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Inhibition of influenza A virus infection by Galectin-9

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Abstract

Galectin-9 (Gal-9) inhibited the infection of H1N1, H3N2 and H5N1 influenza A viruses *in vitro* and *in vivo*. Fifty percent effective doses (ED_{50}) of Gal-9 were 0.1–0.5 μM depending on virus strains in the plaque reduction assay. Gal-9 but not Gal-1 bound to the virus particles of A/Puerto Rico/8/34 (H1N1) (PR/8), resulting in inhibition of virus attachment to the host cells. Lactose but not sucrose inhibited the binding of Gal-9 to the viruses. Endogenous Gal-9 expression was detected and increased with the course of infection with influenza A viruses in mice. Fifty percent of Gal-9-transgenic mice survived after the challenge with PR/8, while all of the wild-type mice died. Gal-9 treatment of mice affected diminishing influenza virus replication in the lungs, body weight loss and the expression level of inflammatory cytokines. Combined administration of Gal-9 and oseltamivir was more effective than the use of single compound in mouse model. The present results indicate that Gal-9 is a candidate compound for influenza A virus infection therapy.

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Introduction

Influenza A virus belongs to the *Orthomyxoviridae* of negative-strand RNA virus, infecting birds and mammals, including humans. Humans experience influenza epidemics every year, so-called "seasonal influenza", and sometimes "pandemic influenza"[9]. Attachment of influenza A virus to the host cell receptor, terminating with sialic acid on an oligosaccharide side chain of a glycoprotein or glycolipid, is mediated by the viral glycoprotein, hemagglutinin (HA). The other viral glycoprotein, neuraminidase (NA), is responsible for the release of progeny viruses from the host cell. A compound that specifically recognizes a carbohydrate chain of the viral glycoprotein should be a good antiviral drug candidate to inhibit the infection of influenza A viruses.

Galectin is a soluble form of lectin family molecule that recognizes carbohydrate chain-containing galactose by its carbohydrate recognition domain (CRD)[14,27]. It has been identified in various species of animals, including cellular slime mold, sea sponge, nematode, frogs, fish, birds and mammals. Fifteen members of the galectin superfamily have been identified in mammals and ten in humans. The galectin family proteins are divided into three types based on their structure. These are proto-type galectins which have a single CRD, chimera-type galectins which have a single CRD fused with another functional domain, and tandem-repeat-type galectins. Gal-9 forms an oligomer through the spacer region between the two CRDs. On the other hand, Gal-1 has only a single CRD[5].

Previous studies showed that mannose-binding lectin, one of the collectin family, has a CRD at C-terminus and inhibited influenza A virus. Ficolins of oligomeric lectins with subunits consisting of both collagen-like and fibrinogen-like domains, showed anti-viral activities[3]. Collectins, a C-type lectin known as a pulmonary surfactant protein, prevent the dissemination of infectious microbes by their biological activities, including agglutination and growth inhibition[12,13].

Since Galectin is known as a β-galactoside binding protein by its CRD, it is speculated that Galectin should bind carbohydrate chain of virus glycoprotein or host cell membrane and inhibit adsorption. In addition, Gal-9 is expressed in the lungs, it is postulated that Gal-9 should inhibit the infection of the cells lining the mucosal surface with influenza A viruses.

In the present study, we focused on galectin family proteins and investigated their inhibitory activity against influenza A virus infection *in vitro* and *in vivo*.

Materials and Methods

Viruses: A/Puerto Rico/8/34 (H1N1) (PR/8) and A/Aichi/2/68 (H3N2) (Aichi/68) influenza viruses were handled in a biosafety level 2 facility, A/Hong Kong/483/97 (H5N1) (HK/483) was handled in a biosafety level 3 facility under the protocols according to the Guidelines of Hokkaido University. PR/8 was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 48 hrs. Concentration and purification of viruses were performed according to Kida and Yanagawa[11]. A/Aichi/2/68 (H3N2) (Aichi/68) and A/Hong Kong/483/97 (H5N1) (HK/483) were from the repository of the OIE Reference Laboratory for Avian Influenza, Hokkaido University.

Compounds: Recombinant human Galectin-1 (Gal-1) was prepared according to Matsushita *et al.*[15]. Mutant forms of recombinant human Galectin-9 lacking the entire linker region, hG9NC (null), were expressed and purified as described by Nishi *et al.*[19].

Mice: The present experimental protocols were reviewed and approved by the Hokkaido University Animal Care and Use Committee (08-0231). C57BL/6 male mice (5 to 7 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Generation of Gal-9-transgenic mice were

prepared according to Tsuboi *et al.*[26]. To generate transgenic mice that overexpress mouse Gal-9, the cDNA encoding full-length of Gal-9, 969 bp was cloned into the mammalian expression vector, pCXN2 that contains the chicken β-actin promoter (CAG) and rabbit β-globin polyadenylation site. Then, a *Nhe*I/*Sma*I fragment containing the Gal-9 coding sequence was subcloned into the multi-cloning sites of the pCXN2 construct, yielding CAG-Gal-9.

Experimental infection of mice with influenza viruses: Gal-9-transgenic and control mice were anesthetized by inhalation of isoflurane and inoculated intranasally with 25 μl of infectious virus using pipettes. The mice were observed for mortality for 16 days. Gal-9 was administrated intranasally 7.5 μg/mouse every four days after virus inoculation. Mice were observed and weighed for 5 days after virus inoculation. Combined administration of Gal-9 and Oseltamivir started 48 hrs post-inoculation with PR/8. Administration of Gal-9 alone was done with 7.5 μg/mouse every day for 5 days. Oseltamivir alone administration was done orally 10 mg/kg/day per mouse for 5 days. In case of the combined administration, Gal-9 was administered intranasally 7.5 μg/mouse every four days and Oseltamivir was administrated orally 10 mg/kg/day per mouse for 5 days, respectively. Mice were observed and weighed for 21 days after virus inoculation.

Cells: Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Tokyo, Japan) supplemented with 10% fetal calf serum.

Assay of Gal-9 and cytokines in bronchoalveolar wash of mice: Concentration of Gal-9 in mouse bronchoalveolar wash was quantified by enzyme-linked immunosorbent assay (ELISA)[10]. Briefly, 96-well plates (Nunc, Naperville, IL, USA) were coated with monoclonal antibodies to Gal-9 (clone 108A2), blocked with 2% fetal bovine serum containing 0.05% Tween 20 in phosphate-

buffered saline (PBS), and then incubated for 1 hr at 37°C with bronchoalveolar washes of mice. After washing 3 times, Gal-9 remaining in the wells was detected by rabbit anti-mouse Gal-9 antibodies conjugated with biotin using EZ-Link Sulfo-NHS-Biotin reagent (Pierce, Rockford, IL, USA). Biotinated antibodies bound to Gal-9 were detected by streptavidin-conjugated horseradish peroxidase (Invitrogen). To stop the reaction, colorimetric substrate tetramethylbenzidine (KPL, Gaithersburg, MD, USA) was added and the optical density was read with a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). Cytokines in mouse bronchoalveolar wash were detected by quantitative ELISA using murine cytokine-specific kits for GM-CSF, INF-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α (R & D Systems, Minneapolis, MN, USA)[1].

Galectin binding assay: Ninety-six-well flat bottom plates (Corning, NY, USA) were coated with PR/8 or Aichi/68 (1×10^4, 1×10^5, 1×10^6 PFU/ml), cell culture supernatants, or PBS overnight at 4°C. Each fluid was aspirated and then blocked by 5% BSA in PBS with 0.01% sodium azide overnight at 4°C. Gal-9 was conjugated to biotin (Pierce)[18]. The plates were washed five times with PBST (PBS containing 0.05% Tween20)[10] and Biotinated-Gal-9 (final concentration 0.2, 0.4 mM) was added and incubated for 1 hr at room temperature. Wells were washed and then streptavidin-conjugated horseradish peroxidase (Bio Legend, San Diego, CA, USA) was added and incubated for 1 hr at room temperature. TMB solution (KPL) was added and incubated in the dark for 30 min. The colorimetric reaction was stopped by the addition of 0.5 M sulphuric acid. Finally, the plates were measured at OD_{450nm} with a microtiter plate reader (Immuno Mini NJ-2300; MICROTEC Co., Ltd, Chiba, Japan). Competitive binding assay was performed according to Niki *et al.*[18].

Plaque reduction assay: Plaque reduction assay was performed using MDCK cells[25]. Three-fold

serial dilutions of Gal-9 were mixed with 100–200 plaque-forming units (PFU) of virus and incubated for 15 min at room temperature. The mixture was inoculated on confluent monolayers of MDCK cells on 6-well plates. After 1 hr adsorption, the inoculum was removed and the cells were overlaid with MEM containing 1% Bacto Agar (Invitrogen) and trypsin (5 mg/ml) (Invitrogen). Plaques were counted after incubation at 35°C at 5% CO_2 for 48 hrs. The $TCID_{50}$ was determined according to the Reed and Muench method[22]. Briefly, 10 times dilutions of Gal-1 or Gal-9 were added on MDCK cell monolayers on 96-well tissue culture plates. After 30 min adsorption at 35°C at 5% CO_2, cells were washed with MEM and infected with PR/8 at 35°C at 5% CO_2 for 30 min. The supernatants were removed and the cells were washed with MEM, MEM containing 0.00025% trypsin (5 μg/ml) was overlaid on the cells, and incubated at 35°C at 5% CO_2 for 72 hrs.

Titration of virus in the lungs: Lung tissue samples were collected 1, 3 and 5 days after infection and 10% suspensions of lung homogenates were examined for virus infectivity. Serial dilutions of the samples were inoculated on MDCK cell monolayers on 12-well tissue culture plates. After 1 hr adsorption, the supernatants were removed, and MEM containing 1% Bacto Agar and trypsin (5 μg/ml) was overlaid on the cells. The plaques were counted after incubation at 35°C at 5% CO_2 for 48 hrs.

Far-Western blotting: Far-Western blotting was performed according to Asanuma *et al.*[2]. Briefly, virus proteins were separated by 10% SDS-polyacrylamide gel electrophoresis in the reducing or non-reducing condition and transferred to cellulose acetate membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 3% skimmed milk powder in PBST, exposed to Biotinated-Gal-9 (20 μg/ml) in skimmed milk in PBST, and reacted with streptavidin-conjugated horseradish peroxidase,

and the formed bands were visualized by Lightcapture (ATTO Corporation, Tokyo, Japan).

Immunofluorescence and imaging of the cells: This experiment was performed according to Fujioka *et al.*[4]. MDCK cells were plated on a collagen-coated 35 mm diameter glass-based dish (Asahi Techno Glass Co. Tokyo, Japan). After 24 hrs, cells were infected with PR/8 at a MOI and incubated with AlexaFluor488-conjugated to Gal-9 at 35°C at 5% CO_2 for 1 hr. Cells were washed with PBS and cultured with MEM at 35°C at 5% CO_2 for 3 hrs. Cells were washed with PBS and fixed in 3% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% TritonX-100 in PBS for 4 min at room temperature, and then incubated with 1% bovine serum albumin to block non-specific binding of antibodies. The cells were incubated with primary antibodies (anti-NP monoclonal antibody 2S347/3 was provided by Dr. Takada, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan) at 4°C overnight, followed by AlexaFluor594-conjugated secondary antibody (1 : 300 dilution, Molecular Probes; Invitrogen) for 1 hr at room temperature in the dark. Cells were visualized using an FV-1000 confocal microscope (Olympus, Tokyo, Japan), then analyzed by MetaMorph image processing software (Universal Imaging, Downingtown, PA, USA), followed by quantification of their fluorescence intensities.

Transmission electron microscopy: PR/8 was placed on the collodion membrane of copper grids (NISSIN EM Co., Ltd, Tokyo, Japan). After washing with PBS, the grids were incubated with Gal-1 or Gal-9 and then washed with PBS and incubated with a rabbit anti-human Gal-1 or Gal-9 polyclonal antibodies (Abcam, Cambridge, MA, USA). They were washed with PBS and incubated with goat anti-rabbit immunoglobulin conjugated to 5-nm gold particles (Sigma-Aldrich, St Louis, MO, USA). After washing with PBS, samples were stained with 2% phosphotungstic

acid solution (pH 5.8) and examined with a JEM-1210 electron microscope at 80 kV.

Hemagglutination tests: Fifty microliters of twofold serial dilutions of Gal-9 in PBS were prepared in microtiter plates. Fifty microliters of 0.5% chicken red blood cells (RBCs) were added to each wells, mixed and incubated at room temperature for 30 min. Then hemagglutination pattern was read and the titer was expressed as the reciprocal of the highest dilution of galectin that cause complete hemagglutination.

Statistical analysis: For statistical comparisons, Student's t-test and the log-rank test were used. Analysis was performed using Prism 5.0 software (GraphPad Software, Inc.).

Results

Induction of Galectin-9 in mice upon influenza A virus infection

To examine the secretion of galectin upon influenza A virus infection, the expression level of galectin was examined in the bronchoalveolar wash during the course of influenza A virus infection. PR/8 was inoculated intranasally into nine 6-week-old C57BL/6 mice and the expression level of Gal-9 in the bronchoalveolar wash was quantified by ELISA. The expression level of Gal-9 on day 5 was seven times more than that of control mice (Fig. 1).

To assess the significance of Gal-9 expression, the survival of Gal-9 transgenic (Tg) mice was tested after challenge with a lethal dose of PR/8. Four of 8 Gal-9 Tg mice challenged with 600 PFU PR/8 survived while all of the wild-type mice died by 9 days post-challenge with PR/8 (Fig. 2).

Inhibition of influenza A virus infection by Gal-9

To assess the effect of Gal-9 against influenza A virus infection, the plaque inhibition test was performed. Plaque number of PR/8 (H1N1) treated with Gal-9 reduced in a dose-
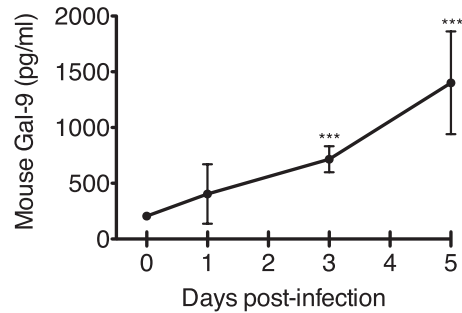

Fig. 1. Response of Gal-9 to influenza A virus infection in mice. Concentration of Gal-9 in the bronchoalveolar wash of mice infected with PR/8 was quantified by ELISA. One thousand plaque-forming units (PFU) of PR8 were inoculated intranasally into nine 6-week-old C57BL/6 male mice. Three mice were sacrificed on 1, 3, and 5 dpi and subjected to Gal-9 quantitation in the bronchoalveolar wash. P-values were calculated using Student's t-test. ***, $P < 0.001$ vs. day 0.
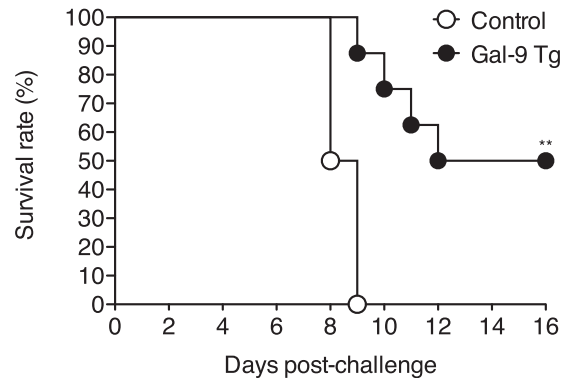

Fig. 2. Survival of Gal-9 transgenic mice after infection with PR/8. Six hundred PFU PR/8 were inoculated intranasally into six-week-old C57BL/6 wild or transgenic mice and observed clinically until 16 dpi. All 8 wild-type mice had died by 9 dpi, while transgenic mice survived until 16 dpi. Open circles: Wild-type mice, closed circles: Gal-9 transgenic mice. P-values were calculated using the log-rank test. **, $P < 0.01$ vs. control mice.

dependent manner, while treatment with Gal-1 did not affect plaque formation (Fig. 3A, 3B, 3C). Gal-3 and Gal-8 failed to inhibit binding to the virus (data not shown). In addition, the specificity of the inhibitory activity of Gal-9 against influenza A viruses of other subtypes was analyzed. Aichi/68 (H3N2) or HK/483 (H5N1) and those treated with Gal-1 or Gal-9 were inoculated onto MDCK cells. As shown in Fig. 3, Gal-9 inhibited plaque formation of H3 and H5

influenza viruses, but Gal-1 did not.

Inhibitory activity of Gal-9 against influenza A virus infection was assessed in MDCK cells. No toxic effect of Gal-9 on MDCK cells was found at a concentration of 30.3 µg/ml. Treatment of MDCK cells with Gal-9 before infection with PR/8 resulted in inhibition of virus growth in the cells (Fig. 4). The present data indicate that Gal-9 inhibits infection of host cells with influenza A virus.

Binding of Gal-9 to influenza A viruses

Gal-9 has two carbohydrate recognition domains (CDRs) with an affinity for β-galactosides. To investigate whether the binding activity to viral particles of influenza A virus is dependent on the CRDs of Gal-9, competitive binding assay was performed using biotinated-Gal-9. As shown in Fig. 5, binding activity of Gal-9 to influenza A virus was significantly inhibited by addition of lactose, but not by addition of sucrose.

To confirm that the inhibitory activity of Gal-9 to influenza A virus is associated with binding to β-galactoside in the carbohydrate chain of the glycoproteins of the viruses, a binding assay was carried out using biotinated-Gal-9 or -Gal-1 (Fig. 6). The results indicate that both H1N1 (PR/8) and H3N2 (Aichi/68) viruses, which were plate-coated, trapped Gal-9, but not Gal-1. These results indicate that Gal-9 binds to influenza A viruses, but Gal-1 does not.

It was analyzed whether Gal-9 binds to PR/8 by electron microscopy using colloidal gold. The results showed that viruses treated with Gal-9 bound colloidal gold, while those treated with Gal-1 did not. These results indicate that Gal-9 binds influenza A virus particles, but Gal-1 does not (Fig. 7). Gal-1 agglutinated 0.5% chicken RBCs at the titer of 1 : 256, and Gal-9 at 1 : 512 (data not shown).

To identify the virus protein to which Gal-9 binds, Far-Western blotting analysis was carried out. Membrane-bound Gal-9 was detected as a band at the same mobility as influenza A virus HA (Fig. 8), indicating that Gal-9 binds to virus
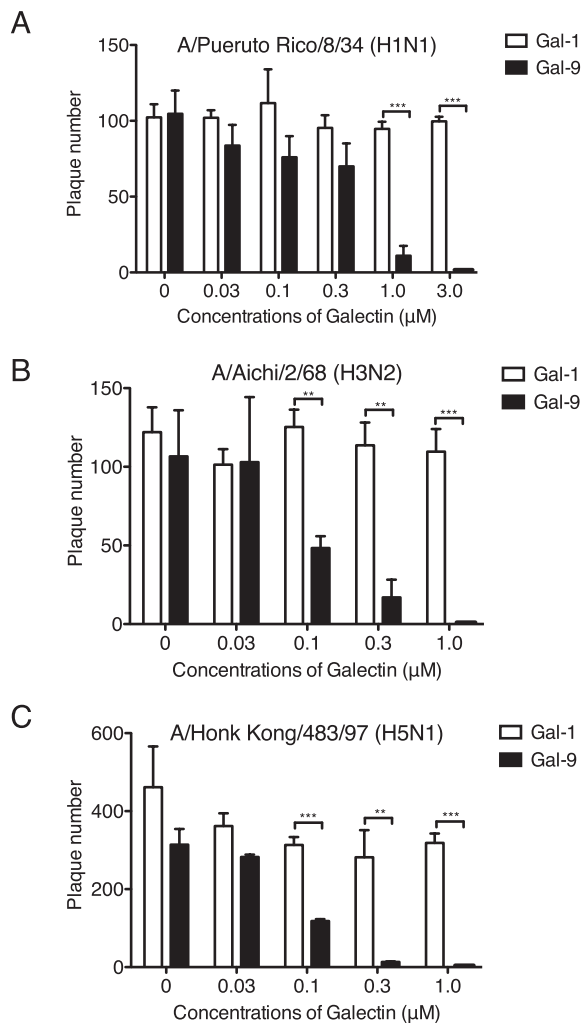

Fig. 3. **Inhibitory effect of Gal-1 and Gal-9 on the growth of influenza viruses in MDCK cells.** The cells were infected with (A) moi 10 of PR/8, (B) moi 0.01 of Aichi/68 and (C) moi 1 of HK/483. (A) (B) (C) were evaluated by plaque assay, and then plaque number counted after 48 hrs. Mean plaque numbers $\pm$ SD from triplicate tests. Open column: Gal-1, closed column: Gal-9. P-values were calculated using Student's t-test. **, P < 0.01; ***, P < 0.001.

particles through recognition of the carbohydrate chain of HA.

Inhibition of influenza A virus infection in MDCK cells

Influenza A virus binds to host cells through recognition of a sialic acid carbohydrate chain, and then the virus internalizes into the cells by endocytosis. Viruses uncoat in endosomes by membrane fusion mediated by the virus HA, and
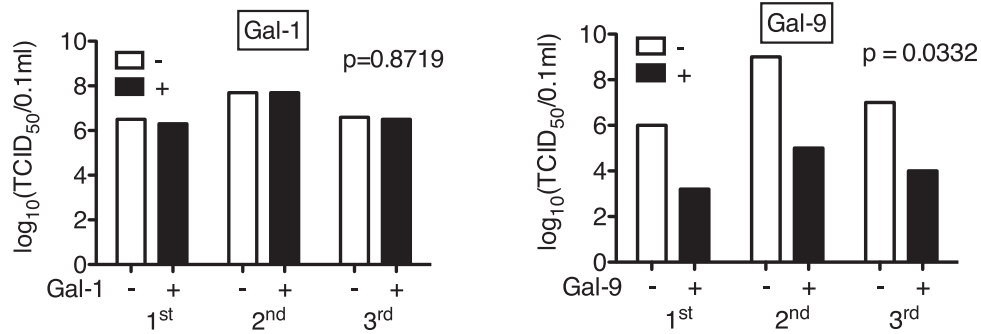
Fig. 4. Effects of pre-treatment of MDCK cells with Gal-1 and Gal-9 on the replication of PR/8 virus. Virus infectivity titers in the culture supernatants were measured by $TCID_{50}$ assay. The assay was performed 72 hrs post infection, three times. Closed column: infectivity titers of the supernatants of the MDCK cells; galectin was pretreated of the MDCK cells before infection, open column: infectivity titers of the virus not pre-treated with galectin. P-values were calculated using Student's t-test.
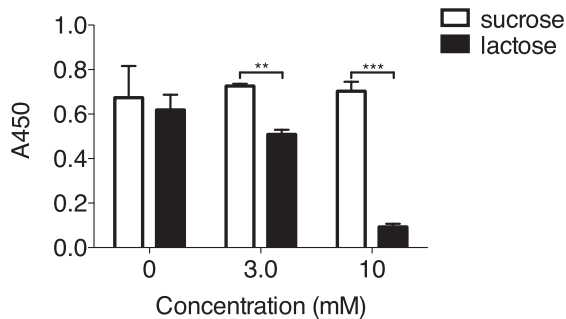
Fig. 5. Comparison of inhibition ability between biotinated-Gal-9 mixed with lactose and biotinated-Gal-9 mixed with sucrose and 10^6 PFU PR/8. This assay was performed by ELISA, in triplicate, and expressed as the mean $\pm$ SD. Open column: sucrose, closed column: lactose. P-values were calculated using Student's t-test. **, $P < 0.01$; ***, $P < 0.001$.

nucleocapsids are released into cytoplasm. To study the inhibitory mechanism of Gal-9 against influenza A virus infection, confocal laser scanning fluorescence microscopy was performed using anti-NP monoclonal antibodies to monitor the effect of Gal-9 treatment on influenza A virus entry into the cells (Fig. 9). The results showed that NP was not observed in the nuclei of cells treated with Gal-9, while they were found in control cells. The present data suggest that Gal-9 inhibits infection of cells with influenza A virus.

Protection of mice from infection with PR/8 by Gal-9

The effect of Gal-9 treatment on influenza A virus infection was then assessed using a mouse model. PR/8 was inoculated intranasally into five 7-week-old C57BL/6 male mice with or without treatment with intranasal administration of 7.5 µg Gal-9/mouse every four days, followed by monitoring virus titers in the lungs and the body weight. The results showed that virus titers in the lungs of mice treated with Gal-9 were low, especially on the day 3, and those in Gal-9 untreated mice were high (Fig. 10A). Body weight loss showed no significant change in Gal-9-treated mice, but was observed in Gal-9-untreated mice (Fig. 10B).

It is known that the expression level of inflammatory cytokines correlates with the severity of the clinical signs of mice infected with influenza A virus. Inflammatory cytokines in bronchoalveolar washes were investigated after inoculation with PR/8 (Fig. 10C). The results day 3 and day 5 showed that the expression levels of IL-6, TNF-α and IFN-γ were low in Gal-9-treated mice, but those in control mice were high. These differences were statistically significant. The present results suggest that Gal-9 protects the mice from inflammation caused by influenza A virus infection and the inhibition of infection by Gal-9 is thought to be responsible for suppression of these inflammatory cytokines.

The effect of combined administration of Gal-9 and oseltamivir treatment on influenza A virus infection after 48 hrs post-inoculation was then assessed using a mouse model. PR/8
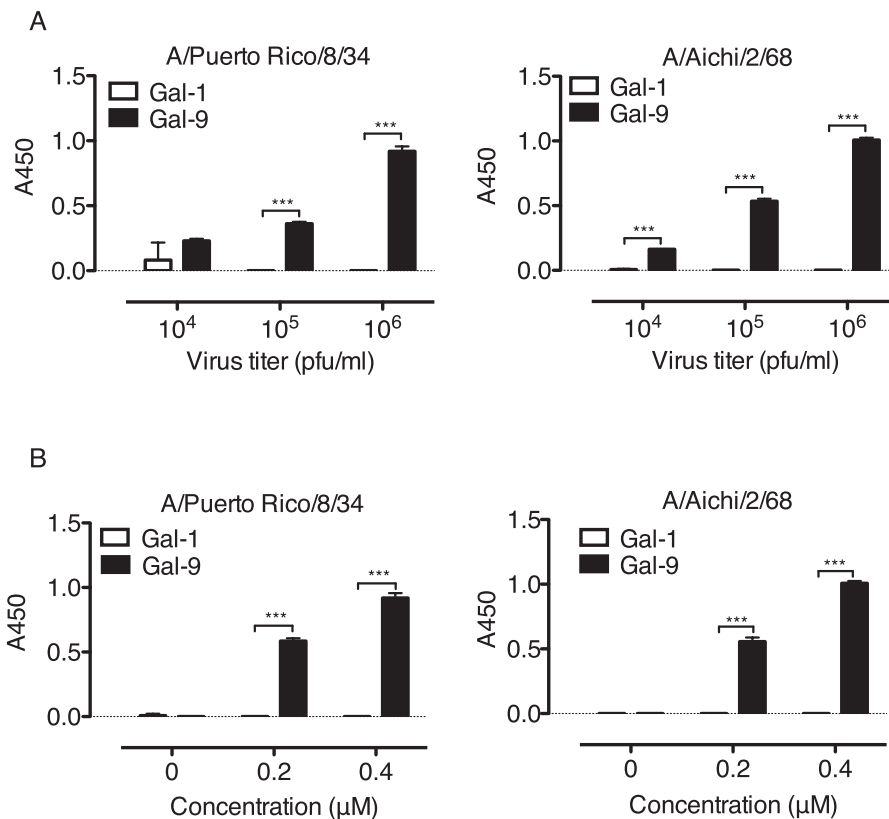
A

Fig. 6. (A) Binding of 0.4 mM biotinated-Gal-9 to different infectivity of PR/8 or Aichi/68. (B) Binding of different doses of biotinated-Gal-9 to 10^6 PFU PR/8 or Aichi/68. This assay was performed in triplicate by ELISA. The average absorbance values are expressed as the mean $\pm$ SD. Open column: biotinated-Gal-1, closed column: biotinated-Gal-9. P-values were calculated using Student's t-test. ***, P < 0.001.
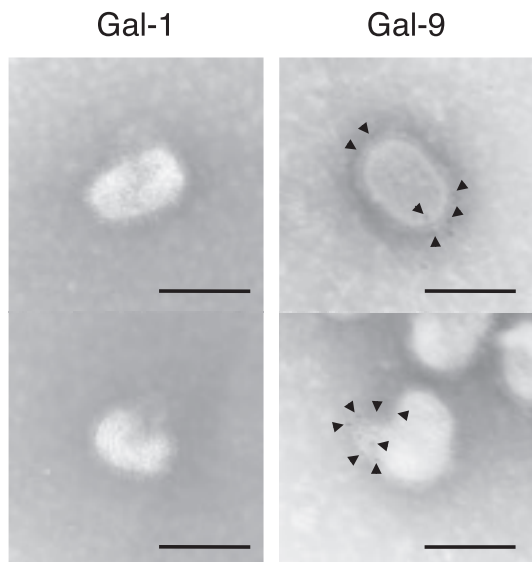

Fig. 7. Transmission electron microscopy images of influenza A virus treated with Galectins. Top and bottom images were observed in different areas of the same sample. Arrowheads, 5-nm gold particles. Bars, 100 nm.
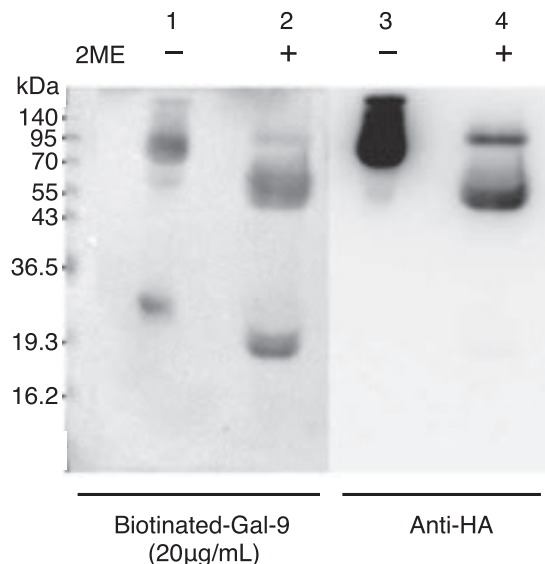
Fig. 8. Binding of Gal-9 to the virus HA. Far-Western blot showing the binding of biotinated-Gal-9 to the HA protein of PR/8. HA protein from influenza virus was resolved by SDS-PAGE, as described in Materials and Methods.
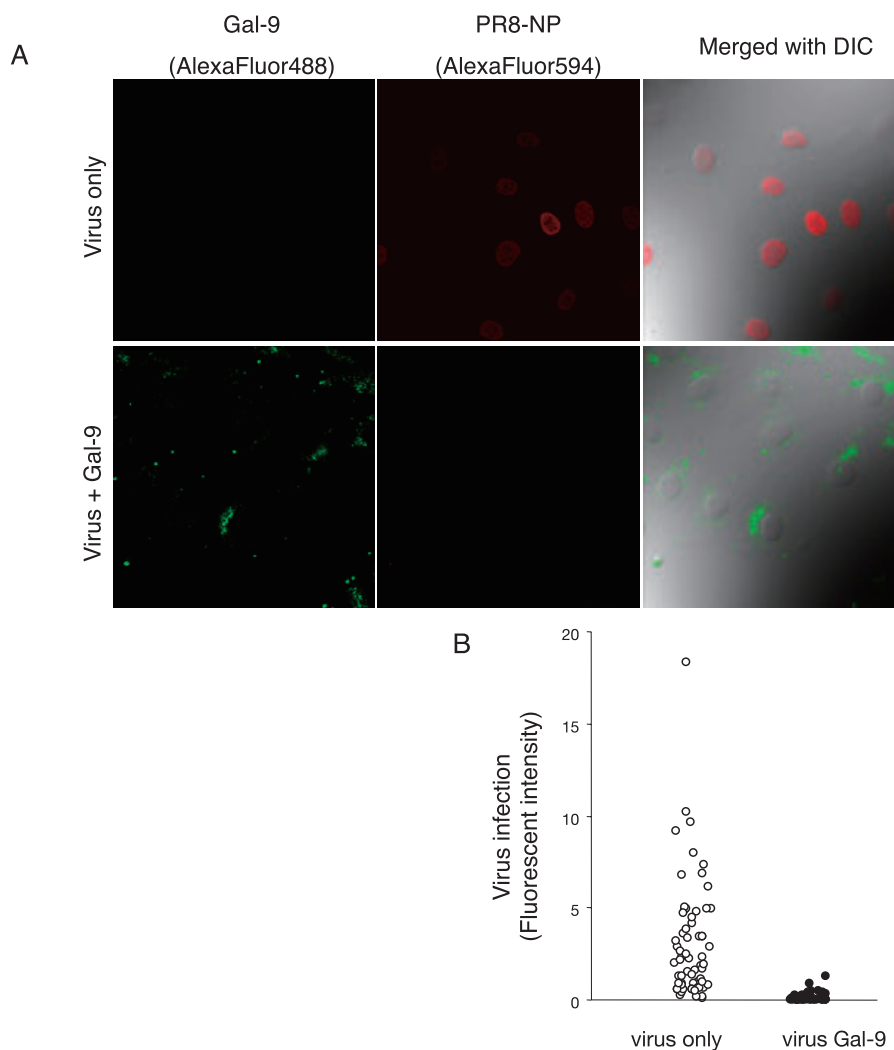
A

Gal-9
(AlexaFluor488)

PR8-NP
(AlexaFluor594)

Merged with DIC

Virus only

Virus + Gal-9

B

Fig. 9. Inhibition of internalization of PR/8 in MDCK cells with Gal-9. Gal-9 was labeled with AlexaFluor488. (A) MDCK cells were infected with PR/8, virus nuclei stained with an anti-NP monoclonal antibody, and observed by fluorescence microscopy. (B) The fluorescence intensity of AlexaFluor594 (NP) of each cell was plotted. Open circles: virus only, closed circles: virus and Gal-9.

was inoculated intranasally into three or five 7-week-old C57BL/6 male mice with or without treatment with intranasally administration of 7.5 µg Gal-9/mouse or with oral administration of 10 mg/kg/day/mouse oseltamivir everyday, followed by observation and weighing every day after inoculation. The results showed that the body weight loss showed a small change in combined administration mice. All of the combined administration mice survived. Slight body weight loss was observed in mice administrated with oseltamivir, but two of 5 mice died. The body weight loss showed large in control mice and

administration of Gal-9 alone mice. All of the control mice and administration with Gal-9 alone showed significant body weight loss and died by 11 days and 16 days, respectively (Fig. 11).

Discussion

Recently, it has been indicated that a β-galactoside binding protein, Gal-9, exhibits diverse effects on human disease, including autoimmunity[24], asthma[8,28], cancer[7,17,20,21] and infections. So far, Gal-9 has been studied for its
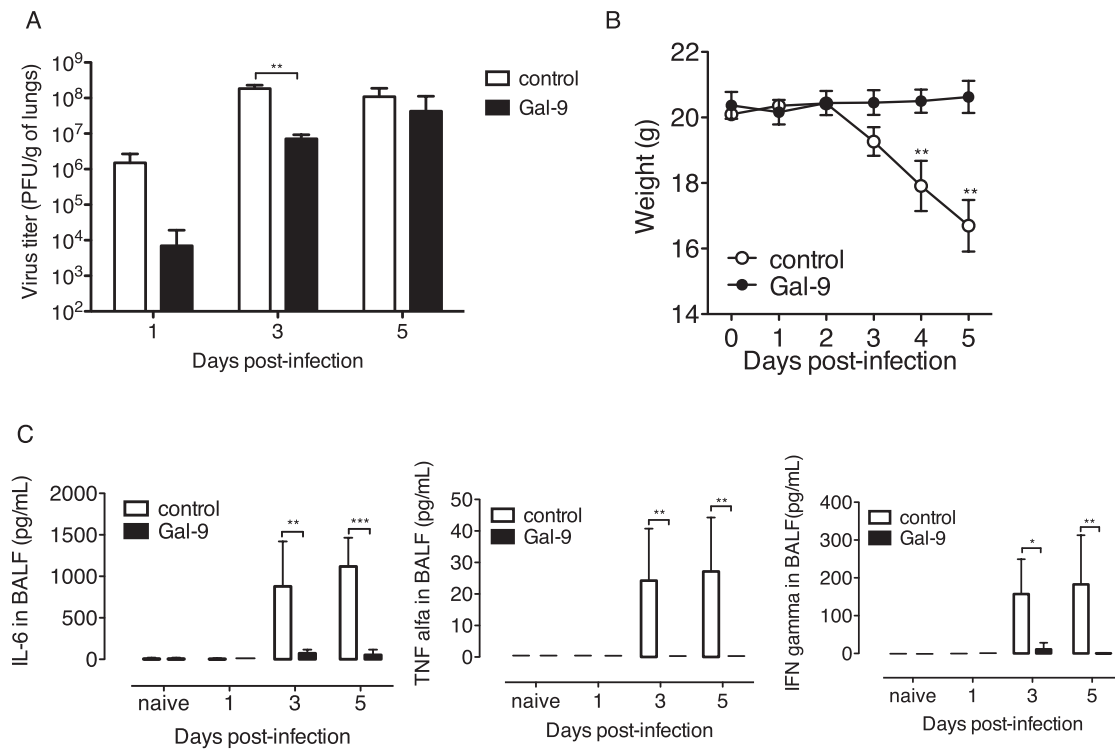
Fig. 10. Effect of Gal-9 on virus replication in mice. Mice were treated intranasally with 7.5 μg/mouse Gal-9 every four days. (A) Virus titers in the lungs. Three mice were sacrificed on 1, 3, and 5 dpi and subjected to plaque assay. Open column: Wild-type mice, closed column: Gal-9-treated mice. (B) Body weight change of 6-week-old C57BL/6 male wild-type mice that were inoculated with five hundreds PFU of PR/8 intranasally. Open circles: Wild-type mice, closed circles: Gal-9-treated mice. (C) Five mice were sacrificed on 1, 3, and 5 dpi to determine the quantity of cytokines in bronchoalveolar wash. Naïve mice were not infected with influenza virus. Open column: Wild-type mice, closed column: Gal-9-treated mice. P-values were calculated using Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

effects on infections with microbes such as hepatitis C virus (HCV)[16], herpes simplex virus (HSV)[23] and *M. tuberculosis*[6]. In the present study, we demonstrated that Gal-9 binds to the surface of influenza A virus particles, inhibiting their attachment to the host cell receptor.

This study showed that Gal-9 expression increased upon influenza A virus infection (Fig. 1). The survival rate of Gal-9 Tg mice was high compared to the wild type (Fig. 2). The present results suggest that Gal-9 contributes in host defense and mitigates the clinical severity of influenza virus infection, but the level of the endogenous Gal-9 expression might not be enough for the complete prevention of the death of the hosts.

Gal-9 bound PR/8 and Aichi/68 (Fig. 6). The binding of Gal-9 to PR/8 was inhibited by lactose, but not by sucrose (Fig. 5). It has been reported that the CRDs of Gal-9 for binding to β-galactosides is competitively inhibited by addition of lactose, but not by addition of sucrose. The results support this report[18]. These results indicate that the binding of Gal-9 to the virus depends on the recognition of β-galactoside on virus particles. On the other hand, Gal-1, which has one CRD and may bind to the carbohydrate chain to β-galactoside, did not bind influenza A virus particles or inhibit the replication of influenza A viruses. The results indicate that Gal-9 inhibits H1N1, H3N2 and H5N1 virus replication (Fig. 3). It is presumed that Gal-9 should inhibit replication of influenza A viruses regardless of the HA subtypes. Gal-9 bound to virus HA (Fig. 8) and influenza A viruses did not invade Gal-9-treated cells (Fig. 9). These results
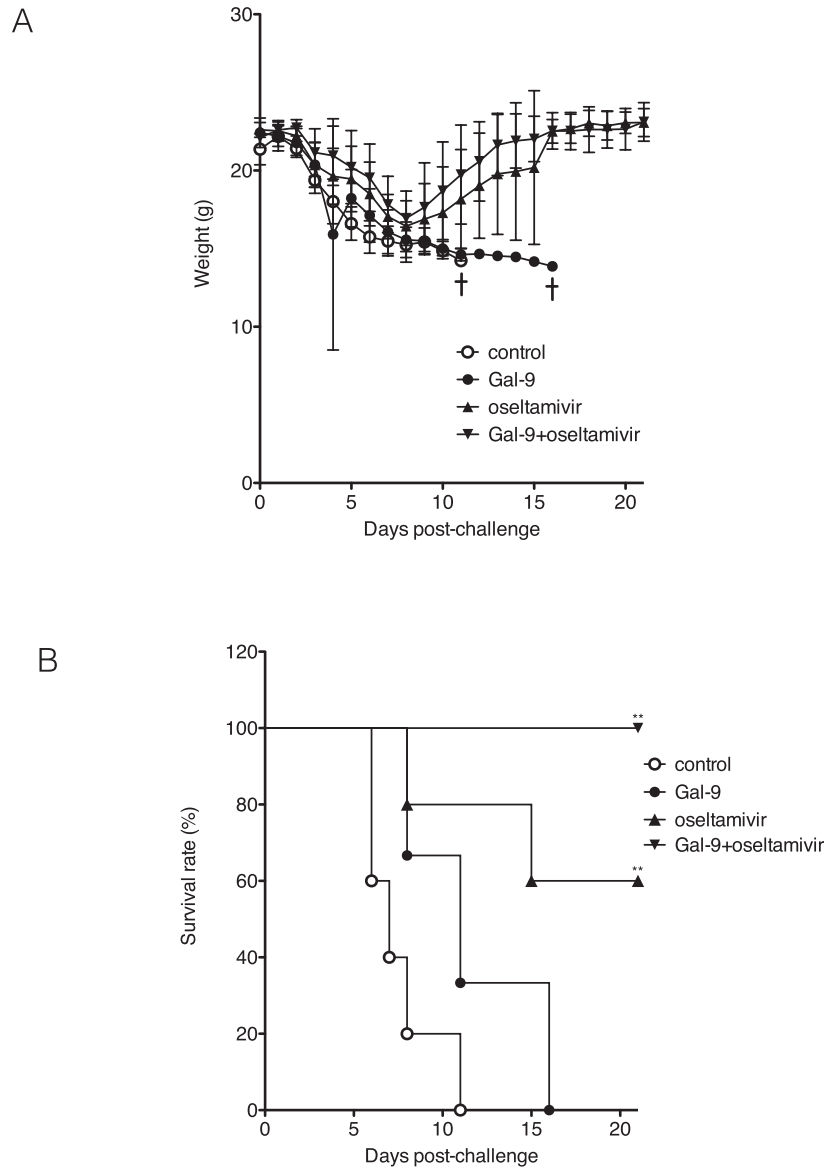
Fig. 11. Effect of combined administration with Galectin-9 and oseltamivir on mice. (A) Body weight change, (B) survival rate. Five thousand PFU of PR/8 were inoculated intranasally into eighteen 7-week-old C57BL/6 male wild-type mice. Body weight changes are expressed as the mean $\pm$ SD. Open circle: control mice, closed circle: Gal-9-treated mice, triangle: oseltamivir, inverted triangle: Gal-9 and oseltamivir-treated mice. P-values were calculated using the log-rank test. **, $P < 0.01$ vs. control mice.

suggest that Gal-9 recognizes β-galactoside in carbohydrate chains on virus glycoproteins. It is postulated that Gal-9 inhibits the internalization of influenza A virus into host cells. In addition to the results of the present study by hemagglutination of chicken RBCs, previous studies indicated that Gal-9 binds not only to viruses but also to the host cell surface to protect from virus infection.

Gal-9 suppressed virus replication in the lungs, body weight loss, and the inflammatory cytokine expression level in mice (Fig. 10). These results suggest that Gal-9 plays a role in protecting the body against influenza A virus infection, however, the response of endogenous Gal-9 against influenza A virus is not enough for protection. The reason why the expression level of cytokine was suppressed may be due to the

direct effects of Gal-9 on immunocompetent cells (Fig.10C). Additional administration of Gal-9 should be effective to prevent the host from the infection with influenza A virus. Oseltamivir and Zanamivir are known as neuraminidase inhibitors. Amantadine is known to block the M2 proton channel of influenza A virus. Gal-9 binds carbohydrate chains of the HA of influenza viruses. Gal-9 and oseltamivir inhibit replication of influenza viruses through different mechanisms; Gal-9 inhibits adsorption of virus to the host cell, while Oseltamivir inhibits release of the influenza virus from the host cell. Combined administration of Gal-9 and oseltamivir to mice resulted in their survival with no clinical signs (Fig. 11). The present results suggest that combined administration of Gal-9 and neuraminidase inhibitor is more effective than single-drug administration. Even after 48 hrs post infection, combined administration completely suppressed the manifestation of disease sign.

In conclusion, the present results revealed that Gal-9 bound to influenza virus particles and inhibited the infection of MDCK cells. Gal-9 should be a useful therapeutic medicine for seasonal and highly - pathogenic avian influenza virus infection. Since Gal-9 is a naturally induced molecule in the host, toxic adverse reactions should be minimal; thus, it is expected that Gal-9 will be developed as an anti-influenza drug.

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