

## Expression of LacZ Gene in Canine Muscle by Intramuscular Inoculation of a Plasmid DNA

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**ABSTRACT.** DNA immunization induces systemic humoral and cellular immune responses to the antigen encoded by cDNA in a plasmid DNA. In the present study, a plasmid DNA encoding cDNA of  $\beta$ -galactosidase ( $\beta$ -gal), pCAGGS-lacZ, was inoculated intramuscularly to a healthy dog in order to evaluate location and duration of the gene expression. On day 7, the plasmid DNA was found by PCR in the muscle where the plasmid was injected. Furthermore,  $\beta$ -gal expression was detected in the same muscle sample by  $\beta$ -gal staining. However, the plasmid DNA was not detected in any samples collected on days 14, 21 and 28. The present results suggest that duration of the gene expression of  $\beta$ -gal by the plasmid DNA is limited in the muscle in dogs and an efficacy for a gene expression should be evaluated depending on the gene inserted in the plasmid DNA for immunotherapy.

**KEY WORDS:** canine, DNA immunization, expression of  $\beta$ -galactosidase.

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Immunization with a plasmid DNA is known to induce alternation of immune responses to the antigen expressed in cells transfected by the plasmid DNA [8]. Gene expression in the cells inoculated with a plasmid DNA has been thought to be important to induce antigen-specific cytotoxic lymphocyte (CTL) responses [3]. In a mouse model of asthma, antigen-specific IgE synthesis and airway hyperresponsiveness were suppressed by an immunization with a plasmid DNA encoding  $\beta$ -gal [4]. These results suggested that DNA immunization might have a potential to provide therapeutic immune responses as a treatment of allergic diseases. Among various routes of a plasmid DNA immunization, intramuscular inoculation appears to be convenient for gene delivery because the gene expression was easily detected within the muscle cells in mice [9] and monkeys [1]. In dogs, however, there has been no report to demonstrate a gene expression in the cells transfected by a plasmid DNA *in vivo*. In advance of clinical applications of DNA immunization in dogs, therefore, it is necessary to evaluate whether a gene expression of a plasmid DNA can occur in dogs. In this study, a plasmid DNA encoding  $\beta$ -gal was inoculated intramuscularly into a healthy dog and dissemination of the plasmid DNA and  $\beta$ -gal expression in various tissues were examined.

**Preparation of the plasmid:** The  $\beta$ -gal expression vector, pCAGGS-lacZ, was constructed by inserting the lacZ gene into the pCAGGS (a kind gift of Toshihiro Tsukui, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) backbone, which contains a cytomegalovirus enhancer and a chicken  $\beta$ -actin promoter [6]. Briefly, the lacZ gene was amplified by PCR from pcDNA3.1D/V5-His/lacZ (Invitrogen Corp., Carlsbad, CA, U.S.A.) using primers: forward primer containing *Xho*I site 5'-CCCCTCGAGATGATAGATCC-3' and reverse primer containing *Not*I site 5'-ACTGCGGC-

CGCTCATTTTTTGACACCAGACC-3'. After a digestion with *Xho*I and *Not*I, the PCR product was ligated into pCAGGS using a commercially available kit (DNA Ligation Kit ver. 2, TAKARA BIO Inc., Shiga, Japan). The plasmid was grown in *Escherichia coli* competent cells (Top10: Invitrogen Corp.) and purified using a commercially available kit (Qiagen Endotoxin Maxi kit, Qiagen, Chatsworth, CA, U.S.A.). The plasmid was finally resuspended in endotoxin-free saline at a concentration of 1 mg/ml.

**Procedure of plasmid inoculation:** A healthy dog kept for experimental purposes was used in the present study under an approved protocol by the Institutional Animal Care and Use Committee at the University of Tokyo. Inoculation of the plasmid DNA and biopsy of muscle were performed under the sedation with intramuscular injection of medetomidine (Domitor®, Orion Corporation, Espoo, Finland) at a dose of 0.04 mg/kg and midazolam (Dormicum®, Roche, Basel, Switzerland) at a dose of 0.3 mg/kg. Hair on the right and left femoral regions was clipped, followed by routinely surgical preparation. For the plasmid inoculation into the biceps muscle of thigh, a small skin incision was performed on both left and right lateral thighs. A total of 4 injection sites were obtained in each biceps muscle on both thighs (2 injection sites in each thigh) and 0.5 ml of the plasmid solution was injected into each site. After the injection, the sites of the injection were sutured with non-absorbable surgical suture, which was used as a marker for identification of the sites.

**Procedure of biopsy:** Biopsy of the muscle was performed on days 7, 14, 21 and 28, using a 4-mm disposable punch (DERMAPUNCH®, Nipro Medical Industries Ltd., Tokyo, Japan). The half of the muscle samples was immediately snap-frozen in liquid nitrogen and stored at -80°C until use of DNA extraction. Another sample was embed-

ded in OCT compound (Tissue-Tek®, Sakura Finetechnical Co., Ltd., Tokyo, Japan) in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until staining of  $\beta$ -gal. On day 28, the dog was euthanized by intravenous administration of approximately 200 mg/kg of thiopental sodium (Ravonal, Tanabe Seiyaku Co., Ltd., Osaka, Japan) and 10 ml of 15% potassium chloride (K.C.L INJECTION No. 1, MARUISHI Pharmaceutical Co., Ltd., Osaka, Japan) and samples of various tissues including lung, thymus, heart, liver, spleen, stomach, small intestine, colon, lymph nodes (mesenteric, popliteal and inguinal) and skin were obtained for PCR analysis. These tissues were handled the same as described in the muscle samples.

**PCR analysis of tissues:** Polymerase chain reaction was performed to detect the plasmid DNA in all tissue samples obtained in this study. Total DNA was extracted from the muscle (4 injection sites), heart, lung, thymus, liver, spleen, stomach, small intestine, colon, lymph nodes (popliteal, inguinal and mesenteric) and skin with a commercially available kit (DNA Purification kit, Promega, Madison, WI, U.S.A.). A set of primers specific to the plasmid backbone of lacZ gene were made to amplify the region between the 5' and 3' end of the lacZ gene: forward primer 5'-CGCG-TAATACGACTCACTATAG-3' and reverse primer 5'-TGCGGCCGCTCATTTTGTACACCAGACCAA-3'. As an internal control, canine GAPDH DNA was amplified, using a primer pair: forward primer, 5'-CTCATGACCA-CAGTCCATGC-3' and reverse primer, 5'-TGAGCTTGACAAAGTGGTCA-3'. The PCR amplifications consisted of pre-denature ( $95^{\circ}\text{C}$ , 2 min) and 35 cycles of denaturation ( $95^{\circ}\text{C}$ , 1 min), annealing ( $60^{\circ}\text{C}$ , 1 min) followed by extension ( $72^{\circ}\text{C}$ , 7 min) with a commercially available kit (RNA PCR Kit: PERKIN ELMER, Branchburg, NJ, U.S.A.). The PCR products were electrophoresed through agarose gel (1%) and stained with ethidium bromide for visualization.

**$\beta$ -gal staining:** Using the samples embedded in OCT compound, serial sections at a thickness of  $5\ \mu\text{m}$  along a transverse direction were made with a cryostat (Leica CM1900, Leica Microsystems, Nussloch, Germany). The sections were stained with a commercially available kit ( $\beta$ -gal Staining Set, Roche Diagnostics Corporation, Indianapolis,

IN, U.S.A.) to visualize expression of  $\beta$ -gal.

During the study period, the dog showed no abnormal findings on physical examination, routine blood test and hematochemistry (data not shown). To examine distribution of plasmid DNA, DNAs were extracted from muscle and various tissues. Results of the PCR analysis revealed that the plasmid was detected only in the muscle sample that was collected on day 7, whereas no plasmid was found in any muscle samples that were obtained on days 14, 21 and 28 (Fig. 1). Furthermore, the plasmid was not detected in any other tissue samples obtained at necropsy on day 28. Since the plasmid was present in the muscle sample biopsied on day 7,  $\beta$ -gal expression was histologically evaluated on a section originated from the same muscle sample. Results of the  $\beta$ -gal staining revealed that small number of muscle cells (2/100 cells) expressed  $\beta$ -gal (Fig. 2).

In the present study, PCR analysis showed that the plasmid DNA remained in the inoculated site on day 7, but did not exist on days 14, 21 and 28. At the necropsy on day 28, the plasmid was not detected in any tissues examined, suggesting that the plasmid was excluded from the body or fully destructed. A recent study in sheep indicated that the plasmid DNA with intramuscular inoculation was rapidly disseminated into regional lymph nodes via blood within 10 min and the plasmid was detected by PCR at the site of inoculation up to 54 days after the inoculation, however, none of the gene expression was confirmed in these samples [5]. In the present study, the gene expression was detected in the muscle sample on day 7, where the presence of the plasmid was confirmed by PCR, however, the number of cells expressing  $\beta$ -gal was extremely low. Since the amount of the plasmid inoculated into each site was  $500\ \mu\text{g}$  and was the same amount used in sheep [5], our dose should have been appropriate. Therefore, the limited gene expression by the plasmid DNA in a small number of the cells and a short period of 7 days may be due to low efficacy of transfection or degradation of the plasmid DNA by deoxyribonuclease distributed in tissues [7]. It is also possible that the transfected muscle cells might be immunologically destructed by CD8+ CTLs as reported in mice [2]. Thus, various issues regarding gene expression by a plasmid DNA still remain

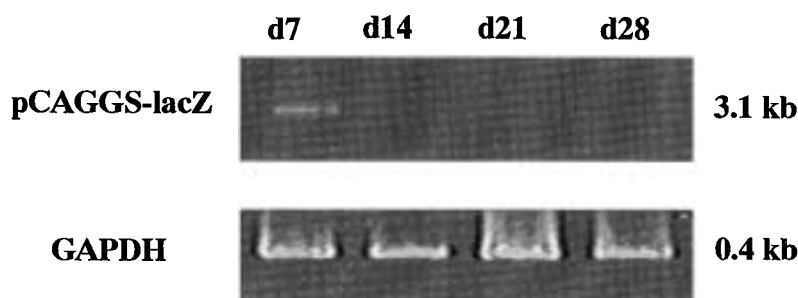


Fig. 1. Polymerase chain reaction (PCR) analysis of muscle samples biopsied from a dog on 7 (d7), 14 (d14), 21 (d21) and 28 (d28) days after the inoculation of the plasmid (pCAGGS-lacZ). Plasmid (upper lanes) and canine GAPDH (lower lanes) DNAs were detected by PCR using primers specific to pCAGGS-lacZ and canine GAPDH DNAs, respectively.

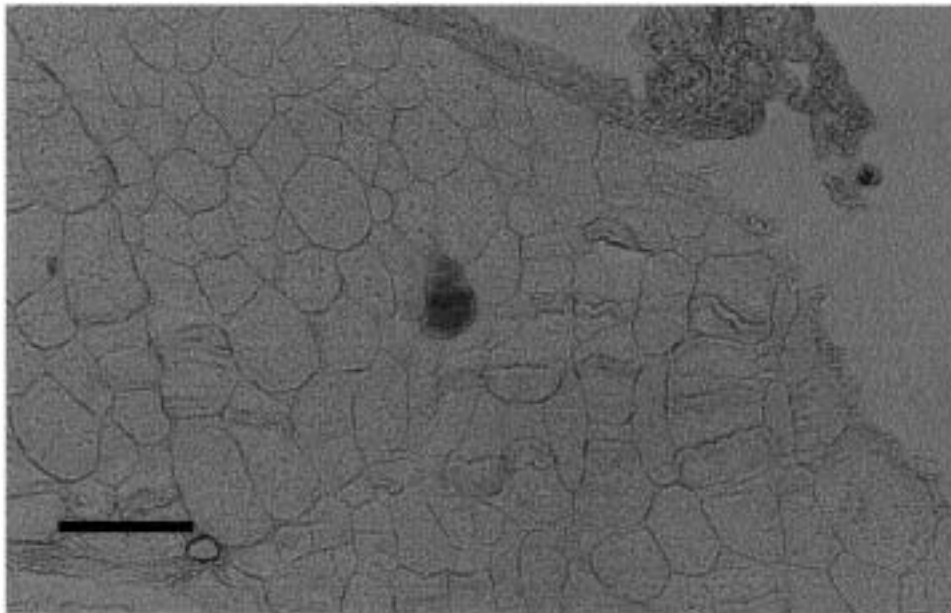


Fig. 2. Staining of  $\beta$ -galactosidase ( $\beta$ -gal) in a muscle sample biopsied from a dog on 7 days after the inoculation of the plasmid (pCAGGS-lacZ). A  $\beta$ -gal-positive-cell is indicated with gray. Magnification,  $\times 100$ . Bar, 100  $\mu$ m.

unclear. *In vivo* immune responses to a protein expressed by a plasmid DNA might be induced regardless of distribution of the plasmid DNA. In advance of clinical applications in dogs, antibody responses and profiles should be evaluated to assure that antigen specific immune responses can occur without a long-sustained gene expression.

In conclusion, the present study revealed that the duration of  $\beta$ -gal gene expression in the muscle cells transfected by the plasmid DNA was limited as long as 7 days after the inoculation in the dog. As a further investigation, it is necessary to evaluate the distribution of the plasmid DNA and the gene expression in other tissues during the first 7 days following the inoculation in order to know a balance between transfection and exclusion of the plasmid DNA in dogs.

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#### REFERENCES

1. Boyer, J. D., Wang, B., Ugen, K. E., Agadjanyan, M., Javadian, A., Frost, P., Dang, K., Carrano, R. A., Ciccarelli, R. and Coney, L. 1996. *J. Med. Primatol.* **25**: 242–250.
2. Davis, H. L., Millan, C. L. and Watkins, S. C. 1997. *Gene Ther.* **4**: 181–188.
3. Gregersen, J. P. 2001. *Naturwissenschaften* **88**: 504–513.
4. Horner, A. A., Nguyen, M. D., Ronaghy, A., Cinman, N., Verbeek, S. and Raz, E. 2000. *J. Allergy Clin. Immunol.* **106**: 349–356.
5. Mena, A., Andrew, M. E. and Coupar, B. E. 2001. *Immunol. Cell Biol.* **79**: 87–89.
6. Niwa, H., Yamamura, K. and Miyazaki, J. 1991. *Gene* **108**: 193–199.
7. Peitsch, M. C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H. R., Mannherz, H. G. and Tschopp, J. 1993. *Embo J.* **12**: 371–377.
8. Raz, E., Tighe, H., Sato, Y., Corr, M., Dudler, J. A., Roman, M., Swain, S. L., Spiegelberg, H. L. and Carson, D. A. 1996. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 5141–5145.
9. Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner, P. L. 1990. *Science* **247**: 1465–1468.