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INFORMATION

Hokkaido University confered the degree of Bachelor of Veterinary Medicine to the following 38 graduates of the School of Veterinary Medicine on March 24, 2006.

The authors summaries of their theses are as follows :

Characteristics of brown-like adipose tissue in mice with chronically elevated activity of the sympathetic nervous system.

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Mitochondrial uncoupling protein-1 (UCP1), a key molecule for metabolic thermogenesis, is usually expressed only in brown adipose tissue (BAT). White adipose tissue (WAT) converts into brown-like adipose tissue expressing UCP1 when animals are subjected to chronic sympathetic nerve stimulation, such as after cold acclimation. To clarify the thermogenic activity of brown-like adipose tissue, in this study, C57BL6 mice were injected with a highly selective β3-adrenergic agonist, CL316, 243 (CL) or exposed to cold environment at 4 ℃. After 2 weeks under these conditions, inguinal-WAT (I-WAT) turned into brown-like adipose tissue expressing UCP1, but epididymal-WAT (E-WAT) did not. Adipocytes were isolated from I-WAT, E-WAT and BAT, and their oxygen consumption was measured in vitro, as an index of thermogenetic activity. In adipocytes from brown-like I-WAT and BAT, but not those from E-WAT, oxygen consumption was increased in response to norepinephrine stimulation. Oxygen consumption per UCP1 protein showed that UCP1 in brown-like I-WAT had much higher activity than that in BAT. Gene expression of cell death-inducing DFF45-like effector-A (CIDEA), proposed as a UCP1 inhibitor, was also increased in brown-like I-WAT. The amount of UCP1 protein relative to CIDEA protein (UCP1/CIDEA) was higher in BAT than in brown-like I-WAT, suggesting a minor role of CIDEA in the control of UCP1 activity in I-WAT.

Thus, brown-like I-WAT expressing UCP1, in addition to BAT, probably contributes to whole energy expenditure, because of its highly thermogenic activity.

Proinsulin C-peptide modulates nitric oxide production and endothelial nitric oxide synthase expression in the streptozotocin-induced diabetic rats.

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C-peptide is cleaved off from proinsulin molecule in the process of insulin biosynthesis, but stored in the secretory granule and released into the blood circulation, along with insulin. Recent studies have revealed that C-peptide is the biologically active peptide showing ameliorative effects on diabetic complications such as neuropathy and nephropathy, possibly through nitric oxide (NO) production. Indeed, C-peptide stimulates NO production by activating enzyme activity and/or increasing enzyme amount of endothelial nitric oxide synthase (eNOS) *in vitro*. However, in diabetic patients and animal models in which functions of endothelial cells are known to be impaired by hyperglycemia, it is obscure whether C-peptide induces NO production and modifies eNOS. expression *in vivo*. To clarify these issues, in the present study, I examined plasma concentration of nitrogen oxides (NOx), metabolites of NO, and eNOS expression in the kidney and lung in the streptozotocin-induced type 1 diabetic model rats, with or without replacement of either C-peptide or insulin by an osmotic pump. Rats treated with streptozotocin showed hyperglycemia with about 90% reduction of endogenous insulin and C-peptide levels, but no effect on plasma NOx concentrations, compared with normal rats. Diabetic rats treated with insulin showed lower plasma glucose levels than that of diabetic rats treated with saline, but without affecting plasma NOx levels. In contrast, diabetic rats treated with C-peptide increased plasma NOx levels by 30% without influencing glucose levels. Diabetic rats treated with saline dramatically increased eNOS expression in the kidney, but not in the lung, compared with normal rats. Diabetic rats treated with either insulin or C-peptide abrogated the increase of eNOS expression in the kidney and did not modify eNOS expression in the lung. However, diabetic rats treated with C-peptide by single bolus injection failed to decrease the increase of eNOS expression in the kidney. Therefore, the results suggest that C-peptide is capable of inducing NO production in diabetic animals, which may be attributed to amend abnormal eNOS expression in the kidney by improving systemic hemodynamics and reducing stress to kidney, while insulin suppresses the abnormal eNOS expression, possibly by reducing hyperglycemic stress to kidney.

The relationship between UCP1 (Uncoupling Protein1) and glucose uptake.

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Brown adipose tissue (BAT) is the major site of sympathetically mediated metabolic thermogenesis, at least in small rodents, during cold acclimation and spontaneous hyperphagia. The principal substrate of BAT thermogenesis is fatty acids, of which energy is dissipated as heat via uncoupling protein 1 (UCP1), a unique mitochondrial protein in BAT. It is known that glucose utilization in BAT is also increased in parallel with thermogenesis. In the present study, to clarify the possible relationship between UCP1 and tissue glucose utilization, I measured 2-deoxyglucose (2DG) uptake into various tissues in mice acclimated to a cold environment (CA) or given a beta-3 adrenergic agonist (CL 316,243) daily (CL) for 2 weeks. Control mice expressed UCP1 only in BAT, whereas CA and CL mice expressed more UCP1 in BAT and ectopically in inguinal white adipose tissue (WAT), but little in epididymal WAT. In control mice, 2DG uptake in BAT was comparable with those in heart under a basal condition and increased after norepinephrine (NE) or insulin stimulation. In contrast, 2DG uptake in both types of WAT was undetectably low and increased only by insulin stimulation. In CA and CL mice, 2DG uptake under the basal and NE stimulation conditions increased in BAT, and remarkably in inguinal WAT, but little in epididymal WAT. Although there were some differences between CA and CL mice, 2DG uptake in adipose tissues was roughly parallel with the expression level of UCP1. Moreover, the stimulatory effect of insulin on 2DG uptake was dramatically enhanced in WAT of CA and CL mice. All these results suggest that UCP1 activation may be beneficial for the improvement of glucose metabolism, particularly under the condition of insulin resistance.

Changes in transient receptor potential V1 function of dorsal root ganglion neurons in mouse model of neuropathic pain

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I made the neuropathic pain model (Chronic Constriction Injury model : CCI model) by sciatic nerve ligation (CCI-operation) using wild and transient receptor potential V1 knockout (TRPV1-KO) mice, and compared the paw withdrawal threshold to mechanical stimuli and paw withdrawal latency to thermal stimuli. To examine the mechanism of hyperalgesia in vitro, I investigated changes in cytosolic Ca^{2+} concentration

($[Ca^{2+}]_i$) in response to various stimuli using fura-2 imaging system in dorsal root ganglion (DRG) neurons isolated from the CCI model mice.

The paw withdrawal threshold to mechanical stimuli was not different between wild and TRPV1-KO mice before CCI-operation. CCI-operation decreased the paw withdrawal threshold at the nerve-ligated side (CCI-side), but not with the sham operation side (sham-side) in both mice. At 7-day after CCI-operation, the decline of the paw withdrawal threshold in TRPV1-KO mice was significantly smaller than that in wild mice. The paw withdrawal latency to thermal stimuli was significantly longer in TRPV1-KO mice than wild mice before CCI-operation, suggesting that thermal sensitivity is low in TRPV1-KO mice. In both mice, CCI-operation shortened paw withdrawal latency, the extent of which was smaller in TRPV1-KO mice than wild mice. In DRG neurons isolated from TRPV1-KO mice, neither $[Ca^{2+}]_i$ response to capsaicin nor TRPV1-immunoreactive positive cells were observed. RT-PCR revealed the lack of TRPV1 mRNA in DRG of TRPV1-KO mice. Capsaicin elicited significantly large $[Ca^{2+}]_i$ increase in DRG neurons at CCI-operated side (CCI-DRG) than those at sham-operated side (sham-DRG). The percentage of cells with a large increment of $[Ca^{2+}]_i$ in response to capsaicin was increased, but the total number of cells responding to capsaicin was unchanged by CCI-operation. In CCI-DRG, the increases of $[Ca^{2+}]_i$ in response to thermal stimuli and anandamide were significantly larger than in sham-DRG. On the other hand, there was no difference in low pH-induced $[Ca^{2+}]_i$ increase between CCI-and sham-DRG. High K^+ or adenosine 5'-triphosphate (ATP) induced $[Ca^{2+}]_i$ increase in CCI-and sham-DRG to the same extent. The increase of $[Ca^{2+}]_i$ induced by bradykinin (BK) was significantly larger in CCI-DRG than sham-DRG, and the percentage of cells with a large increment of $[Ca^{2+}]_i$ in response to BK was increased by CCI-operation. $[Ca^{2+}]_i$ responses to BK were suppressed by a B2 antagonist but not a B1 antagonist. A B1 agonist failed to evoke any $[Ca^{2+}]_i$ response in both CCI-and sham-DRG. The potentiation of $[Ca^{2+}]_i$ response to capsaicin induced by BK was significantly larger in CCI-DRG than sham-DRG. The number of IB4-negative cells was increased by CCI-operation. In both CCI-and sham-DRG, there was no correlation between IB4-staining and responsibility to capsaicin or BK. These results suggest that the potentiation of TRPV1 function and changes in BK-induced regulation of TRPV1 through B2 receptor partly involve in neuropathic pain phenotype in vivo.

Characterization of voltage-dependent channels and inositol 1, 4, 5-trisphosphate-induced current in porcine vomeronasal neurons

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Using a whole-cell patch-clamp technique at a holding potential of -90 mV, I examined the property of voltage-dependent channels and the effect of putative second messengers of the pheromone in the vomeronasal neurons of porcine vomeronasal slice preparations.

Voltage-dependent Na^+ channel currents were transient inward currents which began to be activated by depolarizing pulses to -50 mV and reached a peak amplitude at -30 mV. Tetrodotoxin (TTX) inhibited the currents in a dose-dependent manner. Voltage-dependent K^+ channel currents were outward and began to be activated at depolarizing pulses to -40 mV and were increased linearly with increasing depolarization. The currents were inhibited by tetraethylammonium (TEA). Voltage-dependent Ca^{2+} channel currents, mainly transient inward currents, began to be activated by depolarizing pulse to -60 mV and reached a peak at -40 mV. The currents were inhibited by T-type Ca^{2+} channel blockers, Ni^+ and mibefradil, but not inhibitited by an L-type Ca^{2+} channel blocker, nifedipine. Sustained inward currents were observed only 1 out of 8 cells in which transient Ca^{2+} currents occured.

Extracellular application of arachidonic acid or a diacylglycerol analog, and intracellular application of Ca^{2+} or cAMP using caged compounds did not induce any currents. In 8 out of 13 cells dialyzed with caged inositol 1, 4, 5-trisphosphate (IP_3), IP_3 released by photolysis evoked inward currents. The latency of the current responses was 2.0 ± 0.7 sec, and the average amplitude of a maximal current was -72.8 ± 22.4 pA. A reversal potential of IP_3-induced currents was -29.2 ± 3.5 mV. The IP_3-induced current was not observed in cells dialyzed with Cs^+.

These results indicate that porcine vomeronasal neurons have several voltage-dependent channels such as TTX-sensitive Na^+ channels, TEA-sensitive K^+ channels and T-type Ca^{2+} channels. Furthermore, it is suggested that intracellular IP_3 activates channels in porcine vomeronasal neurons.

Hypercapnic acidosis-induced adenosine release from spinal cord of neonatal rat

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In order to reveal the mechanisms of adenosine release induced by hypercapnic acidosis, we examined the effect of artificial cerebral spinal fluid gassed with 20% CO_2 (hypercapnic acidosis ACSF) on adenosine release from the isolated spinal cord of neonatal rats. We also investigated the changes of adenosine kinase activity during hypercapnic acidosis. The pH of the ACSF gassed with 5% CO_2+95% O_2 (normal ACSF) and hypercapnic acidosis ACSF was 7.3 and 6.7, respectively. The hypercapnic acidosis increased adenosine release from the spinal cord but not affected on adenine nucleotide release. Although hypoxia (5% CO_2+95% N_2) increased adenosine release, the ACSF gassed with 5% CO_2+15% N_2+80% O_2 had no effect on adenosine release.

An ecto-5'-nucleototidase inhibitor, ARL 67156, had no effect on hypercapnic acidosis-induced increase in adenosine release. On the other hand, homocysteine, which trapped intracellular adenosine, depressed the increase in adenosine release caused by hypercapnic acidosis. Tetrodotoxin and removal of extracellular Ca^{2+} had no effect on the increase of adenosine release by hypercapnic acidosis. An equilibrative nucleoside transporter (ENT) inhibitor, NBTI, had no effect on basal adenosine release, and inhibited the increase of adenosine release by hypercapnic acidosis. Another ENT inhibitor, dipyridamole, increased basal adenosine release and depressed the increase in adenosine release by hypercapnic acidosis.

Hyperphoshate acidosis ACSF (5% CO_2, pH 6.7) and isohydric hypercapnia ACSF (20% CO_2, pH 7.3) increased adenosine release from the spinal cord. However hypocapnic acidosis ACSF (5% CO_2, pH6.7) and acidified HEPES ACSF (pH 6.7) had no effect on it. An adenosine kinase inhibitor, ABT702, increased adenosine release from the spinal cord. Hypercapnic acidosis or hyperphosphate acidosis on failed to cause further increases in adenosine release in the presence of ABT702. Hypercapnic acidosis (pH 6.7), hyperphosphate acidosis (pH 6.7) and isohydric hypercapnia (pH 7.3) depressed adenosine kinase activity in rat spinal cords.

These results indicate that hypercapnic acidosis causes the accumulation of intracellular adenosine and then adenosine release via NBTI-sensitive ENT, resulting from the inhibits adenosine kinase activity in neonatal rat spinal cords. The inhibition of adenosine kinase activity may be induced by a fall of intracellular pH.

Vaccine efficacy of the common antigens of *Leptospira interrogans*

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Leptospirosis is a zoonotic infection caused by pathogenic *Leptospira interrogans* and occurs worldwide. Vaccines with inactivated whole cells are used in the prevention of this disease, but these vaccines induce a serovar specific immunity and are not effective for infections with different serovars. Thus, outer membrane proteins LipL41, LipL45 and LipL32 that are the common antigens of *L. interrogans* were expressed and purified as recombinant proteins to examine the effects of serovar-independent vaccines.

Genes of LipL41, LipL45 and LipL32 were cloned and inserted into expression plasmids. Recombinant proteins were expressed in a fusion form with a Histidine tag in *Escherichia coli*. The recombinant proteins were purified with histidine-affinity columns. Four-week-old C3H/HeJ mice were subcutaneously immunized, two times, at two-week intervals and were subsequently challenged intraperitoneally with *L. interrogans* serovar Manilae strain UP-MMC for examination of vaccine efficacy.

Expression and purity of the recombinant LipL41, LipL45 and LipL32 proteins were analyzed as anticipated molecular mass. Serum antibody titers of almost all the mice were elevated by immunization with the recombinant protein. In the groups immunized with single recombinant proteins, some mice lived 1 or 2 days longer than the negative controls after challenge. Furthermore, three mice that were immunized with a mixture of three recombinant proteins lived 1 day longer or survived for 14 days. Moreover, the fact that no leptospira was isolated from the kidneys of mice was an indication of the efficacy of the vaccines.

These results suggest that recombinant proteins when used in combination resulted in enhanced vaccine efficacy. Further research is required to determine a new antigen that is more effective and for the development of a method of immunization for the improvement of vaccine efficacy.

Antigenic and genetic analyses of H5 influenza virus isolates from aquatic birds for vaccine and diagnostic use against highly pathogenic avian influenza

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Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 viruses have occurred in fourteen Asian countries including Japan since 2003 and caused serious economic loss. The aim of this study was to produce an effective vaccine prepared with a vi-

rus strain antigenically and genetically similar to the currently circulating HPAI viruses in Asia for the occurrence of outbreaks that would not be able to control by test and slaughter in future in Japan. In order to establish a vaccine strain, genes and antigenicity of H5 influenza viruses isolated from feral ducks and swans, the natural reservoir of influenza A virus, were analyzed. Effects of the test vaccines were evaluated by the animal experiment with chickens.

Twelve H5 influenza viruses were isolated from the fecal samples of aquatic birds in Hokkaido and Mongolia during1996-2004, and their hemagglutinins (HAs) were analyzed antigenically and genetically determined. Phylogenetic analysis of H5HA genes revealed that eleven isolates were classified into Eurasian type and the other was classified into North American type and that cleavage site of all isolates were LP profile. Homology of amino acid sequences of the HAs between isolates and HPAI viruses was over 90%. A panel of monoclonal antibodies against H5HA was used for the antigenic analysis of isolates and HPAI viruses. It was indicated that a close similarity in the antigenicity between HPAI viruses and isolates of aquatic birds-origin. Inactivated test vaccines with oil adjuvant were prepared from A/R/Hokkaido/1/04 (H5N1), whose HA gene was derived from A/duck/Mongolia/54/01 (H5N2), one of the parent viruses used for the production of the reassortant. The other parent viruses were A/duck/Mongolia/47/01 (H7N1) and A/duck/Hokkaido/49/98 (H9N2). Effects of the test vaccines were investigated by challenge experiment against chickens. Antibody titers of immunized chickens were dependent on the concentration of antigen in the test vaccines. The chickens which had over 4HI antibody titers by vaccination survived challenge with HPAI virus, A/chicken/Yamaguchi/7/04 (H5N1).

From these results, low pathogenic H5 influenza viruses isolated from aquatic birds were useful as development of vaccines.

Characterization of male factor derived from *Rhipicephalus appendiculatus*

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Tick feeding activities and transmission of pathogens cause great losses in the livestock industry. Since ticks play important roles as vectors of various pathogens, suppression of their population is the most effective way to control the disease which they transmit, particularly protozoan diseases. At present, ticks can be effectively controlled by the use of acaricides, which have many disadvantages. Hence, it is necessary to develop alternative tick-control methods such as immunological way, which is currently considered as a major sustainable and practical method.

Previously, it was reported that Voraxin derived from *Amblyomma hebraeum* was transferred from male to female ticks during copulation as a male factor (MF), and stimulated the females to engorge. In addition, rabbits immunized with the recombinant Voraxin showed anti-tick immunity. Thus, the objec-

tive of this study was to obtain Voraxin-like MFs from *Rhipicephalus appendiculatus*, which is a vector for East coast fever.

Total of 287 cDNAs were cloned from a cDNA library constructed from fed male ticks. One cDNA, named as RAMF1, has a sequence similarity to the *Is5* gene expressed in male *Ixodes scapularis,* but not in female. RT-PCR analysis showed that RAMF1 was expressed specifically in the gonads of fed males. Microinjection of the recombinant RAMF1 (rRAMF1) into the hemocoel of virgin female ticks resulted in prolonged feeding time and slight increase in body engorgement of these ticks compared to those of control virgin females. Vaccination of guinea pigs with rRAMF1 did not show any effects on ticks except that the average of the egg weight per engorged body weight was smaller than that of the control ticks. These results suggested that RAMF1, which is not a Voraxin-like molecule, might be one of the factors that support females to engorge.

Epidemiological investigation of Newcastle disease virus and avian influenza virus isolated from fecal samples of wild ducks and geese

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Newcastle disease virus (NDV) and avian influenza virus (AIV) have been responsible for serious economic losses in the poultry industry. These viruses were isolated from various free-living-birds (ducks, pigeons, parrots *etc*). Therefore, wild birds are considered as reservoirs and vectors of NDV and AIV. This work aims to investigate the distribution of these viruses in wild ducks at Hokkaido.

In this study, from total 2,927 fecal and cloacal swab samples of wild ducks and geese collected in Hokkaido in 2003 and 2004, 6NDV strains, 8AIV strains (1 of H3N2, 6 of H3N8, 1 of H4N6 serotypes), 5 avian paramyxovirus 4 (APMV4) strains and 2 APMV 6 strains were isolated. These AIVs and APMVs, except for NDV, are usually isolated from wild ducks, and are non-pathogenic to chickens. Although all 6 isolated NDV strains showed low pathogenicity to chicken embryos, 2 out of 6 isolated NDV strains contained a pair of dibasic amino acids at the cleavage site of the fusion protein, which is a characteristics of a virulent type of NDV, suggesting that they are potentially pathogenic. The result of phylogenetic analysis indicated that these NDV isolates belong to Genotypes Ⅰ and Ⅱ, suggesting lentogenic and old types of NDVs are maintained in wild ducks. Further investigation is expected for detailed understanding of these viruses in wild birds.

Application of simple genotyping method for bovine viral diarrhea virus, analysis of tissue distribution of the virus in the in the infected cattle and seroepidemiology of free-living deer in Japan

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Bovine viral diarrhea virus (BVDV) infection induces various clinical symptoms in the infected cattle including diarrhea, abortion, and respiratory and neurological symptoms. BVDV infection during the first semester of gestation period may result in the birth of persistently infected (PI) cattle. Four genotypes of BVDV, 1a, 1b, 1c and 2, are popular in Japan, and BVDV lc can be isolated from cattle showing neurological deterioration. In the present study, application of a simple genotyping for BVDV was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) instead of a general nucleotide sequence analysis.

Using the combination of restriction enzymes, *Eco*O109I and *Nsi*I, BVDV lc was distinguished from other genotypes. However, a few BVDV la viruses, which were mutated at the restriction enzyme sites, were classified into BVDV lc by PCR-RFLP. This simple genotyping method could be useful for easy and rapid screening of BVDV lc although there is disadvantage of misclassification of some BVDV la strains.

Tissue distribution of BVDV lc in PI cattle was also analyzed to know the relation of BVDV lc with the occurrence of neurological disorder. Results of virus quantification of revealed that there was no difference between BVDV lc and other genotypes, and that all BVDV genotypes were present in all the tested organs. Interestingly, immunohistochemical staining showed the presence of the viral antigen in neurocytes of cerebrum of two PI cattle which were infected with BVDV lc.

Since BVDV lc was isolated from free-living deer, serological survey of BVDV in Japan was conducted by using 147 Sika deer (*Cervus nippon*) samples. BVDV was not isolated from those samples, but 2 of those samples had low titers of neutralizing antibody against BVDV la.

Diagnosis of *Echinococcus multilocularis* infection in definitive hosts by copro-DNA detection

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Echinococcus multilocularis is an important zoonotic parasite which causes alveolar echinococcosis in humans. The increasing risk of the infection to companion animals as well

as humans is anticipated in Hokkaido, Japan, because the prevalence of the parasite in wild foxes has increased dramatically in the last two decades. To evaluate the risk of *E. multilocularis* infection and to take effective preventive measures, it is necessary to accurately diagnose the infection in the definitive hosts, especially in companion animals which are in closer contact with humans. At present, standard diagnostic procedure for the definitive hosts is detection of antigen and eggs in their feces, followed by detection of the parasite DNA from the eggs. However, no eggs can be detected while the coproantigen is positive, in case of prepatent or light infection. Positive results of the coproantigen test alone cannot be conclusive evidence because of its cross reactivity to other taeniid parasites and occasional false positive result. Hence, to confirm the diagnosis, it is essential to detect the parasite DNA from the feces even when the eggs cannot be detected.

In Chapter Ⅰ, a method to detect the parasite DNA from eggs was evaluated using three canine feces, which were positive for coproantigen and taeniid eggs in the screening test for *E. multilocularis* infection. Their EPG (eggs per gram) were 1, 4 and 80, respectively. The eggs were separated from one gram of the each feces by floating and sieving technique. DNA was extracted from the eggs using QIAamp DNA Mini Kit and amplified by a species-specific single tube nested PCR for *E. multilocularis*. The target sequence for amplification was a part of the *E. multilocularis* mitochondrial 12S ribosomal RNA gene. All three samples were positive for the PCR and their infections were confirmed. This method was sensitive enough to detect the parasite DNA from the sample which contained only one egg per gram.

In Chapter Ⅱ, detection of DNA from feces of infected animals which contained no eggs, especially feces during the prepatent period of the infection were evaluated. Nine dogs and two cats were orally given 1,000 to 1,000,000 protoscoleces and the feces of these animals were collected daily. DNA was extracted from the feces using QIAamp DNA Stool Mini Kit and amplified by the nested PCR. At 0-2 days after infection, feces of five dogs and two cats were positive for the PCR. The detected DNA was probably derived from the unestablished protoscoleces or debris of the cysts in the inoculated materials. However, DNA was detected only sporadically after the early phase of the infection, indicating that DNA examination with this technique is not reliable enough to detect the infection during the prepatent period.

Finally, in Chapter Ⅲ, detection of DNA from feces during the prepatent period in combination with praziquantel treatment was examined. It was expected that feces after the treatment would contain more tissues of the parasite than that of non-treated dogs. Three dogs were orally given 10,000 or 100,000 protoscoleces and were treated with praziquantel at 12 or 14 days after infection. Soon after the treatment, the DNA was detected from the feces of all three dogs.

These results suggested that the DNA examination from feces in combination with anthelminthic treatment could become a new option for the diagnosis of *E. multilocularis* infection in definitive hosts.

Genetic analysis of heat shock resistance in AKR/N mouse spermatogenesis

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Generally in mammalian testis, a temperature lower than the abdominal temperature by 2 - 8℃ is required for the normal spermatogenesis. It is known that in cryptorchid testis, germinal cell loss caused by the heat-stress results in male sterility. However, the mechanism of germinal cell loss in cryptorchidism is not well understood. Recently, it has been found that strain difference is present in the response to heat stress in mouse testis (Kazusa et al., 2004). The MRL/MpJ mouse has an abnormal *exonuclease 1* (*Exo1*) gene, which was suggested to be the cause of heat-stress-resistant phenotype of the testis (Namiki et al., 2005). The AKR mouse, which is one of the originated strains of the MRL/Mp mouse, also shows heat-stress-resistant phenotype. Thus, we attempted to identify genetic loci responsible for the heat-stress-resistant phenotype in the AKR/N mouse by quantitative trait loci (QTL) analysis.

Testis weight ratio at 3 weeks after experimental cryptorchidism in C57BL/6J mice was significantly smaller than AKR/N mice. We investigated the expression of *Exo1* gene in AKR/N mice to check whether the heat-stress-resistant phenotype in the AKR/N mouse was caused by mutation of the *Exo1* gene. However AKR/N mice showed normal *Exo1* gene expression. Testis weight ratio in (C57BL/6J×AKR/N) F_1 mice was also significantly smaller than that of AKR/N mice. In contrast, there was a remarkable variation in testis weight ratio in F_2 mice, suggesting that heat-stress-resistant phenotype is controlled by multiple genetic loci. A genome-wide analysis of linkage with testis weight ratio revealed two weak QTL on chromosome (Chr) 10 near *D10Mit16* (16 cM) and *D10Mit42* (44 cM) loci. We could not detect the significant QTL on Chr 1 near the *Exo1* gene in consistent with the result that the expression of *Exo 1* gene was normal in the AKR/N mouse testis. There are some genes involved in apoptosis and spermatogenesis, such as *Tbpl1, Apaf1* and *Perp* on Chr10. Some of these genes may have mutations or polymorphisms as causing the heat-stress-resistant phenotype in the AKR/N mouse. We could not detect other QTL except for those in Chr 10. These results indicate that multiple genes, including those on Chr 10 and those on the other chromosomes, cooperatively contribute to the phenotype of the AKR/N mouse.

We expect that further investigation would contribute to the understanding of the mechanism of spermatogenesis and to the development of the therapy for the infertility caused by heat stress in both human and animals.

Epidemiological survey and pedigree analysis of GM1 gangliosidosis in Shiba dogs and neuronal ceroid lipofuscinosis in Border collies using rapid mutation screening.

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In hereditary diseases in animals, if the mutation responsible for a disease is identified and a rapid mutation screening method for the disease is developed, the genotypes of individuals can be determined, and heterozygous carriers can be found. Inherited diseases can be prevented and controlled by eliminating heterozygous carriers used for breeding purposes. In this study, as part of the preventive program for GM1 gangliosidosis in Shiba dogs and neuronal ceroid lipofuscinosis (NCL) in Border collies, we simplified the mutation screening methods for both diseases, and an epidemiological survey and pedigree analysis were carried out using these methods.

In mutation screening (PCR-RFLP : polymerase chain reaction-restriction fragment length polymorphism) for GM1 gangliosidosis in Shiba dogs, the conditions of the method using buccal swab specimens were examined. PCR products from specimens are sufficient for genotyping when using a specialized commercial kit for collecting buccal mucosal cells, even if buccal mucosa are swabbed only once, and if the swabs are stored at room temperature for 5 days after swabbing. We collected buccal swab specimens from 81 Shiba dogs that belong to the Shiba Dog Club in the Czech Republic. As a result of genotyping these dogs, all the genotypes of the dogs were normal. This method was considered suitable for mass analysis of the genotypes.

For mutation screening for NCL in Border collies, we established PCR-RFLP and PCR-PIRA (primer-introduced restriction analysis) methods. The PCR-RFLP method was useful for determining NCL-affected dogs, but not for distinguishing normal dogs from heterozygous carriers, in contrast with the PCR-PIRA method. We therefore concluded that it was necessary to use these methods together for genotype analysis.

Genotyping of 8 of the 11 Border collies diagnosed with NCL in Japan was performed using the 2 methods described above. As a result, all the dogs were shown to have the mutation homozygously, which has been reported in other countries as a causative mutation of NCL. Moreover, as a result of analyzing pedigree information, it was shown that most of these dogs were blood relatives. The results of this study suggested that the NCL mutation has been distributed in Japan due to the inbreeding of imported dogs.

Induction of phase II detoxification enzymes by alk(en)yl thiosulfates derived from Allium plants

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Two alk(en)yl thiosulfates, sodium *n*-propyl thiosulfate (NPTS) and sodium *2*-propenyl thiosulfate (2PTS), are natural constituents of onion and garlic, respectively, and were identified originally as causative agents of onion-and garlic-induced hemolytic anemia in dogs. Recently, it has been demonstrated that onion and garlic have an inhibitory effect on carcinogenesis in experimental animals due to the increased tissue activities of phase II detoxification enzymes. In this study, we measured the activities and mRNA expression of phase II detoxification enzymes in cultured H4IIE rat hepatoma cells which were treated with several concentrations of NPTS and 2PTS. The effects of diallyl trisulfide (DATS) and *tert*-buthlhydroquinone (TBHQ), known as phase II detoxifying inducers, on the enzymes were also examined and compared with those of alk(en)yl thiosulfates. Furthermore, NPTS and 2PTS were examined for their ability to prevent X-ray-induced DNA damage using the comet assay.

As a result, 2PTS was found to significantly increase quinone reductase (QR) activity, and QR and epoxide hydrolase 1 (EH1) mRNA expressions compared with the control. However, glutahione *S*-transferase (GST) activity, and GSTA5 and UDP-glucuronosyl transferase 1A1 (UDPGT1A1) mRNA expressions were not changed by 2PTS. In contrast, NPTS did not induce any phase II enzymes. DATS and TBHQ significantly increased the mRNA of QR, GSTA5 and EH 1.2 PTS has an equal or higher potential for inducing phase II enzymes compared to DATS and TBHQ. In addition, NPTS and 2PTS protected against X-ray-induced DNA damage in H4IIE.

From these results, it was demonstrated that 2PTS could induce phase II enzymes, and that NPTS and 2PTS had a radioprotective effect, suggesting that alk(en)yl thiosulfates have an effect in preventing cancer.

Effects of ionophore compounds, valinomycin and nystatin, on intracellular *Babesia gibsoni* viability *in vitro*.

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The effects of two ionophore compounds, valinomycin and nystatin, on babesia parasite (*Babesia gibsoni*) viability were investigated *in vitro*.

B. gibsoni was cultured with normal dog erythrocytes containing high Na^+ and low K^+

concentrations and lacking Na, K-ATPase (LK dog erythrocytes). When valinomycin and nystatin were added to the culture, respectively, the number of intracellular parasites was decreased, indicating that each ionophore had an anti-babesial effect on *B. gibsoni in vitro*. On the other hand, the concentration of K^+ and Na^+ in LK dog erythrocytes was unchanged by the ionophores. These results suggested that valinomycin and nystatin might directly injure *B. gibsoni* within LK dog erythrocytes.

To clarify the mechanism of ionophores against the parasite, the effects of valinomycin and nystatin on HK dog erythrocytes were examined. HK dog erythrocytes have high K^+ and low Na^+ concentrations through the function of Na, K-ATPase, which presents genetically in their membranes. When HK dog erythrocytes were incubated with the ionophores, the concentration of K^+ was decreased and that of Na^+ was increased with a decrease of intracellular adenosine 5'-triphopahate (ATP) concentration. Furthermore, HK dog erythrocytes were hemolyzed during incubation. When Na, K-ATPase was inhibited with ouabain, however, the concentration of intracellular ATP did not change and no hemolysis of HK dog erythrocytes was observed during the incubation of HK dog erythrocytes with each ionophore. These results suggested that each ionophore might activate an ion pump, such as Na, K-ATPase on the cell membrane, resulting in the depletion of ATP in the cells. ATP depletion seemed to affect cellular viability.

Finally, to clarify whether the cytosol of *B. gibsoni* contained high K^+ and low Na^+ concentrations, the anti-babesial effect of each ionophore against *B. gibsoni* parasite within LK dog erythrocytes was examined using a culture medium consisting of high K^+ and low Na^+ concentrations. As a results, no anti-babesial effect of each ionophore was observed in that medium. This result might suggest that the ion pump in the cytoplasm of *B. gibsoni* might not be activated in medium containing high K^+ concentration, and that the cytosol of *B. gibsoni* might contain high K^+ and low Na^+ concentrations, indicating that the parasite might have its own sodium pump to maintain high K^+ and low Na^+ concentrations.

In this study, it was suggested that valinomycin and nystatin might activate the ion pump of *B. gibsoni* through perturbation of the cation gradient in the parasite cytosol, resulting in ATP depletion of the parasite. This may be a mechanism of the ionophores against *B. gibsoni*.

Functional assessment of equine nectin-1 as an entry receptor for equine herpesvirus 1

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Equine herpesvirus 1 (EHV-1) is known as a causative agent of equine rhinopneumonitis, abortion, and encephalomyelitis. EHV-1 has caused serious problems in horse industries worldwide. The effect of inactivated vaccine currently used in Japan is short-

termed. In addition, the vaccine is not able to induce a cellular immune response that is strong and effective. To conquer EHV-1 infection, more effective prophylaxes and treatments have been desired. Therefore, it is necessary to identify viral and host factors involved in the development of the EHV-1 infection.

Although the receptor for EHV-1 has not been identified, five receptor molecules that mediate the entry of other alphaherpesviruses have been well characterized so far. Among those receptors, nectin-1 act as a common receptor for Herpes simplex virus-1, Herpes simplex virus-2, Pseudorabies virus, and Bovine herpesvirus-1. It has been reported that the human nectin-1 molecule does not mediate entry of EHV-1 into cells. However, the function of equine nectin-1 as an receptor for EHV-1 remains to be elucidated. In this study, a cDNA of equine nectin-1 (eNec-1) open reading frame was cloned from equine brain microvascular endothelial cells. The deduced amino acid sequence of eNec-1 showed high degree of similarities (greater than 90%) with that of human, porcine, bovine and murine nectin-1. The eNec-1 had two unique amino acid substitutions (P56L and I80L) within a putataive virus-binding region of the V domain.

In order to assess a function of eNec-1 as an entry receptor for EHV-1, we transfected eNec-1 into NIH3T3 cells that were unsusceptible to EHV-1 infection, and then transfected cells were exposed to the recombinant EHV-1 expressing GFP. This virus did not infect the NIH3T3 cells expressing eNec-1. These results suggest that EHV-1, unlike other alphaherpesviruses, is not able to use nectin-1 as an entry receptor.

Age-dependent resistance of developing rat to Japanese encephalitis virus infection

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Fischer rats infected intracerebrally with Japanese encephalitis virus (JEV) show age-specific mortality; rats infected at 12 days of age or younger result in 100% mortality and rats infected at 14 days of age or older result in 100% survival. This age-related resistance has been shown to be related to neuronal maturity, since developing rat neurons, but not mature ones, were demonstrated to be the target of JEV. However, the events underlying this dramatic change in susceptibility have been poorly understood.

Fischer rats were infected with JEV JaGAr-01 strain intracerebrally at the age of 4, 8, 10, 12 and 17 days. Histologically, distributions of necrotic foci and JEV antigen-positive cells were almost the same and were more restricted in older rats. Brain virus load correlated inversely with increasing age at the time of inoculation. Clearance of infectious virus from the CNS delayed in younger animals. Although all rats except the ones inoculated at 4 days of age eventually developed serum antibody response after infection, comparisons showed no significant differences in antibody titer among rats inoculated at 8, 10, 12 and 17 days of age. In some rats infected at 8 or 10 days of age resulted in moderate to se-

vere thymic atrophy, indicating systemic stress response. These data suggest that the mortality of rats from JEV infection is related to brain virus load, and also suggest the possibility of involvement of systemic stress response in the mortality.

Experimental demonstration of transneural spread of *Listeria monocytogenes*

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Listeria (*L.*) *monocytogenes* causes listeriosis in humans and domestic animals. In sheep and other ruminants which have developed encephalitis after *L. monocytogenes* infection, the lesions are usually confined to the brain stem, especially the pons and medulla oblongata. One of the explanations for the predilection to the brain stem is that *L. monocytogenes* spreads directly from peripheral sites to the brain stem via cranial nerves.

In this study, *L. monocytogenes* was inoculated to right cheek muscles of mice and the brains, cranial nerves and visceral organs of the mice were examined histologically and immunohistochemically. To evaluate the transaxonal spread of *L. monocytogenes in vitro,* nerve cells from murine dorsal root ganglia were cultured in compartmentalized culture system, inoculated with the bacteria to axons, and then investigated using confocal laser microscope whether the bacteria were able to ascend the axons. *Erysipelothrix* (*E.*) *rhusiopathiae* was used as negative control.

Inoculation of *L. monocytogenes* to murine right buccinator induced ipsilateral rhombencephalitis and neuritis of the trigeminal and facial nerves. Bacterial antigen was detected in those lesions by immunohistochemistry. In the compartmentalized culture system, total number of *L. monocytogenes* moved from axon terminal to nerve cell body increased with time and many of them appeared to be located within axons. *E. rhusiopathiae* also moved to the compartment of nerve cell body along axons, but many of them appeared attaching to the axonal surface under the laser microscope.

In conclusion, it was suggested that *L. monocytogenes* was able to spread to central nervous system via peripheral nerves such as trigeminal and facial nerves, and the transaxonal passway might be one of the route of transneural spread of *L. monocytogenes.*

Simplified diagnostic procedures for an alteration of endometrial epidermal growth factor profile during the estrous cycle in repeat breeder cows

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Repeat breeder cows that fail to conceive after three or more inseminations without apparent abnormalities in their genital tracts and estrous cycles have been a major source of economic loss in dairy herds. Changes in endometrial epidermal growth factor (EGF) concentrations are altered in some of repeat breeder cows. Although normal cows have two peaks of endometrial EGF concentrations in the estrous cycle, some repeat breeder cows do not have them. Detection of the alteration has been used to segregate this type of repeat breeders from those with unknown causes of infertility to choose the type of treatment. However, the current diagnostic protocol needs repeated biopsy of endometrial tissues and EGF assay requires tissue processing which is time consuming and labor intensive. Thus, the present study evaluated, first, the potential of vaginal curage materials as alternate materials to estimate endometrial EGF concentrations and, then, use of Sep-Pak C_{18} cartridge to simplify the EGF separation protocol.

EGF concentrations of vaginal curage materials per total protein showed a similar change to endometrial EGF concentrations that had 2 peaks on Days 2 to 4 and 14 of estrous cycle and EGF concentrations between the 2 materials highly correlated ($y=1.21x-0.73$, $R=0.9832$, $P<0.05$). Separation of EGF in endometrial tissues using Sep-Pak C_{18} cartridge (Sep-Pak method) was equally effective to the conventional method using Sephadex G-50 column when 50 or 100% methanol or 80% acetonitrile were used for elution. There was a positive correlation between EGF concentrations of vaginal curage and endometrial materials separated by the Sep-Pak method when 50% methanol was used for elution ($y=0.90x+3.47$, $R=0.6154$, $P<0.05$). These results indicate that endometrial EGF profile can be estimated by using vaginal curage material and the EGF separation protocol with Sep-Pak C_{18} with 50% methanol for elution.

In vitro ovulation of *in vivo* grown follicles isolated from eCG-treated mice : effects of hCG dose in culture medium and initial follicle size.

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The objective of this study was to examine the effects of human chorionic gonadtrophin (hCG) dose supplemented with culture media and initial size of follicles on efficiency

of ovulation *in vitro*. *In vivo* grown follicles (350-650 μm of diameter) were isolated from the ovaries of mice treated with equine chorionic gonadtrophin (eCG). Some of the follicles were recovered after 48 h of eCG treatment (eCG group), and the others were recovered 3 h after hCG injection given 48 h after eCG treatment (eCG/hCG group). The isolated follicles were cultured for 21 h (eCG/hCG group) or 24 h (eCG group) in media containing 5, 10 or 20 IU/ml of hCG to examine their ovulation and nuclear status of ovulated and non-ovulated oocytes. The concentrations of sex steroid hormones in the culture media for eCG group were also measured.

In the eCG/hCG group, ovulation rate of the follicles cultured in a medium containing 5 IU/ml of hCG was lower than that of 10 and 20 IU/ml. Follicles with lager initial size showed lower ovulation rate, and follicles with more than 600 μm of diameter did not ovulate. In the eCG group, no difference was observed in ovulation rate between the follicles cultured with 10 and 20 IU/ml of hCG, but follicles with more than 500 μm of diameter did not ovulate. There was no difference in the ovulation rate between eCG/hCG and eCG groups when the follicles with 350-500 μm of initial diameter were subjected to culture. Most ovulated oocytes were at the metaphse II in the eCG/hCG (74%) and eCG (94%) groups. Some (30%) of the oocytes of non-ovulated follicles in eCG/hCG group, but none of the oocytes in eCG group, showed the resumption of meiosis. Estradiol concentrations of the follicle culture media of ovulated follicles was higher than those of non-ovulated follicles. Eighty percent of ovulated follicles produced high level of estradiol or estradiol/progesterone, and 70% of non-ovulated follicles produced high levels of progesterone or testosterone, suggesting that non-ovulated follicles were in the process of atresia. The present results indicate that supplementation of follicle culture media with 10 IU/ml of hCG is necessary to induce *in vitro* ovulation of the follicles recovered from eCG/hCG-treated mice. A low ovulation rate in the follicles with larger initial sizes indicates the necessity of modification in the follicle culture conditions.

A novel posttranslational modification with 4-hydroxyl-2-nonenal of the major red cell membrane skeletal protein β-spectrin

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Red cell membrane proteins are known to be susceptible to form the adducts with a lipid peroxidative product 4-hydroxyl-2-nonenal (4-HNE). However, it remains to be clarified which proteins are the targets of this modification and how it occurs. The present study reports a novel finding that spectrin, the major constituent of the red cell membrane skeleton, is the primary protein accessible to 4-HNE. When human red cell membrane proteins and crude spectrin preparations were analyzed for the 4-HNE adducts by immunoblotting, profound signals were observed in α-and β-spectrin and were remarkably increased after exposure of the membranes to 4-HNE. No significant differences were ob-

served for the signals of 4-HNE adducts of spectrin polypeptides in immunoblots of membranes from red cells separated on an arabinogalactan-density gradient centrifugation. The MALDI-TOF mass spectrometry analysis for tryptic peptides derived from intact β-spectrin demonstrated that 4-HNE was added to some peptides including $Ile^{110}-Lys^{118}$, $Val^{293}-Lys^{302}$, $Ala^{480}-Lys^{497}$, and $Leu^{1234}-Lys^{1244}$ localized in the N-terminal actin/protein 4.1-binding domain (βN), the segment 1, segment 2, and segment 10, respectively, of β-spectrin. The modification of $Ile^{110}-Lys^{118}$ with 4-HNE was also found in the 45-kDa polypeptide produced by limited digestion of β-spectrin with V8 protease followed by mass spectrometric analysis. Glutathione S-transferase-fused proteins of the βN, segments 2-4 (β[2-4]), and segments 5-7 (β[5-7]) of β-spectrin were generated and were analyzed by mass spectrometry. Surprisingly, the 4-HNE adducts of the peptides $Ile^{110}-Lys^{118}$ and $Ala^{480}-Lys^{497}$ were found in the βN and β[2-4], respectively, while no adducts were detected in β[5-7], suggesting that the modification also occurred in bacterial cells. Considering the previously supposed interactions of the βN and β[2-4] regions with phosphatidylserine, these findings suggest that the posttranslational modification of β-spectrin is a spontaneous event subsequent to polypeptide synthesis, presumably by the association of the protein with membrane lipids, rather than a change during the red cell senescence.

Molecular mechanisms for down-regulation of the α-hemoglobin stabilizing protein (AHSP) gene expression in prion diseases

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α-Hemoglobin stabilizing protein (AHSP) is an erythroid-specific molecular chaperone that stabilizes newly synthesized α-globin. The previous study demonstrated that mRNA levels of AHSP was specifically reduced in hematopoietic tissues of PrP^{Sc}-infected animals. The purpose of the present study was to clarify the mechanisms for down-regulation of the AHSP gene expression in erythroid precursor cells. The MELhide8 clone, exhibiting high levels of hemoglobin production in response to erythroid differentiation induced with N,N'-diacetyl-1, 6-diaminohexane (HMBA), was established from the parental MEL cells and was found to possess PrP^{C} and generate AHSP when induced by HMBA. The effects of PrP^{Sc} and several inflammatory cytokines on the expression of some erythroid-specific genes including AHSP were examined in this clone and the cells over-expressing PrP^{C} (MELhide8-MoPrP). The addition of brain homogenates from scrapie-infected mice to HMBA-induced MELhide8 cells had no effect on the expression of AHSP, α-, and β-globins, GATA-1, EKLF, NF-E2, and band 3. No accumulation of PrP^{Sc} in MELhide8-MoPrP cells was found even after 16 passages, while N2a cells over-expressing PrP^{C} showed profound accumulation of PrP^{Sc}. Interleukin (IL)-1β, Tumor necrosis factor (TNF)-α, and IL-6 principally caused suppression of AHSP gene expression at the concentrations reported for sera in hu-

mans and animals suffered from prion diseases, while these cytokines had no effect on the expression of the band 3 gene. However, the decrease in the AHSP gene expression appeared not enough to cause the reduction in AHSP production, since no significant change was found in AHSP contents in erythroid cells from the animals inoculated with PrP^{Sc}. These findings suggest that some inflammatory cytokines, but not the PrP^{Sc}, can cause down-regulation in expression of the genes characteristic to erythroid cells including AHSP in prion diseases.

Physiological roles of band 3 (anion exchanger1) in assembly of the red cell membrane skeleton : a study for production of transgenic mice with band 3 expression at an early stage of erythroid development

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Little is known about the molecular mechanisms for the assembly of red cell membrane skeleton during the development and maturation of erythroid cells. Previous studies have shown that membrane skeletal proteins are stably assembled into the membrane in late erythroblasts concomitant to the onset of band 3, anion exchanger 1, expression. The purpose of the present study was to establish the transgenic mice with enforced expression of band 3 in early stages of erythroid differentiation under the regulation of a novel erythroid-specific promoter of GATA-1, a transcription factor characteristic of erythroid linage. Unfortunately, various murine band 3 mutants, containing the HA- or FLAG-tag sequence in the 3rd or 4th extracellular loop, failed to target the plasma membrane when transfected into HEK293 or MEL cells. Thus, the cDNA for the wild-type mouse band 3 or the mouse-bovine chimeric band 3 was inserted downstream the GATA-1 promoter IE3.9int and was transferred into embryos. Subsequent analyses for 17 individual mice produced showed that the transgenes were incorporated in their genomic sequences. Quantitative PCR analyses for bone marrow cells from F1 animals showed that the two out of 17 parental transgenic mice had increased expression, by 6 to 12 times, of the transgenes compared with the endogenous band 3 gene expression. It was also demonstrated that, under transgenic situation, both $lin^-TER119^-$ and $lin^-TER119^+$ erythroid cells exhibited expression of the band 3 gene, consistent with the expression pattern of GATA-1, whereas band 3 expression was predominant in the $lin^-TER119^+$ population and negligible in $lin^-TER119^-$ cells in normal mice, suggesting that the IE3.9int promoter displayed its proper function. However, no red cell phenotypes and/or no significant expression of the band 3 protein derived from the transgene were observed in these individuals, suggesting that the promoter function was not enough to induce the gene expression sufficient to cover large amount of band 3.

Changes in the expression of claudins along with the developmental stages in the murine mammary epithelia

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Claudins are the major constituents of the tight junction in the epithelial cells. As the first step to establish the molecular bases for different susceptibilities to mastitis in dairy cows, the changes in the expression of various claudins in distinct stages of mammary glands were investigated in mice by RT-PCR, quantitative PCR, immunofluorescence microscopy, and immunoblotting. RT-PCR analysis demonstrated that claudins 1, 3-12, 15, 17, and 19 were expressed in mouse mammary glands with relatively high levels compared with other claudins. Quantitative PCR exhibited that claudin-8 and -12 had remarkable increase in the transcripts in the lactating period, in which the levels of gene expression of claudin-1, -4, and -10 were reduced. Claudin-3 and -5 appeared to be constitutive, since their expressions were kept at constant levels despite of significant alteration in tissue architecture in different stages. Compatible changes in relative abundance of these claudin proteins were also demonstrated in immunoblotting, i.e., claudin-3 and -8 were predominant in the lactating period, while the content of claudin-8 was remarkably reduced in non-lactating stages. Moreover, immunofluorescence microscopy showed that claudins 3, 4, 5, 8, and 10 were exclusively distributed to the tight junction, while claudin-1 was localized to the basement membrane. These findings demonstrate that claudin-3 and -8 are the major components of theight junctions in murine mammary glands and suggest that the developmental changes of several claudins including claudin-8 play key role in regulation of permeability barrier function in the mammary gland.

Development of RT-PCR to detect tick-borne encephalitis virus genomic RNA

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In October 1993, a human case of encephalitis was diagnosed as tick-borne encephalitis (TBE) in Kamiiso, Hokkaido. Far-Eastern subtype TBE virus strain Oshima 5-10 was isolated from a sentinel dog in the same area. Since the suspected vector ticks and reservoir rodents are commonly found in Japan, TBE virus may be endemic not only in the area where a patient was found but also in other parts of Japan. Seroepidemiological survey for wild rodents is efficient to detect TBE endemic area. Now, virus-neutralizing test (NT) is used for serological survey of wild rodents. But neutralizing antibodies can't be

detected at initial phase of TBE infection. TBE infection at initial phase can be diagnosed by detection of virus RNA. In this study, I developed reverse transcription-polymerase chain reaction (RT-PCR) to detect TBE virus RNA.

There are three main subtypes of TBE virus; the European, Far Eastern, and Siberian subtypes. Therefore I designed the PCR primers to detect all subtypes of TBE virus. This primers set could detect each subtype of TBE virus, specifically. The detection limit of this RT-PCR was at least 10^2FFU/tube. In addition, it was examined whether this method could detect virus RNA in animal tissues. RT-PCR for tissues from experimentally infected mice could detect viral RNA in the blood, spleen, and brain samples.

Next, this RT-PCR was applied to the diagnosis of TBE virus from wild rodents. The spleen and serum samples were collected in Nakagawa, Hokkaido, which is a potential TBE endemic area. There is no positive sample in RT-PCR, but 6 samples had TBE-specific antibodies, indicating that Nakagawa was endemic for TBE virus.

In summary, I developed RT-PCR that can detect TBE viral RNA from tissue of rodent. Combine with serological study, this RT-PCR can be useful to specify TBE endemic area.

Characterization of monoclonal antibodies to nucleocapsid protein of severe acute respiratory syndrome (SARS) coronavirus

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Severe acute respiratory syndrome (SARS) suddenly emerged from the end of 2002, originated from Guangdong Province in China, and expanded to the large part of the world. The epidemic ended in July 2003 with total patient number more than 8,000 and 774 deaths. The causative agent was quickly identified as the distinct coronavirus called SARS-coronavirus (SARS-CoV). Since the reservoir animal has not been determined and no effective vaccine and antiviral agent have been developed, the most effective measure to prevent the expansion of SARS-epidemic may be rapid diagnosis and isolation of SARS patients. Therefore, establishment of specific diagnostic methods is crucially important. To establish a reliable diagnostic method, 9 clones of monoclonal antibodies (MAb) to nucleocapsid protein (NP) of SARS-CoV were generated.

All 9 clones of MAb were produced by immunization of recombinant NP or a synthetic peptide of SARS-CoV NP (SARS-NP) to mice. All MAb reacted with a Western blot analysis (WB). In addition, no MAb had cross-reactivity to authentic NP of human coronavirus (HCoV) 229E strain or recombinant NP of HCoV 229E strain by WB.

To determine the binding region of MAb on NP, four truncated recombinant NP (trNP) were generated and reactivity between MAb and trNPs were analyzed by WB and ELISA. It was revealed that 2 MAb recognized trNP 2 region (111-230aa) and 7 MAb recog-

nized trNP 3 region (221-340aa).

The epitopes of MAb on NP were determined by Fmoc method, in which variety of amino acid sequences of NP were synthesized on a membrane. It was revealed that MAb SN 5 - 25 recognized amino acid sequence "QTVTKK" on SARS-NP (245-250aa) and rSN 122recognize"LPYGAN" (122-127aa). These amino acid sequences of the epitopes were searched in the database and no such sequences were found within proteins of main causative agents of human respiratory illness. Therefore, it is strongly suggested that these 2 MAb are specific to SARS-CoV and useful for diagnostic reagents.

Development of new diagnostic methods of hantavirus and its epidemiological application.

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Hantaviruses are causative agents of severe zoonotic diseases which are hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Various types of hantaviruses are distributed all over the world in their specific natural rodent hosts.

In Hokkaido and Far East Russia, gray red-backed voles 〈*Clethrionomys (C.) rufocanus*〉 have hantavirus that is related to Puumala type hantavirus. In this study, the detailed genetic analysis of Sakhaline strain of hantavirus which was isolated from *C. rufocanus* captured in Sakhaline were performed.

Comparison of nucleotide sequences and phylogenetic analysis among hantaviruses revealed that Sakhaline strain was most related to Puumala type hantavirus but may be classified in a different type within the genus *Hantavirus*.

Antigen detection ELISA and quantitative real-time PCR were developed for new diagnostic methods of hantavirus infection. Hantavirus replication was analyzed in Puumala-infected Syrian hamsters (*Mesocricetus auratus*). Hamsters were infected with Puumala virus at 4 week old as adult. The virus antigen and RNA peaked at 14 days post infection (d.p.i) and the virus RNA was detected even at 70 d.p.i. The mode of infection in Syrian hamster was similar to that of the natural hosts. Therefore, Puumala-infected Syrian hamster is a suitable model for hantavirus infection in vivo.

In addition, antigen detection ELISA and Real-time PCR were applied 48 *C. rufocanus* captured in Nakagawa town, Hokkaido to understand the mode of virus infection in natural hosts. Virus RNA was detected from 5 seropositive animals. In addition, virus antigen and RNA were detected from 2 seronegative animals. Therefore, these methods are suitable to analyze the mode of hantavirus infection in natural host in detail.

Antioxidant activity of mouse prion protein

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The cellular prion protein (PrP^C) is a glycosylphosphatidylinositol (GPI)-plasma membrane-anchored protein. Since octarepeat domain (OR domain) in the amino-terminal region of PrP^C acts as a Cu^{2+}-binding domain, Cu^{2+} ion is considered to modulate various biological functions of PrP^C, i.e., the cellular enzymatic activity of superoxide dismutase (SOD), signal transduction, shedding of PrP^C and conversion to the scrapie isoform of prion protein (PrP^{Sc}). In this study, to clarify further physiological function of PrP^C as an antioxidant, the susceptibility of PrP^C-overexpressed cells to various oxidants was evaluated. In addition, we prepared recombinat mouse PrP and examined its SOD-like activity and the inhibiting activity against Cu^{2+}/H_2O_2-catalyzed oxidative reaction or Cu^{2+}-catalyzed oxidative reaction of ^{t}BuOOH in cell-free system.

Wild-type moPrP ($moPrP_{(23\text{-}231)}$) was overexpressed in mouse embryo fibroblast NIH3T3 cells using pRc/EF-MoPrP vector with Lipofectamine and the cell viability after treatment of various oxidants was assessed by WST-1 assay. In the assay for cell-free antioxidant activity of PrP, recombinant mouse PrP ($rmoPrP_{(23\text{-}231)}$) and truncated PrP ($rmoPrP_{(91\text{-}231)}$), lacking OR domain, were expressed using *E. coli* expression system, and the folded rmoPrP was purified by Ni^{2+}-resin and reverse-phase HPLC. The O_2^--scavenging activity (SOD-like activity) of rmoPrP and the inhibiting activity of rmoPrP against Cu^{2+}/H_2O_2-catalyzed oxidative reaction or Cu^{2+}-catalyzed oxidative reaction of ^{t}BuOOH were measured by spin-trapping technique using electron spin resonance spectroscopy (ESR) with spin trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).

In NIH3T3 cells overexpressed with wild-type PrP, the cell viability after treatment of overload of Cu^{2+}, H_2O_2 and ionizing radiation was measured. The Cu^{2+}-and H_2O_2-induced cell death of NIH3T3 cells were significantly inhibited by overexpression of PrP^C, but radiation-induced cell death was not. On the other hand, in the assay for cell-free antioxidant activity of PrP, ESR-spin-trapping experiments showed that the SOD-like activity of $rmoPrP_{(23\text{-}231)}/Cu^{2+}$ complex was about two times higher than that of Cu^{2+} alone, but 10^3 times lower than that of Cu/Zn-SOD. The oxidative reaction of Cu^{2+} with H_2O_2 or Cu^{2+}-catalyzed oxidative reaction of ^{t}BuOOH was also shown to be significantly reduced by the addition of $rmoPrP_{(23\text{-}231)}$ but not $rmoPrP_{(91\text{-}231)}$ by spin-trapping experiments. These results suggested that PrP^C inhibited the cellular damage induced by Cu^{2+}-and H_2O_2-oxidative stress thorough OR domain of PrP.

Visualization of formalin- and capsaicin-induced pain and assessment of analgesics in rat cerebral cortex.
—Application of high magnetic field BOLD-fMRI—

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Blood oxygen level dependent-functional MRI (BOLD-fMRI) is imaging technique employing the difference in the oxygenation level of hemogrobin which is an endogenetic contrast agent. It is useful as non-invasive way to visualize locally activated area in brain after various stimulations. In this study, the possibility that the fMRI method can visualize a response of pain was examined in rat cerebral cortex. In order to demonstrate that BOLD signal changes actually reflect the pain in the cerebral cortex, we compared BOLD-signals with morphine with those without morphine. Experiment was performed using a 7.05 Tesla superconducting MRI system, Varian Unity INOVA 300/183, and a one-turned surface coil centered over the primary somatosensory cortex in cerebral cortex of rat under the mechanical ventilation. A set of multi-slice gradient echo images was acquired and analyzed using commercially available software for imaging analysis (MEDx). In contrast to α-chloralose anesthetization, which is widely used as an anesthetic in forepaw stimulation in fMRI study of rats, isoflurane (1.0%) provided the stable anesthesia level and the favorable results concerning the fMRI in rat cerebral cortex. Following the subcutaneous injection of 50 μl of formalin (5%) or capsicin (10 μg/head) into the left forpaw, a regional increase in the signal intensity in the MR images was observed in all rats.

Formalin stimulation to the left forepaw of rat displayed that the signal increase (about 6%) was observed in contralateral somatosensory cortex following its injection, it was maintained for 4 minutes. Furthermore, the continuous but weak signal increase (about 2%) in signal was observed for 20 minutes after stimulation. Pre-treatment (3 mg/kg i.v.) with morphine vanished these responses. Capsaicin stimulation in left forepaw displayed that the signal increase (about 5%) was observed immediately after stimulation, but the signal disappeared within one minute. Pre-treatment (3 mg/kg i.v.) with morphine vanished these responses in a similar fashion to the formalin stimulation.

It is well known that BOLD signal depends on the cerebral blood flow. In either case of formalin or capsaicin, temporary rise of systemic blood pressure was observed, but this change of blood pressure was not completely depressed in administration of morphine, whereas BOLD signal was not observed in rats administered with morphine. The rise of systemic blood pressure might not necessarily affect the changes in BOLD signal of brain. Accordingly, it was suggest that the changes in BOLD signals in somatosensory cortex certainly reflected the change of focal blood flow. This BOLD-fMRI technique in anesthetized animal brain is a useful way to study the mechanism of pain and assess new analgesics.

Effects of combined treatment of novel anti-tumor nucleosides with X irradiation on the apoptosis induction in MKN45 cells

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Recently, two anti-tumor drugs, 1-(3-C-ethynyl-β-D-ribo-pentofuranosyl) cytosine (ECyd) and 1-(2-deoxy-2-methylene-β-D-erythro-pentofuranosyl) cytosine (DMDC), were newly developed to induce apoptotic cell death by inhibiting RNA synthesis and DNA synthesis, respectively. Since several anti-tumor drugs such as cisplatin and 5-FU were known to have radio-sensitization effects in clinical radiotherapy, we examined whether ECyd and DMDC have radio sensitization effects on the apoptosis induction in human gastric adenocarcinoma MKN45 cells. Observation of morphological changes in nuclei revealed that treatment with ECyd alone induced the dose-dependent increase of apoptosis in MKN45 cells, although X irradiation alone did not. Moreover, combined treatment of ECyd with X irradiation increased apoptosis in comparison with treatment with ECyd alone. On the other hand, DMDC had no radio-sensitization effects in the apoptosis induction. Flowcytometric and western blotting experiments showed that ECyd abrogated radiation-induced G2/M checkpoint and accumulation of phosphorylated Cdc2, whereas DMDC did not affect these radiation-induced phenomena. Furthermore, ECyd was shown to reduce the expression of Bcl-2 but not Bcl-X_L in MKN45 cells with X irradiation, whereas DMDC did not change the expression of these anti-apoptotic proteins. These results suggest that ECyd as an RNA synthesis inhibitor is preferable to DMDC as a DNA synthesis inhibitor when treatment of anti-tumor drug is combined with X irradiation for inducing apoptotic cell death in therapy for solid tumor.

Novel Mechanism of Warfarin Resistance in Roof Rats (*Rattus rattus*) of Tokyo

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Roof rats are often found in high-rise buildings in urban areas. Attempts to exterminate them with rodenticides are failing and their numbers are increasing in the Tokyo area. An increase in the population of warfarin-resistant rats is proposed to be the reason. Recent investigations report the involvement of vitamin K epoxide reductase complex subunit 1 (VKORC1) gene mutation in this resistance.

Using liver microsomes of warfarin-resistant (R) rats from Shinjuku and warfarin-sensitive (S) rats from a closed colony (originally from the Ogasawara Islands) for the

control, I measured vitamin K epoxide reductase (VKOR) and vitamin K quinone reductase (VKR) activities and their sensitivities to warfarin in both groups of rats. I found no significant difference between them in VKR activity. Resistant rats showed significantly lower VKOR activity than sensitive rats. VKOR activity in sensitive rats was markedly decreased with warfarin, while only a slight decrease in the already-low activity was observed in resistant rats. I found that purified NADPH cytochrome P450 reductase (fp2) has VKOR activity. Fp2 activity determined by cytochrome c reductase activity in microsomes of resistant rats was 3-8 times higher than that in sensitive rats. These results suggest that fp2 functions as a VKOR enzyme in resistant rats to compensate for reduced VKORC1 activity.

The warfarin level in serum (AUC) was significantly lower in warfarin-resistant rats after oral administration than in the control warfarin-sensitive rats. Concentrations of warfarin metabolites in urine were higher in warfarin-resistant rats than those in warfarin-sensitive rats. Hydroxylations of warfarin by cytochromes P450 (CYP) isoforms were significantly higher in warfarin-resistant rats. Western blot analysis indicated that levels of CYP3A2 expression in warfarin-resistant rats were significantly higher than in warfarin-sensitive rats.

It is concluded that the mechanism of warfarin resistance in Tokyo roof rats involves high fp2-dependent VKOR activity compensating for low VKOR enzyme activity and increased clearance of warfarin due to increased microsomal activities of cytochrome P450 reductase and CYP3A2.

Synergistic toxicity between dopamine-derived neurotoxin, norsalsolinol, and transition metals, Implication for etiology of Parkinson's disease

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Parkinson's disease (PD) is one of the neurodegenerative diseases that causes motor disorders. Most of PD is reported to be sporadic. There are three major hypotheses of etiology: the dopamine quinone theory, the inhibition of mitochondrial complex I theory, and the oxidative stress theory. Since Langston *et al.* reported that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes Parkinsonism in 1983, many studies were carried out to find the causative substance, but it remains unknown. Recently, tetrahydroisoquinolines (TIQs), whose structures are similar to MPTP, are suspected as possible causative factors for PD.

Norsalsolinol is one of the TIQs and can be biosynthesized from dopamine and formaldehyde by Pictet-Spengler condensation in the brain. In this study, I investigated the cell toxicity of norsalsolinol and its capability of causing PD with *in vivo* and *in vitro* experiments. According to many reports, metals such as iron or copper are accumulated in the brains of PD patients. Thus, I also investigated the toxicity of these metals with and without norsalsolinol.

Rat midbrain primary cultures and PC12 cells, which were induced to neuron-like cells by NGF, were exposed to these chemicals and/or metals. A marked decrease of cell viability and increased production of ROS were observed. In particular, norsalsolinol and metals showed a synergistic effect in primary cells, producing ROS up to 2.7-fold and reducing cell viability to about 50% compared to 100% in controls.

In vivo studies revealed that spontaneous motor activities were significantly decreased in continuously norsalsolinol + iron-infused rats. Significant decreases of dopamine concentration in the midbrain (68±10%) were observed in these rats. Those changes were not found in iron-infused rats.

A previous report showed that norsalsolinol is actively transported by the dopamine transporter, implying that norsalsolinol produces toxicity in the substantia nigra. Taken together, these results suggest that a synergism between norsalsolinol and metals in neurotoxicity may contribute to the onset of PD.

Pharmacokinetics of Glivec (imatinib) in dogs

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Glivec® (imatinib mesylate) is a rationally designed, potent and highly selective tyrosine kinase inhibitor. It is marketed for the treatments of chronic myeloid leukaemia (CML) and gastrointestinal stromal tumours (GISTs) in human. Mast cell tumors (MCT) are the most common neoplasm in the skin of dogs, and half of these tumors are malignant. Glivec has the possibility to inhibit the function of abnormally activated tyrosine kinase in MCT cells of dogs. However, the pharmacokinetics of imatinib in dogs have not been reported. The aim of this study is to determine *in vitro* and *in vivo* pharmacokinetics of imatinib in dogs.

In Chapter 1, I established the method of determination of Glivec using HPLC (high-pressure liquid chromatography) equipped with a UV detecter.

In Chapter 2, the pharmacokinetics of Glivec in plasma of dogs were investigated, using the method in Chapter 1. Glivec was orally or intravenously administered to eight dogs. The time of the peak concentration (Tmax) of imatinib was 4 to 9 hours, indicating the similarity to Tmax in humans. I found large individual differences in the maximum concentration of imatinib in plasma (Cmax, C_0), the area under the plasma concentration *versus* time curves (AUC) and elimination half-life ($t_{1/2}$). After oral administration, Cmax ranged from 0.52 to 3.0μM (n=6) and average value (±SD) was 1.4±1.0μM, AUC (±SD) was 950±720μM·min, $t_{1/2}$ (±SD) was 135±123min. After intravernous injection, C_0 ranged from 8.1 to 34.4μM (n=8) and average value (±SD) was 15.5±8.3μM, AUC (±SD) was 990±400μM·min, $t_{1/2}$ (±SD) was 196±120min. The pharmacokinetic parameters in dogs exhibited higher similarity with those in the monkeys than parameters in humans. Binding affinities of plasma proteins to

imatinib are lower in dogs and monkeys than that in humans. Moreover, I found that the bioavailability of Glivec in dogs was low (71±51%) compared with that in humans (98%).

In Chapter 3, cytochrome P450 (CYP) molecular species which metabolize Glivec in dogs were identified. The metabolic activities in the reconstruction system of dog CYP3A12 and CYP2C21, and inhibition of CYP3A dependent activity using anti-rat CYP3A antibody and ketoconazol, showed that CYP3A12 mainly metabolized Glivec in the liver microsome of the dog.

It is reported that CYP3A subfamily contributes to the metabolism of numerous clinical medicines in dogs. Vitamin D receives metabolism by CYP3A in human, and it is clinically used for transitional cell carcinoma and osteosarcoma of dogs. Then, in Chapter 4, I studied the possibility of adverse drug interaction between Vitamin D and Glivec. *In vivo*, I did not find any differences in plasma pharmacokinetics between Glivec and Glivec-vitamin D treated dogs. I also showed that there is no effect of vitamin D on Glivec metabolism in dog liver microsomes *in vitro*.

In present study, I report pharmacokinetics of Glivec in dogs, and the P450 molecule (CYP3A12) which mainly contributes to the metabolism of Glivec. In addition, I found that vitamin D does not interfere with pharmacokinetics and metabolism of Glivec. These results may contribute to the establishment of effective therapeutic use of Glivec in veterinary field.

Growth-related changes in gonadal histology and immunohistochemistry of the immature green turtle (*Chelonia mydas*)

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This study aimed to reveal growth-related histological changes of green turtle (*Chelonia mydas*) gonads and to establish a way to identify the growth stages of the sea turtles. The role of steroid hormones in the growth of sea turtle gonads was also discussed from an immunohistochemical study of steroidogenic enzymes (P450scc, 3βHSD, P450 c17, and P450arom) and steroid hormone receptors (AR, ERα, ERβ, and PR).

Testes can be categorized into six stages by histology, and here there was a wide range of straight carapace lengths (SCL) for each stage. However, there were no large changes in efferent ductules and epididymal ducts. Steroidogenic enzymes were immunolocalized in Leydig cells, and different kinds of steroid hormone receptors were expressed within the same cells in both testes and epididymides, while immunoreaction of all receptors was enhanced as the stages advance.

Ovaries and oviducts were distinguishable between immature turtles and those in puberty, as was immunolocalization of steroid hormone receptors within them. Different steroid hormone receptors were expressed in the same cells as observed in male gonads.

These results suggested that size is not a reliable indicator of maturity; however, it was possible to histologically identify the

growth stages in males. Although it was not possible to categorize the growth stages of immature females, there was an indicated that maturation of ovaries and oviducts are synchronized and that a histological search is not essential to distinguish between immaturity and puberty. Since the gonads of both sexes became sensitive to these steroid hormones during growth, it was suggested that interactions between these hormones regulate gonad maturation and function.

Prevalence of antibodies to the hepatitis E virus (HEV) in pigs and cattle in Hokkaido

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Recently, hepatitis E has drawn attention as a zoonosis, and there have been several cases reported in Japan that suggest food-borne transmission from pigs, deer, and boar. These animals are thought likely to be reservoirs of HEV. Some cases suggesting HEV infection from pigs to humans have been reported in Hokkaido ; thus we tested pigs and cattle in Hokkaido for the presence of anti-HEV IgG and analyzed the associated public health risks. Some previous studies have been undertaken utilizing ELISA, but without the use of proper control serum. In this study, experimentally immunized sera from pig and deer were prepared and used as the respective positive control sera for ELISA, and Western blot assay was used as the secondary test. The results showed a prevalence of 3.6% in pigs ; suggesting that the actual prevalence and risks of food-borne transmission were not as great as previously reported. Nevertheless, invasion of HEV was confirmed and thus it is necessary to pay attention that raw meat is not consumed. In cattle, there was no anti-HEV IgG among those tested ; suggesting that cattle present little risk of HEV infection. However, if cattle were to be infected with HEV, it would be transmitted to deer as they share the same pastureland and so would become widespread. HEV infection in cattle deserves further attention, and periodic inspection of cattle together with surveillance of deer is required. As ELISA is likely to include some false positive reactions, we propose that a combination of ELISA screening with Western blot assay confirmation is a good technique for large-scale serological surveillance of HEV.

Frequencies of PrP genotypes in Japanese sheep flocks —for breeding programs for TSE resistance in Japanese sheep—

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There are many amino acid polymorphisms in ovine PrP. Two of them, polymorphisms at codons 136 (A/V) and 171 (Q/R) greatly influence the susceptibility to scrapie. Sheep possessing AQ/VQ or AQ/AQ genotype are susceptibile to scrapie, while sheep possessing AQ/AR genotype are relatively resistant and those possessing AR/AR are highly resistant to scrapie. To reveal the susceptibility of Japanese Suffolk sheep to scrapie, in this study, the author analyzed the frequencies of PrP genotypes in Suffolk sheep. In addition, to understand the efficiency of establishing scrapie-resistant sheep flocks by selecting the sire based on their PrP genotypes, the author analyzed the transition of the frequencies of PrP genotypes before and after the implementation of the selective breeding at Shintoku Hokkaido Animal Research Center (HARC). To investigate the amino acid polymorphisms in PrP of Suffolk sheep, DNA fragments corresponding to the open reading frame of the PrP gene were amplified from genomic DNA isolated from 98 sheep and their nucleotide sequences were determined by direct sequencing. Nucleotide substitutions resulting in amino acid substitution were found at codons 112 (M/T), 136 (A/V) and 171(Q/R). Thus, in the following analyses, the author carried out a single nucleotide polymorphisms analysis for codons 136 and 171 that are related to scrapie-susceptibility. The frequencies of PrP genotypes varied among regions in Japan ; the frequencies of sheep possessed scrapie-susceptible genotypes AQ/VQ or AQ/AQ were ranging from 42.9 to 63.1%. Moreover, genotype frequencies for AQ/VQ or AQ/AQ varied from 30.9 to 100% among the private sheep flocks in Hokkaido. These results suggested that the ratio of scrapie-susceptible Suffolk sheep differed among regions and flocks. A year after initiating the selective breeding using rams carrying scrapie-resistant AQ/AR or AR/AR genotype at Shintoku HARC, the frequencies of scrapie-resistant PrP genotypes in lams significantly increased compared to those before the selective breeding. Three years after the initiation, the ratio of lams carrying AQ/AR or AR/AR genotypes was shifted up from 47.9 to 80.0%. These results demonstrated that the breeding with rams selected by PrP genotypes, without selection of ewes, was efficient in increasing the ratio of scrapie-resistant sheep in the flocks during short periods. The results in this study will provide the useful data for taking measures for controlling scrapie.

Analysis of suitable PrP^{Sc} fraction for process validation and the verification of its utility

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Process validation is one of the methods for evaluating the risk of pathogen contamination in medical and pharmaceutical products, cosmetics, and their raw materials. In process validation of the prion contamination, it is expected that the removal efficiency will vary and the results will be overestimated depending on physicochemical properties of abnormal isoform prion protein (PrP^{Sc}), the major component of prion. In this study, the author examined preparations of suitable PrP^{Sc} fraction for process validation, and verified their utility. PrP^{Sc} aggregates with small particle size that were not precipitated by the centrifugation at 100,000 x g, could be extracted with Sarkosyl from brain homogenates of mice infected with scrapie Obihiro strain and hamsters infected with scrapie 263 K strain. Such PrP^{Sc} aggregates could also be extracted from brain microsomal fraction. Thus, I attempted to verify the utility of the PrP^{Sc} preparation extracted from brain microsomal fraction with 3% Sarkosyl, using the filtration steps in manufacturing process of human immunoglobulin product as a model. When microsomal fraction, microsomal fraction treated with 1% Sarkosyl and the 3%-Sarkosyl extract of microsomal fraction were spiked into PBS as PrP^{Sc} preparation, PrP^{Sc} filtered through a 0.22 μm membrane filter with similar removal efficiency; removal factors (Rfs) were 0.6-0.8 Log_{10}. No difference was observed in the removal efficiency by the 0.22 μm membrane filter when the 3%-Sarkosyl extract of microsomal fraction was spike into immunoglobulin solution. In contrast, the Rfs increased up to 2.4log_{10} when the immunoglobulin solution inoculated either microsomal fraction or 1%-Sarkosly-treated microsomal fraction was filtrated through the 0.22 μm membrane filter. PrP^{Sc} passed through Planova 35N membrane with Rfs below 1 Log_{10}, when the 3%-Sarkosyl-extract of microsomal fraction was spiked into PBS, suggesting this fraction contained the PrP^{Sc} aggregates with particle size less than 35 nm. However, PrP^{Sc} was not detected in the filtrates of Planova 35N when the same fraction was spiked into immunoglobulin solution. These results suggested that the PrP^{Sc} fraction with small particle size allows us to perform more precise process validation and that the evaluation of removal efficiency will depend on properties of the spike materials and solution to be examined.