

Vaccination with Melanoma Cells Infected with Recombinant Newcastle Disease Virus Suppresses Tumor Metastasis

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Abstract

Newcastle Disease Virus (NDV) is an RNA virus, which infects several tumor cells and shows cellular toxicity towards them. The antitumor activity of NDV has been reported in several tumors. In this study, we evaluated the antitumor effect of a NDV-infected melanoma cell vaccine on lung metastasis based on tumor-specific immune responses in a mouse model.

B16 mouse melanoma cells were infected with the GFP-expressing recombinant NDV (rNDV) to prepare the vaccine (rNDV-BV). C57BL/6 mice were then immunized twice with rNDV-BV and intravenously inoculated with B16 cells, and the number of metastasis dots in the lungs was evaluated 21 days later. The mice were divided into three groups: pre-inoculation (mice were vaccinated before inoculation with B16 cells), post-inoculation (mice were vaccinated after inoculation with B16 cells), and control (mice were inoculated with DMEM) groups. To evaluate the immune responses, the mouse splenocytes were monitored for lymphocyte subsets and dendritic cells, and IFN- γ and IL-10 gene expression after metastasis was measured.

The mice receiving rNDV-BV showed prolonged survival and a lower number of metastasis dots. Furthermore, lung metastasis was significantly decreased upon post-metastasis vaccination with rNDV-BV. In pre-inoculation group, cytokine responses against tumor antigens were also significantly affected: IFN- γ levels were increased, but IL-10 levels were decreased. The vaccination also increased the T cell population along with the number of CD8+ dendritic cells during early metastasis.

These results indicated that rNDV-BV induced an IFN- γ response against the tumor antigen and suppressed metastasis in the mouse model.

Keywords: Autologous-tumor Vaccine; Newcastle Disease Virus; Melanoma Lung Metastasis Mouse Model; Cell-Mediated Immunity; IFN- γ ;

Abbreviations: NDV: Newcastle Disease Virus; rNDV: Recombinant Newcastle Disease Virus; rNDV-BV: Recombinant Newcastle Disease Virus to Prepare the Vaccine; B16: B16 Mouse Melanoma Cell; IFN: Interferon; IL: Interleukin.

Introduction

Tumor metastasis is a grave issue after surgical operations, and tumor vaccination protocols have been used to manage such tumor metastasis [1]. Some tumor vaccination using an autologous-tumor application method is currently being in clinical trials [2,3]. Autologous-tumor vaccines are supposed to induce tumor-specific cellular immunity, but their antigenicity is usually insufficient to induce a high enough level of such immunity [4,5]. The presence of some immunosuppressive environments in cancer patients has a negative effect and is inefficient to induce cellular immunity against the tumor antigen [6]. Based on this principle, the immunogenicity of tumor vaccines using autologous tumor cells can be enhanced by modifying antigens or applying effective adjuvants to the tumor cells. One of the effective adjuvant for improving the immunogenicity of tumor cell vaccines is using microorganisms such as viruses and bacteria, which has been investigated in some studies for immune stimulation against tumor antigens [7,8].

Newcastle disease virus (NDV) is a non-segmented negative RNA virus belonging to the *Paramyxoviridae* family and causes respiratory disease in chickens [9]. Interestingly, NDV selectively proliferates in mammalian tumor cells and shows oncolytic activity against several tumors [10,11]. In addition, tumor cells infected with NDV induce the production of cytokines such as type I interferon (IFN) and TNF- α and increase the expression of MHC class I and class II molecules [12,13].

The immune response of anti-tumor effects leading to the virus infection is much hope for another choice of tumor therapy. Previously, we generated a recombinant infectious virus using reverse genetics from the cDNA clone of the genome of a NDV vaccine strain (B1) and showed that this recombinant virus could be an effective vaccine vector [14]. In another study, we then modified this recombinant virus to express green fluorescent protein (GFP) (rNDV-GFP). The rNDV-GFP showed cytotoxicity against B16 mouse melanoma cells (B16) in vitro as well as direct cell killing activity against canine tumors, without producing any infectious virus particles, since the cleavage site in the amino acid sequences of the F protein had been transformed to an attenuated

virus type (G-R-Q-G/S-RJL) [10].

In the present study, we aimed to evaluate the efficacy of rNDV-GFP infected B16 melanoma cell vaccine against lung metastasis in a mouse model and to examine the subsequent tumor-specific cellular immune responses.

Methods

Mouse Model of B16 Melanoma Lung Metastasis

To generate the mouse models of melanoma lung metastasis, C57BL/6 mice were transfused intravenously with 1×10^5 B16 cells and reared in BBH isolator units. All the mice were controlled access to sterilized feed and water during the observation period.

Tumor Cell Vaccine

B16 mouse melanoma cells were cultured with DMEM (Sigma, USA) supplemented with 10% fetal calf serum (Biological industries, USA). To prepare the vaccine, B16 cells were infected with rNDV-GFP at a MOI of 2. Next, the infected cells were incubated for 24 hours and washed with PBS (Sigma, USA), followed by irradiation with UV-C (40 mJ/cm^2) using HL-2000 Hybri Linker (BM bio, JAPAN) to inactivate the tumor cells to generate the vaccine (rNDV-BV).

Evaluation of the Anti-Metastasis Effect of the rNDV-BV Vaccination

To generate a mouse model of melanoma metastasis, B16 cells (1×10^5 /mouse) were inoculated intravenously into C57BL/6 mice (8-10 weeks of ages). To evaluate the efficacy of the rNDV-BV vaccination, the mice were divided into three groups (pre-inoculation group, post-inoculation group, control group; 5 mice in each group). The pre-inoculation group was vaccinated with rNDV-BV (2×10^5 /mouse) twice at a 1-week interval before B16 inoculation. The control group was inoculated with DMEM. The mice from both these groups were evaluated for survival and metastasis in the lungs at 21 days after tumor inoculation. To examine post-metastasis vaccination, the rNDV-BV vaccination was carried out at 3 and 10 days post-metastasis in the post-inoculation group, and on day 21, the mice in this group were evaluated for lung metastasis. The number of metastasis dots in the lungs was counted under a stereo microscope to evaluate the efficacy of the rNDV-BV vaccine. Lymphocyte subsets in the splenocytes were also examined by flow cytometry using anti-mouse CD4-FITC monoclonal antibody (BECKMAN COULTER, USA), anti-mouse CD8a/Lyt-2-PE antibody (BECKMAN COULTER, USA), and anti-mouse CD11c-FITC antibody (MiltenyiBiotec, Germany). Flow cytometry analysis was performed using an argon ion laser at 488 nm (FITC) and 565 nm (PE). FITC (PE) were measured on the FL1 (FL2) channel and a histogram of the log FL1 (FL2) (X-axis) versus the number of cells (Y-axis) was generated.

Cytokine Gene Expression

RT-realtime PCR was used to examine the splenocytes (1×10^5) from the immunized mice for IFN- γ and IL-10 gene

expression under stimulation with $1 \mu\text{g/ml}$ of the melanoma antigen, gp100 (PepTivator gp100/Pmel17, MiltenyiBiotec, Germany), for 6 hours. We confirmed IFN- γ and IL-10 gene expression levels in both CD4+ and CD8+ cells. Magnetic separation method (auto MACS Separator, MACS MiltenyiBiotec, Germany) was applied to separate the cells using CD4 (Mouse CD4 (L3T4) MicroBeads, MiltenyiBiotec, Germany) and CD8 (Mouse CD8a (Ly-2) MicroBeads, MiltenyiBiotec, Germany). RNA was extracted from the separated cells using an RNA extraction kit (Qiagen RNeasy Mini kit, Qiagen, Japan), and then the RNA was used for the evaluation of gene expression by Rotor-GeneSYBER green RT-PCR kit (Qiagen Rotor-Gene SYBR Green PCR Kit, Qiagen, Japan) with the Roter-Gene Q. The data were normalized with GAPDH gene expression. The following PCR primers were used for measuring cytokine gene expression: IFN- γ , sense 5'-TGAAAGCCTAGAAAGTCTGAATAA-3', antisense 5'-GTTGTTGCTGATGGCCTGAT-3'; and GAPDH sense 5'-CGTGAGTGGAGTCATACTGGAA-3', antisense 5'-AACGGATTGGCCGTATTG-3'. IL-10 primers (RT2 qPCR Primer Assay, Qiagen, JAPAN) were purchased for measuring IL-10 gene expression.

Statistical analysis

The data were evaluated for the significance of difference using Student's t-test by comparing the vaccinated and unvaccinated groups.

Results

Comparison of survival rate and the number of lung metastases upon vaccination

Melanoma metastases in the lungs were observed in all the inoculated mice, and the mice began to die after 24 days. All the mice were dead at 32 days post-metastasis (Figure 1A). On an average, 85 metastasis dots were observed in the mouse lungs on day 21 after B16 metastasis.

The survival rates in the pre-inoculation mice significantly increased from 0% to 80% in 32 days; the number of lung metastases significantly decreased to 17.4 dots at 21 days (80% decrease, 85 to 17.4 dots on an average) (Figure 1A and 1B, $P < 0.01$).

In the post-inoculation group, vaccination with rNDV-BV significantly reduced the number of metastases dots in the lung by about 64% (85 to 31 dots on average) (Figure 1C, $P < 0.01$).

Tumor antigen-specific cytokine responses in the mouse splenocytes

We then examined IFN- γ and IL-10 gene expression in the splenocytes of the control and pre-inoculation groups under stimulation with the melanoma antigen, gp100. IFN- γ expression was significantly increased in the pre-inoculation group compared to that in the control group (Figure 2A, $P < 0.01$), but IL-10 expression was significantly decreased (Figure 2B, $P < 0.05$).

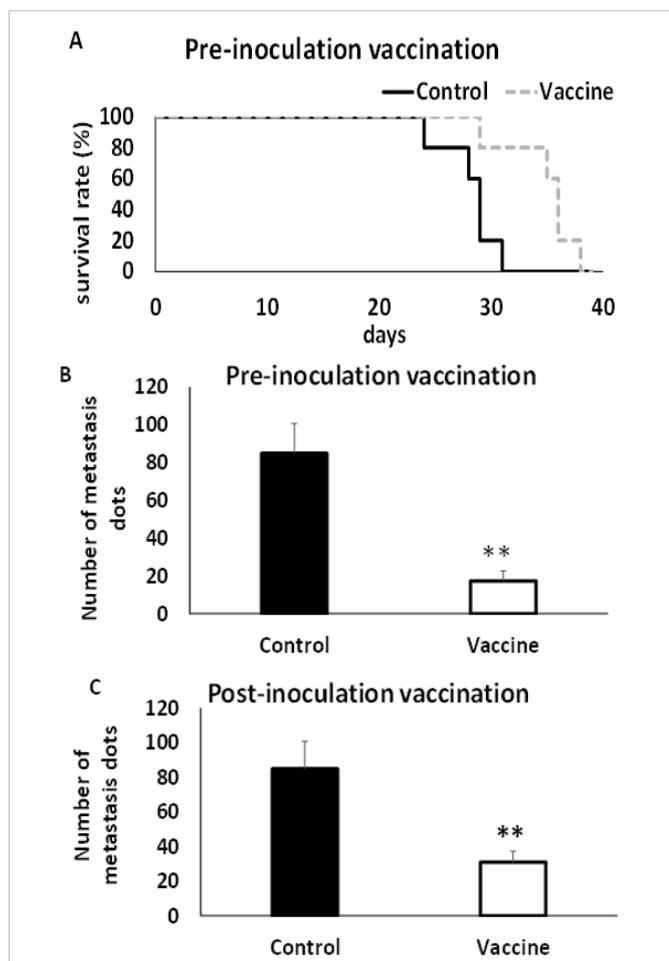


Figure 1: Comparison of survival rate and the number of lung metastases dots with and without vaccination. The pre-inoculation group was immunized twice with rNDV-BV (2×10^5 / mouse) before B16 inoculation, and the post-inoculation group was vaccinated with rNDV-BV (2×10^5 / mouse) at 3 and 10 days after B16 inoculation; the control group was inoculated with DMEM. The mice were intravenously injected with B16 melanoma cells (10^5 / mouse). The survival rate in the pre-inoculation group is shown in (A). The number of

Comparison of lymphocyte subsets during the early stage of tumor metastasis

Comparison of lymphocyte population between the control and pre-inoculation groups revealed that the percent of T cells (CD4+ and CD8+) tended to increase upon vaccination at 3 days post-metastasis. While the increase in CD4+ cells was only slight, the increase in the percent of CD8+ cells during the early stage of melanoma metastasis upon rNDV-BV vaccination was significant (Figure 3A and 3B, respectively, $P < 0.05$ for CD8+ cells).

IFN- γ and IL-10 gene expression in T cells and the ratio of CD8+ dendritic cells in the splenocytes

We next compared cytokine gene expression in the T cells between the control and pre-inoculation groups at 3 days after metastasis and found that IFN- γ gene expression increased in the CD4+ and CD8+ cells in the pre-inoculation group (Figure 4A), whereas IL-10 gene expression significantly decreased upon

vaccination (pre-inoculation) in the CD4+ cells (Figure 4B, $P < 0.05$). However, IL-10 gene expression in the CD8+ cells did not change upon vaccine treatment (Figure 4B).

Furthermore, flow cytometry analysis revealed that the ratios of CD8+ dendritic (CD8+ and CD11c+) cells in the splenocytes significantly increased upon vaccination (pre-inoculation group) (Figure 4C, $P < 0.05$).

Discussion

Autologous-tumor vaccines are one method of prevention against tumor metastasis [1]. These vaccines induce tumor-specific cellular immunity, but this approach is often inefficient due to inadequacy of the tumor vaccine to induce a high enough level of tumor-specific cellular immunity [4,5]. Therefore, autologous-tumor vaccines need effective adjuvants to enhance their immunogenicity. Here, we employed rNDV as an adjuvant of the autologous-tumor vaccine with an aim to improve the immunogenicity of the tumor vaccine to a level high enough to induce cellular immunity without producing any infectious virus particles [10]. To evaluate the antitumor effect of NDV as an adjuvant, we used a B16 melanoma metastasis mouse model with high lung metastasis [15].

We found that the survival rate in the melanoma-bearing mice was prolonged and that the number of metastasis dots was significantly decreased upon treatment with rNDV-BV.

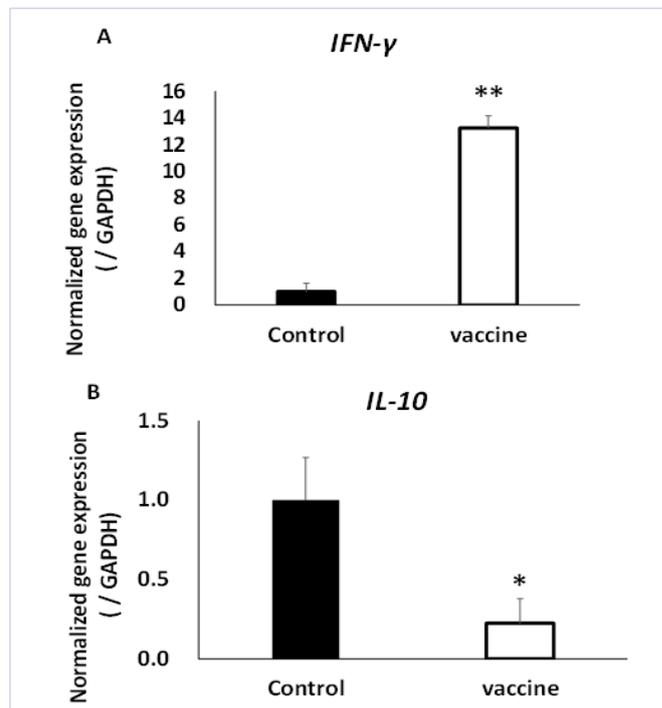


Figure 2: Tumor antigen-specific cytokine response in mouse splenocytes. Mice were divided into the pre-inoculation group (immunized twice with rNDV tumor vaccine (2×10^5 / mouse) before B16 inoculation) and the control group (inoculated only with DMEM). Splenocytes from each group were isolated 1 week after vaccination and co-cultured with gp100 antigen for 6 hours. The stimulated cells were analyzed for IFN- γ (A) and IL-10 (B) gene expression by real-time PCR. * $P < 0.05$, ** $P < 0.01$.

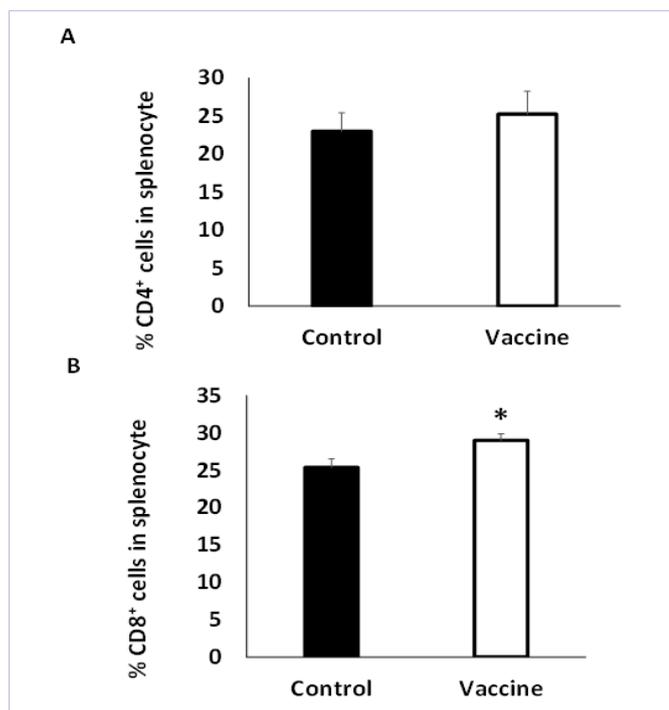


Figure 3: Comparison of lymphocyte subsets during the early stage of tumor metastasis. Mice were divided into the pre-inoculation group (immunized twice with rNDV-BV (2×10^5 / mouse)) and the control group (inoculated only with DMEM) and intravenously injected with B16 melanoma cells (10^5 / mouse) 1 week after rNDV-BV vaccination. Splenocyte were then isolated at 3 days post-metastasis. CD4⁺ cells (A) and CD 8⁺ cells (B) in the splenocytes were measured by flow cytometry. * P < 0.05.

Further, to evaluate the potential for clinical application of rNDV-BV, we performed post-metastasis vaccination and evaluated the efficacy of the vaccine to induce an anti-tumor response in the mouse model. The rNDV-BV vaccination was carried out at 3 and 10 days post-metastasis, and the number of the metastases dots was measured on day 21. The vaccination group showed a significant decrease in the number of metastasis dots in the lungs.

Next, to understand the anti-tumor effect of the vaccination, lymphocyte subsets and cytokine expression were examined in the vaccinated mouse model [16]. In general, antigen-specific cytotoxic T cell induction plays an important role in tumor exclusion by vaccine therapy, and those cells are IFN- γ producing CD4⁺ and CD8⁺ T cells [17,18].

Therefore, we monitored the change in the number of CD4⁺ and CD8⁺ T cells and the IFN- γ gene expression in these cells from mice receiving vaccine treatment (pre-inoculation group). The populations of CD4⁺ and CD8⁺ T cells were increased in the splenocytes from the vaccinated mice at 3 days after tumor metastasis. Moreover, IFN- γ gene expression in the splenocytes significantly increased in response to the tumor-specific antigen, gp100, in the pre-inoculation group. These results suggested that rNDV-BV vaccination induced cell-mediated immunity against the tumor-specific antigen.

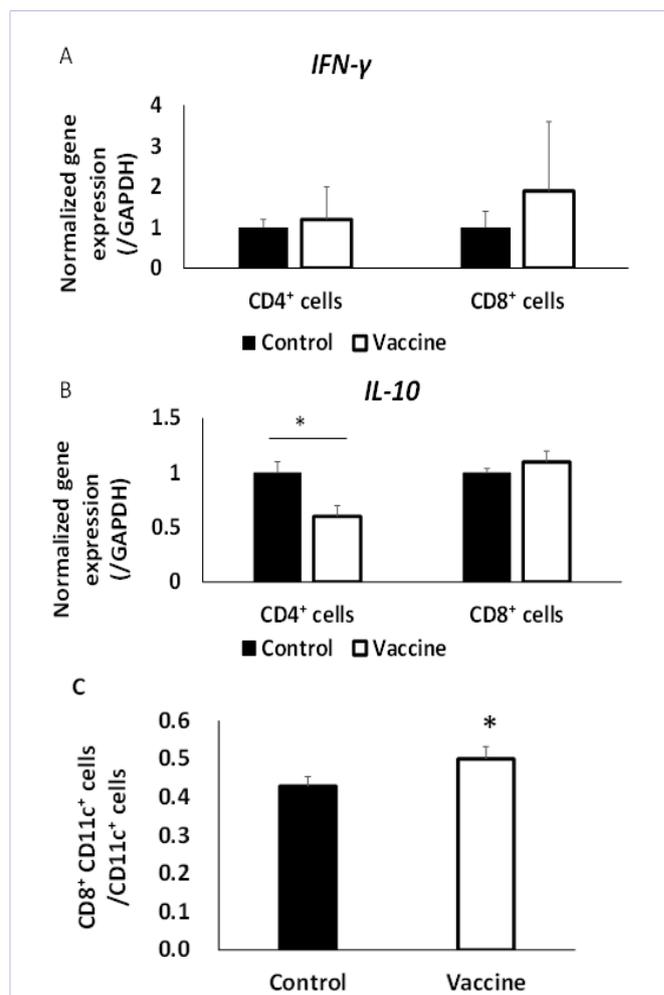


Figure 4: IFN- γ and IL-10 gene expression in T cells and the ratio of CD8⁺ dendritic cells in the splenocytes. Mice were divided into the pre-inoculation group (immunized twice with rNDV tumor vaccine (2×10^5 / mouse)) and the control group (inoculated only with DMEM) and intravenously injected with B16 melanoma cells (10^5 /mouse) 1 week after rNDV-BV vaccination. Splenocytes from each group of mice at 3 days post-metastasis were divided into CD4⁺ cells and CD8⁺ cells using the magnetic bead method. Expression of IFN- γ (A) in CD4⁺ cells and CD8⁺ cells, and IL-10 (B) in CD4⁺ cells and CD8⁺ cells was measured by real-time PCR. The ratio of CD8 and CD11c double-positive cells in the CD11c⁺ cells is shown in (C) to indicate the change in the number of dendritic cells in the splenocytes upon rNDV-BV vaccination, determined using flow cytometry analysis. * P < 0.05.

IL-10 inhibits tumor-specific T-cell activity in cancer patients [19,20]. In the present study, we found that IL-10 gene expression in the splenocytes was increased in the melanoma metastasis mouse model. Thus, we presumed that the induction of immunosuppressive is related to melanoma metastasis. However, in the vaccination group (pre-inoculation group), IL-10 gene expression in the splenocytes stimulated with gp100 was significantly decreased. A decrease in IL-10 gene expression was also observed in the CD4⁺ cells from the metastasis mouse model after vaccination. These results implied that rNDV-BV treatment repressed IL-10 gene expression in the tumor-bearing mice,

which is likely the mechanism by which the rNDV-BV vaccine suppressed metastasis.

Tumor immunotherapy is known to increase the number of CD8⁺ dendritic cells and thus induce antitumor effect [21,22]. In our study, during the early stage of tumor metastasis (3 days post-metastasis), the ratio of CD8⁺ dendritic cells increased in the vaccinated group (pre-inoculation group) compared with that in the control group. This result suggested that the treatment with rNDV-BV increases the number of CD8⁺ dendritic cells in the metastasis mouse model.

Although there were several reports regarding the virus therapy for tumor patients [23,24], some of the virus therapy have pathogenicity to the patients following the propagation of infectious virus particles [25]. On the other hand, the rNDV-BV could not propagate the virus particle due to the mutation of F protein cleavage site [10]. It is relatively safe and easy to prepare for various tumors. Furthermore, our results indicated that the rNDV-BV vaccine has an effect on tumor-bearing mice.

In summary, we showed that the rNDV-BV vaccine effectively induces tumor-specific cell-mediated immunity by increasing IFN- γ expression and represses melanoma metastasis and growth in the mouse model. The use of rNDV-BV provides an opportunity to develop various applications such as for gene insertions according to the modification purpose. The limitation of the study is that we carried out only melanoma mouse model and one of vaccination schedule. In the future, therapeutic treatment with rNDV-BV needs to be examined in various tumor cells with a focus on determining the optimal dose and vaccination schedule. The results of this study will serve as a platform for future research on recombinant virus vaccine therapy.

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Declarations

Ethical Approval

All the experiments were performed according to the animal care and use committee control guidelines by the Committee on the Ethics of Animal Experiments of Rakuno Gakuen University (approved number: VH21A28, VH14A5).

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