Cooperation and Collaboration Programs with ASEAN Universities

Thailand - Japan Research Seminar on Global Health and Infectious Diseases

Date: 18 October 2013, 15:00-18:00

Venues: VOD conference room, Chamlong building, Mahidol University
Room E803, Kobe University Graduate School of Health Sciences
Main Lecture Hall, Kobe University Graduate School of Medicine

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Program:

- **Opening remarks** 15:00
  Yasuhiro Minami, Vice Dean, Kobe University Graduate School of Medicine
  Yaowalark Sukthana, Dean, Faculty of Tropical Medicine, Mahidol University
- **Session 1, Faculty of Tropical Medicine, Mahidol University** 15:10-15:40
  1-1. Chonlatip Pipattanaboon, (Cloning and expression of Dengue recombinant proteins for Dengue vaccine design.)
  1-2. Phanthila Sirichaiyakul, (Gambicin: an antimicrobial peptide as the therapeutic option for treatment of antibiotic-resistant bacteria and tropical pathogens.)
- **Session 2, Mahidol-Osaka Center for Infectious Diseases, Osaka University** 15:40-16:10
  2-1. Orapim Puiprom, (Characterization of chikungunya virus infection of a human keratinocyte cell line: Role of mosquito salivary gland protein in suppressing the host immune response.)
  2-2. Panjaporn Chaichang, (Sequence variation of Dengue virus 2 premembrane and envelope derived from patient plasma shows significantly different biological characteristics in human K562 cells.)
- **Session 3, Kobe University Graduate School of Health Sciences, Kobe University Graduate School of Medicine** 16:10-16:55
  3-1. Eriko Iwasaki / Graduate School of Health Sciences (Bone mineral density and bone turnover among young women in Chiang Mai, Thailand)
  3-2. Shuhei Ueda / School of Medicine Faculty of Health Sciences (Detection of anti-dengue virus antibodies and viral RNA in serum samples derived from Thai patients with febrile illness)
  3-3. Chyntia Jasirwan / Graduate School of Medicine (The human herpesvirus 6 U21-U24 gene cluster is not essential for virus growth)
- **Session 4, Lecture seminar** 17:00-18:00
  4-1. Pongrama Ramasoota / Faculty of Tropical Medicine, Mahidol University (Thailand-Japan research collaboration on development of therapheutic products against Dengue virus)
  4-2. Masanori Kameoka / Kobe University Graduate School of Health Sciences (HIV/AIDS research at oversea research collaboration centers)
- **Closing remarks** 18:00
  Satoshi Takada, Dean, Graduate School of Health Sciences, Kobe University

1-1

CLONING AND EXPRESSION OF DENGUE RECOMBINANT PROTEINS FOR IDEAL VACCINE DESIGN

Chonlatip Pipattanaboon[1], Tadahiro Sasaki[2], Mitsuhiro Nishimura[2], Chayanee Setthapramote[1], Pannamthip Pitaksajjakul[1], Tamaki Okabayashi[3], Panjaporn Chaichana[3], Kazuyoshi Ikuta[2], Pongrama Ramasoota[1]

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Dengue virus (DV) is composed of structural (C, prM, and E) and nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The envelope (E) protein is the principal target of neutralizing antibody and consists of three discrete domains (DI, DII, and DIII). Antibodies against prM, NS1, NS3, and NS5 proteins also have been detected in people. Mostly, neutralizing epitopes have been identified and localized on DII and DIII of E protein. Studies of epitopes on E protein are not only necessary for understanding virus-antibody interactions, but also in terms of helping to design an ideal dengue vaccine that can provide life-long protection, strong neutralizing activity against all four serotypes, and low/no enhancing activity.

Since, 136 HuMAbs were successfully generated using hybridoma technique from peripheral blood mononuclear cells (PBMCs) in acute and convalescent phases of Thai dengue patients at CEAR. Characterization of these HuMAbs is required for future therapeutic use and vaccine development. The aim of the study is to accurately identify target proteins, antigenic domains, and epitope regions of a variety of these HuMAbs. Here, four recombinant proteins (C, prM, E, and NS1) and nine truncated E proteins (E1-394, E1-296, E1-192, E1-132, E1-98, E52-132, E60-121, E74-118, and E74-109) were cloned and expressed in HEK293T cell and *E. coli.* A series of the expressed proteins was used to determine binding reactivity of 136 HuMAbs by immunofluorescence assay (IFA) and western blotting (WB).

From 136 HuMAbs, 101 (74.26%), 10 (7.35%), and 12 (8.82%) HuMAbs were determined target proteins as E, prM, and NS1 proteins, respectively. Noticeably, 71 (52.21%) HuMAbs recognizing the viral E protein were able to neutralize all four serotypes of DV. For E-specific HuMAbs, the epitope regions were assigned to E52-132 (1st DII) by 51 HuMAbs, E192-296 (2nd DII) by 2 HuMAbs, and E296-394 (DIII) by 1 HuMAb. Among these 51 HuMAbs, the minimum epitope sites were defined as E60-121 by 22 HuMAbs, E74-118 by 14 HuMAbs, and E74-109 by 14 HuMAbs. The results implied that 1st DII containing the fusion loop is the predominantly immunogenic target in humans.

According to several studies, dengue vaccine developed from full-length prM/E protein might induce neutralizing activity, but it may also induce enhancing activity. The short truncated protein, E74-118 or E74-109, may be the candidate to construct epitope-based immunogen present in the highly conserved region of fusion loop, with high neutralizing and less enhancing activities compared to full-length proteins.

KEY WORDS: DENGUE / ENVELOPE / DOMAIN II / EPITOPE / RECOMBINANT / TRUNCATE

1-2

Expression and characterization of the antimicrobial peptide gambicin from *Culex quinquefasciatus*

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ABSTRACT

Antimicrobial peptides (AMPs), which can be found in many organisms, are generally low molecular weight peptides containing less than 100 amino acid residues. Due to ineffective conventional antibiotic treatments causing numerous mortalities of infected patients in the world, AMPs are an interesting approach to the development of novel therapeutics against pathogens, particularly antibiotic-resistant bacteria, because they are safe, non-toxic to mammals, no bacterial resistance and showing broad-spectrum antimicrobial activity. Interestingly, an antimicrobial peptide gambicin isolated from *An. gambiae* cell lines possess antimicrobial activity against gram-positive and -negative bacteria, filamentous fungi, and *Plasmodium* species. However, the isolation of *Culex* gambicin and expression of recombinant mosquito gambicins remain unreported. This study aimed to express recombinant *Culex* gambicin in *Pichia pastoris* and/or baculovirus expression system, and successfully cloned and expressed. SDS-PAGE of *Culex* gambicin in *Pichia pastoris* revealed an obvious protein band with a molecular weight of 7.0 kDa, indicating high production of recombinant *Culex* gambicin. Testing of antimicrobial activity against *E. coli* DH5α strain by agar-well diffusion assay exhibited no activity. A refolding approach to the recovery of antimicrobial peptides is required for further studies, while the expression of recombinant gambicin in the baculovirus expression system is on the process which will be also discussed.

2-1

Characterization of chikungunya virus infection of a human keratinocyte cell line: Role of mosquito salivary gland protein in suppressing the host immune response

Orapim Puiprom[1], Ronald Enrique Morales Vargas[2], Rutcharin Potiwat[2], Panjaporn Chaichana[1], Kazuyoshi Ikuta[5], Pongrama Ramasoota[3,4], and Tamaki Okabayashi[*1,5]

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The chikungunya virus (CHIKV) is a mosquito-borne virus that has recently re-emerged in several countries. On infection, the first vertebrate cells to come into contact with CHIKV are skin cells; mosquitoes inoculate the virus together with salivary gland protein into host skin while probing and feeding on blood. However, there is little known about the susceptibility of human skin cells to CHIKV infection. To clarify this, we investigated the kinetics of CHIKV in the human keratinocyte cell line, HaCaT. CHIKV actively replicated in HaCaT cells, with virus titers in the supernatant increasing to 2.8 × 10(4) plaque-forming units (PFU) ml(-1) 24 h post infection. CHIKV infection suppressed production of interleukin-8 (IL-8) in HaCaT cells. The function of IL-8 is to recruit immune cells to virus-infected sites, a process known as chemotaxis. Furthermore, we assessed the role of mosquito salivary gland protein in CHIKV infections by comparing the levels of CHIKV gene expression and chemokine production in HaCaT cells with and without salivary gland extract (SGE). SGE enhanced both the expression of the CHIKV gene and the suppression effect of CHIKV on IL-8 production. Our data suggest that the HaCaT cell line represents an effective tool for investigating the mechanism of CHIKV transmission and spread in skin cells. At the mosquito bite site, CHIKV works together with SGE to ensure the virus replicates in skin cells and escapes the host immune system by suppression of IL-8 production.

2-2

SEQUENCE VARIATION OF DENGUE VIRUS 2 PREMEMBRANE AND ENVELOPE DERIVED FROM PATIENT PLASMA SHOWS SIGNIFICANTLY DIFFERENT BIOLOGICAL CHARACTERISTICS IN HUMAN K562 CELLS

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BACKGROUND: Dengue virus (DV), like most RNA viruses, has been shown to exist as a population of closely related genome, so-called quasispecies within individuals. However, the biological significance of quasispecies in virus infection and immunopathogenesis remains unclear.

OBJECTIVE: In this study, we aimed to determine the biological significance of sequence variation of DV2 structural region (prM-E) derived from patients in Thailand.

METHODS: The prM-E region of dengue virus from 4 clinical samples were amplified by RT-PCR, cloned into plasmid. The sequences of these genes were determined and their variations were analyzed by ABI automated sequencer and MEGA5, respectively. For analyze an effect of sequence variation of prM-E regions, the prM-E region of DENV-2 infectious cDNA plasmid clone R05-624 was replaced with the representative prM-E gene from each variants derived from one DHF patient D30. Virus replication kinetics, neutralizing and infection-enhancing effect of the variants were analyzed.

RESULTS: The extent of sequence diversity of prM-E region revealed that DV2 from 4 patients demonstrated sequence diversity with the mean pairwise distance ranging from 0-0.046%. For further investigation, we selected five representative variants from DHF patient D30 to construct infectious cDNA clones by replacing prM-E region. Variants showed significantly different replication rate in human leukemia K562 cells, but not in C6/36 or Vero cells. It is noteworthy that the variant containing substitution at amino acid position 365 in domain III of E protein showed strong enhancing effect, and low FRNT50 titer by mouse anti-E monoclonal antibody (MAb) 4G2 compared with other variants.

CONCLUSIONS: Our result suggested that substitutions of DV2 prM-E region derived from patient plasma affect virus production in human cell line either in the presence or absence of antibodies.

KEYWORDS: dengue virus, quasispecies

3-1

Bone mineral density and bone turnover among young women in Chiang Mai, Thailand.

Eriko Iwasaki, Nuntana Morakote, Somsak Chaovitsaree, and Hiroya Matsuo

Presenter; Eriko Iwasaki (Kobe University, Graduate School of Health Sciences, Department of International Health)

The present study was carried out to investigate the influence of lifestyle on bone mineral density (BMD) and bone turnover among young women in Chiang Mai, Thailand. A total of 177 young women affiliated with Chiang Mai University hospital were enrolled. Questionnaires about their lifestyle and the Osteoporosis Knowledge Test (OKT) were examined. The measurement of BMD was assessed by Quantitative Ultrasound (QUS). Based on the measurement of BMD, the subjects were divided into 2 groups, a Low BMD group (L group: less than YAM-1.0SD) and a Normal BMD group (N group: more than YAM-1.0SD) . L group (n= 23) and N group (n= 23) were examined using OC, NTx and ucOC as bone turnover markers, and serum Ca, 1,25-$(OH)_2$VitaminD, VitaminK1 and Vitamin K2(MK-4) as bone turnover related factors. The percentage of Low BMD group was 23.2%. Concerning lifestyle and BMD, the BMD of the low cheese intake group was 99.7±17.0 and the BMD of the high cheese intake one was 110.0±23.3 ($p < 0.05$). The BMD of the fracture experience group was 82.5±11.6 and the BMD of no-fracture group was 103.3±19.6 ($p < 0.05$). There were significant differences in ucOC and 1,25-$(OH)_2$VitaminD between L and N groups ($p < 0.05$). It was suggested that BMI, food and fracture experience might affect BMD level and suppression of bone formation might be contributed to the low BMD in young women in Chiang Mai, Thailand.

3-2

Detection of anti-dengue virus antibodies and viral RNA in serum samples derived from Thai patients with febrile illness

Ueda Shuhei
Kobe University School of Medicine Faculty of Health Science

Abstract

In southeast Asia, there are several mosquito-borne viral infectious diseases which are prevalent in tropical and subtropical regions. Among them, dengue fever is an important one that needs to be paid attention. Dengue virus is transmitted by two strains of mosquitoes, *Aedes aegypti* and *Aedes albopictus*. Dengue virus is classified into 4 serotypes, and causes dengue fever in 50-100 million infection per year. 2.5 billion people live in dengue-endemic tropical and subtropical areas. In this study, dengue suspected patients with febrile illness in Nakhonsawan, Thailand were diagnosed by detecting viral RNA by RT-PCR and by detecting anti-dengue antibodies by ELISA. In addition, viral serotyping was also carried out by RT-PCR. Twenty-six of 28 samples were positive for dengue viral RNA. In addition, the most epidemic serotype was dengue type 3; however, the patients infected with dengue virus type 1, 2 and 4 were also present, indicating the co-circulation of all 4 serotypes of the viruses. The ELISA of paired, acute-convalescent serum samples revealed that anti-dengue antibodies, IgG and/or IgM, were positive at acute phase in all samples. In addition, the higher titer of the antibody was detected at convalescent than acute phase in most samples. In a paired samples, IgG was negative at acute phase, while it was positive at convalescent phase, indicating this patient was primary infected with dengue virus. Two samples were positive for anti-dengue antibodies, but were negative by PCR, suggesting that those patients were infected with other virus in the Flaviviridae, such as Japanese encephalitis virus (JEV) and Zika virus. The superinfection of different serotypes of dengue viruses potentially causes severe illuness; therefore, more epidemiological studies including viral serotyping is required in dengue endemic area.

3-3

The human herpesvirus 6 U21 to U24 gene cluster is not essential for virus growth

Chyntia Jasirwan, Huamin Tang, Mayuko Hayashi, Megumi Ota and Yasuko Mori
Division of Clinical Virology, Center for Infectious Diseases, Kobe University Graduate School of Medicine.

Background

Human herpesvirus 6 (HHV-6) is a T-lymphotrophic virus belonging to the genus *Roseolovirus* within betaherpesvirus subfamily. The U20–U24 gene cluster is unique to *Roseoloviruses*; however, it is not known how they function or whether they are essential for virus growth. In addition, we found that CD6 which is one of the immunological synapse components involved in lymphocyte activation and differentiation processes, was specifically downregulated from the cell surface of HHV-6-infected cells. Therefore the aims of our study are to analyze the function of this gene cluster in HHV-6 infection and to elucidate whether it plays roles in the downregulation of CD 6 from the cell surface.

Methods

We used two step Red Recombination procedure to generate the Bacterial Artificial Chromosome (BAC) and reconstitute the recombinant virus with a deletion in full length of U21 to U24 gene and also the revertant. We named the resultant BAC, HHV-6ABACΔU21-U24, and HHV-6ABACΔU21-U24rev. We isolated the BAC DNA and confirmed the digestion pattern with BamH1 enzyme. Then we reconstituted the infectious virus by transfection into the JJHAN cells and coculturing to CBMCs. The titre of virus was measured using $TCID_{50}$ method in JJHAN cells. The growth curve of these viruses was determined by real-time PCR, and was compared. In addition, the CD6 downregulation in infected cells was examined using the viruses.

Results

Here we successfully generated recombinant viruses of a U21–U24 deletion mutant and the revertant BAC. There was a similar proportion of GFP-positive cells within the rHHV-6ABAC- and rHHV-6ABACΔU21-U24-infected CBMC populations. This was confirmed by the expression of gQ1 in the cell lysates. The viral growth curves showed that there was no significant difference in the growth of the viruses (wild-type, recombinant and its revertant), suggesting that U21–U24 is not required for virus propagation in vitro. The flow cytometric analysis showed tha CD6 was not downregulated from the cell surface in HHV-6A U21-U24 deletion mutant virus-infected Molt3 cells, indicating that U21-U24 region may contribute to the CD 6`s downregulation from the cell surface.

4-1

"Thailand-Japan research collaboration on development of Therapeutic Human Monoclonal Antibodies against four serotype of Dengue virus"

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Dengue virus infection a re-emerging virus, constitute the largest vector-borne disease virus, with 50-100 million cases reported every year, has been very important public health problem in Tropical and sub-tropical countries. Thailand is one of Dengue affected country. Recently, from January till September 2013, there are big outbreak of Dengue in the northern and southern provinces of Thailand, which lead to 120,000 cases with 110 dead cases. Even though several Dengue vaccine and therapeutic products have been developed and tested. But until present, there have no effective vaccine and specific treatment for dengue virus (DENV), especially against all 4 serotype.

From the years 2009-2013, with the supported from Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA), Professor Kazuyoshi Ikuta from Research Institute for Microbial Diseases (RIMD), Osaka University and Thai Researchers groups from Department of Medical Sciences, Ministry of Public Health, together with Faculty of Tropical Medicine, Mahidol University had started the research collaboration project entitle " Development of Therapeutic products against Dengue virus"

One of the objective of this research project was to develop the therapeutic human monoclonal antibodies (HuMAbs) with strong neutralizing activity against all four serotypes of DENV using novel myeloma fusion partner namely SPYMEG. Peripheral blood mononuclear cells (PBMC) obtained from acute and convalescent dengue patients that were used for fusion with SPYMEG cells. Then, fused hybridoma cells were cultured, screening of DENV specific antibody using Immuno fluorescence Assay (IFA), followed by limiting dilution, Finally, culture fluids containing HuMAbs were tested by IFA and viral neutralization (NT). Total 136 hybridomas producing anti-dengue huMAbs were successfully generated. Interestingly, highly efficacy in number of hybridoma clones producing specific huMAb was obtained from PBMCs of acute phase (121 HuMAbs) than those from convalescent phase (15 HuMAbs). After characterization using IF and NT assay, 20 out of 136 HuMAbs potentially showed 85-100% NT activity against all 4 serotypes of DENV. Three candidates from these 20 HuMAbs were further tested and found that it could neutralized 20 clinical DENV isolated (5 isolates per each serotype) with higher NT than those of laboratory isolates. DENV inoculated suckling mouse that treated with these three candidate NhuMAbs could survive longer than untreated group significantly, and also DENV were clearly eliminated within 2 days among NhuMAb treated marmoset groups, in contrary with control group (using non dengue human IgG and PBS) that DENV copy still very high within 2-3 days. These 3 candidate NhuMAbs are now ongoing for large scale IgG stable expression in Chinese Hamster Ovary (CHO) cell, which aiming for use in human clinical trial in the near future..

*Present in video conference seminar of Kobe University and Mahidol University on 18 October, 2013 at Faculty of Tropical Medicine, Mahidol University.

4-2

HIV/AIDS research at oversea research collaboration centers

Masanori Kameoka, Division of Infectious Diseases, Department of International Health, Kobe University Graduate School of Health Sciences

Human immunodeficiency virus type 1 (HIV-1) is a major causative agent of acquired immunodeficiency syndrome (AIDS). HIV-1 is prevalent all over the world, and according to the report from the Joint United Nations Programme on HIV/AIDS (UNAIDS), 34 million individuals are living with HIV-1, while 2.5 millions are newly infected with HIV-1 and 1.7 millions died from AIDS during 2011. Among Southeast Asian countries, the annual incident rate of HIV-1 infection has declined in countries including Cambodia, Malaysia and Thailand, whereas it has continuously increased in countries such as Indonesia, Bangladesh and the Philippines. HIV is a blood-borne virus that spreads through contaminated blood and other body fluid. Antiretroviral therapy using multiple anti-HIV drugs is currently available, while the development of vaccines has not been succeeded. As a collaborative research with the Ministry of Public Health of Thailand, we studied the immunological characteristics of Thai strains of HIV-1, and found the mechanism of how the virus is escaped from the inhibitory effect of a neutralizing monoclonal antibody on viral replication. In addition, as a collaboration with the Institute of Tropical Disease, Airlangga University of Indonesia, sero and molecular epidemiology of HIV have been carried out in Indonesia, and the high prevalence of HIV among commercial sex workers in Surabaya was revealed. In this talk, I will present the overview of global HIV/AIDS endemic and HIV-1 pathogenesis, as well as these research outcomes produced at research collaboration centers in Thailand and Indonesia.