HOKKAIDO UNIVERSITY

Title	INFORMATION: Theses for the Doctor of Veterinary Medicine (equivalent to Ph.D.)
Citation	Japanese Journal of Veterinary Research, 56(3), 167-169
Issue Date	2008-11
Doc URL	http://hdl.handle.net/2115/35116
Type	bulletin (article)
Note	Hokkaido University confered the degree of Doctor of Philosophy (Ph.D.) in Veterinary Medicine on June 25, 2008 to 1 recipient and September 25, 2008 to 1 recipient.
File Information	56-3_p167-169.pdf

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Japanese Journal of Veterinary Research 56(3): 167-169, 2008

INFORMATION

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The titles of theses and other information are as follows:

A basic study on clinical application of canine antitumor immunotherapy using activated lymphocytes.

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Conventional tumor therapy, including surgery, radiotherapy, and chemotherapy, effectively prolongs the survival time of tumor-bearing patients. However, critical adverse effects frequently occur during conventional tumor therapy, disturbing the quality of life (QOL) of these patients. Immunotherapy is therefore expected to be an alternative therapy for accelerating antitumor immunity, while inducing less adverse effects.

In tumor-bearing animals, the activation of spontaneous cellular immunity to tumorous tissue is relatively limited, and this suppression of immune function is not easily compensated even after the removal of the tumor mass. The induction of both specific and nonspecific immunity, especially T cell function, is imperative for the activation of antitumor immunity, which is suppressed in tumor-bearing animals. Passive immunotherapy by administration of artificially proliferated effector cells that are activated *ex vivo* is considered to be applicable for these immunosuppressed animals. Activated lymphocyte therapy is a type of passive immunotherapy; it involves sequential administration of autologous lymphocytes stimulated and cultured with anti-cluster of differentiation (CD)-3 antibody and human recombinant (hr) interleukin (IL)-2. The majority of these cultured cells are speculated to be $CD3^+$ T cells and natural killer (NK) cells, which can induce both specific and nonspecific immunity against the tumor mass. It is essential to determine the optimum conditions for lymphocyte culture and cell administration and to evaluate this method, which is not yet well established. The purpose of the present study was to establish the methodology for the administration of activated lymphocyte therapy in tumor-bearing dogs and to evaluate this therapy.

In the first part of this study, the dynamics of canine $CD56^+$ cells in peripheral blood collected from healthy beagles were investigated. CD56 is considered to be one of markers for NK cells in human beings; thus, canine CD56 is expected to be a candidate marker for canine NK cells. Quantitative polymerase chain reaction (PCR) method was used to determine canine CD56 messenger ribonucleic acid (mRNA) expression in normal tissues, peripheral blood mononuclear cells (PBMCs), and stimulated lymphocytes. The highest CD56 mRNA expression was observed in the brain. The CD56 mRNA expression in stimulated lymphocytes was higher than that in PBMCs. To localize CD56 mRNA, *in situ* hybridization was performed. The number of $CD56^+$ cells of stimulated lymphocytes was extremely higher than that of PBMCs. CD56 mRNA expression in canine lymphocytes was considerably lower; most lymphocytes were $CD56^-$. However, in the lymphocytes activated by stimulated PBMCs, both CD56 mRNA expression and number of $CD56^+$ cells were increased.

In the second part of the study, PBMCs collected from clinically healthy dogs were activated using anti-CD3 antibody and hrIL-2 and reacti-

vated using human recombinant interferon (IFN)-α and hrIL-2. The properties of cells thus obtained were analyzed in order to investigate the adaptation of canine activated lymphocyte therapy. In the obtained cells, the proportion of $CD4^+CD8^-$ cells and $CD4^-CD8^+$ cells was significantly increased; the cytotoxicity of the cultured cells was reinforced; and CD56 mRNA levels tended to be increased. These cells were confirmed to be activated lymphocytes. Furthermore, we investigated the effect of sequential administration of these cells in healthy dogs. This sequential administration was shown to increase the cell proliferative activity of PBMCs, the proportions of $CD4^+CD8^-$ cells and $CD4^-CD8^+$ cells, and the serum IFN-γ concentration; no severe adverse effects were observed. We concluded that activated lymphocytes could be administered to healthy dogs, and sequential administration of these cells reinforced the recipient's immunity.

In the third part of the study, autogenous activated lymphocytes, which were initiated as the second part of the study, were sequentially administered as an adjuvant antitumor therapy to 10 dogs with spontaneous malignant tumors, in Veterinary Teaching Hospital of Hokkaido University. Peripheral blood lymphocytes from tumor-bearing dogs were proliferated and activated for 14 days in culture. After sequential administration of these activated lymphocytes, the proliferation activities of PBMCs, proportions of $CD4^+CD8^-$ cells and $CD4^-CD8^+$ cells, and serum IFN-γ concentration increased in some cases. The general conditions of all 10 patients remained stable, and the patients were comfortable during the entire period of lymphocyte administration. The owners of these dogs considered this new concept of antitumor therapy acceptable. This shows that the QOL of these tumor-bearing dogs was maintained in a relatively favorable state, with no evidence of any adverse effects. Activated lymphocyte therapy was therefore thought to be applicable and effective as an adjuvant antitumor therapy; moreover, it maintained the QOL in immunosuppressed tumor-bearing dogs.

In conclusion, $CD56^+$ cells existed in canine peripheral blood, and these cells proliferated during the process of activation to lymphocytes. Expression of canine CD56 mRNA possibly indicates the activation of lymphocytes. The conditions of activation of lymphocytes were then optimized, using anti-canine CD3 antibody, hrIL-2, and hrIFN-α in healthy and tumor-bearing dogs. Sequential administration of these activated lymphocytes was an applicable and effective therapy for tumor-bearing small animals with immunosuppression induced by malignant tumors.

Original papers of this thesis appeared in *J. Vet. Med. Sci.,* **70**: 309-312 (2008) and *J. Vet. Med. Sci.,* **70**: 581-588 (2008).

Studies on the diagnosis and molecular epidemiology of avian influenza

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On the outbreak of avian influenza, rapid diagnosis is critical not only for the control of HPAI but also for human health. In the present study, a rapid diagnosis kit based on immunochromatography for the detection of H7 hemagglutinin (HA) antigen of influenza A virus was developed using 2

monoclonal antibodies that recognize different epitopes on the H7 HAs. The kit detected each of the tested 15 H7 influenza virus strains and did not react with influenza A viruses of the other subtypes than H7 or other avian viral and bacterial pathogens. The kit detected H7 HA antigen in the swabs and tissue homogenates of the chickens experimentally infected with HPAIV strain A/chicken/Netherlands/2586/2003 (H7N7). The results indicate that the present kit is specific and sensitive enough for the diagnosis of HPAI caused by H7 viruses, thus, recommended for the field application as a pen-site test kit.

During 2000-2007, 218 influenza viruses of 28 different combinations of HA (H1-H13) and NA (N1-N9) subtypes were isolated from fecal samples of free-flying water birds at two distant lakes in Hokkaido, Japan. Phylogenic analysis of the matrix (M) genes of 67 strains, selected on the basis of their subtype combinations, revealed that A/duck/Hokkaido/W95/2006 (H10N8) was a reassortant whose M and NA genes belonged to North American non-gull-avian and the other 6 genes to Eurasian non-gull-avian lineages. The M genes of other 65 strains belonged to Eurasian non-gull-avian and the one to Eurasian-gull lineages. The M genes of 65 strains were grouped into 3 different sublineages, indicating that influenza viruses circulating in different populations of free-flying water birds have evolved independently in nature.

A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04 (H5N1)) do not replicate in pigs (Isoda *et al.* 2006). The genetic basis for such host-range restriction was determined using swine influenza virus as a donor virus, since swine influenza viruses have been reported to be circulating in pigs which can act as potential donors. The inoculum containing the reassortant viruses was prepared between A/swine/Hokkaido/2/1981 (H1N1) (Sw/Hokkaido/81 (H1N1)) and Ck/Yamaguchi/04 (H5N1) in embryonated eggs. The reassortant viruses capable of replication in pigs were selected by administering the inoculum intranasally into pigs. Two single gene reassortant virus clones of H5N1 subtype (clones1 and2) whose PB2 genes were derived from Sw/Hokkaido/81 (H1N1) and the remaining 7 genes from Ck/Yamaguchi/04 (H5N1), were recovered. These virus clones replicated in pigs upon re-inoculation. This finding was further supported by using rg-H5N1-PB2H1N1virus of same gene constellation as virus clones 1 and 2.

Original papers of this thesis appeared in *Virus Genes,* **37**: 144-152 (2008) and *J. Vet. Med. Sci.,* **70**: 557-562 (2008).