Long-term surveillance of people immunized with tetravalent dengue vaccine in Thailand. Presenter: Dr Arunee Sabchareon, Mahidol University, Bangkok, Thailand

Goals: To begin to assess the long-term safety profile of live-attenuated dengue tetravalent vaccination by long-term follow-up of vaccinees. The question needs to be answered whether
or not vaccination is a predisposing risk factor for the development of severe dengue upon subsequent natural infection, e.g., mediated by antibody dependant enhancement (ADE).

Progress and Future Plans: This work stems from 2 studies, one in adults (n = 49) in 1998, and one in children age 5-12 years (n = 82) in 1999. Following 2 doses of seven tetravalent vaccine formulation administered 6-months apart, the adult vaccinees were followed for 4 years. After 2 primary doses of two tetravalent vaccine formulations (3-5) months apart) and a booster dose a year later, the children were followed for 3 years. Neutralizing antibody titers were measured once a year. The subjects were monitored for febrile illnesses and hospital admissions. During the period of observation, no individuals were hospitalized with a diagnosis of severe dengue, i.e., no DHF or DSS. However, 3 of 82 children required hospitalization for febrile illnesses, although no dengue disease was diagnosed. Three of 42 adults had febrile illnesses but did not require hospitalization; one of them was diagnosed as mild dengue fever. This individual was a 25 year-old male who presented about 4 years post-vaccination with fever and rash. Dengue-2 virus was detected in blood from this individual by PCR, and convalescent anamnestic neutralizing antibody was measured. Serological studies performed shortly after tetravalent vaccination demonstrated that this individual had developed neutralizing antibodies only against dengue types 1 and 3 but no antibodies against types 2 and 4. Sometime later, prior to onset of his illness, he developed antibodies to all 4 serotypes, apparently as a result of a sub-clinical dengue infection. Evidence for a putative sub-clinical dengue infection was also seen in other 8 adult volunteers, whereas other 4 of 42 apparently had an asymptomatic exposure to JE virus. Eight of 73 children also had serologic evidence for an asymptomatic dengue infection and 1 of 73 had an antibody response to JE. There was no evidence (by either serology or virus isolation) for dengue-3 infection after vaccination due to solid protection against this serotype or, alternatively, to low virus circulation. The dengue infection rate observed in unvaccinated individuals was three times that in vaccinees. The conclusion was that the vaccine formulation did not increase the risk for severe disease.

Questions, Discussion, and Recommendations: It was emphasized that the tetravalent vaccine formulation 3232 used for the subject with subsequent mild DF was the formulation that induced poor antibody response, resulting in low immunogenicity for types 2 and 4. It was suggested that in terms of assessing vaccine safety, comparison with a placebo group is really needed. It was stressed that the nature of the post virus-exposure antibody responses should be carefully evaluated. Gladys Sather’s early studies of secondary dengue in Puerto Rico also demonstrated significant anamnestic antibody responses upon second infections. Data from the WRAIR group was mentioned, which showed that rhesus monkeys infected with wild-type dengue-2 virus can be boosted with the same virus after their antibodies from the earlier infection have waned, however, this occurred in the absence of detectable viremia suggesting that the animals were still protected. It was pointed out that repeated natural boosting may also occur in human populations in dengue endemic areas; it just needs to be looked for.

3.6. Information on the development and current status of WHO Guidelines for production and control of live attenuated dengue vaccine (LAV)

Presenters: Dr D. Wood and D. Y. Pervikov, WHO, Geneva

Goals: To formulate a set of guidelines for the production and quality control of live attenuated dengue vaccine candidates.
Progress and Future Plans: The first set of draft guidelines was prepared in 1998. At this time, however, the WHO ECBS determined that dengue vaccine development was not sufficiently advanced to establish formal guidelines as part of a WHO document. In March 2003, the Task Force for dengue clinical trials recommended renewed development of a set of guidelines and a small advisory panel was convened by the WHO/QBS to review the existing draft recommendations and suggest changes for new draft guidelines. It was agreed that the guidelines should cover all LAV made by classical and molecular approaches. The passage histories of seed viruses and cell substrates should be well documented and include published references, which should aid in the establishment of a testing program for adventitious agents, the traceability of source materials, and risk assessment for TSE. Guidelines for master and production seeds should also cover virus characterization (e.g., sequence, attenuation markers). Current recommendations and requirements for virus neurovirulence testing should be reviewed following consultation with experts in this area, and such testing should be justified, based for example on the neurovirulence or neurotropism of the parent virus, or previous isolation or passage of the virus in neural tissue or cells. Cell substrate guidelines include the recommendation to follow existing WHO guidelines for vaccine production in FRhL diploid cells. For vaccines produced in certified Vero cells, guidelines on permissible residual levels of cellular DNA should be applied. For vaccines produced in primary PDK cells, testing for adventitious agents should be applied to each batch of cells as well as testing and monitoring of the source animals. It was agreed that standardized tests for infectivity (e.g., virus focus or pfu assay) be performed on monovalent and tetravalent vaccine lots; and that there could be standard cells and viruses for this assay. The development of assay reagents and their standardization by international collaborative study could be considered by WHO. Guidelines for the stability (e.g., thermostability) of vaccine products should be established, including tests to determine the stability of the individual virus serotypes in the freeze-dried tetravalent vaccine formulation and after reconstitution of the vaccine. A timeline was proposed to have the new draft guideline recommendations completed by June 03, to have the guidelines reviewed by expert consultants by December 03, and finally to submit the revised draft recommendations to the WHO ECBS in 2004.

Questions, Discussion, and Recommendations: The issue was raised as to whether or not monkey neurovirulence testing should be performed on dengue LAV lots and vaccine MS or PS seeds. If so, should there be a standardized test and reference viruses? The possible use of YF 17D vaccine virus as a provisional neurovirulence standard was suggested. It was also suggested that wild-type parent isolates should be compared with vaccine seeds in order to demonstrate that derivation of the vaccine did not increase its neurovirulence. It was brought up that, unfortunately, for dengue neurovirulence testing there appear to be no good, generally agreed upon reference standards. While some argued that there was justification for dropping this requirement because dengue is not a neurotropic virus, others maintained that it would be prudent to continue doing the testing because of the occasional findings of CNS involvement after natural dengue infections. Further study and consultation is needed before a recommendation can be made.

3.7. Consultation on the monkey model for dengue: Information from the meeting held recently at Erasmus University, Rotterdam. Presenter: Dr. V. Deubel, Institut Pasteur, Lyon, France

Goals: To discuss non-human primate (NHP) models for dengue and the development of a standardized NHP model that is more useful for vaccine development. There is a need to develop a better understanding of dengue pathogenesis and the immunological correlates of
protection. While the NHP model is potentially a good model for dengue infection (although without apparent disease), it should be better standardized in order to be most useful for vaccine development. Several issues were discussed, such as, which species should be used, the methods used for vaccination and challenge, the standardization of assays (e.g., neutralizing antibody, T-cell, viremia) and reagents, and the kinds of data that should be collected.

Progress and Future Plans: It was agreed that the Cyno macaque model developed at Erasmus was a good candidate for evaluation. For this evaluation, standard reagents (e.g., challenge viruses) and assay SOPs are being assembled, and study plans and protocols are being drafted, which are geared to the evaluation of parameters such as vaccine dose, inoculation route and schedule, measurements of antibody and T-cell responses and viremia after virus challenge, and the potential of the vaccine for inducing protection, or conversely, antibody-dependant enhancement (ADE) of challenge virus replication.

Questions, Discussion, and Recommendations: A recommendation was made that as much previous data as possible on NHP models should be assembled before embarking on new efforts to develop and adopt a standard model. This may allow one to build upon successful experiments and to avoid past mistakes. It was also mentioned that new assays, reagents, and better ways for monitoring the animals are now available. The issue of which viruses to use for challenge was raised. Some human isolates induce only low titers of viremia in NHP, which makes them of limited usefulness for assessing protection. There were suggestions that natural monkey isolates might be useful as challenge viruses and the possibility was raised for adapting human viruses to replicate to higher titers in macaques. Another suggestion was to use reverse genetics in order to define virus mutations linked to virus replication and virulence. This approach could be possible given the availability of dengue infectious cDNA clones. An opinion was voiced that in vitro neutralization assays might be superior to NHP models for predicting vaccine efficacy. The general consensus, however, was that both assays are potentially useful and should be developed. The usefulness of the NHP model for studying ADE was questioned; further research is required in this area.

3.8. Standardization of the neutralization test for dengue and JE viruses.
Presenter: Dr. M. Ferguson, NIBSC, UK

Goals: The NIBSC will serve as Reference Center to make protocols and reference reagents available to laboratories performing dengue and JE neutralizing antibody assays. These materials will include a standardized SOP, Vero cells, viruses, and dengue and JE reference antibody standards for the assay.

Progress and Future Plans: Three mycoplasma-free Vero cell banks of 500 ampoules have been produced using a Vero cell line obtained from Erasmus University, Rotterdam (Dr J. Groen’s Lab). These cells were evaluated by Dr I. Kurane and shown to be suitable for plaquing dengue and JE viruses. Multi-laboratory collaborative studies have been launched to evaluate the neutralization assay and reference reagents. Participants in the evaluation are requested to perform three independent assay runs using the standard assay or their own assay (both if possible). The raw data generated will be used to calculate neutralizing antibody concentrations in each test sample relative to an appropriate antibody reference standard. Progress towards this goal has been to distribute the study materials to most participants. These materials include the standard assay SOP, sufficient ampoules of freeze-dried viruses (JE and DEN, see below), antisera (standard positives and negatives,
including BIKEN JE plasma, antisera to NAK, BEJ, and SA14-14-2, dengue antisera from natural immunes against all four serotypes, and a dengue tetravalent antiserum pool). The antibodies have been titered against JE and dengue in 2 laboratories and have been tested and shown to be free from markers for HBV and HIV infection. Low passage dengue viruses for the dengue neutralization assay have been produced in the Vero cells, bottled and freeze-dried (300 ampoules of each serotype, DEN-1 WP 74, DEN-2 S16803, DEN-3 CH53489, DEN-4 TVP-360). The JE viruses for the JE neutralization assay are SA14-14-2 (the PDK and Vero cell-adapted attenuated virus), and wild-type virus strains NAK and BEJ for use in labs with BL-3 capability.

Questions, Discussion, and Recommendations: A question was asked whether the cells were clean and whether they will plaque all wild-type virus isolates. The cells should be free of mycoplasma and were demonstrated by Dr. Kurane to plaque several dengue and JE isolates. The usefulness of being able to obtain, in theory, standard titers and derive standard antibody units was pointed out. The possibility to use monoclonal antibodies as standards was raised because these reagents can be produced in virtually inexhaustible supply.

3.9. Information on the status of the Pediatric Dengue Vaccine Initiative (PDVI)

Presenter: Dr. D. Gubler, CDC, Fort Collins, CO, USA (for Dr. S. Halstead)

Goals: The PDVI is an alliance of stakeholders (incl., WHO, research institutes, foundations, etc.) with the goal to facilitate and accelerate the development of safe, effective and affordable dengue vaccines for children.

Progress and Future Plans: Dr. S. Halstead is the initiator and Acting Director of the PDVI. The PDVI Steering Committee with 8 councilors held its first meeting in Bethesda, MD, in June 2003. The task of the Committee centers on vaccine-related issues, e.g., to identify and stimulate the development of the best candidate dengue vaccines and field sites for eventual Phase 3 trials. Immediate priorities: (1) establish field sites that can be set up to perform both disease surveillance and clinical research, (2) establish a funded research program to study burden of illness and dengue biology, and (3) examine ways to increase dengue laboratory and scientific capabilities in underdeveloped areas, especially in those countries where dengue disease is endemic. Start up funds for the project were secured from the Rockefeller Foundation; the Gates Foundation will provide additional funding for 5 years.

Accomplishments include establishment of a PDVI website, surveying the ministries of health of various countries where dengue is endemic in order to get their support, arranging for meetings in endemic areas to discuss clinical research protocols. In the future more fundraising is needed. An Advisory Committee will be formed with the role to advance ‘calls for proposals’ in the areas of basic research and field studies.

4. DEVELOPMENT OF JAPANESE ENCEPHALITIS (JE) VACCINES

4.1. Identification of Cell mediated immune responses after natural JE infection and vaccination Presenter: Dr. F. Ennis, University of Massachusetts, Wooster, MA, USA

Goals: The PI for this Study is Dr. S. Green. The aim is to measure T-cell responses against all 4 genotypes of JE virus using PBMCs collected from recipients of BIKEN vaccine (n=12)
and from JE natural immunes (n=12, children from a prospective study in Thailand who have neutralizing antibody to JE but not to dengue).

Progress and Future Plans: The viruses used in the assay (inactivated JE lysates from each of the 4 genotypes) were provided by Dr. A. Barrett. A proliferation (3H-thymidine uptake) assay was performed using donated PBMCs, pulsed with inactivated JE antigens in order to measure genotype cross-reactive responses. Thus far, PBMCs from 7 individuals have been tested using this assay; all 7 demonstrated genotype cross-reactive T-cell proliferative responses. Representative data from a BIKEN JE-VAX vaccine recipient (otherwise flavivirus non-immune) was presented. Good T-cell proliferative responses were seen vs. most strains of JE antigen tested. A somewhat lower response was seen against JE strain KPO 439-84. The response to control YF 17D antigen was minimal, and the response to DEN-2 antigen approximated that of the medium negative control, demonstrating a good deal of JE specificity in this individual. JE-specific T-cell responses were then assayed by ELISPOT and CTL assays using a library of overlapping peptides representing NAK E antigen. Recombinant vaccinia virus expressing JE prM-E was used for stimulation of T-cell bulk cultures from the JE immune donors prior to assay. Data were presented from a JE-VAX recipient, showing IFN-gamma production and CTL activity (measured as percent lysis) following exposure of stimulated PBMCs to JE peptide pools. The future plans are to complete testing of the PBMCs from the remaining JE immunes by T-cell proliferation and ELISPOT assays, identify JE-specific T-cell epitopes, and compare the sequences of the T-cell epitopes to E antigen sequences from other JE genotypes.

Questions, Discussion, and Recommendations: A question was asked about expected CD4+ vs. CD8+ responses. A CD4+ response may be more likely in the case of vaccination with JE-VAX inactivated vaccine. Nevertheless, high levels of soluble CD8 were seen, and one sometimes sees CD8+ responses with non-replicating antigen-based vaccines given at high concentration. Additional discussions related to endocytosis and Class II antigen presentation with formalin-inactivated virus; the nature of the overlapping peptides (15-mers), and the possibility that one may dispense with stimulation of cells with recombinant vaccinia when using peptides.

4.2. Genetic variation of JE virus and its possible role in vaccination.
Presenter: Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA

Goals: There are four, or possibly five, genotypes of JE virus, but with the exception of genotype III, little genetic sequence information is available. A goal of this project is to sequence, by RT-PCR methods, representative isolates from genotypes I, II, and IV. A second goal is to address the issue of cross neutralization among genotypes, with sera from vaccinees (genotype III).

Progress and Future Plans: The sequencing results demonstrated that genotype IV is the most divergent in terms of its nucleotide and amino acid differences. Isolate JKT 6468 was found to be the most divergent. The genetic variation among JE isolates appears to represent true differences found in nature, not merely insufficient sampling or sampling error. There are currently 290 JE sequences in the database. Phylogenetic analysis suggests that JE may have originated in Indonesia and spread out from there to the other endemic regions in Asia and S.E. Asia, the Western Pacific, Australia and New Guinea, and India. There is now geographic separation of the genotypes. The effect of this separation on our vaccination
strategy should be considered, since all the current vaccines are made from genotype III viruses (NAK, BEJ, SA14). Another important question, therefore, is whether the antibodies induced with the current vaccines will neutralize virus strains from genotypes other than type III. To address this, sera obtained from persons after natural infection and sera from BIKEN vaccinees were assayed vs. viruses representing each genotype in a PRNT50 neutralization assay in Vero cells. Neutralization was demonstrated for all genotypes, with some variation in titer according to the particular serum assayed. The highest titers tended to be against genotype III. Higher titers were sometimes seen with the Vero-WHO cell line (from the NIBSC cell bank and originally from Dr. J. Groen, Erasmus Medical Center), than with the Vero-76 line (from ATCC), suggesting that perhaps cell origin, passage history and handling are important variables. Sera provided from the NIBSC for use in evaluating the standardized JE PRNT assay were also titered. With the exception of one serum from a supposedly naturally JE immune Thai donor, which failed to neutralize, all the other sera had titers > 1:20.

Questions, Discussion, and Recommendations: It was brought up that there are other WHO designated cell banks, which could lead to confusion when requesting or working with the Vero-WHO cell line use in the neutralization assay. Therefore, it was suggested that the name “Vero-WHO” should be changed to make it more specific and avoid confusion with other Vero cell banks. The question was asked how much JE sequence divergence was observed. There was approximately 6.5% amino acid maximum divergence between genotypes. The question of cross-protection experiments in mice was raised, as was the relative virulence of the challenge viruses. A passive protection experiment has been carried out in mice passively immunized with sera from JE-VAX recipients and there was cross-protection observed. A question about evidence for recombination between genotypes was asked. Unfortunately, the answer to this is not known. The importance of looking carefully at well-defined sera from primary natural infections was emphasized; however, the reagents required to do this are limited. The question was posed whether genotype distribution might explain some epidemics. The answer is not clear-cut, although it appears that genotype differences may not be directly linkable to endemic or epidemic disease. At this time it is also unclear why genotype IV viruses appear not to be so widely distributed. It was emphasized, however, that all genotypes that co-circulate in a given area are probably able to cause human disease.

4.3. Neutralization of JE strains by sera from recipients of four different JE vaccines.
Presenter: Dr. S. Yoksan, Mahidol University, Bangkok, Thailand

Goals: To evaluate the immunogenicity of four different JE vaccines (AvP formalin inactivated mouse brain derived JE-VAX vaccine, AvP Vero cell derived inactivated JE vaccine, Glovax SA14-14-2 live attenuated vaccine, Acambis ChimeriVax-JE vaccine) by measuring neutralizing antibodies in vaccinees’ sera to different viruses (NAK, BEJ-1, BEJ-3, 902/97, SA14-14-2, ChimeriVax-JE) in a PRNT50 assay in LLC-MK2 cells.

Progress and Future Plans: All vaccines gave seroconversion rates of 70% or greater against all virus strains tested in the PRNT assay. Antibody GMTs ranged from around 1:20 to several hundred, according to the particular vaccine and dose schedule. The highest GMTs tended to be against the homologous virus. The conclusion was that all virus strains tested as dose viruses in the LLC-MK2 cell PRNT50 assay could be neutralized by antibodies induced by each vaccine, although the PRNT50 endpoint titers varied.
Questions, Discussion, and Recommendations: The difficulty was mentioned in comparing different vaccine types (e.g., live vs. killed), especially, when different inoculation doses and schedules are used.

4.4. Information on the Meeting of the Global Alliance For Vaccines And Immunization (GAVI), South Asia and Asia Pacific Regional Working Groups on Immunization against Japanese encephalitis (JE), June 2002, Bangkok, Thailand.

Presenter: Dr. J. Jacobson, Global Alliance for Vaccines And Immunization (GAVI)

Goals: To address the need at the national level for current information on JE to provide a platform for at-risk countries to share updates on disease burden and control programs. To obtain a consensus from the community of experts on the steps needed to control JE in the region. The objective is to eliminate JE disease by vaccinating in JE endemic areas, where affordable.

Progress and Future Plans: Several recommendations were reached at the meeting. The following is a brief summary, however, a complete set of minutes was distributed. Global action points were: (1) to include JE on the agenda of the 2003 World Health Assembly; (2) to get GAVI to support JE vaccine as a new and underutilized vaccine; (3) to establish a working group to coordinate JE prevention and control issues; (4) to plan a follow-up GAVI meeting. National action points: (1) to establish JE surveillance; (2) to discuss implementation of JE vaccination programs; (3) to define at-risk populations; (4) to perform advocacy activities. Specific action points: (1) the need for better JE disease burden analysis, including making JE a notifiable disease in Asian surveillance programs; (2) facilitate interdepartmental collaboration to advance control initiatives; (3) commit to JE prevention and develop vaccination strategies. Other action points: the need for reliable, affordable, commercial diagnostics at the point-of-care, which are JE virus specific and easy to use; (2) the need for JE surveillance and control, including introduction of WHO pre-qualified vaccines that are safe, effective and affordable; (3) the need to develop new vaccines and international standards or to improve existing ones to meet requirements, (4) to establish guidelines and models for evaluating vaccine efficacy, and (5) to address the need for better clinical care, especially for children, and for the development of standardized guidelines for case management.

Questions, Discussion, and Recommendations: The requirement for better diagnostic reagents, e.g., IgM assays and sera were discussed. The need to set budget priorities was raised; possible considerations should include a model for controlling JE, and a model for moving vaccines into the PVI. For this to happen there must also be a commitment on the part of host countries to buy JE vaccines and implement vaccination programs. It is not clear which vaccines should be adopted by GAVI, but such vaccines must be sufficiently available and inexpensive. The question of asymptomatic infection vs. symptomatic disease was raised, with the recommendation that the primary focus be to prevent disease, and to do this effectively in endemic areas one must vaccinate.

5. OTHER FLAVIVIURUSES VACCINES
5.1. Prevention of the disease burden from flavivirus infections
Presenter: Dr. V. Deubel, Institut Pasteur, Lyon, France

Goals: Determine how we can prevent or reduce the burden of disease from flavivirus infections, with emphasis on dengue (DEN), Japanese encephalitis (JE), yellow fever (YF), West Nile (WN), and tick borne encephalitis (TBE) viruses.

Progress and Future Plans: A summary was given on the current status of flavivirus disease burden and the ways for reducing disease burden. For DEN, the disease burden is very high, primarily in Tropics and sub-Tropics, including S.E. Asia, Africa, and the Americas. Two and one-half Billion people worldwide are at risk in ca. 100 countries (with 0.6 Billion is S.E. Asia), 50 Million infections/year (26 Million in S.E. Asia), 6 Million clinical cases, 380,000 cases of DHF, and 3,000 deaths, mainly in S.E. Asia. The ultimate aim is to develop a tetravalent dengue vaccine that will greatly reduce the disease burden, but the cost of such a vaccine should be affordable. Each country must weigh the cost of continued disease burden vs. the cost of vaccinating the susceptible population. As we work toward developing a safe, effective, and affordable vaccine, the immediate goals are for better assessment of disease burden, fund raising for continued vaccine development, including Phase 3 clinical trials, development of improved vaccines, more research on dengue biology, vector control, immunology, and pathogenesis. For YF, the disease is endemic in S. America and Africa. There are an estimated 300,000 clinical cases and 30,000 deaths per year. The actual number of infections may be greatly under reported. The case fatality rate can be over 50%. The cost of hospital treatment varies. Although there is a good live attenuated vaccine, recently the number of cases has begun to increase due to reduced vaccination and mosquito control. More than 20 outbreaks have been reported in Africa over the past 20 years, where different genotypes circulate in the Eastern and Western parts of the continent. There is a possibility for future outbreaks in the American Tropics and potentially in Asia, where there is insufficient vaccine for all susceptibles. More funding is required to implement vaccination programs, more basic and applied research and improved disease surveillance. For JE, where at least 4 genotypes circulate widely in large parts of Asia, S.E. Asia and the Western Pacific, there are an estimated 30,000 to 50,000 clinical cases per year, up to 30% of which may develop encephalitis with a mortality rate that can exceed 30%. However, the disease risk is low for travelers who tend to stay in urban areas. Improved vaccines are required, which are safe, inexpensive and effective, e.g., a live virus vaccine. For WN, 3 genotypes circulate, 2 in Africa and 1 in Asia. Until recently, the primary disease burden was in Africa and the Middle East. However, last year there were over 4,000 reported cases and 200 deaths in the U.S. due to WN, and the virus has been introduced into the Caribbean and S. America. Otherwise, the risk factors for WN appear fairly low, with generally low mosquito transmission rates, and probably a low rate of CNS involvement. Vaccines for animals (horses) have been developed, and vaccines for humans are under development. How this development should proceed is unclear, as is the continued emergence of the virus. For TBE virus, there are Eastern (Far Eastern, i.e., RSSE) and Western subtypes. There is a high degree of homology and probably cross-protection among genotypes. These viruses cause more than 10,000 case of encephalitis per year, with an increasing prevalence for the Far Eastern type. Safe and effective killed vaccines have been commercially available for two decades; these are used widely in Europe where the disease burden has been greatly reduced. Overall, flavivirus infections, e.g., WN, are on the increase, possibly due to changes in climate, and movements of host and vector populations. More support, including support and recommendations from the WHO, is needed to sponsor vaccine development (taking first generation vaccine into
Phase 3 clinical trials; developing new vaccines), and research into the virus/host/vector biology, immunology (neutralization, ADE assays), risk factor reduction, and field research.

Questions, Discussion, and Recommendations: A question was raised about the effect that pre-existing yellow fever immunity has on subsequent dengue infection or vaccination. There is not a lot known, however, live dengue vaccines tend to replicate better and to give higher secondary-type immune responses in YF immunes compared to non-immunes. The potential for dengue ADE aside, broad exposure to flaviviruses may be beneficial with regard to cross-protection, at least in the short term (timing is probably critical). More study of this problem is needed. A question was asked about the subclinical infection to clinical case rate for WN. It was estimated to be about 150 to 1.

5.2. Do we need a new yellow fever (YF) vaccine? Presenter: Dr. R. Barwick, CDC, USA

Goals: By using clinical data to examine the safety profile and side effects of the current YF 17D vaccine, begin to assess (1) whether there is a continued need for a YF vaccine, (2) whether there is a problem with the existing YF vaccine, (3) whether a new vaccine is needed, and (4) whether there are strategies for developing alternatives.

Progress and Future Plans: From epidemiological data and disease burden assessment, it is clear that there is a continued need for a vaccine against YF. YF virus is endemic in parts of Africa and S. America, with continued risk to unvaccinated persons. However, the YF 17D vaccine strain, although comparatively safe, may have rare but serious side effects. These serious side effects include viscerotropic and neurotropic syndromes. During the period 1996 to 2002, approximately 16 cases of presumptive vaccine-associated viscerotropic disease were reported, 8 of which were in the US and 9 of which were fatal. All cases were in primary vaccinees; the majority of patients were over 50 y/o. YF 17D vaccine-type virus was isolated from 9 patients with typical YF symptomatology and histopathology. The onset of illness 2-5 days post-vaccination also implicated the vaccine. There were 9 cases of neurotropic disease in YF vaccinees in the US, all in primary vaccine recipients, during the period 1995 to 2002. Illness occurred 4 to 23 days after vaccination. All patients survived. Some viral isolates from clinical cases were sequenced to determine whether or not reversion mutations had occurred. In those instances presented, no significant back mutations appear to have occurred, and therefore, there was no evidence that the vaccine virus had reverted to wild-type. The described adverse effects were associated with all vaccine strain subtypes and all of the commercially available live-attenuated YF vaccines. In the absence of case controlled studies, however, the risk from vaccination is difficult to determine with accuracy. Estimates of the cumulative risk were made using the number of doses of vaccine that were distributed. The estimated risk averaged over time is 3 to 5 serious adverse events (SAEs) per million vaccine doses. The risk by age was calculated as adverse events/total vaccine doses x age group distribution. The trend was for a higher risk for viscerotropic disease with increasing age. The need to more accurately estimate the vaccine’s risk to benefit ratio was emphasized; here, vaccine coverage and exposure to the virus must be taken into account. There is no clear answer at this time. Comparison with some other vaccines was made, where the risk from YF vaccine (taking into account severity and frequency of side effects) appears to be greater than flu vaccine, approximately equivalent to polio vaccine, and less than smallpox vaccine (vaccinia). The risk factors, other than possibly age, are also not well defined. The possibility was proposed that administration of immune serum globulin (ISG) containing anti-YF antibody to vaccinees might reduce the risk for vaccine-associated SAEs
in some high-risk individuals, since boosted vaccinees do not have detectable YF viremia and vaccine-associated SAEs occur mainly after primary vaccination.

Questions, Discussion, and Recommendations: A concern was raised with including GB syndrome in the adverse event analysis. The question of how many non-vaccinees experience similar syndromes was also raised. There is a need for case controlled studies in order to better assess risk. In addressing the question of whether the risk of vaccination outweighs the benefits, it was mentioned that this would be expected to vary from person to person depending upon the particular circumstances. The possible role for host factors that might lead to increased risk was discussed, including the fact that 3 individuals with SAEs had thymectomies before being vaccinated. Whether HIV infected individuals are also at increased risk is an important question, which needs further study. However, one study in Thailand was mentioned in which vaccination was safely carried out in persons with low CD4+ cell counts. The question was raised concerning medications (e.g., Statins, immunosuppressives), which might increase risk. The consensus was that all this information should be collected and considered in the risk assessment. With respect to the negative genomic sequencing results, it was brought up that these studies should also be interpreted cautiously and that other studies have demonstrated patient isolates that contained significant mutations and even some that appeared to be wild-type YF virus. The recommendation was made by the presenter that the WHO again include YF vaccine on the research agenda, with emphasis on understanding and limiting vaccine related SAEs.

5.3. Development of new vaccines against West Nile: A WN DNA vaccine
Presenter: Dr. J. Roehrig, CDC, Fort Collins, CO, USA

Goals: To develop vaccines to immunize and protect against West Nile encephalitis and other flaviviruses, and to develop flavivirus diagnostic antigens.

Progress and Future Plans: An update was presented on the status of flavivirus DNA vaccine and recombinant subviral particle (RSP) cell line research and development at CDC, Ft. Collins. The plasmid used for the flavivirus DNA vaccine constructs was typical, i.e., the prM-E gene under the control of the CMVi/e promoter, except for the use of a JE virus signal sequence upstream of the prM gene. The DNA vaccine research focused first on JE. It was demonstrated that the expressed E antigen was conformationally correct. In addition to its potential use in a vaccine, the expressed E antigen was found to be a useful diagnostic antigen, e.g., for ELISAs. The same expression system could also be applied for making DEN, WN, TBE, SLE and YF antigens. The DNA vaccines were then evaluated in animal models, mice for DEN-2 and JE, and mice, crows, condors and horses for WN. The DEN-2 and JE DNA vaccines were shown to be effective in mice. The WN DNA vaccine (administered at a dose of 10 to 100 ug for mice and 100 mg for horses) resulted in the induction of neutralizing (PRNT90) antibody titers of 1:40 – 1:160 in mice (n=10) and 1:10 to 1:40 in horses (n=4) and 100% protection against challenge (including a mosquito challenge of the horses). A cooperative development agreement was established with the Fort Dodge Laboratories, KS, in order to in order to further demonstrate the safety and efficacy of the WN DNA vaccine in horses. These studies are (1) a Phase 1 safety/dose-ranging trial, (2) a Phase 2 efficacy trial with vaccine plus adjuvant, with a boost at week 3 and a virus challenge at week 12, and (3) a multi-State Phase 3 field trial, with the potential for a veterinary biological product license following the field trial. Additional USDA/Ft. Dodge-sponsored studies are planned; if successful, this may be the first DNA vaccine to be approved for veterinary use. The status of work to create cell lines for producing recombinant subviral
particles (RSPs) was also presented. Recombinant cell lines have been made, which produce RSPs for JE, WN, DEN-1, 2, and 3; work to make additional lines, which produce RSPs for DEN-4, SLE and YF is ongoing. Secretion of RSPs is constitutive. A DEN-2/JE chimera was found to be the most effective secretor. The RSPs can be used as diagnostic antigens, e.g., in IgM/IgG ELISAs. Some companies have already expressed interest in commercializing these products.

Questions, Discussion, and Recommendations: A suggestion was made that flavivirus RSPs might replace suckling mouse brain (SMB) antigens, which are becoming more difficult to produce, due mainly to increasingly restrictive animal use regulations. It was mentioned that Dr. Konishi in Japan also has a cell line that makes JE RSP. It was recommended that reagents and assays to test for WN virus in blood, e.g., RT/PCR assays, should also be developed.

5.4. Development of new vaccines against West Nile: A chimeric YF/WN vaccine.

Presenter: Dr. T. Monath, Acambis, Inc., Cambridge, MA, USA

Goals: To develop a vaccine to immunize and protect against West Nile encephalitis virus.

Progress and Future Plans: There is an increasing medical impact of WN, especially in the U.S. where there were over 4,000 cases and 200 deaths last year in 43 States. The virus is also in the Caribbean and in Mexico. There is an urgent need for a vaccine. The candidate vaccine, a YF/WN chimeric construct, is similar to the ChimeriVax™ vaccines developed previously for JE and DEN. The YF prM-E gene region was substituted by the WN genes, in this case from a wild-type virus. The recombinant virus was propagated in Vero cells. Safety evaluation included neurovirulence testing, where the original version of the YF/WN chimera was found to be similar to YF 17D. Therefore, the chimera was further attenuated by introducing several mutations in the E gene (i.e., E-107 in domain I, E-316 in domain III, and E-440 in the anchor region), which made it more closely resemble attenuated JE virus. The re-engineered construct exhibited much reduced neurovirulence in mice; in monkeys the new chimera was much less neuroviral than YF 17D vaccine virus. The chimera has been tested for immunogenicity in hamsters, where a control group received the Ft. Dodge inactivated WN vaccine. The chimera elicited 100% seroconversion vs. 60% seroconversion with the killed WN vaccine. Monkeys vaccinated with the chimera showed low levels of viremia (ca. 1.4 to 2.4 log10) lasting for 3 to 5 days, similar to YF 17D. Vaccinated monkeys seroconverted to WN, with neutralizing antibody titers of 1:60 to >1:640 (GMT 1:190), and all were protected from encephalitis and death after intracranial challenge with 5.4 log10 of WN NY99 virus. Mosquito transmission studies with the YF/WN chimera (though not with the triple mutant) were presented. Although the chimera infected mosquitoes after intra-thoracic inoculation, virus replication was reduced. There was no infection of the usual vector mosquito species after oral feeding, and only a small amount of virus replication in A. aegypti but no dissemination. Manufacturing issues were addressed: The candidate vaccine production seed was made at Vero cell passage-3, the master seed at P-4, and the vaccine at P-5. For vaccine production, the Vero cells were grown in a bio-fermentation unit; more than 8 log10 of virus was produced, which was purified by nuclease treatment, ultrafiltration and diafiltration. Phase 1 and Phase 2 clinical trials are scheduled for 3 quarter 2003; a Phase 3 trial is anticipated by 2004-2005, with the possibility for commercial use by 2006-2007.

Questions, Discussion, and Recommendations: There was discussion on the role that the WHO should have in making recommendations for WN vaccination. As an emerging
disease, it was argued that WN vaccine development should be treated similarly to DEN and JE vaccine development. However, it was also considered that more disease risk assessment and sero-epidemiological data is needed before a recommendation can be made. It was emphasized that although WN is an emerging disease, the future transmission of the virus, incidence of infection, and spread are difficult to predict with any certainty. With respect to testing the new vaccine for efficacy, the issue of large-scale field efficacy trials was raised. Since attack rates appear to be low in most outbreaks, however, these trials would appear to be impractical (requiring too many volunteers) for measuring protection. This suggests exploring alternatives such as efficacy testing in animal models or the use of surrogates of protection such as neutralizing antibody titers. Regarding efficacy testing in an animal model, the question of the LD50 for wild-type WN in monkeys was brought up. Although this parameter still needs to be determined, an approximation was made of 1-2 log10 pfu of virus. The issue of whether the chimera will be used as a veterinary vaccine was brought up. Apparently, it has been licensed for veterinary use in horses and other mammals. A study conducted by CDC showed that it does not immunize birds, presumably due to host restriction of the YF vector. A question was asked whether the construct with the triple attenuating mutation in E results in any measurable changes in antigenicity or immunogenicity, and whether there are any differences in glycosylation compared with the wild-type virus. It appears that the chimera is immunogenically and antigenically similar to wild-type virus, and there is thought to be no difference in glycosylation, although these studies are still incomplete.

5.5. Vaccines and vaccination against tick-borne encephalitis (TBE): TBE and the impact of vaccination. Presenter: Dr. F. X. Heinz, University of Vienna, Vienna, Austria

Goals: To address the effectiveness of the current TBE vaccines.

Progress and Future Plans: The TBE viruses comprise Eastern and Western genotypes with three subtypes (European, Siberian, and Far Eastern). There is about 2.2% genetic variation among the subtypes and about 5% variation between types. However, all TBE strains appear to belong to a single serotype. Importantly, current TBE vaccines induce cross-protection against the different types in animal challenge models. There is approximately 40% homology between the TBE serocomplex and other flaviviruses, and TBE is closely related to other tick-borne flaviviruses, e.g., Louping Ill and Langat viruses. TBE is endemic, with several thousands of cases in Eastern Europe and Russia and a case fatality rate of around 0.5%. However, in Russia case fatality rates greater than 20% have been reported, perhaps suggesting increased virulence for some Far Eastern strains. The TBE viruses continue to exist in stable, endemic areas in Europe. There is also evidence for virus migration to new areas. Vaccination is required in order to effectively contain the spread of disease. Different inactivated vaccines are in use in Russia (see following presentation) and in Europe. The European vaccines are purified, inactivated viruses (PIVs) grown in primary chick embryo cells, highly purified, and adjuvanted with aluminum hydroxide. In Austria, the Chiron and Baxter vaccines are used primarily, with a coverage of 87% (of the general population) and up to 94% (of the population in endemic areas). Disease rate comparisons with countries that do not vaccinate have allowed for vaccine efficacy assessment. The protection rate of the vaccine is estimated to be about 98%. Since the introduction of vaccination to Austria about 20 years ago, there has been a significant decline (about 90%) in disease, from about 700 cases/year to less than 60 cases/year. However, countries that do not vaccinate may still experience high rates of disease.
Questions, Discussion, and Recommendations: A question was asked about the number of vaccine failures. There have been few reported vaccine failures in Austria and the estimated protection rate is about 98%. Additional questions were deferred until after the following presentation.

5.6. Vaccines and vaccination against tick-borne encephalitis (TBE): Development of a new purified, inactivated vaccine in Russia against TBE (strain 205).

Presenter: Dr. I. Krasilnikov, Joint Stock Company, Moscow, Russia

Goals: To present results for a new TBE vaccine developed in Russia and a comparison with existing TBE vaccines.

Progress and Future Plans: The R&D Center in Moscow has been active in the development of TBE vaccines. The history of TBE vaccine development in Russia was reviewed. The virus was isolated in 1938 and in 1939 the first inactivated vaccine was made in mouse brain. In 1963, an inactivated vaccine was produced from the “Sophin” strain in Chick embryo cell (CEC) fibroblast culture, followed in 1976 by a strain 205 vaccine. In 1981, a freeze-dried, purified inactivated vaccine (PIV) was made with the “Sophin” strain. However, TBE rates in Russia remained high, especially in Siberia with 20-50 cases per 1,000. Therefore, an effort was started to produce a new TBE vaccine, which could afford better coverage. For making the new vaccine, TBE virus (strain 205) was propagated in CEC culture, inactivated with formalin, then concentrated 20-30-fold by silica adsorption or ultrafiltration, and purified to >99% by gel filtration. The resulting virus preparations averaged 9 log10 particles per ml, and had low CEC protein levels. Adsorption of the inactivated virus to aluminum hydroxide formed the basis for a liquid vaccine. Testing was performed in animals where the vaccine showed high immunogenicity and low reactogenicity; then the vaccine was tested in 25 adult volunteers. Two 0.5 ml doses each containing 3-4 ug of viral antigen, administered 2 months apart, induced neutralizing antibodies in all volunteers with low associated reactogenicity. Further clinical trials were then carried out in endemic areas. The study involved two groups of adults. The first group was vaccinated at 0, 2, and 12 months and the second group at 0, 5, and 12 months. Vaccinees were monitored for local (injection site) and systemic (e.g., febrile) reactions. More reactions were seen after the first dose, where about 15% to 22% had low-grade fever or other systemic reactions and 75% exhibited local reactions. The reactogenicity decreased for the second and third doses. Immunogenicity was measured by ELISA and HAI assays. Anti-viral IgG was detected in all vaccinees one month after the second dose, and the antibody persisted. Higher titers were seen with the 0, 2 month schedule, however, all vaccinees developed high antibody titers after the third dose at month 12. The new TBE vaccine was compared to the old vaccine in immunogenicity. The capacity for producing the new vaccine was estimated at 2 to 4 million doses/year, therefore, providing ample vaccine coverage. The new vaccine has now been approved for use in adults by the Russian Ministry of Health. Clinical trials in children 3 to 9 years old are beginning.

Questions, Discussion, and Recommendations: Questions were addressed to both presenters concerning the existing vaccination schedule with the recommendation for a booster at 3 years (or more) to ensure protection. It was mentioned that the new European vaccine exhibited high potency vs. Far Eastern viruses, even compared with homologous Far Eastern TBE vaccine. The issue of cost was raised. The cost of the Chiron TBE vaccine was estimated to be about $5 per dose. It was not clear whether this is the production cost or the cost to the patient, which could be higher. It was mentioned that the Russian vaccine might be less expensive, perhaps $1 per dose, due to high manufacturing capacity. The possibility
was raised for comparing the European and Russian vaccines, to include comparison and standardization of GMP production processes. It was suggested that dose-ranging studies could be performed in order to determine the lowest effective vaccine dose, potentially reducing both cost and vaccine-associated side effects.

6. FUTURE MEETINGS

Scientific meeting on dengue virus cell entry: structure and function relationships, 25-27 June 2003, Vienna, Austria

The Task Force on clinical trials of dengue vaccines, December 2003, Philadelphia, USA

The annual meeting of the Steering Committee on dengue and other flaviviruses vaccines, April 2004
7. CONFIDENTIAL MATTERS DISTRIBUTION: RESTRICTED

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Project title: Contribution of genotypic variation to the degree of protection conferred by JEV vaccine-induced immunity, V22/181/132

Principal Investigator: Dr Alan D.T. Barrett, University of Texas Medical Branch (UTMB), Galveston, Texas, USA

This project has the following objectives: 1. deposit of JE strains appropriate for NT and T cell tests in the World Arbovirus Reference Centre at the UTMB; 2. develop a panel of JE viruses and compare the strains by sequencing and neutralization; 3. characterize JEV genotype representative in cross-protection studies in mice using panel of viruses developed in 2 above.

The panel of JE virus strains was established. The PI sequenced the genome of strain JKT6468, a representative of genotype IV, considered to be the most divergent of the JEV genotypes. The genome of strain JKT6468 differed between 17.4-19.6% at the nucleotide level and 4.8-6.4% at the amino acid level from the other three genotypes. This confirms that genotype IV is the oldest genotype of JEV and that the other three genotypes diverged more recently. Phylogenetic analysis indicates that genotype IV is the oldest genotype of JEV and that the other three genotypes diverged more recently. Geographic distribution and geographic distribution of JEV suggests that JEV originated in Indonesia/Malaysia and spread to north and south.

Studies on cross-neutralization involving representatives of the four genotypes of JEV and human sera from recipients of some inactivated JEV vaccines shows that although the strains are neutralized by the vaccines sera samples, in vitro neutralization titers vary between JEV genotypes. Similar results have been found in passive protection studies in mice.

SC comments: The progress is satisfactory. The report provides results that confirm some variability in biological and antigenic features of JE virus strains. Cross-protection studies in mice were completed using a panel of JE viruses (intracerebral challenge) and inactivated vaccine from Nakayama strain (genotype III). Sera passively protected mice against genotypes III and II while protection was poor against challenge with viruses in genotypes I and IV. Attempt to improve mouse model by breaking the blood-brain barrier was unsuccessful. Obviously, the mice are not the best animal model to evaluate JE vaccine efficacy and monkeys should be envisaged.

The SC accepted the report.

Project title: Specificity of Human T Cell Responses to Japanese Encephalitis Virus, V22/181/131

Principal Investigator: Dr Sharone Green, University of Massachusetts Medical School, Worcester, MA 01655, USA

The purpose of this project is to study human T cell response to JE virus. Although the inactivated JE vaccine is highly efficacious, neutralizing antibody responses are of low titer.
The presence of cross-reactive memory T cells to the different genotypes of JE virus is a plausible explanation for the dissociation between efficacy and seroconversion rates. Based on this hypothesis, the PI identified the following objectives for her study: 1) to determine if the T cell responses in JE vaccine recipients cross-reactive with different JE virus strains belonging to different genotypes and different countries to those measured in individuals with serologic evidence of natural JE infection; 2) to compare T cell proliferative responses in vaccine recipients to those measured in individuals with serologic evidence of natural JE infection; 3) Develop standards for the measurement of T cell proliferative responses as a marker of immunogenicity for clinical trials of second-generation JE vaccines.

As indicated in the original work plan, the PI proposed to enrol a total of 9 subjects for study purposes. However to date, the PI has already enrolled 12 volunteers and have obtained pre-vaccination, pre-travel and day 60 bleeds on all of them. Additionally, the PI has identified specimens from 12 children enrolled in an ongoing prospective study of schoolchildren in Thailand that demonstrate evidence of neutralizing antibody to JE but no evidence of neutralizing antibodies to dengue virus.

The PI has been establishing reagents for the studies by propagating the 8 different viruses belonging to the 4 different genotypes of JE virus and preparing inactivated antigens for detection of CD4 cell responses. They have been able to measure CD4 cell proliferative responses to inactivated antigens produced from viruses of the same genotype as the parent vaccine (Nakayama, SA14-14-2) in recipients of the inactivated JE vaccine. Studies are ongoing to measure these responses against antigens produced from viruses of the other 3 genotypes of JE virus.

The Researchers received lyophilized JE virus strains from four different genotypes and different geographic regions as outlined in the original proposal (KPP-034, Nakayama NIH, Korea, P-20779, Beijing-1, JKT 1724 and JKT 6468). In addition, they received lyophilized JE SA14-14-2 vaccine virus. The study is in progress to identify an appropriate treatment for inactivation of selected viruses. The PI proposes to perform TC proliferation assay in future with all bleeds in a single experiment in order to avoid the problem of inter-assay variation.

The PI developed IFN gamma ELISPOT assay to quantitate JEV-specific T immune response using a panel of 20 amino acid overlapping JE peptides that span the envelop region of the virus.

**SC comments:** The PI has made considerable progress. The project appears to be only slightly behind schedule. Important assays have been developed and evaluated. The viral inactivation problems have been solved since stock viruses have been prepared and proliferation assays have been developed. The SC noted that the experiments using peptides are promising for quantitative analysis of immune responses.

The SC accepted the report.

**Project title:** Improving clinical endpoint for phase I and II clinical trials of currently available JE candidate vaccine, V22/181/145

**Principal Investigator:** Dr Y. Sutee, Mahidol University, Salaya, Thailand
The principal objective of this study is to develop a sensitive, standard MT assay to detect and quantitative JE virus-neutralizing antibody in sera of volunteers receiving JE candidate vaccines. The another objective is to select suitable JE stains appropriate to be used as reference strains for NT assay. Three vaccines against JE are in the current study: inactivated mouse-brain derived vaccine, inactivated vaccine produced in primary hamster kidney cell, and a live attenuated vaccine SA 14-14-2.

Standard and sensitive plaque reduction neutralization test was developed. Serum samples were obtained from adults who had been immunized with four JE candidate vaccines, and their neutralizing antibodies against different JEV strains were included in the study. Selection of JE viruses used in the study included two vaccine strains and four wild viruses.

Seroconversion levels against all tested strains were similar in group vaccinated with the same vaccine. However, some differences in GMT were identified when antibodies were tested against selected strains. Inactivated mouse brain vaccine produced from Nakayama strain elicited stronger immune responses against homologous virus than the heterologous strains. Antigenic differences in the same JE viral genotypes (between Beijing, P3 and Nakayama) was confirmed in study with sera from people immunized with inactivated Vero cell derived vaccine.

**SC comments.** The PI developed the standard sensitive PRNT_{50} to detect neutralizing antibody in most vaccinees after immunizations with various JE vaccines. Wild- type and vaccine strains of JE viruses were explored as potential source of JE antigen with improved PRNT_{50} specificity and sensitivity. The results obtained show that Beijing-P3 and Nakayama might be the optimal reference JE strains of choice for using in PRNT_{50} assay of candidate vaccines.

The SC accepted the report provided that the PI provides missing information in Table 2 of the final report and send revised version to WHO by 1 September 2003

### 7.2 Progress reports.

**Project title:** Monoclonal antibody competitive ELISA to determine the role of cross-reactive antibodies in dengue virus infection-dengue virus monoclonal antibody, (V22/181/150)

**Principal Investigator:** Dr J. Aaskov, School of Life Sciences, Queensland University of Technology, Brisbane, Australia

The objectives of this project are to prepare and characterize a panel of anti-dengue virus monoclonal antibodies (Mabs) for deposition at the WHO reagent bank for use in diagnostic assays, epidemiological investigations and vaccine production.

The logistical phase of the project has been began. Virus isolation has been attempted form approximately 200 dengue patients from whom paired sera have been obtained. Viruses of all serotypes have been recovered and there is sufficient convalescent phase serum in each case for use in competitive ELISAs.
Twenty-one hybridomas producing cross-reactive, neutralizing, monoclonal anti-dengue virus antibodies have been used to produce antibodies free of other protein. These antibodies will be concentrated and their immunoglobulin content standardized. These antibodies will be used to identify cross-reactive neutralizing epitopes in dengue viruses and in competitive ELISAs with sera from dengue patients.

Many of the viruses recovered for use in this study will not produce plaques in a standard dengue virus plaque assay. An infectious focus assay has been developed in which "plaques" of infected cells in cell monolayers are detected by indirect ELISA with HRP-labelled antiflavivirus antibody. This assay is being validated as an alternative to the standard assay in which viral plaques are detected by staining monolayers with neutral red, crystal violet or napthol black.

**SC comments:** Project was initiated and progress is satisfactory taking into account a short period of time after transfer of funds to the PI (February 2003). The SC confirmed that if successful the project would provide a valuable panel of reagents to be available for vaccines manufacturers and developers of diagnostic tests.

The SC accepted the report and recommended funding at the level of US$ 35 000 for the second and final year.

**Project title:** Efficacy and safety of a tetravalent dengue vaccine in a macaque model, (V22/181/149)

**Principal Investigator:** Dr A. Osterhaus, Institute of Virology, Academic Hospital Rotterdam, The Netherlands

The project focuses on the evaluation of vaccination with a candidate tetravalent live attenuated dengue vaccine (LADV) in a cynomolgus macaque model. The PI proposes the following subjects to be addressed: 1.) The immunogenicity, efficacy and safety of tetravalent live attenuated dengue vaccine in the absence and presence of dengue virus serotype specific antibody, 2.) Dengue virus specific antibodies responses following vaccination and/or challenge, 3.) Dengue virus specific cell-mediated immune responses following vaccination and/or challenge.

Discussions with the industry (GlaxoSmithKline Biologicals and Aventis/Acambis) are ongoing about tetravalent vaccine to be included in the study. The representatives of GSK visited the Erasmus University and an agreement was reached to revise some components of a study format. The PI has obtained wt-DEN strains serotype 1-4 form Dr V. Deubel, to be used as challenge viruses. The researchers have grown these viruses by two rounds of passaging in insect cells and determined the titers (TCID50/ml) in Vero-E6 cells (DEN 1: 10^{6.38}, DEN 2: 10^{4.75}; den 3: 10^{6.63}; DEN 4: 10^{6.2}). At least 85 ampoules of each virus stored at -80°C. The real-time PCR assay was optimized and standard curves were generated for all for viruses. The set of specific primers and probes for mRNA quantitative detection is now extended for up to 25 inflammation and fibrinolysis markers. For analysis of dengue-specific T cell responses, the PI has generated recombinant vaccinia viruses expressing the E protein and NS2A protein of all four serotypes.

The above mentioned wt-DEN viruses are now being tested in the cynomolgus macaque model.
The researchers have infected one animal with DEN 1, one animal with DEN 2 and one animal with DEN 3 via the intravenous route. Preliminary results show that all animals became viremic and seroconverted.

SC comments: Progress is satisfactory. Challenge viruses were selected and preliminary characterized. The PI established conditions for real time RT-PCR for virus RNA and can measure 25 inflammation and fibrinolysis markers. Recombinant vaccinia viruses that express E and MS2A proteins for all four dengue viruses have been generated to measure dengue virus-specific T cell responses. The PI is not including NS3 the major protein that T cell epitopes. The SC recommended that NS3 be included in the vaccinia constructs. The SC is also concerned about the number of different wild-type dengue viruses being used for challenge studies, and recommend to standardize wild-tripe viruses used in vaccine studies such that vaccine candidates can be compared.

The SC accepted the report and recommended funding at the level of US$ 50 000 for the second and final year.

Project title: Characterization of protective neutralizing antibodies following vaccination with tetravalent live-attenuated dengue vaccine, (V22/181/148)

Principal Investigator: Dr W. Sun, Walter Reed Army Institute of Research, Silver Spring, MD, USA

Neutralizing antibody to one or more of the dengue serotypes has been considered as the surrogate marker for protective immunity. However, in recent animal studies as well as in prospective human studies, the presence of neutralizing antibody did not necessarily protect against infection or disease. The validity of neutralizing antibody as a surrogate marker for protective immunity is less certain than previously appreciated. There may be other more specific markers of protection including antibody isotypes, IgG subclass and avidity of antibody.

Objectives of the proposed project are to: 1). Describe serotypes specificity of neutralizing antibodies after primary vaccination and revaccination with monovalent and tetravalent live attenuated dengue virus vaccines, 2). Determine the avidity of the antibodies after primary vaccination and revaccination with monovalent and tetravalent LAV, 3). Determine if tetravalent LAV protects against disease and viremia following dengue virus challenge and then determine if protection correlates with neutralizing antibody titer and/or antibody avidity, 4). Describe the antibody isotypes and IgG subtypes and respective avidity of the protective antibody response.

The dengue virus human challenge study, which forms the basis of this study, was completed. Five subjects previously vaccinated with the WRAIR tetravalent dengue vaccine were challenged with DEN-1 low-passage virus and another five volunteers were challenged with DEN-3. There were 2 flavivirus naïve control volunteers who each received DEN-1 or DEN-3.

Pre-challenge and post-challenge sera were collected from each volunteer and will be used in the next phase to measure Ig subclass and avidity of antibody to the challenge serotype dengue virus.
The majority of investigators in this study consists of U.S. Army military investigators. Due to deployment and redirection of key investigators the development of antibody avidity measurements by surface plasmon resonance have been delayed. It has been necessary to train co-investigators on the use of the BIACORE technology. This training is in progress.

Because of late transfer of the funds to the PI the reports will be submitted to the SC consideration in July 2003.

**SC comments:** Progress is satisfactory. Vaccination with tetravalent vaccine provided protection against challenge with type 1 virus. However, symptoms of dengue disease were shown in vaccinees challenged with type 3 virus. More information on clinical manifestation of dengue fever in vaccinees should be presented in the next progress report.

The SC recommended funding at the level of US$ 50 000 for the second and final year.

**7.3 New Proposals**

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<th>Project title:</th>
<th>Protective abilities of specific and cross-reactive Dengue antibodies analysed at clonal levels (V22/181/153)</th>
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<tr>
<td>Principal Investigator:</td>
<td>Dr Eiji Konishi, Kobe School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Japan</td>
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The objectives of this study are (1) to analyze antibodies induced by a tetravalent dengue DNA vaccine at a clonal level in terms of specificity/cross-reactivity and protective/pathogenic roles, and (2) to assess the effect of preimmunity with yellow fever (YF) 17 D vaccine and/or Japanese encephalitis (JE) vaccine on vaccine-induced antibodies in a murine model.

The PI proposes to study qualitative and quantitative aspects of specific neutralizing antibodies and non-neutralizing cross-reactive antibodies induced by a tetravalent dengue DNA vaccine in relation to their protective and pathogenic roles. The researchers plan also to evaluate the effect of previous flavivirus infection on vaccine-induced antibodies. To achieve this purpose, they will analyze antibodies at a clonal level in mice with or without preimmunization with yellow fever 17D vaccine and/or Japanese encephalitis vaccine. A series of monoclonal antibodies raised against the tetravalent vaccine will be examined for specificity and cross-reactivity against each of 4 serotypes of dengue virus in vitro and for protective and pathogenic capacities in murine models.

**SC comments.** The project is well presented with clear description of objectives and methodology. The proposal is relevant to the WHO research priorities in the area of dengue vaccines and the PI has the experience to carry out the proposed study. If successful, the study could contribute in our knowledge on pathogenesis of DF and DHF. Nucleotide sequencing of the genes encoding the variable regions of immunoglobulin molecules is potentially an important outcome of this project and may have important applications for therapeutics in the future.
However, the SC made some critical notes. The PI proposes using DNA vaccines based on prototype strains of the four dengue viruses. Since these are not vaccine strains, it raises questions if the DNA vaccines will have the same immunodominant epitopes as the candidate live dengue vaccine viruses. It is not clear how the virus/antigenic specificity of the antibodies will be determined. There is little evidence to support the applicant’s hypothesis of a “rare” important clone. The methodology is not explained in detail. The PI should take into account critical notes of independent referees and SC members.

The SC recommended financial support for two years at the level of USD 50,600 for the first year.

**Project title:** Further characterization of four human macrophage putative receptors for dengue virus and their role in the immune system, V22/181/156

**Principal Investigator:** Dr M. M. B. Moreno-Altamirano
Depto. De Genetica Y B. Molecular Centro de Investigacion Y Estudios Avanzados-IPN, Mexico, D.F. Mexico

The objectives of the proposed study are to assess the identity specific receptors for dengue virus and characterize the signal pathways activated by dengue virus binding to its cell surface receptor. In previous work the PI has already identified four putative receptors for dengue virus on human macrophages. He has also identified numerous cytokine-related genes that are deregulated upon dengue infection.

In proposed study the PI plans that human monocyte-derived macrophages will be used as target cells for dengue virus serotype-2. Macrophage cell membranes will be obtained, and surface proteins will be solubilized and subjected to affinity chromatography. The proteins retained by a dengue virus-bound sepharose column will be separated by double dimension (DD) gel electrophoresis. Separated proteins will be transferred onto nitrocellulose membranes for virus overlay binding assays. Once identification of the dengue virus binding proteins is completed, replicate DD gels transferred onto PVDF sheets will be used to cut the relevant spots (virus-binding proteins) and the task of aminoacid microsequence by mass spectrometry will be assigned to a specialized laboratory, on contract. Protein sequence data banks will be searched for homology with the identified proteins. The PI propose to use a short panel of commercially available monoclonal antibodies with known specificity for *in vitro* inhibition of dengue virus infection, inhibition of overlay binding assays and western blotting over nitrocellulose membranes which contain separated proteins. The PI suggested that taken together results of the study will quite likely identify surface proteins involved in dengue virus infection of human macrophages.

**SC comments:** The project is interesting and innovative. However, it is highly ambitious in the time frame of one year. The approach proposed to identify the signals of activated pathways resulting from virus binding to receptors identified on the surface of macrophages is not well designed. It may be more relevant to differentiate signals of quiescent macrophages or after incubation with inactivated dengue virus rather than using monoclonal antibodies, if available, interacting with the receptors. This project is relevant to assess the role of cellular immunity.
against dengue, but may be far away from any application to the prevention or the treatment of the disease.

The SC did not recommend the project for financial support.

**Project title:** Assess efficacy of currently available vaccines against the three existing subtypes of TBE, V22/181/154

**Principal Investigator:** Dr I.V. Krasilnikov, Research unit of Microgen, Moscow, Russia,

The aim of this project is to carry out a comparative assessment of the efficacy of the existing tick-borne encephalitis (TBE) vaccines against the different subtypes of TBE virus. There are four manufactured TBE vaccines, based on killed purified viruses: two vaccines used in Russia are based on far eastern strains and two other used in Europe are based on western strains. The question to resolve is whether these vaccines are cross-protective against heterologous western, central or far eastern subtype strains.

The PI proposes two subproject to be completed. Under the first one he intends to vaccinate BALB/c mice with one of each available vaccines and to test the cross-reactivity against the three subtypes before challenging the mice with each of the subtypes of TBE viruses. In another set of study, the PI intends to evaluate neutralization antibodies in sera of individuals vaccinated with different TBE vaccines against strains representing the three existing subtypes of TBE. Finally, the cross-reactivity of a panel of anti-TBE envelop protein monoclonal antibodies is proposed to be tested against the three TBE subtypes.

**SC comments:** The project is relevant to the WHO research priorities in the area of flavivirus vaccines. If successful, the study could provide important information on the efficiency of TBE vaccines against different subtypes of TBE viruses. However, the SC noted some deficiencies in the project description. More details on the study design should be presented. For instance, under the proposed study with human sera data should be presented on a number of samples, procedure of neutralization assay and statistics. No tests indicated to be used for evaluation of cross-reactivity of antibodies in mice.

The SC recommended that the PI revises the application and focuses it on the study with human sera and not on subproject on mice. The budget could be consequently decreased. The revised application should be received in WHO by 1 January 2003.

**Project title:** Integral study of the kinetic of the humoral and cellular immune response in dengue 3 infected adults patients with implications for vaccine design, V22/181/155

**Principal Investigator:** Dr Maria Guzman, Pedro Kouri Tropical Medicine Institute, Habana, Cuba
The aim of the proposed study is to investigate the immune responses during natural dengue infection that leads to protection against dengue hemorrhagic fever (DHF) accompanying a succeeding dengue infection. This project will focus on the kinetic measurement of the humoral and cellular immune response in adult patients with dengue fever (DF) and DHF who were infected secondarily with dengue 3 virus.

To reach this aim, the PI identified the following objectives: 1) To characterize the kinetic humoral immune response including a) neutralizing antibody development, b) IgA, IgM and IgG enzyme-lined antibodies in serum and urine c) the IgG subtypes d) ADCC antibodies targeted against dengue virus infected cells. 2) To partially characterize the cellular immune response including: a) Th1 /Th2 response, b) occurrence of apoptosis of T cells during the evolution of the disease. 3) To compare results obtained in objectives 1 and 2 with the clinical picture of the disease (DF or DHF) and the virological findings.

The PI proposes to evaluate above-mentioned immune parameters in 22 adult patients with DF or DHF following DEN-3 infection in 2001. Th1/Th2-cell responses will be evaluated by measuring levels of IL-10, IL-13, TGF-beta, IFN-gamma, CD25, and beta-2 microglobulin, using commercial kits. In addition, CD4/CD8 levels will be determined by FACS analysis, and plasma levels of FAS (CD95) and sFASL (CD95-L) will be determined using commercial kits. Virological analysis will be performed using PCR and virus isolation in C636 cells.

SC comments: The overall objectives of the study are important and the potential value of the results is high. The study is addressed on important questions concerning the role of immune factors in enhancement of dengue infection. If successful, the study could improve on the knowledge in pathogenesis of DMF and safety of dengue vaccines. However, the SC has some critical notes: since this is retrospective analysis, specific breakdown of diseases (DF vs DHF) in the patients should be presented. It will allow determining whether the sample size will be large enough to make any conclusions. The SC feels that the establishing of commercial tests to measure cytokines in the laboratory of the PI is not necessary because these kits can be quite expensive. The SC would like to have more information on the capabilities of the laboratory in ADCC assay and on the availability of appropriate T-cell specimens for measuring apoptosis in T cells during this course of diseases.

The SC recommended that the PI revise the application taking into consideration by 1 January 2004. The PI should reply to questions raised by WHO SCRIHS.

Project title: Cohort natural dengue infection and study of dengue disease mechanism, (V22/181/152)

Principal Investigator: Dr Watanaveeradei Veerachai, Phramongkutklao, Hospital, Bangkok, Thailand

The objectives of this project are to 1). Characterize the dengue neutralizing antibodies after primary dengue infection and their kinetics by serotype-specific and cross-reacting antibodies. 2). Compare the incidence of secondary dengue infection between children who experienced severe and non-severe primary dengue infection and 3). Screening and evaluation of children who experienced primary dengue infection as the potential volunteers for dengue candidate vaccines.

To reach the objectives the PI plans that subjects enrolled in the study will be followed up into two groups with severe and non-severe primary dengue infection. Sixty cases at least will be enrolled in each group during 2 years. The information from the kinetics of neutralizing dengue
antibody will be analysed to better understand pathogenesis of disease. The PI plans to evaluate kinetics of type-specific and cross-reacting antibodies after primary and secondary infections in order to evaluate its role in incidence of DHF. The PI suggests that there is potential risk that vaccination of children after primary infection could cause disease if the level of immunity after the first contact with dengue virus is unprotective and proposed study could be useful in terms to evaluate this potential risk.

The cohort could provide a specific group of population who experienced primary dengue infection and could be invited to participate in future clinical trials of dengue vaccine.

**SC comments.** Project is relevant to the WHO strategic plan in dengue vaccines research. However, while the objectives of the proposed study are important, there seems to be no clear roadmap for reaching them. One flaw is a weak or poorly defined study design. It is not clear how the methods will be used to test the hypothesis. For example, from the description of the PRNT50 assay it is not clear which virus serotypes and strains will be used. The members of the SC noted that it may be difficult to always assign with certainty, those patients with primary dengue and those with secondary dengue. A better definition of disease severity should be attempted, over and above the need for patient hospitalisations, which is too subjective and may vary among clinicians.

The proposal was not recommended for support.