北海道大学グローバルCOEプログラム

Global COE Program
Progress Report FY 2012
Presentation

2012年度 PD･RA研究成果発表会

Programme & Abstract

■場所：北大 獣医学研究科　講堂
■日時：　2013年3月22日(金)13:30～17:00

Venue: Conference Hall
Graduate School of Veterinary Medicine, Hokkaido University
Time & Date: 13:30～17:00, March 22 (Fri), 2013

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13:30-13:35 Opening speech:

■Chairperson■ Jung-Ho Youn

13:35-14:00

1. **The significance of two recombinant antigens base ELISA assay to diagnose leptospirosis**
   Chandika D. Gamage
2. **Production of recombinant LipL32 protein of a major outer membrane protein of Leptospira for the diagnostic antigen**
   Kanae Shiokawa

■Chairperson■ Masayoshi Isezaki

14:00-14:30

3. **Comparative genomic analysis of eight *Leptospira* isolates from Japan and the Philippines revealed the existence of four putative novel genomic islands/islets in *L. interrogans* serovar Lai strain 56601**
   Jung-Ho Youn
4. **Detection of potential zoonotic pathogens in ticks using a metagenomic approach**
   Ryo Nakao

■Chairperson■ Ryo Nakao

14:30-14:55

5. **Epidemiological study of infectious diseases in wild birds in Hokkaido and molecular characterization of expressed sequence tags for *Dermanyssus gallinae* for the development of a novel control method of the mite**
   Masayoshi Isezaki
6. **The role of Hokkaido sika deer in the transmission of zoonoses**
   Kyunglee Lee

14:55-15:15 Coffee break

■Chairperson■ Manabu Hashimoto

15:15-15:40

7. **West Nile virus replication is inhibited by autophagy gene *Atg5***

Shintaro Kobayashi

8**. The role of mouse oligoadenylate synthetase-1 family**

Enas Hamed Mahmoud Elkhateeb

9**. Excessive cytokine respose to rapid proliferation of highly pathogenic avian influenza viruses leads to fatal systemic capillary leakage in chickens**

Saya Kuribayashi

■Chairperson■ Shintaro Kobayashi

15:40-16:05

10**. Molecular mechanism of intracellular rabies virus dybamics**

Yusuke Sakai

11. **Protooncogene TCL1b functions as an Akt kinase co-activator which exhibits oncogenic potency *in vivo***

Manabu Hashimoto

■Chairperson■ Hiroki Yamaguchi

16:05-16:25

12. **Characterization of Hokkaido virus, genus hantavirus**

Takahiro Sanada

13. **Application of new world hantaviruses pseudovirions to an alternative method to plaque reduction neutralization test (PRNT)**

Sanae Nishio

■Chairperson■ Takahiro Sanada

16:25-16:45

14**. Examination of adjuvant effects of gold nanoparticles combined with West Nile virus E protein**

Hiroki Yamaguchi

15. **Efficient delivery of epitope peptides to antigen-presenting cells using phosphatidylserine for induction of epitope-specific T cell responses**

Toru Ichihashi

16:45- Certificate Awards Ceremony - Zoonosis Control Expert

Best Presentation Awards Ceremony & Closing speech

The significance of two recombinant antigens base ELISA assay to diagnose leptospirosis

Chandika D. Gamage
Department of Microbiology
Hokkaido University Graduate School of Medicine

Introduction

Leptospirosis is a significant serious zoonotic disease with veterinary and public health impacts. Laboratory diagnosis of the disease is largely based on serological methods such as enzyme-linked immunosorbent assays (ELISA), in most of resource limited settings where leptospirosis is an endemic. In this study we have evaluated the diagnostic sensitivity of two recombinant antigens for ELISA to detect anti-leptospiral antibodies in different species.

Materials and methods

Whole sequence of LipL32 and partial segment of the LigA gene were amplified using PCR. Those sequence segments were subcloned into pET-43.1a. *E.Coli* BL21 was transformed by the recombinant plasmid and both fusion (NUS) proteins expression was induced by ITPG. Humans, rodents, cattle and dogs serum samples which were collected from Sri Lanka, Tokyo and Vietnam were diagnosed to detect anti-leptospiral antibodies employing recombinant antigen bases ELISA system which has capable of detecting IgG class antibodies. Each antigen's cutoff point optical density (OD) value for ELISA was determined by considering the control group's OD values or based on previous evidences. All samples were tested duplicate and average values were used for further calculations and plotting. Positive samples' data were grouped as follows; a) Only LipL32 antigen positive; b) Only LigA antigen positive; c) Both antigen positive and d) Either antigen positive. Grouped data used to determine the assay's sensitivity.

Results

In this study a total of 72 leptospirosis suspected patients, 125 healthy people, 58 rodents, 110 cattle and 30 dogs sera were examined. Of 395 serum samples, 94 (24%) and 115 (29%) were detected as containing anti-lepstospiral antibodies by LipL32 recombinant assay and LigA recombinant assay respectively. Grouped data indicates a higher detection rate (134, 34%) than the individual antigen based assay's data. Furthermore, only 77 (19%) of samples were positive for both antigens, which is clearly indicates the variability and complexity of anti-leptospiral antibodies.

Conclusion

The summarized study results indicate that recombinant antigens of LipL32 and LigA are useful for the detection of anti-leptospiral antibodies. However, it is advisable to use more different types of recombinant antigens in an assay to increase the sensitivity.

Production of recombinant LipL32 protein of a major outer membrane protein of Leptospira for the diagnostic antigen

Kanae Shiokawa
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Graduate School of Medicine, Hokkaido University

[Introduction]

Leptospirosis is a febrile disease caused by infection with *Leptospira interrogans*, and known as important zoonoses. Although leptospirosis has been reported in worldwide underestimated incidence might be existing in tropical and subtropical countries among fever patient with unknown etiology. Therefore, a rapid, and simple diagnosis is strongly required for differential diagnosis as well as obtaining correct epidemiological information. Leptospira is a gram negative bacteria expressing several antigens as outer membrane proteins (OMP) that have been studied as diagnostic antigens. Among them, the protein LipL32 is found exclusively in pathogenic leptospires as highly conserved and immunogenic OMP. Therefore, recombinant LipL32 antigen expressed with NUS-tag was prepared by pET 43.1 system and applied for serodiagnostic antigen. However the antigen was not stable because of its large molecular mass. Here, we have firstly attempted to determine a basic condition of purification method of LipL32 and secondary antigenic regions were examined by comparing the reactivity of truncated LipL32 antigens with immune serum.

[Materials and Methods]

For preparation of recombinant LipL32 (rLipL32; 273 amino acid (aa)) and truncated LipL32 (N-rLipL32; 1–155 aa, I-rLipL32; 155-200 aa, C-rLipL32; 200-273 aa) of *L. interrogans*, serogroup Pyrogenes, serovar Manilae, coding information were amplified by PCR. The DNA amplicons were cloned into pET43.1 and pRSET.A vectors. rLipL32 and truncated LipL32 were expressed in BL21(DE3) *Escherichia coli* and were purified from the extract of *E.coli* using Histrap column with Tris-HCl elution buffer containing 20 mM, 30 mM, 40 mM Imidazole. Obtained recombinant proteins were evaluated by SDS-PAGE, Western blotting, and ELISA. For Western blotting and ELISA, rabbit anti-LipL32 antibody and immune rat sera inoculated with *L.interrogans*, serover Manilae were used.

[Result]

The result from SDS-PAGE, rLipL32 and N-rLipL32, I-LipL32, and C-rLipL32 expressed as expected molecular sizes. Analyses by Western blotting showed LipL32 antigen had multiple linear epitopes throughout the molecule. Following ELISA study showed rLipL32 reacted stronger than truncated rLipL32 proteins with antisera. For the use of serological diagnosis, entire LipL32 antigen was required. The use of pRSET vector giving shorter fusion protein gave less degraded antigen. And fragmentation of antigen was reduced depending on higher concentration of imidazole during purification process.

[**Discussion**]

Expression and purification methods of rLipL32 antigen were established. In the future, we will apply this antigen for immnochromatography (ICG).

Comparative genomic analysis of eight *Leptospira* isolates from Japan and the Philippines revealed the existence of four putative novel genomic islands/islets in *L. interrogans* serovar Lai strain 56601

Jung-Ho Youn
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Research Center for Zoonosis Control, Hokkaido University

Introduction

Leptospirosis is the most widespread zoonotic disease worldwide which is of concern to both human and animal health. Most studies conducted on leptospires have been related with the pathogenesis of *Leptospira* species. Although 50% of more than 230 serovars among pathogenic leptospires belong to the species *interrogans* and *borgpetersenii*, only three full genome sequences of *L. interrogans* serovars are currently available. Therefore, comparative genomic analyses across serogroups and serovars are limited. The advancement of the next-generation sequencing (NGS) technology, a faster and affordable alternatives to conventional sequencing methods, has allowed for a detailed analysis of *Leptospira* serogroup, serovar, strain-specific and virulence related genes.

Materials and Methods

Leptospira interrogans strains were sequenced by NGC technology GS 454 Junior, GS FLX titanium. Various analysis programs were used to analyse the obtained data.

Result

Our study revealed two novel genomic islands and two novel genomic islets (GIs) in the strain 56601. In addition, the O-antigen gene (*rfb*) cluster region mapping results imply high similarities between isolates belonging to the same serogroup as well as to different ones. The neighor joining phylogenic tree based on concatenated sequence of SNPs demonstrated close relation between isolates within the same serogroup.

Conclusion

Our results revealed that the *rfb* region was similar not only among strains of the same serogroup but also among different ones. The results obtained on the novel genomic islands/ islets indicates the possibility of a horizontal gene transfer (HGT) between isolates and may also lead to a better understanding of the dissemination of genes, especially related to virulence in pathogenic *Leptospira* species. In addition, this study has also demonstrated a novel method for serotyping using SNPs.

Detection of potential zoonotic pathogens in ticks using a metagenomic approach

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Hokkaido University Research Center for Zoonosis Control

Ticks can transmit a variety of viral, bacterial and protozoal pathogens, which are often zoonotic. The increasing number of novel tick-borne pathogens has been reported during recent decades. The aim of this study was to identify diverse tick microbiomes that may contain as-yet unidentified pathogens using a metagenomic approach.

Seven tick species (both field-collected and laboratory-reared) were used in the present study. Purified bacteria/archaea-enriched fractions prepared from tick homogenate were subjected to DNA extraction and pyrosequencing after whole genome amplification. Resulting sequence reads were phylotyped using a Batch Learning Self-Organizing Map (BLSOM) program, which allowed phylogenetic estimation based on the similarity of oligonucleotide frequencies, and functionally annotated by BLASTX similarity searches.

We showed that ticks harboured a variety of bacteria including those previously associated with human and animal diseases such as genera *Anaplasma*, *Bartonella*, *Borrelia*, *Ehrlichia*, *Francisella*, and *Rickettsia*. In addition, BLSOM analysis detected microorganisms belonging to the phylum Chlamydiae as potential zoonotic pathogens. Gene sequences associated with bacterial pathogenesis were identified and were suspected to originate from horizontal gene transfer events.

Our efforts to construct a database of tick microbes may lead to the empowerment to predict emerging tick-borne diseases. A comprehensive understanding of the tick microbiomes will also be useful to understand tick biology including vector competency and interactions with pathogens.

Epidemiological study of infectious diseases in wild birds in Hokkaido and molecular characterization of expressed sequence tags for *Dermanyssus gallinae* for the development of a novel control method of the mite

Masayoshi Isezaki
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Wild birds including waterfowls play important roles as the sources of infections for the epidemiology of many infectious diseases including the zoonosis, such as the influenza virus and West Nile virus (WNV) infections. We have previously established detection methods for Japanese encephalitis virus (JEV) and WNV from feather tips using RT-PCR and nested RT-PCR. Then, the molecular epidemiological surveillance of JEV and WNV were performed by using feather tips, organs collected from the wild birds in Hokkaido. However, no JEV- and WNV-positive birds were found in the survey of 2009-2012 in the Hokkaido area. We also carried out epidemiological surveillance of other viruses, influenza virus, Newcastle disease virus (NDV), and Marek's disease virus (MDV) using either the cloacal swabs or feather tips from the wild birds. From some of these samples, influenza viruses, NDV, avian paramyxovirus, and MDV, were isolated, showing that these viruses are prevalent in wild bird populations. In addition to the molecular biological methods, we were also developing serological method to detect specific antibodies to pathogens using feather tips. Fractions containing antibodies were successfully extracted from the feather tips, and anti-sera specific to domain III peptide from JEV and WNV envelope protein were prepared. Using these anti-sera and antigens, we are constructing an ELISA method to detect WNV- and JEV-specific antibodies from the feather tips.

The poultry red mite, *Dermanyssus gallinae*, is regarded as the most economically important ectoparasite for the poultry industry all over the world. In addition, the red mite has been suggested as a potential vector for several pathogens. Currently, acaricide resistance is a major problem for the control of the mite in poultry farms. However, information on the molecules of this mite was scarcely available for the development of novel control methods. Hence, in this study, analysis of expressed sequence tags of the red mite has been performed. The cDNA library was constructed from the red mite collected from a poultry farm, and cDNA clones were randomly sequenced. We have analyzed a total of 2,466 cDNA clones, and 373 sequences were identified with those known of functions. Then, we focused on homologues of peroxiredoxins (Prx4 and Prx2) and type II allergen (Der-g2), which are suggested as possible vaccine candidates for other ticks, and their functions were analyzed by using recombinant proteins. Moreover, we obtained anti-sera specific to these recombinant proteins. We are testing vaccine efficacy with *in vitro* feeding assay using these anti-sera.

The role of Hokkaido sika deer in the transmission of zoonoses

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Sika deer (*Cervus Nippon yesoensis*) population in Hokkaido was estimated about 640 thousand in 2010. Those overpopulated deer have caused many problems not only in forests but also in the cultivation of farms. As the contact between the wild animal and cattle increased, the possibility to share infectious pathogen raised. However, the status or prevalence of zoonotic pathogens in wild deer are still left unknown well. So far, deer are not considered as so significant in the link of zoontic disease infection but their potential should be counted in the unpredictable global climate changing situation.

To define the role of deer in the transmission of spirochetic pathogen; *Borrelia spp*. and *Leptospira spp*, deer tissue and blood samples were devoted in molecular and serological analysis. Deer sample were collected from 206 Hokkaido wild deer caught from nuisance control or traffic accident in Hokkaido.

In total, 15 from 193 blood DNA were positive in *Borrelia fla*B detecting PCR. *Borrelia miyamotoi, B.japonica* and *B.lonestari* were confirmed with the number of 1, 2 and 12, respectively. Lyme borrelia were not found in deer blood but checked in skin DNA (2/87).

2 of 174 deer kidney samples were positive in Leptospira flaB gene detecting PCR. In MAT, 9 from 206 serum samples were checked as positive.

West Nile virus replication is inhibited by autophagy gene *Atg5*

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ATG5 is necessary for autophagy, which is highly conserved process of intracellular homeostatic regulation. It has been reported that autophagy plays a role in virus infection process. For instance, autophagy has antiviral activity for herpes simplex virus type 1, while it is required for efficient viral replication for poliovirus and dengue virus infection. West Nile virus (WNV) belongs to the *Flaviviridae* family of viruses and has emerged as a significant cause of viral encephalitis in humans, animals, and birds. In this study, we examined the effect of autophagy on the WNV replication.

Following WNV infection, the extra- and intra-cellular WNV titer of the *Atg5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) was significantly higher than that of *Atg5*$^{+/+}$ MEFs. After transient expression of ATG5 in *Atg5*$^{-/-}$ MEFs, the extra-cellular WNV titer was decreased. These results suggested that an antiviral role of autophagy in WNV replication. To determine the step of WNV life cycle affected by autophagy, *Atg5*$^{+/+}$ or *Atg5*$^{-/-}$ MEFs were inoculated with the pseudo-infectious WNV reporter virus particles (WNV-RVPs) that represent infection as a function of GFP reporter gene expression. WNV-RVPs are capable of only a single round of infection and offer a quantitative approach for detecting early steps. The GFP positive rate in the *Atg5*$^{-/-}$ MEFs was higher than that in *Atg5*$^{+/+}$ MEFs. This result suggested that autophagy process affects early steps in WNV life cycle.

Taken together, we show that the cellular autophagy process acts as a defense system against WNV infection. We are currently examining the detailed mechanism of antiviral role of autophagy.

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The role of mouse oligoadenylate synthetase-1 family

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West Nile Virus (WNV) is an enveloped, positive-sense single-stranded RNA virus, a member of the *Flaviviridae* family. The natural transmission cycle of WNV involves birds and mosquitoes of the genus *Culex*. However, it occasionally involves horses and humans as incidental hosts.

One of the most important immune response proteins to the viral infection is the interferon-induced oligoadenylate synthetase (OAS) family. The double-stranded RNAs produced in virus-infected cells activate OAS enzymes. The 2-5-oligoadenylates produced by activated OAS bind to latent RNase L, leading to the activation of RNase L, followed by degradation of cellular and viral RNA.

In mice, the *Oas* gene family locates on Chr 5 and forms *Oas* gene cluster consisting of *Oas1a* through *Oas1h* (8 genes), *Oas2*, *Oas3* and two *OasL* genes. Among the mouse *Oas1* gene family, the *Oas1b* is a critical determinant for genetic susceptibility to the WNV infection in mice. The *Oas1b* gene is shown to possess an anti-flavivirus activity, although it does not possess enzymatic activity.

On the other hand, the Oas1a protein is shown to possess the enzymatic activity. However, the anti-viral activity is unknown. Furthermore, the roles of other *Oas1* gene families are little known. In this study, therefore, I will focus on 1-Evaluate the relationship between antiviral and enzymatic activities in both mouse Oas1a and Oas1b. 2-Elucidate the function of other Oas1 families in the mouse.

Results:

Flag-conjugated mouse *Oas1a* and *Oas1b* gene constructs were produced by cloning PCR products into mammalian expression vector, pCAG- IRES-GFP. Accuracy of gene constructs was confirmed by PCR and digestion with several restriction enzymes.
Plasmid containing *Oas1a* and *Oas1b* was transiently transfected to BHK-21cells stably expressing WNV-replicon conjugated with secreted alkaline phosphatase (SEAP), and measured SEAP activity.
The SEAP activity was inhibited in BHK21cells by *Oas1b* but not by *Oas1a.*
The*Oas1b* possess antiviral activity but not possess the enzymatic activity.
BHK-21cells, HEK293T and NIH3T3 cell lines stably expressing Oas1a and Oas1b were established to confirm the antiviral activity.

Future plan:

1- Infection of theses stable cell lines expressing *Oas1a* and *Oas1b* gene constructs by WNV-SEAP replicon as well as wild type WNV to measure the antiviral of both gene products.
2- Elucidate the relationship between antiviral and enzymatic activities in both Oas1a and Oas1b by using site-directed mutagenesis technique.

Excessive cytokine respose to rapid proliferation of highly pathogenic avian influenza viruses leads to fatal systemic capillary leakage in chickens

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Highly pathogenic avian influenza viruses (HPAIVs) cause lethal systemic infection in chickens. In the previous study, the HPAIV, A/turkey/Italy/4580/1999 (H7N1) (Ty/Italy) and the low pathogenic avian influenza virus (LPAIV), A/chicken/Ibaraki/2/2005 (H5N2) (Ck/Ibaraki) were intanasally inoculated into chickens. Only Ty/Italy replicated rapidly and extensively in systemic tissues of the chickens, and induced excessive mRNA expression of cytokines, especially IL-6, in proportion to its proliferation. In order to elucidate the mechanism of high pathogenicity of HPAIV in chickens, cytokine producing cells and vascular permeability in multiple organs were examined after the infection with Ty/Italy.

Based on the previous results that IL-6 mRNA significantly expressed in brains of the chickens, *in situ* hybridization were performed with the brain sections of the chickens at 2 and 4 days post infection with Ty/Italy. To assess vascular permeability of tissues, evans blue (EB) was intravenously injected into the chickens at 4 days post inoculation with Ty/ Italy or Ck/Ibaraki. Three hours later, chickens were exsanguinated and the brains, lungs, hearts, spleens, kidneys, and colons were collected. The amounts of EB dye etracted from the tissues were calculated from the absordance at 630nm using a standerd curve.

Using *in situ* hybridization, IL-6 mRNAs were detected mainly in microglial cells in the brain of the chickens infected with Ty/Italy. The mRNAs were most abandantly detected in the sections from the chickens at 2 days post infection, and were rarely detected at 4 days post infection. EB concentrations in the all tissues of the chickens infected with Ty/Italy were significantly higher than those of the chickens infected with Ck/Ibaraki, except for those in the lungs.

In conclusion, microgrial cells are dominant producer of IL-6 in the brains of the chickens infected with Ty/Italy. It is assumed that bone-marrow macrophages play some role in driving the excessive cytokine response in other tissues. The present results indicate that excessive cytokine response induced by proliferation of HPAIV causes fatal multiple organ failure in chickens.

Molecular mechanism of intracellular rabies virus dybamics

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[Background]

Although rabies is an important zoonosis, the molecular biology of its etiologic pathogen, rabies virus (RV), has not been well clarified. Thus, the molecular mechanism of RV life cycle, especially interaction of RV and ubiquitin-mediated intracellular protein transport machinery of host cell, were studied in this study.

[Materials and methods]

Inhibitor of E1 ubiquitin ligase, UBEI41, was used to analyse the role of ubiquitin in the life cycle of RV. NA cells, a murine neuloblastoma cell line, were treated with UBEI41 at various time points and infected with RV. The amounts of RV were measured by immunofluorescent staining, PCR and western blotting on RV proteins.

Further, to investigate the role of ubiquitin in entry process of RV, we focused on the clathrin-mediated endocytosis mechanism for ubiquitinated membrane proteins. The expression vectors of epsin1 and its mutants which lacks ubiquitin interacting domain were constructed. NA cells were infected with RV at 48 hours after transfection of these vectors. The amounts of RV were analysed as described above.

[**Results]**

UBEI41 treatment significantly decreased RV positive cells and production of viral components in NA cells. The sizes of viral inclusion were also reduced by UBEI41. Especially, pretreatment of UBEI41 before RV markedly suppressed RV infectivity.

Inhibition of clathrin-dependent endocytosis and forced expression of epsin1 mutant dramatically decreased RV components in NA cells.

[Conclusion]

This study demonstrated that 1) ubiquitination is required at the entry process of RV and production of RV components in NA cells, 2) RV utilize clathrin-dependent endocytosis for entry to the host cells and 3) recognition of ubiquitinated protein by epsin1, an adaptor protein for clathrin cargo complex, might be involved in this process.

Protooncogene TCL1b functions as an Akt kinase co-activator which exhibits oncogenic potency *in vivo*

Manabu Hashimoto[1], Futoshi Suizu[1], Wataru Tokuyama[3], Hiroko Noguchi[4], Noriyuki Hirata[1], Mami Matsuda[1], Mikio Masuzawa[6], Noriko Gotoh[5], Shinya Tanaka[2], Masayuki Noguchi[1]

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We have previously demonstrated that protooncogene TCL1 (T cell leukemia 1) implicated in human T cell prolymphocytic leukemia (T-PLL), interacts with Akt, enhances its kinase activity, and functions as an Akt kinase co-activator. The TCL1 protooncogene family consists of three members; TCL1, TCL1b and MTCP1 with relatively high sequence and structural homology. Both TCL1 and TCL1b gene are located close each other on human chromosome 14q32. In human T-PLL, both TCL1 and TCL1b genes are activated by the T cell receptor promoter, secondary to chromosomal translocations. Therefore it remains unclear whether TCL1b, independent of TCL1, bears oncogenicity. In this study, we investigated whether and how TCL1b might have similar function as an Akt kinase co-activator. In co-immunoprecipitation assays, TCL1b interacted with Akt. In in vitro kinase assays, TCL1b enhanced Akt kinase activity. Bioinformatics approach using Agilent DNA Microarray Analysis of TCL1b, TCL1, and Myr-Akt, a constitutive active form of Akt, showed that TCL1b showed highly homologous gene induction signature as Myr-Akt or TCL1. Soft agar colony transformation assay demonstrated that TCL1b exhibited oncogenic potency. Two lines of TCL1b-transgenic mice resulted in hemangiosarcoma. Consistently, 8 out of 8 cases of human angiosarcoma stained positively by both anti-TCL1b and anti-phospho-Akt antibodies. In human cancer tissue arrays, 69 out of 146 cases stained positively by anti-TCL1b antibody, out of which 46 were positive by anti-phospho-Akt antibody. Moreover, TCL1b structure-based inhibitor "TCL1b-*Akt-in*" suppressed Akt kinase activity in in vitro kinase assays and PDGF-induced Akt kinase activities, in turn, "TCL1b-*Akt-in*" inhibited cellular proliferation of sarcoma. The current study disclosed TCL1b bears oncogenicity, and hence serves as a novel therapeutic target for human neoplastic diseases.

Characterization of Hokkaido virus, *genus* hantavirus

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Hantaviruses cause two severe human illnesses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Hokkaido virus (HOKV) is a hantavirus maintained in *Myodes rufocanus* and its association with human disease remains unknown. In the previous study, we reported the isolation of HOKV Kitahiyama128/2008 strain. In this study, to analyze the isolated HOKV in more detail, we determined the full-length sequences of S, M, and L segments and analyzed the viral growth of HOKV in various cultured cells. In addition, we generated reassortants between HOKV and Puumala virus (PUUV) to analyze the role of each segment in propagation.

To determine the full-length sequences of S, M, and L segments of the HOKV, each segment of the HOKV was amplified using specific primers and then sequenced directly. To assess the growth of HOKV, the HOKV was inoculated to VeroE6 (green monkey origin), A549 (human origin), and MRK101 cells (*Myodes rufocanus bedfordiae* origin), and the culture fluids of these infected cells were subjected to focus-forming assays. Reassortants were obtained from the culture fluid of VeroE6 cells co-infected with HOKV and PUUV. Genotyping of the obtained clones was performed by RT-PCR.

Analysis of genetic characteristics showed that HOKV was closely related to PUUV which causes HFRS. However, the propagation of HOKV in cultured cells was quite different with that of PUUV. HOKV propagated well in MRK101 cells, but not in VeroE6 or A549 cells. In contrast, PUUV propagated well in all three cell lines. We successfully generated the reassortant containing the M segment from HOKV and the other genomic segments from PUUV. This reassortant propagated in MRK101 and VeroE6 cells. Thus, the inability of HOKV propagation in VeroE6 cells seems to be associated with S and/or L segments.

This is the first report on hantavirus which propagates in a cell line that originated from the natural host but not VeroE6 or A549 cells. No propagation of HOKV in human cell line may be associated with no human disease cases so far. Further analysis of this phenomenon would provide us important information regarding on host specificity and hantavirus pathogenicity in humans.

Application of new world hantaviruses pseudovirions to an alternative method to plaque reduction neutralization test (PRNT)

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Andes virus (ANDV), Sin Nomber virus (SNV) and Laguna Negra virus (LANV) are members of the *Hantaivurs* genus of the *Bunyaviridae* family, and causative agents of hantavirus pulmonary syndrome (HPS). The fatality rates are tended to varied depending on the viruses. Particularly, only ANDV caused human to human transfer of infection. Thus, the typing of the infected hantavirus provides important information for control and treatment. The neutralization test (NT) is a gold standard to define virus types. However, NT require infectious virion, BSL3 facility to perform, complexed technique and need several days to get results. Therefore, safe, rapid and simple diagnostic method, which substitute ordinary NT, has been required.

Pseudovirions are not replication competence as it has exogenous envelope proteins. Therefore, in the present study, we have aimed to apply New World hantavirus pseudovirions for safe and rapid NT.

Coding region of ANDV glycoprotein (GP) was cloned into pCAGGS and expressed in 293T cells. Expression of GP was confirmed by IFA test. The pseudovirions which have VSV GP and reporter gene GFP (VSVΔG*G) was infected to ANDV GP expressing cells. After incubation 24 hours, the pseudovirions with ANDV GP (VSVΔG*AND) were produced in the culture supernatant. By using the VSVΔG*AND, NT was performed. Based on the 80% focus reduction, NT antibody titers of sera from ANDV, SNV and LANV virus patients were 640~>1280, 40~320 and 40~160, respectively. Thus, the VSVΔG*AND is considered useful for NT. For the next step, production of pseudovirion of SNV and LANV are necessary to serotype.

Examination of adjuvant effects of gold nanoparticles combined with West Nile virus E protein

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West Nile virus (WNV) is a mosquito-borne, neurotropic flavivirus. WNV is comprised of an enveloped spherical virion with a diameter of approximately 50 nm. Entry of WNV into cells is thought to occur *via* interaction of the viral envelope protein (WNVE) with cellular receptors. After invading the hosts, WNV causes viremia and febrile illness that can lead to fatal meningitis or encephalitis in humans (particularly the elderly) and horses. Currently, prophylactic vaccine against WNV and specific treatment for WNV encephalitis is not available.

Gold nanoparticles (AuNPs) have been utilized as carriers for drug delivery, photo-activated therapeutics, and antigen carriers for antibody generation, because of their biocompatibility and easy control of its surface, size, and shape. In fact, it has been reported that AuNPs can be used to enhance the immune response in mice as a vaccine adjuvant. In collaboration with Dr. Niikura's group (Research Institute for Electronic Science, Hokkaido University), we examined the adjuvant efficacy of AuNPs combined with WNVE.

We synthesized spherical (20 and 40 nm in the diameter), cubical (40 x 40 x 40 nm), and rod (40 x 10 nm) AuNPs. The synthesized AuNPs were coated with anionic polymer and WNVE *via* electrostatic interaction. We initially examined the cellular uptake of AuNP-WNVE complexes (AuNP-E) using different type of AuNPs. We also measured the antibody titer against WNVE after inoculation of AuNP-E to mice.

Among the different AuNPs, including spherical 20, spherical 40, cubic, and rod, AuNP (rod)-E was most efficiently uptaken into the cells. In contrast, AuNP (cube)-E was least incorporated. Enzyme-linked immunosorbent assay (ELISA) revealed that the highest level of antibody titer was detected in the serum of mice inoculated with AuNP (spherical 40)-E which was most similar to the native WNV virion in shape and size. The relatively lower levels of antibody titer was detected in the serum of mice inoculated with AuNP (cubic, rod, and spherical 20)-E. These results suggested that AuNPs might be applicable as an effective vaccine adjuvant.

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Efficient delivery of epitope peptides to antigen-presenting cells using phosphatidylserine for induction of epitope-specific T cell responses

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[Background] To induce potent epitope-specific T cell immunity by a peptide-based vaccine, epitope peptides must be delivered efficiently to antigen-presenting cells (APCs) *in vivo*. Therefore, selecting an appropriate peptide carrier is crucial for the development of an effective peptide vaccine. In this study, we explored new peptide carriers which show enhancement in cytotoxic T lymphocyte (CTL) induction capability.

[Methodology/Principal Findings] Data from an epitope-specific *in vivo* CTL assay revealed that phosphatidylserine (PS) micelles have a potent adjuvant effect among candidate materials tested. Further analyses showed that PS micelle-conjugated antigens were preferentially and efficiently captured by professional APCs, in particular, by $CD11c^{+}CD11b^{+}MHCII^{+}$ conventional dendritic cells compared to conventional liposome-conjugates or unconjugated antigens. In addition, PS micelles demonstrated stimulatory capacity of peptide-specific helper T cells *in vivo*.

[Conclusion] This work indicates that PS micelles are the easily preparable efficient carrier with a simple structure that deliver antigen to professional APCs effectively and induce both helper and cytotoxic T cell responses *in vivo*. Therefore, PS micelles are a promising novel adjuvant for T cell-inducing peptide vaccines.

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