北海道大学グローバルCOEプログラム

Global COE Program
Progress Report FY 2010
Presentation

2010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：　2011年3月10日(木)10:00～15:40

Venue: Conference Hall
Graduate School of Veterinary Medicine, Hokkaido University
Time & Date: 10:00～15:40, March 10 (thu), 2011

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10:00-10:05 Opening speech:

■Chairperson■ Takanori Kooriyama

10:05-10:15

1. Leptospisosis in Smallholder Dairy Cattle and peridomestic Rodents in Kandy, Sri Lanka

   Chandika D. Gamage

10:15-10:25

2. Leptospira infection among febrile patients at the University of Peradeniya Teaching Hospital in Sri Lanka: Clinical, epidemiological and laboratory investigations

   Chinyere Nwafor-Okoli

■Chairperson■ Chandika D. Gamage

10:25-10:40

3. Bovine Tuberculosis in Lechwe: Prescreening for domestic cattle around Liuwa National Park in the Western Province, Zambia. Diseases of wild chimpanzees in Mahale Mountains National Park, Tanzania

   Takanori Kooriyama

4. Isolation and characterization of serine proteases of tsetse and stable flies, and their roles in the differentiation of bloodstream forms of *Trypanosoma brucei* into procyclic forms

   Satoshi Miyazaki

10:40-10:55

5. Towards the elimination of rabies in Sri Lanka: Laboratory practice and field study

   Koji Kanda

10:55-11:10

6. Epidemiological study of infectious diseases in wild birds in Hokkaido and biological characterization of molecules from poultry red mite, *Dermanyssus gallinae*

   Masayoshi Isezaki

■Chairperson■ Masayoshi Isezaki

11:10-11:25

7. Detection and characterization of a novel polyomavirus in wild rodents

Yasuko Orba

11:25-11:35

8. Whole genome next-generation sequencing reveals polymorphisms in *Theileria parva*

Kyoko Hayashida

■Chairperson■ Yosuke Nakayama

11:35-11:50

9. Development of the method for monitoring cytotoxic T lymphocyte (CTL) responses to hantavirus in laboratory rats

Shumpei P. Yasuda

11:50-12:00

10.Persistent Puumala virus infection in Syrian hamsters (*Mesocricetus auratus*) resembling Hantavirus infection in natural rodent hosts

Takahiro Sanada

12:00-13:00 ==== Lunch ====

■Chairperson■ Yasuko Orba

13:00-13:10

11. Analysis of pulmonary edema in hantavirus infected SCID mouse

Takaaki Koma

13:10-13:20

12. Functional analysis of murine Flavivirus resistance gene *Oas1b*

Kanako Moritoh

13:20-13:35

13.Studies on Molecular Pathogenesis in Borna Disease Virus Infection

-Comparative Characterization of Avian Bornavirus Glycoprotein-

Yayoi Otsuka

■Chairperson■ Yayoi Otsuka

13:35-13:50

14. Characterization of differentiated and prion infected neurospheres that were derived from wild-type mice

Yukiko Sassa

13:50-14:05

15. Analysis of the role of TIMP-1 after influenza A virus infection

Yosuke Nakayama

■Chairperson■ Naoki Yamamoto

14:05-14:15

16. Host response to infection with highly pathogenic avian influenza viruses in chickens

Saya Kuribayashi

14:15-14:25

17.The role of antibodies in heterosubtypic protective immunity against influenza virus infection

Mieko Muramatsu

14:25-14:35

18. Development of a CTL-based human influenza vaccine

Toru Ichihashi

14:35-14:50 Coffee Break

■Chairperson■ Shumpei P. Yasuda

14:50-15:05

19. The K627E amino acid substitution of the PB2 of A/Hong Kong/483/1997 (H5N1) influenza virus alters the efficiency of RNA synthesis of NP gene

Naoki Yamamoto

15:05-15:15

20. Potency of an inactivated avian influenza vaccine prepared from a non-pathogenic H5N1 virus against the challenge with an antigenically drifted virus of clade 2.3.4

Shintaro Shichinohe

■Chairperson■ Yukiko Sassa

15:15-15:25

21. Characterization of the interaction of influenza virus NS1 with Akt

Mami Matsuda

15:25-15:35

22. Functional Analysis of Filovirus Glycoprotein

Osamu Noyori

15:35-15:40 Closing Speech

Leptospisosis in Smallholder Dairy Cattle and peridomestic Rodents in Kandy, Sri Lanka

Chandika D. Gamage
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Hokkaido University Graduate School of Medicine

Introduction: Leptospirosis is an important zoonotic disease globally and one of the notifiable diseases in Sri Lanka. Other than human leptospirosis, little information is available on bovine leptospirosis in Sri Lanka. Thus, this study attempted to determine the prevalence and carrier status of leptospirosis in smallholder dairy cattle and peridomestic rodents by identifying the circulating leptospiral serogroups and species using microscopic agglutination test (MAT) and polymerase chain reaction, respectively.

Materials and Methods: In September and October 2009, cattle and rodent biological samples were collected from the Yatinuwara and Udunuwara divisional secretaries in Kandy. Serum samples were analyzed for the presence of anti-leptospiral antibodies using MAT; the test consisted of a panel of 18 pathogenic live Leptospira serovars. DNAs were extracted from cattle urine pellets and rodents' kidneys. Polymerase chain reaction was used to detect the Leptospira *flaB* gene; amplicons were sequenced and phylogenic distances were calculated

Results: Out of 113 cattle serum samples, 23 (20.3%) were positive (titer of ≥ 1:100). Of the 23, sixteen (69.5%) and 6 (26%) reacted with serogroup Sejroe and Hebdomadis, respectively. Out of 74 rodents, thirteen (17.5%) were positive (titer of ≥ 1:25), of which 8 (61.5%) and 4 (30.7%) had reactions to serogroups Javanica and Icterohaemorrhagiae, respectively. Leptospiral DNA, specifically *Leptospira interrogans*, was detected in one urine samples of cattle.

Conclusions: This study revealed the prevalence of leptospirosis among cattle and rodents in the study sites. These animals were found to have been infected with a wide array of leptospiral serovars. Bovine leptospirosis needs to be controlled due to its zoonotic nature.
Acknowledgments: This study was supported by Global COE program "Establishment of International Collaboration Centers for Zoonoses Control", MEXT and National Institute of Infectious Diseases, Japan.

Leptospira infection among febrile patients at the University of Peradeniya Teaching Hospital in Sri Lanka: Clinical, epidemiological and laboratory investigations

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Hokkaido University Graduate School of Medicine

Introduction

Leptospirosis is an emerging infectious disease in Sri Lanka because of the rising number of infections in recent years. The incidence of Leptospirosis based on notifiable cases was 35.7 per 100,000 in 2008. In Kandy District, the number of reported cases also rose from 147 in 2007 to 501 in 2008. To help formulate local intervention, the study describes the socio-demographic characteristics and the circulating leptospiral serogroups and serovars among the clinically diagnosed cases; deduces the leptospiral species among laboratory-confirmed positive cases; and critically examines the characteristics of laboratory confirmed positive cases vis-à-vis negatives.

Methods

Patients were identified by physicians according to the surveillance case definition of the Epidemiology Unit of the Ministry of Health Sri Lanka from 1 April 2009 to 31 March 2010. We interviewed 97 patients clinically diagnosed with Leptospirosis and requested for blood samples from these cases. We examined serum samples for distribution of serovars using microscopic agglutination test (MAT) with a battery of representative leptospiral serogroups; and polymerase chain reaction test (PCR) was applied to the serum samples to detect *flaB* gene. Data were analyzed by SPSS version 14 and χ^2 test was employed for the test of significance.

Results

Among others, the 97 clinically diagnosed cases were mostly men (92.8%); nearly two-thirds were 35 years of age or older and had secondary or higher education (61.8%); half were farmers or laborers; and more than half were acute cases (57.7%). Of these 97 cases, 17 (17.5%) were MAT-confirmed positives, while 80 (82.5%) were negatives. The predominant serogroups among the confirmed positives were *Sejroe* and *Tarassovi*. Of 8 (8.2%) PCR-positives detected among 97 clinical cases, 1 PCR-positive occurred in the serum with MAT titre $\geq$200 but the remaining PCR-positives (7) were observed among those with MAT-negative. The 7 leptospiral species among 8 PCR-positives were *L. interrogans.* Having a pet (dog) seemed to be statistically associated with laboratory-confirmed positives.

Conclusions

Leptospirosis is relatively common in Kandy District and the most common leptospiral species is *L. interrogans*. Of 97 clinically diagnosed patients, only 17(17.5%) or 8 (8.2%) were confirmed by MAT or PCR, respectively. All 8 PCR-positives were observed in MAT-negative sera. The laboratory results by MAT and PCR depend on the time of blood collection after onset of fever. Laboratory-confirmed positives seem to be associated with those who played with a dog. Further studies are warranted for choice and development of appropriate assay methods as well as identification of potential reservoirs in the region.

Bovine Tuberculosis in Lechwe: Prescreening for domestic cattle around Liuwa National Park in the Western Province, Zambia
Diseases of wild chimpanzees in Mahale Mountains National Park, Tanzania

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Our goal of this research is to compare the genetic characteristics of Mycobacteria isolated from three subspecies of Lechwe in Zambia. A previous study of Kafue lechwe in Lochinvar National park showed a relationship between the *Mycobacterium bovis* of wild lechwes and domestic cattle. Our next target, red lechwe lives in Liuwa National Park, western province. The isolated Mycobacteria will be compared with that isolation for local domestic cattle. We surveyed Mycobecterial infections in domestic cattle at abattoirs in the western province city, Mongu. The main type of cattle in this region is the traditional 'Zebu'species. Cattle were brought into abattoirs in Mongu from the local towns of Mongu, Senanga and Kalabo, near Liuwa NP. The typical caseous necrosis was not found from 793 cattle. However, liver flukes (80%), abscesses in lymph nodes and lungs, hygromas and pneumonia were found. One positive sample was noted from a lymph node cultured in Ogawa media.

The chimpanzee is the closest primate species to humans genetically, and it has been used as an experimental animal for human disease models. The wild chimpanzee is an endangered animal and their habitat has been protected as National parks. However, ecotourism has increased, and with this, the number of people in close proximity to wild chimpanzees has also increased. Therefore the risk of human born infection in chimpanzees has also increased, and monitoring their health is essential. Suffering of chimpanzees from Flu-like symptoms have been observed previously in Mahale Mountains National Park, Tanzania, which was suspected to be human borne infection. Disease outbreak was not recorded this year, so we changed our target to baseline diseases. Our continuing study showed not only zoonotic but also newly identified parasites in Mahale. We also surveyed SIV and STLV in wild chimpanzee fecal and urine samples by detecting excretion IgA by western blot assay. SIV infection in 9-18% chimpanzees has been reported in Gombe National Park 100km north of Mahale, but no cases of these two retroviruses were detected.

Isolation and characterization of serine proteases of tsetse and stable flies, and their roles in the differentiation of bloodstream forms of T*rypanosoma brucei* into procyclic forms

Satoshi Miyazaki
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Trypanosoma brucei, which causes a zoonotic African trypanosomiasis, is biologically transmitted by tsetse flies (*Glossina* spp.). When a tsetse fly sucks the mammalian blood containing the bloodstream forms (BSF) of *T. brucei*, the parasites can differentiate into the insect forms (procyclic forms: PCF) only in the tsetse midgut. Elucidation of mechanisms of PCF differentiation is hallmark for the novel strategies of control of trypanosomiasis. The treatment of BSF with a recombinant serine protease of *G. fuscipes* (*Glossina proteolytic lectin*: *Gpl*) induced the PCF differentiation at 27°C *in vitro* (Abubaker et al., 2006). Therefore, serine protease(s) may play a role in triggering the PCF differentiation, but the molecular mechanism of the PCF differentiation remains unknown.

On the other hand, BSF of *T. brucei* does not differentiate into PCF in blood-sucking stable flies (*Stomoxys* spp.), although these flies can mechanically transmit some *Trypanosoma* species. The involvement of serine proteases of stable flies in the PCF differentiation has not been studied. Thus, the present study aims to isolate and characterize serine proteases of tsetse and stable flies and to examine their roles in the PCF differentiation.

First, *Gsp1* (*Glossina serine protease 1*), which was reported as an ortholog of *Gpl*, was isolated from *G. morsitans*, whereas *Sspx* (*Stomoxys serine protease x*) was newly isolated from *S. calcitrans*. Although the *Sspx* was similar with some *Ssp* genes reported before, the *Sspx* showed higher similarlity (77.8%) with *Gpl* than other *Ssp* genes, suggesting that *Sspx* is an ortholog of *Gpl*. Next, *in silico* characterizations of *Gsp1* and *Sspx* showed that both genes shared all domains and that all residues at cleavage, active and substrate binding sites were completely conserved. Now, expressions of the genes using a bacterial expression system and evaluations of the PCF inducibilities of its recombinant proteins are performed. Furethermore, transgenic *Drosophila melanogaster* strains expressing each of *Gsp1* and *Sspx* were developed and will be used for *in vivo* functional analyses of these genes. Based on the results of these experiments, the roles of fly's serine proteases in PCF differentiation mechanism will be discussed.

Towards the elimination of rabies in Sri Lanka: Laboratory practice and field study

Koji Kanda
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Vaccination of dogs against rabies and dog population control activities were reinforced to a greater extent in Sri Lanka during the recent years and it resulted in gradual declining of human rabies cases from 337 in 1975 to 58 in 2009. However, despite the fact that approximately 1.2 million domestic and stray dogs were vaccinated in 2009, the nationwide coverage rate still records under 50% and the current interventions have been insufficient to interrupt the natural cycle of rabies transmission among dogs. Therefore, we conducted several research and public health activities in the Central Province of Sri Lanka.

Laboratory practice

Rabies Control Unit in the Faculty of Veterinary Medicine and Animal Science at the University of Peradeniya has significantly contributed to decentralizing the capabilities of rabies diagnosis in suspacted animals in the Central and adjacent provinces. In 2010, 81 animal specimens submitted by government sectors and general public were examined by using negri body test, fluorescent antibody test (FAT), and histopathological procedures. It was found that 21 (25.9%) of the specimens were infected with rabies. Of 60 dog specimens submitted during this period, 19 (31.7%) were found to be positive for rabies.

Field study

In collaboration with universities, local government health and community representatives, several community surveys were carried out extensively in Kandy and Nuwara Eliya Districts of the Central Province to assess the level of knowledge, attitude, and practice among general population and health-seeking behaviors of animal bite victims. Information and Education Campaign (IEC) materials such as awareness leaflets and in-class entertainment activities were also introduced to propose cost-effective, sustaiable interventions for rabies prevention.

Epidemiological study of infectious diseases in wild birds in Hokkaido and biological characterization of molecules from poultry red mite, *Dermanyssus gallinae*

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Last year, RT-PCR and nested RT-PCR methods were established to detect West Nile virus (WNV) and Japanese encephalitis virus (JEV) from feather tips of the wild birds. Thus far, no positive birds were found in the survey of 2010 in the Hokkaido area.

As the next step to establish the methods for complete epidemiological survey, an attempt was made to develop serological method to detect specific antibodies using feather tips. Western blot analyses were performed to detect IgG proteins extracted from feather tips of wild birds using several commercial antibodies to avian IgG. Heavy and light chains of IgG were detected in most bird species using rabbit anti-chicken IgG antibodies, showing that feather tips can be used for the serological survey of wild birds. At the present time, a serological method to detect WNV- or JEV-specific antibody from feather tips of wild birds are being constructed.

The poultry red mite, *Dermanyssus gallinae* is distributed worldwide, and an economically important parasitic pest of domestic chickens. The red mite could be responsible for the decrease in egg production, weight loss, and anemia, and the multiple infestation of red mite can cause the death of the host. In addition, red mite has been suggested as a potential vector of several pathogens. (e.g., *Salmonella*). However, little is known on the molecules of the mite, and acaricide-resistant mite is also widely present in farms, which could be a serious problem to poultry industry in the future. Therefore, in this study, a global analysis of gene expressions has been performed in this red mite.

A plasmid cDNA library was constructed from the red mite collected from a poultry farm. A total of 987 expression sequence tags (ESTs) were sequenced from the plasmid cDNA library. When these sequences were compared to NCBI databases, 286 sequences were identified as the genes of known function. Many of these clones have high-homology with the genes of *Ixodes scapularis*, known as a vector of the Lyme disease. In addition, one clone was found to be homologous to anti-oxidant, thioredoxin peroxidase, which is suggested as a possible vaccine candidate for other ticks. Currently, functional analysis of these clones is in progress to identify new vaccine candidates and target molecules for acaricide. Moreover, the role of red mite as a vector is also analyzed for the transmission of various pathogens.

Detection and characterization of a novel polyomavirus in wild rodents

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Polyomaviridae is a family of non-enveloped viruses that carry circular, dsDNA genomes. At present, eight human and 18 non-human polyomaviruses are known. Most mammalian polyomaviruses cause subclinical infections with life-long persistence in their natural immunocompetent hosts. However, when host immunity is compromised, the virus can reactivate and cause disease.

To investigate polyomavirus infection in wild rodents, we analyzed DNA samples from the spleens of 100 wild rodents from Zambia using a broad-spectrum PCR-based assay. A previously unknown polyomavirus genome was identified in a sample from a multimammate mouse (*Mastomys* species) and the entire viral genome of 4899 base pairs was subsequently sequenced. This viral genome contained potential open reading frames for the capsid proteins, VP1, VP2 and VP3, and early proteins, small t antigen and large T antigen. Phylogenetic analysis showed that it was a novel member of the family *Polyomaviridae*, and thus the virus was tentatively named mastomys polyomavirus. After transfection of the viral genome into several mammalian cell lines, transient expression of the VP1 and large T antigen proteins was confirmed by immunoblotting and immunocytochemical analyses. Comparison of large T antigen function in mastomys polyomavirus with that in rhesus monkey polyomavirus SV40 and human polyomavirus JC virus revealed that the large T antigen from mastomys polyomavirus interacted with the tumor suppressor protein pRb, but not with p53.

Whole genome next-generation sequencing reveals polymorphisms in *Theileria parva*

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East coast fever and Corridor disease, caused by protozoan parasite *Theileria parva*, is one of the most serious cattle diseases in Africa. The genome of *T. parva* Muguga strain, isolated in Kenya has already been sequenced and satellite-marker based analyses have been used for the genetic study. However, as the numbers of the genetic marker used in these study were limited, construction of more high resolution genetic map has been awaited, which is essential for epidemiological survey or vaccine development.

To reveal comprehensive polymorphisms, we performed whole genome re-sequencing of 15 *T. parva* strains with Illumina Genome Analyzer (Solexa), which include two vaccine strains (Serengeti -transformed and Kiambu), field isolates from such as Zambia, and buffalo-derived strains. The obtained short DNA reads were aligned to approximately 8.3 Mb of the *T. parva* Muguga reference sequence, and over 90 % of the genome was re-constructed. Comparison with the reference Muguga strain revealed 37,000-140,000 single nucleotide polymorphisms (SNPs), which was highest in buffalo derived strains. We further analyzed dN/dS ratio (non-synonymous substitutions per non-synonymous site) to estimate selective pressure. The possible genes under positive selection (>1) were selected, which have a potential as immunogenic or vaccine candidates.

T. parva Marikebuni strain St72 progeny, which is known to have heterogeneous population showed mosaic pattern with Muguga strains, indicating this Marikebuni St72 strain is the resultant of sexual recombination in vector ticks. The chromosomal rearrangement was observed at some limited locus, suggesting the existence of hot and cold spot in the genome of *T. parva*.

Thus, our extensive SNP data generated in this study with next generation sequencer will provide a powerful tool for future studies of genetic diversity and biology of *T. parva* parasite.

Development of the method for monitoring cytotoxic T lymphocyte (CTL) responses to hantavirus in laboratory rats

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Seoul virus (SEOV), one of the serotypes of hantavirus, is a causative agent of hemorrhagic fever with renal syndrome. SEOV is maintained in *Rattus norvegicus* with persistent infection. However, the mechanism by which causing persistent infection is still unclear. To examine the relationships between hantavirus infection and suppression of cytotoxic T lymphocytes (CTLs), I attempted to establish methods for immunological studies using transiently infected laboratory rats, a laboratory strain of *R. norvegicus*.

Rats (WKAH, male, 6 weeks old) were experimentally infected with SEOV strain SR-11 ($6.0x10^4$ ffu/rat, intra-peritoneal route). Lungs, sera and splenocytes were collected at periodic intervals. IgM and IgG antibody titers were measured using the ELISA method. The amount of virus was examined using a real-time PCR method. The mitogenic responses of splenocytes were evaluated using concanavalin A (ConA response). Hantavirus specific CTLs were detected using ELISPOT assay with 309 kinds of 15-amino acid peptides as epitopes which covered entire glycoprotein and nucleocapsid protein sequences.

IgM antibody appeared at 6 days post-inoculation (dpi) and IgG antibody appeared at 9 dpi. The IgM antibody titers began to decrease at 13 dpi, whereas the IgG antibody titers increased continuously. The amount of virus peaked at 3 dpi and then decreased in lung and disappeared in serum. ConA response of splenocytes from infected rats showed same level to those from noninfected ones. ELISPOT assay defined WKAH strain specific three epitopes. CTLs were activated stronger by epitope #149, located in glycoprotein Gc, than others. ELISPOT assay using epitope #149 showed that CTLs activation were peaked at 6 dpi.

These results show that the ConA response and the ELISPOT assay are applicable for immunological study in SEOV infected rat. Further study for apply these methods to naturally infected *R. norvegicus* will be necessary to examine mechanisms for persistent infection in the natural host.

Persistent Puumala virus infection in Syrian hamsters (*Mesocricetus auratus*) resembling hantavirus infection in natural rodent hosts

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Hantaviruses cause severe human illnesses, such as hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). In contrast to human infection, hantaviruses do not cause any symptoms in host rodents and persist for a long period. However, the mechanisms of hantavirus persistence in natural hosts is poorly understood due to a lack of laboratory animal models of persistent infection. In this study, we report that Syrian hamsters have a high susceptibility to infection of Puumala virus (PUUV), a causative agent of HFRS, and the infected hamsters maintain PUUV for a long period.

Subadult (4 weeks old) and adult (8 weeks old) hamsters were inoculated subcutaneously with 3,300 ffu of PUUV Sotkamo strain. Blood and tissue samples are collected from 3 to 70 days post infection (dpi). Serum samples were used for the measurement of antibody responses, and tissue samples were used for the TaqMan quantitative RT-PCR analysis and pathological analysis.

Although both of IgM and IgG antibodies against the viral nucleocapsid protein were induced in subadult and adult hamsters, IgM responses in subadults were stronger and longer than that in adults. Neutralizing antibody responses in both age groups were almost equivalent. Viral RNA load in lungs peaked on 14 days in subadult hamsters, and the viral RNA persisted for 70 days. The viral RNA was also detected in kidney, spleen, liver, heart, and brain. In adults, viral RNA loads in various organs were much lower than those of subadults. In immunohistochemical analysis, the viral antigen was detected in lung, kidney, cerebellum and adrenal gland of subadults. Slight inflammatory reactions were also observed in lung, cerebellum and adrenal gland. On the other hand, no antigen and any inflammatory reaction were observed in adult hamsters. Infected hamsters in both age groups showed no body weight loss or clinical signs.

Syrian hamsters infected with PUUV showed persistent infection despite the presence of neutralizing antibodies without any clinical symptoms, which is quite similar to the hantavirus infection in natural host rodents. Therefore, PUUV infected hamsters, especially subadults, could be useful model to clarify what factors contribute to hantavirus persistence in the reservoir host.

Analysis of pulmonary edema in hantavirus infected SCID mouse

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Hantavirus causes two important rodent-borne viral zoonoses, hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus pulmonary syndrome (HPS) in North and South Americas. Acute respiratory distress syndrome (ARDS) with pulmonary edema has been reported in severe cases of both HFRS and HPS patients.

We have developed a mouse pulmonary edema model by adoptive transfer of immune splenocytes into Hantaan virus (HTNV)-infected SCID mice. The mice showed severe lung edema of which wet lung weight at 6 days after transfer was more than 2-fold greater than that of mock and histological change was similar to that in HPS patient.

In this study, we examined cell populations in bronchoalveolar lavage fluid (BALF) and lung homogenate (LH) of mice with lung edema at 6 days after transfer by morphological analyses and FACS.

Total number of cells in BALF of the model was markedly increased (3-fold) compared with that in controls. Lymphocytes constituted the major population in BALF (approx. 75%). The majority of lymphocytes were T-lymphocytes (approx. 30%) and the percentage of B-lymphocytes was relatively small (approx. <5%). The number of neutrophils was also increased. The number of macrophages was similar to that in controls. Likewise, the percentage of T-lymphocytes was higher than that of B-lymphocytes in the LH. Neutrophils were prominently increased to approx. 40% (control <20%). The percentages of macrophage and dendritic cells were similar to those in controls. These results indicate that T-lymphocytes and neutrophils might have an important role in inducing lung edema.

Since neutrophils play a key role in the physiopathogenesis of general ARDS, the mouse model might mimic human ARDS caused by hantavirus. Further studies, such as studies in which each lymphocyte subset alone is transferred and neutrophils are depleted, are needed to determine the mechanism of pulmonary edema in hantavirus infection.

Functional analysis of murine flavivirus resistance gene *Oas1b*

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Type1 interferons (IFNs) are produced and secreted from virus-infected cells and render the surrounding cells to induce a number of cellular proteins, including the Oas (Oligoadenylate synthetase). In mice, the *Oas* locus locates on Chr 5 and is composed of the *Oas* gene cluster, consisting of *Oas1a-h*, *Oas2*, and *Oas3*. Among these genes, *Oas1b* has been identified as a flavivirus-resistant gene, of which protein product, Oas1b confers resistance to flaviviruses including West Nile virus (WNV) in mice. Most strains of laboratory mice such as C57BL/6J (B6), BALB/c, and DBA/2 lost the functional *Oas1b* gene during selective breeding. Therefore, laboratory mice are susceptible to flavivirus, whereas wild mouse-derived strains are known to be resistant to flavivirus due to the possession of the intact *Oas1b* gene.

In previous study, we generated *Oas*-congenic strain, in which the *Oas* locus of the MSM/Ms strain was introduced into the most widely used strain, C57BL/6J mice. *Oas*-congenic strain showed higher antiviral specificity than C57BL/6J parental strain possessing the same genetic background. However, *Oas*-congenic mice did not show resistance to influenza virus infection. These data suggest that murine Oas1b proteins inhibit flavivirus multiplication specifically; however, the mechanism of it remains unknown. To explore the anti-WNV activity of Oas1b *in vitro*, we established stably *Oas1b*-expressing cells. Then, we evaluated inhibitory activity of murine Oas1b to flavivirus replicatioin using WNV replicon. WNV replicon analysis revealed that even mirune Oas1b derived from laboratory strain Oas1b inhibits WNV replicon replication inside cells. Molecular mechanism by which Oas1b inhibits replications of flavivirus remains to be elucidated. The discrepancy between the results of *in vivo* experimental infection and *in vitro* analysis might be due to the nonsense-mediated decay of the *Oas1b* mRNA *in vivo*.

Studies on Molecular Pathogenesis in Borna Disease Virus Infection -Comparative Characterization of Avian Bornavirus Glycoprotein-

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Borna disease virus (BDV) causes neurologic disease manifested by behavioral abnormalities in several animal species, and evidence suggests that it may be a human pathogen. BDV glycoprotein (BDVG) is posttranslationally cleaved by the cellular protease furin to generate two subunits of the N-terminal GP1 and C-terminal GP2. GP1 is involved in virus interaction with cell surface receptor and GP2 mediates a pH-dependent fusion event between viral and endosome membrane. BDVG expression is extremely low in BDV persistently infected cells and the cells transfected with eukaryotic expression plasmids, and these findings led to the view that BDV disseminates through cell-to-cell spread rather than virion release, although the details remain unclear. Recently, avian bornaviruse (ABV) was identified in parrots with proventricular dilatation disease. However, no information is available yet about ABV glycoprotein (ABVG). The purpose of the present study was to characterize and compare the expression of ABVG with that of BDVG to investigate propagation and pathogenesis of bornaviruses.

Characterization of ABVG: Expression of ABVG resulted in the generation of two polypeptides with molecular masses of 100 and 65-40 kDa. The 100-kDa and 65-40-kDa bands likely corresponded to the full-length ABVG (GPC) and its C-terminal cleavage product GP2, respectively. Deglycosylation studies demonstrated that both products predominantly contained endoglycosidase H-sensitive *N*-linked oligosaccharides. Consistent with this observation, ABVG accumulatesd in the endoplasmic reticulum (ER) in immunofluorescence microscopy. We also tried to generate a recombinant lymphocytic choriomeningitis virus (LCMV) expressing ABVG. However, incorporation of ABVG into virions was not achieved despite of its high level expression, indicating inefficienct formation and release of virus particles due to limited trafficking to the cell surface.

Differential expression of ABVG and BDVG: We generated a series of chimeric proteins of BDVG and ABVG. Expression analysis, in summary, demonstrated that substitution of the transmembrane segment (TM) of ABVG by that from BDVG remarkably reduced expression of the chimera, while expression of the chimeric BDVG possessing TM of ABVG was significantly insreased. These findings indicate that structure of the TM affects plasma membrane expression of bornavirus glycoprotein, and probably account in part for negligeble expression of BDVG and persistent infection of BDV.

Characterization of differentiated and prion infected neurospheres that were derived from wild-type mice

Yukiko Sassa
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Background: Prion diseases are progressive neurodegenerative diseases that infect both human and animals. The reasons why prions cause nerodegeneration have not been clarified yet. The prion infection in neurons itself does not cause neurodegeneration using cultured neuronal cell lines. In prion infected animal models, astrocytic and microglial activation has been occurred long before clinical stage. Therefore we speculated that neurodegeneration is not caused by prion infection in neurons, but by the concomitance with other cells such as microglia and astrocytes. In order to investigate this possibility, we generated *in vitro* circumstances that are seen in central nervous system *in vivo*. We used neurosphere cultures that defferentiate into neurons, astrosytes and oligodendrocytes in vitro. Recently, some have shown that prion infection in neurospheres that generated from genetically engineered prion protein (PrP) over expression mice. However, no investigation of neurospheres from wild-type mice, nor cellular characterization of disease specific PrP (PrP^{Sc}) in neurspheres has yet been performed.

Objectives: The aim of this study was to investigate prion infection in neurosphres generated from wild-type mice, and to characterize prion infected cells in neurospheres.

Materials and Methods: We isolated cells of brain of embryonic day 13-15 wild-type mice and cultured for generation of primary neurospheres. Neurospheres were differentiated, then infected with prions at the differentiated day 5 to 10. We observed PrP^{Sc} localization by IFA co-stained with neuronal marker (MAP2, bⅢ-tubulin, and Tau) or astrocytic marker (GFAP).

Results and prospects: The differenciated neruspheres that generated from wild-type mice persistently infected with prions over 50 days of post infection. The PrP^{Sc} was firstly and mainly found in astrocytes, then found in neurons at 50 days of post infection. The prion infected neurosphere culture might be the suitable model for investigating neurodegeneration in prion diseases. For future work, I'll use the prion infected neurosphere cultures for investigating neurodegeneration by evaluating the expression of synaptic marker protein.

Analysis of the role of TIMP-1 after influenza A virus infection

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After influenza A virus infection, a variety of inflammatory cells are recruited into the virus-infected sites as a critical step of host defense response. This process is tightly coordinated by the interaction of cells with their surrounding extracellular matrix (ECM) proteins.

In addition, matrix metalloproteinases (MMPs) and their inhibitors play an important regulatory role in the inflammatory response, and are also involved in a number of pathological processes, such as fibrosis, chronic inflammation, and tissue destruction. Thus, alteration of the expression levels of these proteins may affect pathological condition after influenza A virus infection. However, the functional role of these molecules (ECMs, cell adhesion molecules, MMPs and MMP inhibitors) after the viral infection is still unclear.

In this study, we found alterations of gene expression of several MMPs and ECMs in the lungs of mice infected with influenza virus A/Puerto Rico/8/34 (A/PR/8) (H1N1). In particular, the mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), which is a specific inhibitor of MMPs, was remarkably induced in the lung after PR/8 infection. Therefore, we focused on the function of TIMP-1 for the pathogenesis by PR/8 infection, and infected TIMP-1 knockout (KO) and wild type (WT) mice with PR/8 virus. After the viral infection, the survival rate of TIMP-1 KO mice was lower than that of WT mice.

It has been reported that an imbalance in the ratio of MMP/TIMP expression has been implicated in pathological disorders such as pulmonary fibrosis. Therefore, it is suggested that the increased expression of TIMP-1 after influenza A virus infection is correlated to the pathological condition. To address the functional pathophysiological role of TIMP-1, analysis of the expression and activity of TIMP-1 in the tissues or cells using of PR/8 infected TIMP-1 KO and WT mice is currently in progress.

Host response to infection with highly pathogenic avian influenza viruses in chickens

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The reason why chickens die due to infection with highly pathogenic avian influenza viruses (HPAIVs) is not well understood. In our previous study, all of the chickens intranasally infected with $10^{6.0}$ EID_{50} of A/turkey/Italy/4580/1999 (H7N1) (Ty/Italy/99) died in 3-4 days, while a half of the birds infected with the same dose of A/chicken/Netherlands/2568/2003 (H7N7) (Ck/NL/03) died in 6-7 days and the others recovered. To better understand the basis of the pathogenicity of these HPAIVs in chickens, $10^{6.0}$ EID_{50} of each strain was intranasally inoculated into chickens and compared viral growth and host immune response in early stage of infection.

Ty/Italy/99 replicated efficiently in each tissue of the chickens, especially in the brain, since early stage of infection. On the other hand, Ck/NL/03 replicated significantly more slowly than Ty/Italy/99 in each tissue of the infected chickens. The virus titers in the blood of the chickens infected with Ty/Italy/99 rapidly increased, while in the chickens infected with Ck/NL/03 virus was detected transiently. These results suggest that growth of virus in the endothelial cells or immunocompetent cells is different between these strains. In the real-time PCR analysis, brisk expression of anti-viral and pro-inflammatory cytokine mRNA in each tissue of the chickens infected with Ty/Italy/99 increased in correlation with extensive replication of virus. In contrast, those of the chickens infected with Ck/NL/03 moderately increased through the period of infection.

The present results indicate that rapid replication of virus induces hyper expression of cytokine mRNA, leading cytokine storm and multiple organ disorder followed by sudden death of chickens.

The role of antibodies in heterosubtypic protective immunity against influenza virus infection

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Influenza A viruses are divided into subtypes based on the antigenic properties of the membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Neutralizing antibodies against influenza A viruses bind mainly to HAs. Generally, influenza vaccines via subcutaneous (s.c.) injection are protective against homologous virus infection, but less effective against heterologous virus infection. The development of new methods which confer cross-protection against influenza viruses of multiple HA subtypes are required.

It has been reported that intranasal (i.n.) immunization of mice provides a broad spectrum of heterosubtypic protection against influenza virus infections, but the mechanism of cross-protection is poorly understood. In the present study, the role of HA-specific antibodies in heterosubtypic immunity is discussed.

H5 and H9 subtypes were first selected for immunizations. After i.n. or s.c. immunization of mice with inactivated viruses, serum, lung wash (LW), and nasal wash (NW) samples were collected. By enzyme-linked immunosorbent assay (ELISA) using recombinant HAs (rHAs) of each HA subtype as antigens, the relative amounts of IgG and IgA antibodies that can bind to each rHA were investigated.

In the serum, LW, and NW samples of s.c. groups, the amounts of IgG reacting to rHAs of inoculated strains were larger than those in the samples of i.n. groups, while IgA was detected only in those samples of i.n. groups.

Heterosubtypic reactivities of antibodies against multiple rHAs were observed in samples from both H5 - and H9-immunized mice. In the repertoires of cross-reactive antibodies, there was no difference among serum, LW, and NW samples. The IgG and IgA antibodies in each sample showed almost same spectra in the reactivity to multiple rHAs. These antibodies exhibited neutralizing activities against homologous virus strains, but didn't against the heterologous strains.

These results suggest that the IgA response in lung and nasal cavity may play a role in the broad-spectrum of heterosubtypic protection induced by i.n. immunization.

Development of a CTL-based human influenza vaccine

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[Introduction] For the development of a cross-protective influenza vaccine the induction of virus-specific cytotoxic T lymphocytes (CTL) is an important target, since most CTL epitopes are found on internal viral proteins and relatively conserved. Although influenza virus nucleoprotein (NP), which is a major internal structural protein, serves as a target antigen on infected cells for CTL, most of HLA-A*2402 restricted NP-derived CTL epitope peptides showed low immunogenicity. In this study, one or two amino acid substitution was introduced into the anchor motif at position 2 and/or 9 of the natural peptides to enhance the induction efficiency of NP-specific CTL. We evaluated their protective effect against influenza A virus infection using HLA-A*2402 transgenic mice (A24Tg), in which a human CTL immune system have been reconstituted.

[Methods] The modified peptides derived from NP of avian influenza virus strain A/HongKong/483/97 (H5N1) were synthesized. Binding affinity of the each peptide to HLA-A*2402 molecules was measured by HLA class I stabilization assay. In vivo cytotoxicity assay was performed to examine the immunogenicity of the peptides. To evaluate protective effect of the modified NP peptides in human CTL immune system, A24Tg were immunized subcutaneously or intranasally with the peptides, then challenged with lethal doses of A/HK483 virus and monitored for body weight for 14 days.

[Results and Conclusions] This substitution increased the binding affinity of the NP peptides to HLA-A*2402 molecules. As expected from the increased binding affinity, the modified peptides elicited NP-specific CTL more effectively than the natural peptides in A24Tg. More than half of the immunized mice survived after lethal doses of virus challenge. Intranasal administration of peptide vaccine was more effective than subcutaneous administration in survival and body weight reduction. We have demonstrated that NP-specific CTL induced by the modified peptide can effectively protect against lethal virus challenge.

The K627E amino acid substitution of the PB2 of A/Hong Kong/483/1997 (H5N1) influenza virus alters the efficiency of RNA synthesis of NP gene

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Influenza virus is enveloped and has eight negative-sense viral RNA (vRNA) gene segments which are packaged into viral ribonucleoprotein complexes. The viral RNA polymerase is a heterotrimeric complex of PB2, PB1, and PA proteins. This complex synthesizes virus mRNA, cRNA, and vRNA. The role of each protein involved in the regulation of RNA synthesis is yet unclear.

In the present study, the effect of amino acid substitution of Lysine for Glutamic acid at position 627 (K627E) of the PB2 of A/Hong Kong/483/1997 (H5N1) (HK483-K) in the RNA synthesis activity was examined. The mutant virus HK483-E (H5N1) was generated by using site-directed mutagenesis and reverse genetics from HK483-K (H5N1). The amino acid substitution of the PB2 did not affect the virus growth in 293T cells. The polymerase activity of HK483-E (H5N1) was lower compared with that of HK483-K (H5N1) in the replicon assay using the plasmids. To examine the affect on the RNA synthesis activity of the virus, the amounts of vRNA, cRNA, and mRNA of each gene in 293T cells infected with HK483-K (H5N1) or HK483-E (H5N1) were measured at 6 hours post infection by primer extension assay. The relative amounts of mRNA to vRNA of the PB2, PB1, PA, HA, and the NA of HK483-E (H5N1) were lower compared with those of HK483-K (H5N1). On the other hand, the relative amount of mRNA to vRNA of the NP of HK483-E (H5N1) was higher compared with that of HK483-K. The present results suggest that the K627E of the PB2 of HK483-K (H5N1) affected the RNA synthesis starting from the vRNA promoter of the NP gene in those viruses. Since the amino acid substitution in HK483-K (H5N1) was responsible for the pathogenicity against mouse, the comparison of the RNA synthesis efficiency in the lung tissues of mice is under way.

Potency of an inactivated avian influenza vaccine prepared from a non-pathogenic H5N1 virus against the challenge with an antigenically drifted virus of clade 2.3.4

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H5N1 highly pathogenic avian influenza virus (HPAIV) infection has become endemic in 4 countiries; China, Vietnam, Indonesia, and Egypt, where vaccine is used as a tool for the control of H5N1 HPAIV infection. In these countries, antigenically drifted viruses have been isolated from domesitic birds. It is assumed that these antigenic variants have been selected under the antibody selention pressure induced by vaccination.

In the present study, vaccine prepared from a nonpathogenic H5N1 avian influenza virus from the library in our laboratory was assessed its potency to induce protective immunity against antigenically drifted virus belonging to the clade 2.3.4 in chickens.

Antigenic analysis by haemaggulutination-inihibition test revealed that a HPAIV, A/peregrine falcon/810/2009 (H5N1) belonging to clade 2.3.4, is antigenically different from those of clades 2.2, 2.5, 2.3.2. The vaccine, oil-adjuvanted inactivated whole virus particles of A/duck/Hokkaido/Vac-3/2007 (H5N1), was inoculated into thigh muscle of four-week-old chickens. Three weeks later, these chickens were challenged with A/peregrine falcon/Hong Kong/810/2009 (H5N1).

Although vaccinated chickens shed much lower titers of virus compared with those from unvaccinated chickens, two out of 12 chickens died on 4 and 7 post-challenge days and the other 10 chickens survived without showing disease signs.

The present results indicate that antigenically drifted viruses were selected by vaccine used as a tool for the control of HPAIV infection. Thus, since the misuse of vaccine leads silent spread of antigenically drifted viruses, it is recommended that avian influenza vaccine should be very much carefully applied in addition to stamping-out policy. It is urgently needed eradicate H5N1 HPAIV from Asia by stamping-out without misuse of vaccine.

Characterization of the interaction of influenza virus NS1 with Akt

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Avian influenza viruses belong to the genus influenza A virus of the family Orthomyxoviridae. The influenza virus consists of eight segmented minus stranded RNA that encode 11 known proteins. Among the 11 viral proteins, NS1 (non-structural protein 1, encoded on segment 8) has been implicated in the regulation of several important intra-cellular functions. In this report, we investigated the functional interaction of NS1 with serine threonine kinase Akt, a core intra-cellular survival regulator. In co-immunoprecipitation assays and GST pull-down assays, NS1 directly interacted with Akt. The interaction was mediated primarily through the Akt-PH (Pleckstrin Homology) domain and the RNA-binding domain of NS1. NS1 preferentially interacted with phosphorylated Akt, but not with non-phosphorylated Akt. Functionally, the NS1-Akt interaction enhanced Akt activity both in the intra-cellular context and in in vitro Akt kinase assays. Confocal microscopic analysis revealed that phosphorylated Akt interacted with NS1 during the interphase of the cell cycle predominantly within the nucleus. Finally, mass spectrometric analysis demonstrated the position at Thr215 of NS1 protein is primary phosphorylation target site through Akt activation. The results together supported the functional importance of influenza virus NS1 with Akt, a core intra-cellular survival regulator.

Functional Analysis of Filovirus Glycoprotein

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General Introduction

Ebola and Marburg viruses are filamentous, enveloped and nonsegmented negative-strand RNA viruses in the family *Filoviridae*. They cause severe hemorrhagic fever in both humans and non-human primates.

Introduction

1) Filovirus glycoprotein (GP) is the only viral protein expressed on the surface of the virion, and therefore, GP is responsible for the virus entry into target cells. Many host factors have been implicated in the entry mechanism, however, ubiquitous cell-surface receptor(s) or co-receptor(s) responsible for filovirus entry have not been identified so far. To clarify the mechanism of filovirus entry into Vero E6 cells, a cell line highly susceptible for filovirus infection, the putative host cell surface factor(s) required for filovirus entry are being investigated.
2) Immune dysregulation is considered to be one of the critical mechanism responsible for the high pathogenicity of filovirus. Previous reports suggested that the transient Zaire GP expression resulted in impairing CD8 T-cell recognition of MHC class I on antigen presenting cells, since Zaire GP had heavily glycosylated domains which formed steric shield over proteins on the cell surface (steric occlusion). In this study, the difference in the efficiency of steric occlusion among Ebola virus species is being investigated.

Results

1) Monoclonal antibody M224/1 (MAb M224/1) which bound to the surface of Vero E6 cells and blocked filovirus infection was obtained. Thus MAb M224/1 is considered to recognize essential molecule(s) for filovirus infection. To isolate those molecule(s), immunoprecipitation using MAb M224/1 was attempted. In this analysis, unfortunately, the molecule(s) recognized by MAb M224/1 couldn't be immunoprecipitated from Vero E6 cell lysate. It is hypothesized that MAb M224/1 recognized the tertiary structure of the molecule(s) in the membrane, and this tertial structure was somehow disturbed during solubilization resulting in failure in immunoprecipitaion. Hence, in future, some cross-linkers will be used to covalently link the MAb M224/1 with its putative ligand in the membrane of intact cells and then identify the molecules.
2) After transfection of 293T cells with plasmids expressing Zaire or Reston GP, cells were collected, stained with antibodies detecting CD29 or HLA-ABC and analyzed by flow cytometry. Reactivity of these antibodies was decreased in Zaire GP-expressing cells after transfection, most likely because antibody couldn't bind to its target molecules effectively by Zaire GP-induced steric occlusion. In Reston GP-expressing cells, no difference was observed by comparing with mock-transfected cells. As a future plan, I will compare the differences in steric occlusion among the other Ebola and Marburg virus species.

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