*Jpn. J. Vet. Res.* 51(3－4) : 173-177, 2004

# INFORMATION

Hokkaido University confered the degree of Doctor of Philosophy (Ph. D) in Veterinary Medicine on June 30, 2003 to 1 recipients and September 25, 2003 to 3 recipients.

The titles of theses and other information are as follows :

## Characterization of Theileria parasites and their vector ticks in Thailand

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Original papers of this thesis appeared in *J. Vet. Med. Sci.*, 61 : 991-994 (1999) and 65 : 133-135 (2003)

## An experimental study on regeneration therapy for damaged articular cartilage by chondrocytes transplantation using a bovine model

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Articular cartilage is an avascular, aneural and alymphatic tissue with a limited capacity for self-regeneration. Autologous chondrocytes transplantation (ACT) is thought to be an ideal method for the regeneration of hyaline cartilage in human cartilage defects, however a clinical application of ACT has not been reported in animals yet. This study therefore focused on the development of a basis for the clinical application of ACT in veterinary surgery.

In the first study, effects of hyaluronic acid (HA) on the attachment and migration abilities of bovine chondrocytes cultured with or without a supplementation of IL-1 α were evaluated. The attachment and migration of bovine chondrocytes cultured *in vitro* were significantly suppressed by IL-1 α. However, the application of HA recovered significantly the suppressed attachment and migration of chondrocytes.

In the second study, the effects of ascorbic acid (Asc) on the proliferation and biological properties of bovine chondrocytes were analyzed in a alginate culture. Bovine chondrocytes were cultured in alginate beads with or without Asc for 16 days, and cell proliferation and the gene expression of aggrecan, collagen type I and II mRNA were analyzed. Cell morphology and the production of extracellular

matrix (ECM) were also evaluated. The proliferation of chondrocytes was significantly stimulated with Asc on day 16. The expression of the collagen type I mRNA was increased and that of the collagen type II mRNA was decreased by Asc. In alginate beads cultured with Asc for 2 months, proliferating cells were observed mainly at the periphery of the beads, and glycosaminoglycans (GAG) and collagen type II were found around the cells.

In the third study, the attachment and proliferation of chondrocytes and the formation of the ECM were analyzed on the intact and damaged surfaces of cartilage explants. Bovine cartilage explants were co-cultured with or without chondrocytes for 4 weeks. Co-cultured chondrocytes attached to and proliferated on the intact and damaged areas, and created a new layer there. The defect was filled with the ECM produced by the co-cultured chondrocytes. GAG and collagen type II were detected in the newly formed ECM and large numbers of rounded chondrocytes were observed at primitive lacunae in this matrix at the end of the culture period.

In the final study, the effects of ACT by intraarticular injection or injection into blood clots at cartilage defects on the regeneration of the damaged cartilage were evaluated using a bovine model. Chondrocytes were isolated from harvested cartilage pieces and cultured in a monolayer fashion for cell proliferation. Cells were injected into a left femoropatellar joint (G 2, n＝11), and into the blood clot in the cartilage defects (G 3, n＝5). The defects (G 1, n＝16) of the control group were left untreated. After 14 weeks, repaired tissues were evaluated based on gross and histological examinations. In G 3, more repaired tissue and a better interface between the repaired tissue and host cartilage were observed compared with the results for G 1 and 2. In G 1 and 2, repaired tissue contained mainly fibrous tissue with or without some fibrocartilaginous tissue. In G 3, both fibrous tissue and fibrocartilaginous tissue were common, and some amount of hyaline cartilaginous tissue was also observed.

In conclusion, effects of HA and Asc are suggested to be useful to improve ACT. Proliferated chondrocytes have the ability to attach to, to proliferate on and to establish a new cartilaginous matrix in intact and damaged cartilage explants. The blood clot in the cartilage defect is thought to be a useful autogenous scaffold for cell transplantation. ACT by injection into the blood clot could therefore be applicable for the regeneration of damaged cartilage in veterinary surgery. ACT by this method could also be applicable following other type of regeneration therapy such as transplantation of bone-marrow-derived mesenchymal cells.

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The original paper of thesis appeared in *J. Vet. Med. Sci.*, 65 : 427-430 (2003), *Jap. J. Vet. Res.* 51 : 83-94 (2003) and *J. Vet. Med. A* (in press), (2004), J. Vet. Med. sci., 66 : (in press) (2004)

# Studies on major histocompatibility complex and cytokine responses in bovine leukemia virus-infected animals

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Bovine leukemia virus (BLV) is a type C retrovirus which is genetically and structurally similar to human T-lymphotropic virus types (HTLV) -I and II. Resistance or susceptibility to progression to these disease stages has been shown to be determined by several host immune responses. However, the exact mechanism of the disease progression from aleukemic (AL) to persistent lymphocystosis (PL) or lymphosarcoma (LS) in BLV-infected cattle is not known. Thus, I focus on the influence of polymorphism of the major histcompatibility complex (MHC) class II molecule and cytokine responses in BLV-infection. The conclusions obtained by this study are summarized as follows.

1) To investigate the genetic diversity of Ovine MHC (Ovar)-DRB 1, the exon 2 of the Ovar-DRB 1 alleles amplified by polymerase chain reaction (PCR) and their nucleotide sequences were determined. In a total of 97 sheep of three individual breeds, namely, Suffolk, Cheviot and Corriedale, 18 previously published alleles and 17 new alleles were identified. Furthermore, analysis of a total of 106 Ovar-DRB 1 alleles for restriction endonuclease cleavage sites resulted in the selection of Rsa I, Hae III, Sac I, Sac II, Dde I, Nci I, Hin 1 I, EcoR I and BstNI as tools for the study of polymorphism in exon 2 of the Ovar-DRB 1 gene. PCR products from DNA samples of 52 Suffolk sheep were characterized by the RFLP technique using nine restriction enzymes.

2) Although the number of BLV-infected cells and virus titers were maintained at low levels throughout the experimental period, the sheep with the RK/RK genotype induced expansion of CD 5 - B-cells and rapid production of neutralizing antibody in the early phase of infection. Peripheral blood mononuclear cell (PBMC) from the sheep with the RK/RK genotype showed a strong response to the BLV virion antigen and synthetic antigenic peptides corresponding to the T-helper epitope of the BLV envelope glycoprotein, gp 51. In contrast, the sheep with the SR/SR genotype showed a strong response to the BLV virion antigen and synthetic antigenic peptides corresponding to T-cytotoxic and B-cell epitopes. In such cases, interferon (IFN)-γ strongly expressed in the animals with the RK/RK genotype, whereas interleukin (IL)-2 strongly expressed in the animals with the SR/SR genotype.

3) The cells derived from cattle with PL and LS expressed higher levels of tumor necrosis factor-receptor (TNF-R) II but not -RI than those from AL and BLV-uninfected cattle. The proliferative response of PBMC isolated from those cattle with PL in the presence of recombinant bovine TNF-α was higher than those from AL cattle and uninfected cattle. Furthermore, most of cells expressing TNF-α and TNF-RII in the BLV-infected cattle were CD $5^+$ or $sIgM^+$ cells. These data suggest that

imbalance in the expression of TNF receptors could at least in part contribute to the progression of bovine leukosis in animals.

Original papers of this thesis appeared in *Eur. J. Immunogenet.*, 30 : 275-282 (2003), *Microbiol. Immunl.*, 47 : 223-232 (2003), *Vet. Microbiol.*, 94 : 283-294 (2003) and *J. Dairy Sci.*, (2003), (in press).

# The role of erythrocyte 5'-nucleotidase in the multiplication of *Babesia gibsoni* in canine reticulocytes

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The present study was conducted to elucidate the relation between erythrocyte 5'-nucleotidase and the multiplication of *Babesia gibsoni* in canine reticulocytes and to clarify the role of this enzyme in the mechanism of *B. gibsoni*-induced anemia.

First, the characteristics of erythrocyte 5'-nucleotidase was examined in dogs and other domestic animals compared with that of the human enzyme. The activities of canine erythrocyte 5'-nucleotidase measured using cytidine 5'-monophosphate (5'-CMP) and uridine 5'-monophosphate, which are preferentially catalyzed by one of the human pyrimidine 5'-nucleotidase isozymes (P5N-I), were similar to those of the human enzyme. The canine enzyme preferentially catalyzed thymidine 3'-monophosphate, which is catalyzed only by human P5N-II. This suggests that canine erythrocytes have two isozymes similar to human P5N-I and P5N-II. Also, the canine erythrocytes had markedly high activity and preferentially catalyzed purine 5'-monophosphate such as inosine 5'-monophosphate (5'-IMP) suggesting the presence of a purine-specific 5'-nucleotidase as in human erythrocytes. The reticulocyte count in the peripheral blood was approximately proportional to the P5N-I activity in canine and other species showing that the P5N-I activity may play an important role in the morphological maturation of canine erythrocytes.

Next, the relation between erythrocyte 5'-nucleotidase and the multiplication of *B. gibsoni* was examined based on the characteristics of canine erythrocyte 5'-nucleotidase described above. Serum from dogs chronically infected with *B. gibsoni* had inhibitory effects on the maturation of reticulocytes and canine P5N-I and purine-specific 5'-nucleotidase activities *in vitro*. The multiplication of *B. gibsoni* in *in vitro* culture also resulted in a significant decrease of this enzyme activity in erythrocytes in culture. Parasitemia was significantly decreased in the *in vitro* culture of *B. gibsoni* by pretreatment of host canine erythrocytes with lead acetate, which is a specific inhibitor of P5N-I and has an inhibitory effect on the maturation of canine reticulocytes. Furthermore, the *in vitro* multiplication of *B. gibsoni* was significantly inhibited by pyrimidine nucleotides such as 5'-CMP which is

preferentially catalyzed by P5N-I and also inhibits the maturation of canine reticulocytes. Purine nucleotides such as 5'-IMP also had an inhibitory effect on the multiplication of this parasite. From these results, nucleotides such as 5'-CMP and 5'-IMP might accumulate in young erythrocytes and/or serum in dogs infected with *B. gibsoni* as a result of decreased activity of erythrocyte 5'-nucleotidase, and the accumulation of these nucleotides might inhibit the multiplication of this parasite and simultaneously retard the maturation of reticulocytes.

It was previously reported that *B. gibsoni* parasites preferentially invade and multiply in reticulocytes rather than in mature erythrocytes when cultured *in vitro*. However, severe hemolytic anemia with reticulocytosis often occurs in dogs infected with this parasite despite markedly low percentage of parasitized erythrocytes in peripheral blood. The results obtained from *in vitro* examinations in the present study may partly explain the relationship between low parasitemia and simultaneous reticulocytosis *in vivo* in canine babesiosis.

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The original paper of this thesis appeared in *J. Vet. Med. Sci.*, 65 (2) : 193-197 (2003) and *J. Vet. Med. Sci.*, 65 (12) : 1281-1286 (2003).