北海道大学グローバルCOEプログラム

Achievements of Global COE Program

Establishment of International Collaboration Centers For Zoonosis Control

Programme & Abstract

Duration: December 17 (mon), 18 (tue), 2012
Venue: Conference Hall
Graduate School of Veterinary Medicine, Hokkaido University

External Evaluation Meeting for Global COE Program

Duration: December 17 (mon), 18 (tue), 2012
Venue: Conference Hall, Graduate School of Veterinary Medicine, Hokkaido University

========== Time schedule & Contents ==========

Day 1, December 17 (mon), 2012

13:00-13:10
Opening speech: Hiroshi Kida, Leader of Global COE Program
Hiroshi Kida

13:10-13:35
Activities for human resource development
Chihiro Sugimoto

13:35-14:00
Epidemiological research for zoonosis control
Ayato Takada

14:00-14:20 Coffee Break

14:20-14:45
Achievements of immunological and pathological research subgroup
Motohiro Horiuchi

14:45-15:10
Development of diagnostics, therapeutics and prophylactics for zoonoses
Yasuhiko Suzuki

15:10-16:10
Poster session

16:15-17:45
Discussion

- Introduction of GCOE (15 min) — Hiroshi Kida
- Presentation of overseas activities (15 min) — Chihiro Sugimoto, Hirofumi Sawa

Day 2, December 18 (tue), 2012

9:00-14:00 Review

14:00-15:00 Evaluation

Oral Presentation

Oral

Activities for human resource development

Chihiro Sugimoto

Leader, Cultivation of human resource subgroup

With the aim of developing human resources who can lead the prevention and control of zoonoses, the following activities for young researchers and graduate students were promoted:

1) The Zoonosis Control Expert (ZCE) Certification Program was launched, and those regarded as having the knowledge and experience necessary to develop measures to prevent and control zoonoses were certified as ZCEs. As part of the Program, a ZCE certification board was founded, and this organization established certification standards, screened ZCE candidates, and made the final decisions.

2) In the Program, postdoctoral fellows were employed and engaged in the research of zoonoses under the leadership of the persons in charge of the program. Graduate students were also employed as research assistants (RA) to support the research activities. At the end of each school year, a meeting was held in which the postdoctoral fellows and the RAs presented their research results, and their work was evaluated.

3) Japanese doctoral students and young researchers were also encouraged to get involved in practical epidemiological research training outside of Japan. They were also encouraged to make presentations at international conferences, with the Program funding the necessary travel expenses and participation fees.

4) Since 2009, the International Young Researcher Seminar in Zoonosis Control has been held once a year. Young researchers from various countries have been invited to this seminar, and young researchers and graduate students from the University have also been given the opportunity to make presentations, in order to improve their ability to communicate with others about science and technology and develop their research capabilities.

5) The Advanced Training Course for Zoonosis Control, a training course intended to allow young researchers in developing countries to gain the knowledge and skills necessary to control zoonoses, has been held 5 times, and trainees from Asia, Africa, North America, and other developing countries participated in this training. In order to examine the effect of the training as well as strengthen and develop relationships with organizations in other countries, follow-up training for those who completed this course and JICA training courses were conducted in 2012.

Epidemiological research for zoonosis control

Ayato Takada

Leader, Epidemiological research subgroup

Recently, newly emerging and reemerging infectious diseases are frequently appearing worldwide, and have become of major concern to public health. Importantly, most of these diseases are zoonoses. Epidemiological efforts to prevent outbreaks lie mainly in identifying natural host animals and elucidating transmission routes of the pathogens. Increasingly frequent outbreaks and concerns about the global spread of the zoonotic pathogens point to the importance of public health in a way - finding strategies to control and predict disease outbreaks.

The subgroup, "Epidemiological Research", in this GCOE program have conducted global surveillance (influenza, hanta virus, rabies, filovirus, West Nile virus infections, leptospirosis, tuberculosis, Lyme disease, leishmaniasis, trypanosomiasis, and so on) with the aid of the international collaboration research network (Zambia, Mongolia, Indonesia, Philippines, Thailand, Vietnam, Myanmar, Pakistan, Bangladesh, Nepal, Sri Lanka, Mexico, and so on) to understand the epidemiology of these zoonoses. Furthermore, we focused on the influenza A virus as a typical zoonotic pathogen and analyzed massive biological data sets by bioinformatics approaches to predict genetic and antigenic evolution and zoonotic potential of the virus.

Following are the projects conducted by the members and associate members of the subgroup, "Epidemiological Research":

- Toward the Control of Viral Zoonoses, "Influenza virus and Filovirus" (Ayato Takada)
- Studies on diagnoses of hantavirus infection and leptospirosis for epidemiological and epizootiological studies (Jiro Arikawa)
- Epidemiological study of avian infectious diseases (Kazuhiko Ohashi)
- Wildlife ecology and zoonosis control (Toshio Tsubota)
- Development of Bioinformatics Methods for a Large Scale Analysis of Genetic Information of Zoonotic Pathogens (Kimihito Ito)
- Epidemiological study of hantavirus infections in Mexico and Japan (Hiroaki Kariwa)
- Research on arthropod-borne bacterial and protozoan infections (Chihiro Sugimoto)
- Epidemiological Studies on Zoonoses in Sri Lanka (Hidehiko Tamashiro)

Achievements of immunological and pathological research subgroup

Motohiro Horiuchi

Leader, Immunological and pathological research subgroup

Clarification and understanding of host reaction against infectious agents, interaction between cells and pathogens, pathophysiology and pathogenesis of infections, are important for the intervention of diagnosis methods, prophylactics and therapeutics. Under the framework of GCOE program, the immunological and pathological subgroup mainly focused on the basic research on infectious diseases. The research targets are zoonotic diseases that threaten public health and that are society' concern due to the lack of the treatment and/or preventive measures, severity of diseases, or limited scientific knowledge of the diseases. Indeed, this subgroup studied many types of infectious diseases such as rabies, echinococcosis, West Nile virus, Influenza virus, prion diseases, and so on. Followings are major research outcomes of this subgroup.

Dr. Umemura and his colleagues made a great progress in the treatment of rabies. Indeed, intrathecal immunization of rabies vaccine, a direct inoculation of viral antigens into cerebrospinal fluid of rabbits that show clinical signs of rabies, could mitigate the clinical symptoms of the terminal stage of rabies. They also addressed the protective mechanism of intrathecal immunization against rabies. The success of the rabies treatment in rabid animal model encourages further studies on the development of novel treatment of rabies in humans and animals.

Dr. Agui and his colleagues demonstrated the role of Mx1 gene as a disease-resistant gene against influenza virus infection and Oas1b gene as a disease-resistant gene against flavivirus infections using Mx1 or Oas1b congenic mice. They also found loci controlling the Echinococcus cyst establishment by QTL analysis. The findings on inherited disease-resistant traits are important for the understanding of the molecular mechanisms of pathogenesis of these infectious diseases.

Dr. Sawa and his colleagues demonstrated the role of "viroporin" on the spread of polyoma virus from infected cells. They also found the association of ubiquitinated proteins accumulation with neuronal apoptosis caused by West Nile virus infection, and they also addressed the importance of mononuclear cell infiltration into lung in the severity of lung pathology in influenza virus infection.

Dr. Horiuchi and his colleagues established the method for the detection of abnormal isoform of prion protein (PrPSc) from prion-infected cells and tissue sections and using this method, they identified the intracellular compartment where prion propagation takes place and clarified the glial cell response against the prion propagation during the early stage of infection. They also demonstrated the utility of immunotherapy and regenerative medicine/ cell therapy for the treatment of prion diseases.

Development of diagnostics, therapeutics and prophylactics for zoonoses

Yasuhiko Suzuki

Leader, Diagnostic and therapeutic research subgroup

Diagnostics

Genetic information of pathogens obtained in this Global COE program allowed us to develop low cost simple detection methods for influenza virus, rabies virus, Ehrlichia and Leptospira based on Loop mediated isothermal amplification of DNA, LAMP. Genetic information was also used for developing genotyping methods of Ehrlichia and Mycobacteria and used for the molecular epidemiological study. Enzyme-linked immunosorbent assay or immunochoromatograph test or both were developed for hantavirus and leptospira infection using recombinant antigens. These genetic and immunologic tools are useful for the diagnosis, surveillance and quarantine of zoonosis.

Therapeutics

The hemagglutinin (HA) of influenza viruses is a potential target for antiviral drugs because of its key roles in the initiation of infection. The present findings indicate that Stachyflin, obtained from *Stachybotrys* sp., has antiviral activity against not only H1 and H2 but also H5 and H6 viruses by the interaction with the cavity on the HA molecule. The present analyses of the compound HA are useful for the development of HA inhibitors to have a border spectrum. In addition, we generated a novel monoclonal antibody specific to HA, which showed heterosubtypic neutralizing activity against influenza A viruses with multiple HA subtypes which can be a potential therapeutics against influenza. Additionally, we utilized human-mouse chimeric monoclonal antibodies with strong neutralizing activity against Ebola virus to show that anti-Ebola virus neutralizing antibodies may be beneficial in reducing viral loads and prolonging disease progression during Ebola hemorrhagic fever.

Prophylactics

Lactic acid bacteria are known to have a positive effect in maintaining the ideal immune system. We found that *Lactobacillus gasseri* administration exerts a protective effect against influenza virus infection through inhibition of inflammatory response and viral replication.

Poster Presentation

Poster

Research on arthropod-borne bacterial and protozoan infections

Chihiro Sugimoto, Ryo Nakao, Kyoko Hayashida

Research Center for Zoonosis Control, Hokkaido University, Japan

In order to seek control measures for infectious diseases caused by arthropod-borne protozoans and rickettsiae, analyses of their genomes, pathogenesis, and ecology were carried out. Research subjects included pathogens in the genera *Trypanosoma, Theileria*, and *Ehrlichia* found in the African continent, as well as the vector ticks and tsetse flies that transmit them. In particular, gene databases of parasites and vectors were developed and utilized to enable analysis of the triangular relationship between parasites, vectors, and mammalian hosts. We present here our genetic analyses of two tick-borne pathogens.

I. Genetic characterization of *Ehrlichia ruminantium*

Ehrlichia ruminantium is a tick-borne intracellular bacterium that causes heart water in ruminants. The disease is responsible for significant economic losses in endemic areas such as sub-Saharan Africa and several Caribbean islands; however, there is no effective control method available. Genetic characterization of global strains from geographically diverse origins and field samples collected in heart water endemic areas in Uganda was conducted using two different methods, multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA). We also developed a loop-mediated isothermal amplification (LAMP) assays for rapid, simple genetic detection of E. ruminantium.

II. Genome analysis of *Theileria orientalis*

The genome of *Theileria orientalis*, a tick-borne apicomplexan protozoan parasite of cattle, was sequenced and analyzed with a focus on comparative genome analysis of *T. orientalis* relative to other highly pathogenic *Theileria* species, *T. parva* and *T. annulata*. While synteny across homologous chromosomes of the three *Theileria* species was found to be well conserved overall, subtelomeric structures were found to differ substantially. Moreover, expansion of particular gene families by gene duplication was found in the genomes of the two transforming *Theileria* species, most notably the TashAT/TpHN and Tar/Tpr gene families. The *Theileria orientalis* genome database is available at http://totdb.czc.hokudai.ac.jp/.

Epidemiological study of hantavirus infections in Mexico and Japan

Hiroaki Kariwa[1)], Ngonda Saasa[1)], Takahiro Sanada[1)], Haruka Yoshida[1)], Yuka Ozaki[1)] Cornelio Sánchez-Hernández [2)], María de Lourdes Romero-Almaraz[2)], Celso Ramos[3)], Kumiko Yoshimatsu[4)], Jiro Arikawa[4)], Kentaro Yoshii[1)], Ikuo Takashima[1)]

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A variety of hantaviruses are harbored by rodents in the world, some of which can cause hantavirus infections in humans such as hantavirus pulmonary syndrome (HPS) and hemorrhagic fever with renal syndrome (HFRS).

In our recent epidemiological survey conducted in Mexico for hantavirus infection, we identified three distinct viruses circulating in Mexican wild rodents, namely Montano virus (MTNV), Huitzilac virus (HUIV), and Carrizal virus (CARV). To gain a detailed understanding of hantavirus epidemiology and its associated hosts, 410 rodents were captured at eight collecting points in Morelos and Guerrero, Mexico, and examined for anti-hantavirus antibodies and nucleotide sequences of the viruses. Of the 32 species captured, seven species were seropositive for hantavirus: *Peromyscus beatae* (31/127), *Reithrodontomys sumichrasti* (6/15), *R. megalotis* (2/25), *P. aztecus evides* (1/1), *P. megalops* (1/41), *Megadontomys thomasi* (1/9), and *Neotoma picta* (1/6), with an overall prevalence of 10.5%. Virus genome persisted in the majority of seropositive rodents. Nucleotide sequence and phylogenetic analysis showed that the viruses belonged mainly to the three lineages. The data showed that MTNV and CARV were primarily carried by *P. beatae* and *R. sumichrasti*, respectively. In addition, the data revealed an apparent complex interaction between hantaviruses and their hosts, suggesting active transmission and spillover infections within sympatric rodent species.

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To understand the recent epidemiology of hantavirus infection in Japanese rodents, 1,658 rodents were captured in various regions of Japan from 1994 to 2011. All of the 840 *Rattus* spp. and 113 wild rodents captured in southern regions of Japan excluding Hokkaido were seronegative. In contrast, among 705 wild rodents and Soricomorpha species from Hokkaido, 7.67% (27/352) of *Myodes rufocanus* and 1.19% (2/168) of *Apodemus speciosus* had anti-Hokkaido virus (HOKV) antibodies. HOKV was isolated from the lung homogenate of *M. rufocanus* through the MRK cell line originating from a kidney of *M. rufocanus*. These results strongly suggest that the prevalence of hantavirus infection in wild rodents is extremely low in the southern regions of Japan. However, HOKV infection is prevalent in *M. rufocanus* in Hokkaido.

Investigation of genomic factors responsible for resistance or susceptibility to infection by zoonotic pathogens in the laboratory mouse

Takashi Agui

Graduate School of Veterinary Medicine, Hokkaido University, Japan

The laboratory mouse includes a lot of various inbred strains that are useful tools for the investigation of genomic factors rendering a host resistant or susceptible to infection by zoonotic pathogens.

1. Role of Mx1 and Oas1b

Previous publications have reported that both Mx and Oas1b, which play a role as antiviral factors for the influenza virus and Flavivirus, respectively, were deficient in most of the laboratory inbred mouse strains. Because most of the inbred strains such as B6, BALB/c etc. are not suitable for the infection experiment of influenza or Flaviviruses, B6 congenic mice introgressed feral mice (MSM)-derived *Mx1* or *Oas1b* genes were established. B6.M-*Mx* congenic mice became to be resistant to influenza virus infection, but remained to be susceptible to infection by the West Nile virus. On the other hand, B6.M-*Oas* congenic mice became to be resistant to infection by the West Nile virus, tick-borne encephalitis virus, and Omsk hemorrhagic fever virus, all belonging to the *Flaviviridae*, but remained to be susceptible to the influenza virus infection. These results indicate that Mx1 and Oas1b show the specificity against pathogens and these congenic mice are useful tools for the infection experiments of these viruses.

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2. Identification of genetic factors for resistance or susceptibility to zoonotic pathogens

2-1) Sendai virus

Previous publications have reported that some inbred mice are resistant or susceptible to the Sendai virus infection. B6 and D2 mice are representatives of resistant and susceptible mice to the Sendai virus infection, respectively. Using backcrosses between B6 and D2 mice, we performed quantitative trait locus (QTL) analysis and identified three genetic loci, SeV1, SeV2, and Sev3 that were sufficient to determine resistant or susceptible phenotype to the Sendai virus infection.

2-2) *Echinococcus multilocularis*

It has been shown in previous publications that B6 and D2 mice are also resistant and susceptible to *Echinococcus multilocularis* infection, respectively, with respect to the cyst establishment and more advanced protoscolex development. Using backcrosses between B6 and D2 mice, QTL analysis revealed loci controlling the cyst establishment and more advanced protoscolex development.

Epidemiology, pathogenesis and drug development of parasitic zoonoses

Ken Katakura

Graduate School of Veterinary Medicine, Hokkaido University, Japan

1. Epidemiological investigation of zoonotic parasites, including Leishmania, Trypanosoma, Babesia, Theileria, Toxoplasma, Neospora and Fasciola species, were conducted in Myanmar, Thailand, Pakistan and/or Bangladesh. The results revealed new insights of distribution, evolution and control strategies of these parasites as follows. 1) Although visceral leishmaniasis (VL) in the Indian subcontinent is considered as anthroponosis, antileishmanial antibodies and Leishmania donovani DNA were detected in blood samples from stray dogs in VL endemic areas in Bangladesh, suggesting that dogs are a probable animal reservoir for VL in Bangladesh. 2) Certain Sergentomyia species in endemic areas of cutaneous leishmaniasis in Pakistan preferred various mammalian hosts, including humans, dogs, domestic ruminants, donkeys, wild rats and Indian gerbils although Sergentomyia sand flies are known to feed on cold-blooded animals. The epidemiological significance of the zoophilic feeding is required for further studies of the zoonotic transmission of sand-fly-borne pathogens. 3) Surra caused by Trypanosoma evansi infection is an important livestock disease in many developing countries. One donkey sample in the northern part of Myanmar was positive by loop-mediated isothermal amplification of the parasite DNA, suggesting the distribution of T. evansi parasites in Myanmar equids. 4) Results of national wide surveys of Myanmar cattle showed that bovine piroplasm infections are highly prevalent in Myanmar. Toxoplasma and Neospora infections in cattle were serologically detected. 5) Distribution of spermic Fasciola gigantica and aspermic Fasciola sp. was demonstrated in cattle and water buffaloes in Myanmar, suggesting different routes of the introduction of these parasites into Myanmar.

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2. Involvement of CD4+ Foxp3+ regulatory T cells in Leishmania donovani persistence in the liver was demonstrated in alymphoplastic aly/aly mice, an immunodeficient murine model of visceral leishmaniasis.

3. Antitrypanosomal quassinoid compounds were isolated from a medicinal plant, Brucea javanica. This is the first report on the antitrypanosomal activity of isolated quassinoids. In addition, bioactivity-guided fractionation of an ethanolic extract of another medicinal plant, Vitis repens, led to the isolation of resveratrol, 11-O-acetyl bergenin and stigmast-4-en-3-one for their antitrypanosomal activities.

Epidemiological Studies on Zoonoses in Sri Lanka

Hiko Tamashiro[1)], Yoshi Obayashi[1),] Asuna Arai[1)], Romeo B Lee[1)], Chandika D Gamage[1)], Chinyere Nwafor-Okoli[1),] Koji Kanda[1)], SAM Kularatne[2)], JRPV Rajapakse[2)], Panduka Gunawardana[2)], Ananda Jayashinghe[3)], Samath Dharmaratne[3)], Nobuo Koizumi[4)]

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We have carried out several epidemiological studies on rabies and leptospirosis in Sri Lanka to collate scientifically sound data on these diseases and help the country develop cost effective preventive and advocacy programs.

Rabies, one of the oldest NTDs, remains a major public health issue worldwide including Sri Lanka, where more intensive interventions are needed to achieve the national target of its eradication by 2016. We have exchanged the data, and discussed potential intervention strategies with its authorities, and developed important advocacy materials for students and their teachers and trained some of them. Furthermore, a rabies diagnostic laboratory has been installed in UP Faculty of Veterinary Medicine and Animal Sciences, which became a center for its diagnosis in the Central Province and its vicinities. Several young Sri Lankan scientists have been trained for the rabies diagnosis and conduct of epidemiological studies in their own country and in Japan. Other epidemiological studies have also provided valuable information on the development of potential intervention strategies and future studies, which will be carried out in due time.

Leptospirosis is a globally important zoonotic and emerging infectious disease caused by spirochetes of the genus Leptospira, comprising over 200 serovars in 26 serogroups. A variety of wild and domestic animals form natural reservoirs for pathogenic leptospira, which is transmitted to humans by direct contact with animals (e.g., rats) or by exposure to water or soil contaminated with urine of infected animals. To help formulate local intervention for leptospirosis in Sri Lanka, we studied the distribution of common serogroups, and identified circulating leptospiral species among these patients at UP Teaching Hospital. Among the 17 MAT positives, the infections occurred within a wide array of serogroups predominantly Sejroe and Tarassovi. We also attempted to determine the carrier status of pathogenic leptospira in dairy cattle. A high portion of cattle harbors a variety of pathogenic leptospira spp. and could also act as important reservoirs for human disease.

Results indicated that local cultural contexts should be taken into account when developing control programs for rabies. For leptospirosis control, more comprehensive advocacy programs need to be elaborated. Pupils could play important roles in their advocacy and information dissemination.

Clinical research on emerging infectious diseases in humans

Tadashi Ariga

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We have engaged in studies on emerging infectious diseases including human metapneumovirus and human bocavirus infections.

1. Human metapneumovirus (hMPV) has recently been recognized as an aetiologic agent of respiratory tract infections in children and adults. hMPV strains are classified into two genetic groups, A and B, each of which is further divided in two genetic subgroups, A1, A2, B1, and B2. To address the antigenic variation between these genetic subgroups, we developed an immunofluorescence assay (IFA) method using Trichoplusia ni (Tn5) insect cells infected with each recombinant baculovirus-expressed hMPV G (Bac-G) protein of the four genetic subgroups. This assay may be useful for the study of immune responses to different hMPV strains.

2. Human bocavirus 1 (HBoV1) was cloned by molecular screening of pooled human respiratory tract samples in 2005.

    1) HBoV1 encodes two structural proteins (VP1 and VP2) and two nonstructural pro teins (NP-1 and NS1). Titers of specific antibodies against VP1, VP2, NP-1, and NS1 proteins of HBoV1 were measured by IFAs using Tn5 cells infected with re combinant baculoviruses. The highest seroprevalence was observed in the IFA for VP1-specific IgG (71.6%).

    2) HBoV1 VP1- and VP2-specific IgG antibodies were detected in all four convales cent-phase serum samples but not in any acute-phase serum samples, demonstrating that recombinant VP1 and VP2 proteins of HBoV1 produced by a baculovirus sys tem are useful as a source of antigens for serological tests and that seroconversion with IgG antibodies to VP1 and VP2 proteins of HBoV is a reliable marker of HBoV infection.

    3) Between 2009 and 2010, three additional species of human bocaviruses, HBoV2, HBoV3, and HBoV4 (HBoV2-4), were discovered from fecal samples. In our study, HBoV1-4 was detected in 132 (15.5%), 5 (0.6%), 3 (0.4%), and 5 (0.6%) of 850 nasopharyngeal swab samples collected from children with RTIs, respectively. HBoV2 (3 samples) and HBoV4 (4 samples) were detected without codetection of other respiratory viruses in a few NPSs, suggesting that HBoV2 and HBoV4 may play some roles in respiratory tract infections in children.

Toward the control of viral zoonoses, "influenza virus and filovirus"

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Influenza virus

(1) Twelve avian influenza virus (AIV) strains were isolated from wild waterfowls in Zambia. Phylogenetic analyses of all gene segments of the isolates suggested a possible AIV exchange between wild birds and poultry in southern Africa and an AIV gene flow between the Eurasian and African continents.

(2) H5N1 highly pathogenic AIVs were isolated from wild waterfowl in Mongolia during 2005-2010. However, our AIV surveillance of migratory waterfowl in Mongolia every autumn indicated that birds were sporadically infected with the viruses prevailing in southern Asia. On the other hand, in Hokkaido in 2010, a highly pathogenic AIV closely related to the Mongolian 2009 H5N1 virus was isolated from apparently healthy ducks migrating southward, suggesting that the H5N1 virus may have been harbored in the waterfowl population during 2009-2010.

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(3) A novel monoclonal antibody showing neutralizing activities against influenza A viruses with multiple subtypes was generated. This antibody binds to the epitope adjacent to the receptor-binding region of the hemagglutinin. Our study underscores the potential therapeutic utility of cross-reactive antibodies.

Filovirus

(1) We have established an enzyme-linked immunosorbent assay in which the histidine-tagged viral glycoprotein of all known filovirus species is used as antigens. This assay enables us to detect filovirus species-specific antibodies.

(2) By using the enzyme-linked immunosorbent assay, filovirus-specific antibodies were detected in the serum samples collected from orangutans in Kalimantan Island, Indonesia. Our results suggest the existence of multiple species of filoviruses or unknown filovirus-related viruses in Indonesia, some of which are serologically similar to African filoviruses.

(3) We produced two clones of human-mouse chimeric monoclonal antibodies with strong neutralizing activities against Ebola virus and evaluated their protective potential in a rhesus macaque model. Reduced viral loads and partial protection were observed in animals given the antibodies, indicating that the antibody therapy is beneficial in reducing viral replication and prolonging disease progression.

Wildlife ecology and zoonosis control

Toshio Tsubota[1)], Kyle R Taylor[1)], Kyunglee Lee[1)], Satoru Konnai[1)], Ai Takano[2)], Hiroki Kawabata[2)], Sarad Paudel[1)], Takanori Kooriyama[1)], Michito Shimozuru[1)], Chie Nakajima[3)], Yasuhiko Suzuki[3)]

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1. Study on Lyme disease *borrelia* and other *borrelia* spp. prevalence and ecology of wildlife in Hokkaido

In Hokkaido, Japan, Lyme disease *Borrelia* and other *borrelia* spp. are present, but relationships between these prevalence and wildlife ecology have been poorly studied. We collected rodents from two locations in Hokkaido, tested for infection rates with *Borrelia* spp. using PCR for the borrelial *flaB* gene, seroprevalence using ELISA, and attachment of ticks by direct counts and identification. The four rodent species showed differential infection rates for *Borrelia* spp. Moreover, differential nymph and larval burdens were noted. Since seroprevalences by species were in agreement with infection rates and tick burdens, we suggest that the differences in infection rates of *Borrelia* spp. may largely be a direct consequence of differential exposure to vectors. We also compared our results for *Borrelia miyamotoi* infection rates to *Borrelia garinii* and *Borrelia afzelii*. Furthermore, we examined the extent of rodent exposure to *I. persulcatus* nymphs and larvae throughout most of their active season. Whereas *the Lyme borreliaeB. garinii* and *B. afzelii* showed age dependence of infection rates among wild rodents, *B. miyamotoi* infection rates were not age dependentdid not. *B. miyamotoi* infection rates did not show significant month dependence., *B. miyamotoi* did not show significance for such a trend. These results differences in month and age dependence lead us to suspect that *B. miyamotoi* may not develop persistent infections in wild rodents, as *B. garinii* and *B. afzelii* are thought to in the same manner as Lyme borreliae.

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2. Epidemiological study on tuberculosis in elephants in Nepal

Tuberculosis is a common zoonotic and communicable disease in elephants in Nepal, but the type of Mycobacteria i.e. human type or bovine type has not been identified yet. These 2 years we first had been conducting an epidemiological study on elephant tuberculosis in Chitwan and Bardia NP in Nepal. We tried to detect Mycobacterium tuberculosis complex organism in the trunk discharge and milk samples of captive Asian elephants using the acid-fast staining, culture and loop-mediated isothermal amplification after DNA extraction, but no positive Mycobacterium DNA is detected so far. We also collected serum samples for the antibody test using Elephant TB STAT-PAK assay, and did the intradermal tuberculin testing. The current results show that one elephant which was positive on Elephant TB STAT-PAK test was positive on skin test.

3. Parasitological surveillance in primates of Tanzania

Parasitological surveillance in 5 primate species has been performed using coprological observation in Mahale Mountains National Park, Tanzania. Faecal samples were examined microscopically, and parasite identification was based on the morphology of cysts, eggs, larvae, and adult worms. The parasitological data reported for red colobuses, vervet monkeys, and yellow baboons in Mahale are the first reports for these species.

Studies on diagnoses of hantavirus infection and leptospirosis for epidemiological and epizootiological studies

Jiro Arikawa

Graduate School of Medicine, Hokkaido University, Japan

Hemorrhagic fever with renal syndrome (HFRS) and leptospirosis are important rodent-borne zoonoses caused by hantavirus and leptospira infection, respectively. Since the clinical features of HFRS and leptospirosis are similar and rodents are a common reservoir, differential diagnoses are necessary in areas where hantavirus and leptospira co-circulate.

Through the GCOE program, we have studied the serological diagnosis of hantavirus infection and leptospirosis by using recombinant antigens and applied to epidemiological study as follows.

(1) E. coli-expressed hantavirus recombinant nucleocapsid protein (rNP) and baculovirus expressed N-terminal truncated rNP were developed as enzyme-linked immunosorbent assay (ELISA) antigens for screening and serotyping of hantavirus infection, respectively. The availability of the two ELISAs was confirmed by examining the number of patients or animal sera infected with HFRS or HPS causing viruses. Particularly, acute phase HPS patient sera were serotyped by the serotyping ELISA, indicating the availability for early phase diagnoses.

(2) E. coli-expressed N-terminal part of hantavirus rNP was applied to immunochromatographic (ICG) test for the detection of anti-hantavirus antibodies in rat and human sera. Colloidal gold labeled with anti-rat IgG antibody and Protein A was applied to rat and human sera, respectively. The sensitivity and specificity of the ICG test are equal or even higher than IFA and ELISA both in rat and human sera. Furthermore, the ICG test was able to serotype human sera obtained from patients with Seoul, Puumala, and Sin Nombre type hantavirus infections.

(3) The diagnostic utility of recombinant antigens of leptospira was examined. Two major surface proteins, LipL32 (highly conserved among pathogenic leptospira) and 3 regions of LigA (immunodominant and variable among strains), such as LigA-K, -C and –V of the Manila strain were expressed by E. coli and used as antigens in ELISA. Sensitivities and specificities of these antigens were examined with serially collected rat sera from laboratory rats experimentally inoculated with Leptospira interrogans serovar Manilae. Comparison of results by the microagglutination test (MAT) and ELISA indicated that recombinant antigens LipL32 and LigA-K were useful antigens in ELISA for the laboratory diagnosis of a carrier infection of leptospirosis in rats.

Epidemiological study of avian infectious diseases

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Wild birds play an important role as the infection sources in the epidemiology of many infectious diseases including zoonosis. We have developed the nested RT-PCR method to detect the West Nile virus (WNV) and Japanese encephalitis virus (JEV) genomes using feather tips, which are easy to collect from wild birds. By using this method, the viral genomes were detected for a long time after infection in experimentally infected chickens, showing that this method is useful for the detection of WNV and JEV from wild birds. In addition, an attempt was made to establish a serological method to detect JEV- and WNV-specific antibodies from feather tips. It has been shown that the fractions of antibodies can be extracted from feather tips, and these can be used to develop the ELISA method to detect virus-specific antibodies by using recombinant viral proteins.

Marek's disease virus serotype 1 (MDV-1) strains cause malignant lymphoma in chickens, and MDV-1 has been previously reported to be widespread in white-fronted geese. In this study, we found that the MDV-1 was also widespread in wild geese and ducks, but not in other bird species, suggesting that resident waterfowls may be carriers and reservoirs of MDV-1. We also studied the molecular mechanisms of the pathogenesis caused by MDV, and found that both viral (i.e. Meq) and host factors (i.e. PD-1/PD-L1 pathway) are involved in the pathogenesis.

P-10

Poultry red mite, *Dermanyssus gallinae*, is an economically important ectoparasite, which can be a vector for several infectious diseases. Currently, red mites have been controlled by the use of acaricides, but the mites can develop acaricide-resistance, and thus alternative control strategies are required. However, little is known on the molecules of the red mite to search for good candidate antigens for the development of anti-mite vaccines. Thus, EST analysis has been performed. Total of 2,466 cDNA clones were randomly picked, and 1,147 cDNA clones were sequenced, and then 373 sequences were identified as those known of functions. Among these molecules, homologues of peroxiredoxins (Prx4 and Prx2) and type II allergen, which are suggested as possible vaccine candidates for other ticks, were shown to react with sera from chickens infested with red mite, showing that these are exposed antigens of the mite. It will be necessary to check protective efficacy of these molecules against feeding of the red mites.

Development of bioinformatics methods for a large scale analysis of genetic information of zoonotic pathogens

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The rapid development of molecular biology has accelerated the accumulation of results from genetic and experimental studies on zoonotic pathogens. Through a fusion of bioscience and informatics, we conducted comprehensive studies based on large-scale datasets to predict amino acid substitutions on the hemagglutinin (HA) molecules of antigenic variants of human influenza A viruses.

Human influenza A viruses undergo antigenic changes with gradual accumulation of amino acid substitutions on the HA molecule. A strong antigenic mismatch between vaccine and epidemic strains often requires the replacement of influenza vaccines worldwide. The establishment of a practical method to predict the antigenic changes of the influenza viruses is particularly important for the efficacy of vaccination.

P-11

To expose underlying patterns of HA amino acid substitutions in the evolutionary pathway, relative distances of amino acid sequences among past epidemic strains were analyzed by multidimensional scaling (MDS). We found that human influenza viruses have evolved along a gnarled evolutionary pathway, which was reasonably explained by assuming that the rate of amino acid substitutions varied from one position to another according to a gamma distribution. Retrospective prediction tests for 12 years from 1997 to 2009 showed that 70% of actual amino acid substitutions were correctly predicted, and that 40% of predicted amino acid substitutions have been actually observed.

We also showed that HAs of 2009 H1N1 and the 1918 pandemic viruses shared a significant number of amino acid residues in their antigenic sites, suggesting the existence of common epitopes for cross-reactive neutralizing antibodies. We also identified conserved codons, which could produce N-glycosylation sites with a single nucleotide substitution. We thus hypothesized that mutations at these conserved sites could soon be selected by antibody -mediated selection pressure in humans.

A novel algorithm—called the closest-neighbor trimming method—that resamples from a large nucleotide sequence dataset was proposed. The method can thin out densely sampled sequences from a dataset. We anticipate this algorithm can provide a small number of sequence dataset, which could to be used as reference sequences. The resampled sequences could enable researchers to conduct phylogenetic analyses in dramatically shorter time than that with the original dataset.

Toward the understanding of pathogenesis of prion infection

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Prion diseases are characterized by the accumulation of abnormal isoform of the prion protein (PrP^{Sc}) in the central nervous system, neuronal loss, astrogliosis and microglial activation. However, how prions propagate in cells, how microglia and astrocytes recognize and respond to prion propagation, and the mechanism of neuronal death by prion propagation remain to be elucidated. To understand the pathogenesis of prion infection and to contribute to the establishment of therapeutics of prion diseases, our research focused on 1) prion propagation in cells, 2) glial cell activation and neuronal death, and 3) treatment of prion diseases.

1. Mechanism of prion propagation in cells.

We established the method for PrP^{Sc}-specific staining by IFA using mAb 132 that recognizes the region adjacent to the most amyloidogenic region of PrP. By using this method, we found that PrP^{Sc} cycles between peri-nuclear and peripheral regions including the plasma membrane via an endocytic recycling pathway and that such membrane trafficking machinery is important for the generation of PrP^{Sc}. Furthermore, we also found that the transfer of inoculated PrP-res from the endo-lysosomal pathway to the endocytic-recycling pathway is important for the initiation of efficient prion propagation.

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2. Analyses of glial cell activation and neuronal death.

We successfully modified our PrP^{Sc}-specific staining for frozen tissue sections and double-staining of PrP^{Sc} and glial cell markers provided evidence that astrocytes recognize prion propagation directly or subtle changes in neurons by prion propagation and that play an important role in neuropathobiology in the early stage of prion infection.

Lack of the in vitro model for neurodegeneration is one of the obstacles for the elucidation of the mechanism for neuronal death by prion propagation. We found that neurons in differentiated neurospheres reduced the expression of pre-synaptic markers such as synaptophysin by prion infection, which is observed in the brains of prion-infected animals. Thus differentiated neurospheres will be useful for analyzing molecular mechanism for neurodegeneration caused by prion infection.

3. Treatment of prion diseases

To date, treatment of prion diseases is not available. Under the GCOE program, we showed that anti-PrP mAb and MSCs prolonged the survival of prion-infected mice even administrated after the clinical onset. The results provide insight into immunotherapy and regenerative medicine/cell therapy for the therapeutics of prion diseases.

Investigation of the molecular mechanisms of the pathogenicity of viral diseases

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1. Host factors and viral infection:

We previously examined host responses against viral infections, focusing on interactions between viral and host proteins, and reported novel findings (EMBO Rep, 2005, J Biol Chem 2005). In this Global COE Program, we reported that the viral protein of polyomavirus works as "viroporin" and enhances the exit of viruses from host cells (PLoS Pathogens, 2010). This finding was introduced in "Featured Research" in PLoS Pathogens and referred to in Nat Rev Microbiol (2012).

We also found that the viral protein results in G2 arrest in virus infected cells and promotes viral replication (J Biol Chem, 2010).

2. Molecular mechanisms of pathogenicity of viral infection:

In collaboration with many institutions in Japan and overseas, we have performed research on the mechanisms of the pathogenicity of infections by viruses, including influenza virus, polyomavirus, human T-lymphotropic virus, and West Nile virus, which has been reported in journals, such as Blood (2009), J Med Virol (2010), AIDS Res Hum Retroviruses (2010), J Gen Virol (2011), Biochem Biophys Res Commun (2011), Jpn J Infect Dis (2012), Neuropathology (2012), Microbiol Immunol (2012), and PLoS One (2012).

3. Investigation of natural reservoirs of viruses

According to the objective of the Global COE Program, "Cultivation of human resources who contribute to prevention and control of zoonoses", I visited the Republic of Zambia, Africa, 21 times and performed epidemiological research together with young post docs and Ph. D. students in collaboration with other projects. We collected samples from wildlife, including rodents, bats, baboons, and vervet monkeys. After analyses of obtained samples, we isolated and identified known and unknown arenaviruses, polyomaviruses, paramyxoviruses, pox viruses, and retroviruses (Emerg Infect Dis, 2011, J Gen Virol, 2012, J Gen Virol, 2011).

A novel therapy for rabies

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Rabies is almost always fatal when the rabies virus invades the brain. Two human cases survived after developing the symptoms of rabies using a therapeutic coma (Milwaukee Protocol). However, the therapeutic coma was not successful in the other patients, leading to controversy regarding the scientific rationale for the Milwaukee protocol, and we still do not have definitive therapeutic measures against rabies. In this research program, we first demonstrated that a specific antibody can be rapidly induced in the cerebrospinal fluid (CSF) by intrathecal (IT) immunization, a direct inoculation of antigens into the CSF, and that this immunization was effective in preventing intracerebral and intramuscular challenges with the rabies virus. The antibody induced in the CSF originated from the serum and a de novo antibody locally produced in the brain. Subcutaneous immunization given prior to IT immunization induced a more rapid and higher antibody response in the CSF than IT immunization alone and IT immunization alone in rabid rabbits was invalid.

We tried to treat rabbits showing the clinical signs of rabies with IT immunization. All rabbits given subcutaneous immunization 3 days before the virus challenge from the periphery and treated with IT immunization after showing the clinical signs of rabies recovered from the terminal stage and reassumed eating and drinking. They started to respond to external stimuli again from 12-18 dpi and survived until the end of the experiment. Pathological examination of surviving rabbits revealed a few virus antigens in the central nervous system (CNS), whereas rabbits that died without IT immunization showed a massive amount of rabies antigen throughout the CNS. Antibody responses of dead and surviving rabbits were not significantly different at the terminal stages (8-12 dpi), and the lymphocytes that infiltrated into the CNS of dead rabbits were predominated by T lymphocytes. These findings indicated that antibody titers of the serum and CSF were not the crucial or sole key for clearance of the virus from the CNS of rabid rabbits, and both humeral and cellular immunities contributed to the clearance of the rabies virus from the CNS.

We could save lives and clear the rabies virus from the CNS of experimentally induced rabid rabbits using IT immunization. However, our success is limited because (1) damage to CNS tissues of recovered rabbits was severe enough to prevent complete recovery, (2) rabbits should be pre-immunized 3 days before the virus challenge, and (3) we used a neurovirulent fixed virus, the CVS strain, instead of the street virus. Most human cases that recovered spontaneously from rabies had received a pre-exposure vaccination and our treatment protocol may be applicable for individuals pre-vaccinated before exposure to the rabies virus. Nerve tissue damage caused by street virus infections is minimal due to active production of anti-apoptotic and anti–inflammatory proteins in relative to the fixed virus. Therefore, we believe that the present limited success using IT immunization may stimulate the future development of novel therapies widely applicable for rabies in humans and animals caused by exposure to the street virus.

Development of rapid diagnostic system of zoonoses

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1. Establishment of a novel isothermal DNA amplification method
Nucleic acid amplification techniques (NAAT) brought a breakthrough in diagnosis of infectious diseases. Several NAAT including Polymerase Chain Reaction have been developed for this, however, requirement of expensive laboratory infrastructure and sophisticated technical skill make them inaccessible in developing countries. We have developed a novel isothermal DNA amplification method by targeting tandem repeat sequences on the DNA of pathogens and termed TRIAmp. This method successfully detected as small as 10 copy of *Mycobacterium tuberculosis* DNA. Further experiment targeting various bacterial pathogens confirmed the usefulness of this method for the diagnosis of infectious diseases.

2. Development of novel genotyping methods of *M. tuberculosis*
Genotyping of *M. tuberculosis* aims to analyze nosocomial infection, epidemiological analysis of transmissions and others. Classical epidemiology of tuberculosis was base on the analysis of contact to patients, and previous disease history. Recently, genotyping became available by the accumulation of the data on the changes in specific gene arrangement existing in the genome of *M. tuberculosis*. Among genotypings, "Spoligotyping" is a most powerful tool for genotyping *M. tuberculosis*, however, the procedure is tedious intensive and has a risk of contamination. We have developed a method based on Spoligotyping with simple procedure and low risk of contamination and named SpoligoArray. We transferred this technology to Zambia, Nepal, Bangladesh, Myanmar, Thailand, the Philippines and China. And furthermore, we applied this method together with the other genotyping method "Variable Number Tandem Repeat (VNTR) analysis" for the genotypic analysis of multi- and extensively deru resistant *M. tuberculosis* strains.

3. Establishment of sensitive detection methods for pathogenic leptospiras
Leptospirosis is a worldwide zoonosis caused by infection with pathogenic spirochetes of the genus *Leptospira*. Early diagnosis is essential because antibiotic treatment is most effective during the initial course of the disease. However, current diagnostic methods are not useful for early diagnosis (e.g., culture and microscopic agglutination test) or are not widely applicable in developing countries (e.g., PCR). We developed a new loop-mediated isothermal amplification (LAMP) method to detect 16S rRNA gene segment of pathogenic *Leptospira* spp. in urine. The method enabled the detection of two leptospiral cells. The sensitivity of this method is higher than that of culture or conventional *flaB*-nested PCR. This new method can be a powerful tool for practical use to diagnose leptospirosis.

PI3K-Akt signal transduction: implication for the influenza therapy

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The growth factor signals regulate the balance of cell proliferation and cell death to maintain the homeostasis *in vivo*; hence, deregulation of the balance underlies wide varieties of human diseases. The serine threonine kinase Akt, a core intracellular survival regulator, is activated by various cytokines or growth factors, hence, Akt mediated signal underlie various human diseases such as cancers, viral infections, glucose intolerance. Therefore, targeting Akt becomes an attractive goal for drug development for influenza infection.

We have demonstrated the biological significance of functional interaction of Akt with influenza virus NS1 (non-structural protein 1) protein. The results together supported that Akt-NS1 functional interaction as a novel molecular target for the influenza therapy with clinical implications (Matsuda et al., BBRC 2011).

We have demonstrated that protooncogene TCL1, previously unknown biological function, is an Akt kinase co-activator that physically interacts with Akt and enhances Akt kinase activity (Noguchi et al., Faseb J 2007). Further, by structural-functional analysis of TCL1-Akt protein complexes, we have identified a peptide named TCL1-*Akt-in* (Akt inhibitor) interacted with the PH domain of Akt and specifically inhibited Akt kinase activity (Noguchi et al., Curr Sig. Thera 2008). Based on the structural comparison study of TCL1b, another family member of TCL1 protein with TCL1, we designed TCL1b based Akt inhibitor, namely "TCL1b-*Akt-in*"（RLGVPPGRLWIQRPG）to examine the therapeutic potential of the peptide(Hashimoto et al., submitted 2012). Indeed, "TCL1b-*Akt-in*"not only inhibited Akt kinase activity, but also cellular proliferation. The observation prompted us to examine the effect of "TCL1b-*Akt-in*" for the viral replication of influenza infection. Both"TAT-TCL1 *Akt-in*" and"TAT-TCL1b *Akt-in*" showed significant inhibitory effect of viral replication compared to LY294002or wortmannin, the most commonly used pharmacological inhibitors of PI3K. The inhibitory effect of Akt kinase activity by" TAT-TCL1b *Akt-in*" was well correlated with not only the levels of viral replication measured by the plaque assay, but also the levels of phosphorylation of NS1 protein determined by immunoblot. The observation supported the notion that inhibition of Akt kinase activity by the peptide inhibitors showed a therapeutic potential for the influenza therapy. Collectively, the study supported that by down modulation of Akt signaling as a novel molecular target for the treatment of human influenza infection by possibly targeting the influenza NS1 with Akt functional interaction.

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Host defense system against influenza A virus infection

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To develop novel drugs or methods for the prevention and treatment of influenza, we study the mechanisms of the host defense system and viral replication.

(1) Type I interferons (IFNs) are critical factors for protection against influenza A virus infection. The virus sensors, RIG-I and IPS-1 are critical for induction of type I IFNs. We found that the viral polymerase complex exhibited an inhibitory activity on IFNβ promoter activation by these sensors. Especially, PB2 or PB2-containing complex strongly inhibited IFNβ gene transcription by direct binding to IPS-1. Importantly, this inhibitory pathway is different from NS-1 dependent pathway. These findings demonstrate that viral polymerase plays an important role in regulating the host anti-viral response through binding with IPS-1 to inhibit IFNβ production.

(2) We found that Siva-1 is crucial for apoptosis induction caused by infection with influenza A virus and is involved in viral replication. Susceptibility to apoptosis induced by the viral infection was increased in human lung-derived A549 cells, which stably express Siva-1. Furthermore, the viral replication was significantly suppressed in these cells in which Siva-1 expression was inhibited. Effect of Siva-1 knockdown was eliminated by treatment with a caspase inhibitor, Z-VAD-FMK. These findings suggest that the caspase-dependent pathway for the induction of apoptosis is involved in Siva-1-mediated influenza A virus replication.

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(3) Osteopontin (OPN) is critical to determine the symptom of inflammatory diseases and severe pulmonary inflammation is frequently found in influenza A virus infection. We found that expression levels of OPN increased in mice after the virus infection. OPN knockout (KO) mice exhibited a severe pathological phenotype and survival rates were lower than those of wild type mice, while survival rates were higher in OPN transgenic (Tg) mice infected with the virus. The population of NK cells significantly decreased in OPN KO mice at day 5 after the infection, whereas, it increased in OPN Tg mice. These results suggest that OPN plays an important role in host defense against influenza A virus infection through regulation of the NK cell population.

(4) Lactic acid bacteria are known to have a positive effect in maintaining the ideal immune system. We found that the survival rate of mice administered with a water-soluble fraction (SLFK) of lactic acid bacteria was significantly better than that of control mice after influenza A virus infection. Expression levels of anti-inflammatory cytokine, interleukin -10 in lung tissues were enhanced by the administration of SLFK. In addition, oral administration of Lactobacillus gasseri (LG) prolonged the survival time of mice infected with the virus. Interestingly, (i) virus titer in lung tissue (ii) cell infiltration level into the bronchoalveolar lavage fluid (BALF), (iii) produced IL-6 amount in the lung tissue, (iv) lactate dehydrogenase activity in the BALF were decreased in LG-administered mice compared to control mice. These observations suggest that oral administration of lactic acid bacteria exerts a protective effect against influenza virus infection through down-regulation of the excessive inflammatory response and viral proliferation in the lung tissue.

De novo designated molecular for structure-based Anthrax vaccine

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Anthrax is a lethal disease caused by the Gram-positive, spore-forming bacterium Bacillus anthracis. Although anthrax is primarily an epizootic disease, humans are at risk for contracting anthrax. However, a safe and effective vaccine for humans is not available to the general public. With a problem of bacterial resistance to antibiotics, it is necessary to improve our understanding of B. anthracis through surveillance and develop more effective prophylaxis against B. anthracis. Previous report showed that immunization with oligomerized protective antigen (PA) of B. anthracis neutralized B. anthracis toxity effectively compared with monomeric PA. In this study, we examine to construct de novo designed PA antigen that is based on the structural information of PA heptamer. In August and September 2011, about 400 cases of human anthrax were found in Zambia and 5 people died. It was suspected that patients had contact with B. anthracis contaminated hippopotamus meat. We succeeded in isolating B. anthracis from the hippopotamus samples in Zambia, and cloned the B. anthracis PA gene from the isolated strains. To identify the potential structure of the antigen, we calculated the solvent-accessible surface area of the PA heptamer and constructed a fully synthetic de novo designed molecule that is constituted by an oligomerized PA domain1. From results of molecular dynamics simulation, the designed molecule showed stable conformation in hydrophillic solvent. The designed molecule was expressed in E. coli and then purified using column chromatography. We examine the antigenicity and protective the efficiency of designed molecule using animal model and molecular basis-approaches. Based on the information, we will further improve the designed antigen to develop safe and effective vaccine against B. anthracis.

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