Laboratory Animal Research Center

Morbilliviruses in the family Paramyxoviridae including canine distemper virus, rinderpest virus and measles virus are highly infectious among their natural hosts. We have succeeded in establishing a system of reverse genetics for morbilliviruses. The new technology is being applied to the study of the functions of viral components in replication, pathogenicity, and species-specificities. We have also started a study on Hepatitis C virus which affects more than 2 million patients in Japan. In addition, more than 30,000 mice, mainly transgenic and gene-targeted ones, are always kept for researches of IMSUT and the technical staffs members contribute to their maintenance and breeding.

1. The reverse genetical approach to canine distemper virus

Chieko Kai, Ryuichi Miura, Kentaro Fujita, Fusako Shimizu, Hiroki Sato, Athipoo Nuntaprasert, Misako Yoneda, and Masashi Uema

The reverse genetics for Mononegavirales is an innovative technique among those developed to rescue infectious viruses from cDNA. We have developed this system for canine distemper virus (CDV), which is prevalent and causing serious problems in dogs and wild animals in the world. We first determined the complete nucleotide sequence of the Yanaka strain of CDV, one of recent field isolates from affected dogs in Japan. A plasmid was designed to contain the cDNA copy of the full-length CDV with unique restriction enzyme sites between structural genes for further basic and applied studies. Viruses were successfully recovered from 293 cells transfected with the full-genome plasmid and the plasmids encoding transacting proteins of RPV-N, P, and L. A protein called the V protein is fused to the amino-terminal half of P protein, and it has recently been suggested that the protein may be required for *in vivo* pathogenicity of mononegavirus by using the new reverse genetic system. However, the functions of the protein still remain to be clarified. We have also succeeded in recovering a mutated virus without the ability to synthesize the V protein from the cDNA of CDV. Both recovered viruses, maternal and mutated, induced typical cytopathogenic effects in B95a cells and grew well *in vitro* without any significant differences compared to the original Yanaka strain. This new technology will allow us a great progress in the study of the functions of viral components in replication, pathogenicity, and host specificities.

2. Establishment of Recombinant canine distemper viruses with EGFP of luciferase reporter genes

Kentaro Fujita, Ryuichi Miura, Fusako Shimizu, Hiroki Sato, Athipoo Nuntaprasert, Misako Yoneda, and Chieko Kai,

The EGFP and luciferase genes were incorporated into the full-length CDV genome between N and P genes. The recombinant EGFP-CDV showed its specific fluorescence in the early stage of infection and the recombinant luciferase-CDV showed activity after 12 hrs of infection. These results indicate that the full-length CDV may be utilized as a novel vector for the expression of exogenous genes of at least 1.7 kb DNA fragment. These recombinant CDVs should be a powerful tool for the characterization of the tissue

tropism, infection pathways, and host range of CDV.

3. Characterization of M protein in a strain of canine distemper virus causing persistent infection

Toshiya Nishi, Ryuuichi Miura, Hiroki Satoh, Kentaro Fujita, Athipoo Nuntaprasert, Misako Yoneda, Chiaki Wakasa, Masashi Uema, Motohiro Shiotani, and Chieko Kai

We have established a CDV strain (BP) causing persistent infection in B95a lymphoid cell line without inducing fusion-type cytopathic effects. We cloned H and F genes from the parental strain of CDV-BP. Co-transfection of both H and F genes from CDV-BP induced giant cell formation, which was an effect similar to that of the parental strain. These results indicate that the H and F proteins themselves do not play a central role in causing the cytopathic effects. We are analyzing the possibility of the modification in the interaction of H and F proteins of CDV-BP with other viral proteins.

4. Epidemiological survey of morbillivirus infection in aquatic mammals

Kenjiro Ohashi, Masashi Uema, Yoko Goto and Chieko Kai

In the genus Morbillivirus of the family Paramyxoviridae, only four viruses were known as its members until 1987, which were measles virus (MV), rinderpest virus (RPV), CDV and peste des petis ruminants virus. In 1988, a serious infectious disease with a high mortality rate started to spread among seals in northwest Europe, followed by outbreaks of a similar disease among dolphins and porpoises. After the outbreaks, three new morbilliviruses were identified: phocine distemper virus (PDV), dolphin morbillivirus (DMV) and porpoise morbillivirus (PMV). We conducted an epidemiological survey of these new morbilliviruses in aquatic animals along the coast of Japan and in the Caspian Sea and Lake Baikal. Serological analyses of more than 300 animals including cetaceans and pinnipeds indicated that the pinnipeds had been infected with a PDV-like virus by 1988 and cetaceans with a DMV-like virus by 1978 along the coast of Japan. This is the first report of morbillivirus infection in various aquatic mammals from the western Pacific Ocean. A serological survey of Caspian seals revealed that the mass mortality of Caspian seals in 1997 was caused by distemper virus infection and that the 2 types of distemper viruses, the CDV-like and PDV-like viruses, existed in the Caspian Sea. In Lake Baikal, where the CDV outbreak occurred in 1987, it is suggested that infection still continues.

5. A CDV epidemic in raccoon dogs (Nyctereutes Procyonoides viverrinus) in Japan

Kenjiro Ohashi and Chieko Kai

All members of the Canidae and Mustelidas families and certain members of the Procyonidea family are susceptible to CDV infection. Moreover, CDV has recently been isolated from wild animals including large cats and aquatic mammals, which had not been known to be susceptible to CDV

previously. To understand the relation of canine distemper in domestic dogs and that in wildlife in Japan, the distribution of CDV infection in raccoon dogs was surveyed seroepidemiologically. Antibodies against CDV were detected by ELISA and virus neutralization tests in 75 blood samples collected from raccoon dogs from 1982 to 1998 in Japan. The genetic and serological analyses of a newly isolated CDV from a raccoon dog revealed that there are only four mutated portions in the amino acid sequence of the H protein and one mutated antigenic epitope, indicating that the virus spreading among Japanese raccoon dogs belongs to the same group as the CDV strains causing recent outbreaks of canine distemper in dogs.

6. Studies of pathogenicity of rinderpest virus using a lappinized strain

Misako Yoneda, Motohiro Shiotani, Ryuichi Miura, Athipoo Nuntaprasert, Kentaro Fujita, Kenjiro Ohashi, Michae D. Baron[1], Thomas Barrett[1] and Chieko Kai:[1]National institute for Animal Health, UK

RPV causes an acute, febrile and highly infectious disease in cattle and wild bovids and results in serious economic damage in most developing countries. Previously we established an excellent rabbit model experimentally infected with a lappinized strain of RPV (RPV-L), which is considered to be useful for the study of the pathogenesis of RPV. The reverse genetics of the vaccine strain of RPV (RPV-RBOK), which does not cause a severe disease in rabbits, has already been established. In order to analyze the role of the H protein in the pathogenicity, we constructed a full genomic plasmid of the RPV-RBOK strain whose H gene was replaced with that of RPV-L strain, and succeeded in rescuing an infectious virus (RPV-lapH). The recombinant virus did not cause a severe disease in rabbits after infection, suggesting that other genes in the RPV-L are necessary for the pathogenicity in rabbits. In order to further study the pathogenicity of RPV, we attempted the cloning of virulent and avirulent strains of the RPV-L. After three times of plaque purification followed by experimental infection in rabbits, two clones of virulent and two clones of avirulent strains were established.

7. Efficiency of recombinant cytokines expressed by a Sendai virus expression system

Ryuichi Miura, Yasuyuki Endo, Kentaro Fujita, Hiroki Satoh, Kenjiro Ohashi, Atsushi Kato[2], Yoshitsugu Matsumoto[3] and Chieko Kai:[2]National Institute of Infectious Diseases and [3]Laboratory of Veterinary Applied Immunology, The University of Tokyo

We applied a new protein expression system using recombinant Sendai virus (SeV) to the production of canine cytokines used in clinical treatment and as cytokine adjuvants for immunization, since the proteins expressed by this system are expected to have more natural sugar chains than those produced by the Baculovirus expression system. We first cloned and analyzed cDNAs of canine GM-CSF, IL-6, IFNγ and IL-8 from canine PBMC or spleen cells. Using a new reverse genetics method for SeV which was recently been established, we succeeded in rescuing the recombinant SeVs producing these cytokines. The presence of each cytokine in the culture supernatant was confirmed immunologically. Produced cytokines were extracted from the allantoic fluid in embryonated chicken eggs infected with corresponding recombinant SeVs. The recombinant canine IFNγ, GM-CSF and IL-6 were modified by glycosylation. The electromobility of the IFNγ analyzed using SDS-PAGE was similar to that of human natural IFNγ than the canine IFNγ expressed by a Baculovirus system. These cytokines were biologically active. The recombinant GM-CSF bound to lectin sepharose but not to Con A sepharose. The concentration of GM-CSF in the allantoic fluid was 10 μg/ml.

8. Analysis of cytokine expressions at an early stage of MV infection using a monkey model

Hiroki Sato, Yasushi Ami[2], Fumio Kobune[2], Ryuichi Miura, Kazuya Yamanouchi[4] and Chieko Kai: [4]Nippon Institute for Biological Sciences

The mechanisms of virus elimination from patients after natural infection of measles virus have not been elucidated. Using a monkey model of acute MV with characteristic clinical signs of infection which we had successfully established, we have investigated the immunological mechanisms at an early stage of MV infection. IFN and IL-6 activity in plasma increased and reached a peak after 5 days of MV infection, then decreased slowly. However, IL-10 concentration started to increase after 8 days of infection. IL-8 showed three peaks: at days 3, 5 and 11. IFN-γ was expressed in the macrophage-like cells in the early period of infection. Therefore, IL-6, 8 and IFN-γ are considered to have roles in MV elimination.

9. Studies of pathogenesis of HCV infection on a molecular basis

Kyoko Tsukiyama-Kohara, Keigo Machida[5], Chieko Kai, and Michinori Kohara[5]:[5]The Tokyo Metropolitan Institute of Medical Science

Hepatitis C virus (HCV) causes persistent infection in hepatocytes, which has been strongly associated with the development of hepatocellular carcinoma (HCC). To clarify the mechanisms underlying these effects, we have established a Cre/*lox*P conditional expression system to express the HCV genome in human liver cell lines and transgenic mice. Passages of hepatocytes expressing replicable full-length HCV RNA caused an increase in tumorigenicity. An HCV (structural gene) conditional transgenic mouse showed a suppressive effect on Fas-mediated apoptosis after gene expression. It has been revealed that the HCV structural protein has an inhibitory effect on the cytochrome c release from mitochondria, resulting in the inhibition of caspase 9 and 3/7 activities.

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Laboratory of Molecular Genetics

This laboratory has two main activities: developing efficient expression vectors for gene therapy and supporting the researchers by advising on recombinant DNA technology under the safety guideline.

The purposes of our laboratory are concerned about not only research but also support for all researchers in this institute. Our supporting activity is involved in advising service on gene-manipulation experiments under the safety guideline. For the research part, we intend to develop novel methods or new experimental systems leading in the field of gene expression and its regulation. We are concentrating mainly on developing efficient adenovirus expression vectors aiming gene therapy. We are maintaining more than 50 collaborations within and outside of this institute. In these collaborations, we offer and supply our efficient method (COS-TPC method: Miyake *et al*., Proc. Natl. Acad. Sci. USA, 93:1320-1324, 1996) to construct recombinant adenoviruses expressing various genes efficiently. Eight years ago, we constructed 44 recombinant adenovirus for 14 months using this method; this number was more than double constructed in the world per year at that time. More recently we have developed a method for ON/OFF switching of gene expression in mammalian cells using a combination of Cre/*loxP* system and adenovirus vector (Kanegae *et al*. Nucleic Acids Res. 23:3816-3821, 1995; Kanegae *et al*. Gene 181:207-212, 1996). The method will promote many fields of molecular biology and medicine and may open a new field of "intracellular gene manipulation". The research activities in 2000 were shown below.

1. Efficient gene activation in cultured mammalian cells mediated by FLP recombinase-expressing recombinant adenovirus

Masakazu Nakano[1], Kazuhiko Odaka[1], Masakazu Ishimura[1], Saki Kondo, Naoto Tachikawa[1], Yumi Kanegae and Izumu Saito:[1]Faculty of Industrial Science and Technology, Science University of Tokyo

A recombinant adenovirus (rAd) expressing Cre recombinase derived from bacteriophage P1 has already been extensively used for the conditional gene activation and inactivation strategies in mammalian systems. In this study, we generated AxCAFLP, an rAd expressing FLP recombinase derived from *Saccharomyces cerevisiae* and carried out quantitative comparisons with Cre-expressing rAd in both i*n vitro* and in cultured cells to provide another efficient gene regulation system in mammalian cells. In the *in vitro* experiments, the relative recombination efficiency of FLP expressed in 293 cells infected with FLP-expressing rAd was approximately 1/30 of that of Cre even at 30℃, the optimum temperature for FLP activity, and was approximately 1/90 at 37℃. Co-infection experiments in HeLa cells using a target rAd conditionally expressing *LacZ* under the control of FLP showed that an FLP-expressing rAd infected at a multiplicity of infection (MOI) of 5 was able to activate the transgene in almost 100% of HeLa cells whereas the Cre-expressing rAd was sufficient at an MOI of 0.2. Since an MOI of 5 is ordinarily used in rAd experiments, these results showed that the FLP-expressing rAd is useful for gene activation strategies and is probably applicable to a sequential gene regulation system in combination with Cre-expressing rAd in mammalian cells.

2. DNA substrates influence the efficiency of recombination mediated by FLP recombinase

Masakazu Nakano[1], Masakazu Ishimura[1], Yumi Kanegae and Izumu Saito

The FLP recombinase derived from *Saccharomyces cerevisiae* mediates precise site-specific recombination between a pair of FLP recognition targets (FRTs). Like Cre/*loxP* system derived from bacteriophage P1, FLP/FRT system has been widely applied to the gene regulation system both *in vitro* and *in vivo*. However, it still remains to be improved for various applications in mammalian systems, since the recombination efficiency of FLP at 37℃ is much lower than that of Cre. Trying to improve the recombination efficiency of FLP/FRT system by altering DNA substrates, we compared the recombination efficiency among different substrates using a quantitative *in vitro* assay. Unexpectedly, we found that one linearized DNA substrate showed 2- to >10-fold lower recombination efficiency than other substrates, the phenomenon of which have not observed in the Cre/*loxP* system. The quantitative *in vitro* assay using truncated DNA substrates suggested that the recombination efficiency seemed to be influenced by not only the linearized position of the substrate, but also the length between a pair of FRTs. Such substrate preference of FLP should be probably noted when designing versatile applications of FLP/FRT system as a gene regulation system. Fortunately, however, we demonstrated that such substrate preference of FLP was not observed when using a substrate possessing a certain pair of mutant FRTs, which will also be a promising tool for the simultaneous gene regulation in combination with wild-type FRT.

3. Development of a new method for constructing helper-dependent adenovirus vectors using double-reciprocal recombination mediated by Cre recombinase

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Although the first-generation adenovirus vector (E1-substitution type) has been extensively used in various fields of basic research and for gene therapy, the next-generation adenovirus vector has also been reported. The new adenovirus vector, called "gutted vector", is devoid of all the viral genes but retains only about 0.5-kb of the left terminal and 0.2-kb of the right terminal of the viral genome. These terminal regions of the virus genome contain viral replication origins and the signal for packaging into the viral capsid. The gutted vector does not express any viral gene products and is consequently expected to cause only minimum immune reaction against its host and to achieve prolonged gene expression. However, because the gutted vector require helper virus, which supply all the viral gene products *in trans*, the method for construction and production of gutted vector is still complex and can be managed by only a limited number of groups in the world.

We have developed a new method for constructing first-generation adenovirus vector using double-reciprocal recombination mediated by Cre/*loxP* system. We have reported a mutant *loxP* V, which contains two certain transversion mutations in its 34-nucleotide sequences. The mutant *loxP* recombines efficiently with the identical mutant *loxP* but not with wild-type *loxP* (Lee and Saito, Gene, 216:55-65, 1998). A pair of DNA sequences flanked with wild-type *loxP* and mutant *loxP* V can efficiently be exchanged through double-reciprocal recombination mediated by Cre recombinase. We constructed a parent virus, called a recipient virus, which contains an inserted gene at the E1 region flanked with wild-type *loxP* and mutant *loxP* V. In addition, the packaging signal of the virus is flanked with a pair of wild-type *loxP*. We also constructed a donor plasmid containing viral packaging signal and a gene to be transferred onto the adenovirus genome, both of which were flanked as a unit with wild-type *loxP* and mutant *loxP* V. A 293 cell line constitutively expressing Cre recombinase was transfected with a donor plasmid containing a marker gene and then infected with the recipient virus. Some of the recipient viruses lacking their packaging signal by Cre-mediated excision restored the signal together with the marker gene from the donor plasmid through the double-reciprocal recombination. The resulting marker gene-containing recombinant adenovirus became the major population of the virus stock after five cycles of serial passages through a Cre-expressing 293 cell line. Therefore, this method works for construction of the first-generation adenovirus vector. Using the same strategy, we are currently trying to construct gutted vector. We constructed a recipient virus containing mutant *loxP* V at 0.2 kb downstream from the right end of the genome. We also constructed a donor cosmid where 28 kb DNA containing expression units is flanked with wild-type *loxP* and mutant *loxP* V. Transfection of the new recipient virus and the donor cosmid into Cre-expressing 293 cells and subsequent serial passages generated virus containing a marker gene derived from donor cosmid. Further investigation is under way.

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Amami Laboratory of Injurious Animals

The Amami Laboratory of Injurious Animals was established in 1965 at Setouchi-cho in Amami-oshima Island in order to study on endemic diseases involving parasite, arthropods, and venomous snakes in the tropics or subtropics.

The Amami-oshima Island belongs to the Nansei (Southwest) Islands and the fauna is quite different from that in other islands of Japan. Since establishment of the laboratory, trials have been carried out to utilize small mammals found unique in the Amami islands as experimental animals in addition to studies on prevention of Habu bites. As well known, successful eradication of filariasis from this island is one of the monumental works of the laboratory. Our present works are as follows:

1. Research on Habu control

Shosaku Hattori, Yoshihisa Noboru, Hiroshi KIhara[1], Motonori Ohno, Daisuke Tsuru[2], Shigenari Terada[3], Shin Kaneda[4], Yoshihiro Hayashi[5], Michihisa Toriba[6], and Hideki Endo[7] and Tomohisa Ogawa[8]:[1]Bioscience research Institute, Takara Shuzo Co., Ltd., [2]Department of Applied Microbiology, Kumamoto Institute of Technology, [3]Department of Biochemistry, Faculty of Science, Fukuoka University, [4]Department of biochemistry, Faculty of Science, Kagoshima University, [5]Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, [6]The Japan Snake Institute, [7]Department of Zoology, National Science Museum and [8]Faculty of Agriculture, Tohoku university

Snake bites by the venomous snake Habu, *Trimeresurus flavoviridis*, have been reported annually about 100 cases in the population of 100,000 in the Amami Islands. Moreover, there is no indication that the population of Habu itself has decreased, despite a campaign for capture of snakes by the Kagoshima Prefectural Government. Rat-baited box traps have been introduced to catch the snakes and found to be quite effective. However, maintenance of live rats requires man power and its cost is expensive. Therefore, our effort has been focused on the development of attractant for Habu. The attractant extracted from rats seems ineffective if compared with use of live rats.

It was known that Habu survived the injection of Habu venom since early times. Because, some proteins in the serum of Habu blood combine to the elements of Habu venom. The research of these binding proteins has been initiated with an objective of clinical trials. Phospholipase A2 and its isozymes isolated from Habu venom have myonecrotic activity and hemorrhagic activity, and T2 protease has hemorahagic activity. The binding proteins isolated from serum of Habu inhibit myonecrotic activity of phospholipase A2 and its isozymes. We found that protein-HSF and peptide-AHP isolated from the Habu serum effectively control the hemorrhage caused by Habu, Himehabu (*Ovophis okinavensis*), *Calloselasma rhodostoma*, and *Bitis arietans* venoms.

Further, a statistics analysis and the simulation were done with the snakes captured by the Government, and the analysis of population dynamics of Habu was attempted. By the analysis of the measured data of last six years, the snake sizes were miniaturized, and the population of young snakes decreased. According to these investigations, the population of Habu is expected to decrease in the near future.

These studies are supported by grants from the National Bureau of Land Development and the Kagoshima Prefectural Government.

2. Reproduction of squirrel monkeys=

Shosaku Hattori, Yoshihisa Noboru, Yoshitugu Matsumoto[9], Mamoru Ito[10], and Takahisa, Furuta[11]: [9]Department of Molecular Immunology, Faculty of Agriculture, University of Tokyo, [10]Laboratory of Immunology, Central Institute of Experimental Animals and [11]Department of Parasitology

The squirrel monkey, *Saimiri sciurea*, is widely distributed in the tropical rainforest in Central and South America between 10 degrees N and 17 degrees S of latitudes. The advantage of using this species for medical researches resides in its small size and gentle behavior. In this laboratory, about 5 newborns are given annually by 30 adult females.

Recently, primates came to be often used to experiments of parasites. Especially the primates in the South America are the important infectious models. We use the squirrel monkeys to basic experiments on the infection and vaccination models for malaria, toxoplasma, and schistosomiasis.

3. Research of wild mammals

Shosaku Hattori, Yoshihisa Noboru, Hideki Endo[12], Kimiyuki Tsuchiya[13], Nobuo Ishii[14] and Fumio Yamada[15]:[12]Department of Zoology, National Science Museum, [13]Experimental Animal Center, Miyazaki Medical College, [14]Japan Wildlife Research Center and [15]Wildlife Ecology Laboratory, Forestry & Forest Products Research Institute

Amami-oshima Island is a habitat of animals and plants indigenous to the Nansei Islands. These animals occur originally in the Oriental region of Asia and include the Amami rabbit, *Pentalagus furnessi*, the Ryukyu spiny rat, *Tokudaia osimensis*, the Okinawa long-haired rat, *Diplothrix legata*, the Watase's shrew, *Crocidura watasei*, and the Musk shrew, S*uncus murinus*. These mammals are used for researches on comparative anatomy, taxonomy, and development of experimental animals. Besides, these mammals are valuable species biologically as survivors from the Miocene about 10,000,000 years ago. We have initiated the investigation for these species to protect from extinction. We have documented the feasibility of recovering large numbers of oocytes from the Watase's shrew, and some of oocytes can be induced to mature *in vitro*.

Recently, the Java mongoose, *Herpetologica javanicus* grew in the wild as invasive carnivore in the Amami-oshima Island. The population of the mongoose increases every year and the habitat range is extending to south area in the Island. It is necessary to remove the invader to defend nature. Then we are investigating the influence which the mongoose gives to wildlife in the Island.

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