Studies on the prevention of influenza by vitamin D metabolite and vaccine in mice

（マウスにおけるビタミンD代謝物およびワクチンを用いたインフルエンザの予防に関する研究）

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Abbreviations

AAALAC:	Association for Assessment and Accreditation of Laboratory Animal Care
BCA:	bicinchoninic acid
BSA:	bovine serum albumin
dpi:	days post-inoculation
EID_{50}:	50% egg infectious dose
FCS:	fatal calf serum
GM-CSF:	granulocyte-macrophage colony-stimulating factor
HA:	hemagglutinin
HI:	hemagglutination-inhibition
IFN:	interferon
IL:	interleukin
MDCK cells:	Madin–Darby canine kidney cells
MEM:	minimum essential medium
mg:	milligram
mL:	milliliter
MLD_{50}:	50% mouse lethal dose
µL:	microliter
MRM:	multiple reaction monitoring
NA:	neuraminidase
PBS:	phosphate-buffered saline
$TCID_{50}$:	50% tissue culture infectious dose
TNF:	tumor necrosis factor
VDR:	vitamin D receptor

Notes

Contents of the present thesis were published in the following articles.

1. **Hayashi H.,** Okamatsu M., Ogasawara H., Tsugawa N., Isoda N., Matsuno K., Ochbayar E., Suzuki A., Shimizu Y., Ito Y., Sakoda Y. Oral Supplementation of the Vitamin D Metabolite 25(OH)D_3 Against Influenza Virus Infection in Mice. **Nutrients 2000, 5-12, 2020**

2. **Hayashi H.,** Isoda N., Bazarragchaa E., Nomura N., Matsuno K., Okamatsu M., Kida H., Sakoda Y. Potency of an inactivated influenza vaccine against a challenge with A/swine/Missouri/A01727926/2015 (H4N6) in mice for pandemic preparedness. **Vaccines 768, 8, 2020**

Preface

Influenza A viruses belong to the genus *Influenzavirus A* of the family *Orthomyxoviridae*. Influenza A viruses are further divided into 18 hemagglutinin (HA) and 11 neuraminidase (NA) subtypes based on the phylogenetic analysis of their two gene segments [73]. Influenza A viruses are zoonotic pathogens that are widely distributed among mammalian or avian hosts, such as humans, pigs, chickens, and ducks, then wild migratory waterfowls are considered to be natural reservoirs for influenza A viruses [13]. These subtypes (H1–H16 HA and N1–N9 NA) of influenza A viruses have been isolated from waterfowls [40]. Waterfowls are infected with influenza viruses by water-borne transmission at their nesting lakes during their breeding season in summer [35]. Due to a lack of selective pressure in natural hosts, the antigenicity of influenza A viruses is highly conserved in waterfowls [36].

Pigs can become infected with both human and avian influenza viruses, because avian-type α2,3- and human-type α2,6-linked sialylated glycans are expressed in the respiratory epithelium of pigs [13]. This can cause a reassortment between the two different influenza viruses [13]. Influenza viruses that infect pigs are of great interest because the virus is a zoonotic pathogen with the potential to become a new pandemic strain with an antigenic shift for humans. An influenza pandemic is derived from influenza viruses circulating among pigs, actually swine-to-human transmission of swine influenza viruses have been recently confirmed. For example, the Eurasian avian-like H1N1 swine influenza virus was recently isolated and it has an ability to infect humans [62]. Moreover, human H3N2v infection was recently confirmed, and it is supposed to be derived from swine influenza strain [41]. Development of influenza vaccines is mandatory after occurring of a pandemic, but it takes time to develop a new vaccine to select the ideal vaccine strain by antigenicity and to ensure its safety [21]. On the other hands, unlike specific preventive measure requiring the antigenicity matching between the prevalent and vaccine strains, developing nonspecific preventive measures, such as dietary supplements that boost immunity, against a future influenza pandemic is also important.

Since antigenicity of influenza viruses is conserved in waterfowl, monitoring viral circulation in waterfowl is necessary for preparedness against an influenza pandemic in humans via pigs. In previous studies, intensive surveillance of avian influenza was conducted in wild waterfowl in Eurasia, including Hokkaido, Japan, to monitor viruses that are maintained in the nesting lakes of northern territories, and their antigenicity and

proliferation have been evaluated since 1977; thereafter, isolates have been stored in the Hokkaido University Influenza Virus Database System (*http://virusdb.czc.hokudai.ac.jp*).

In emerging of pandemic influenza, pigs play an important role in the transmission of influenza viruses to human hosts [13]. Therefore, it is vital to develop an effective vaccine to prepare for the possible outbreak by an influenza virus infection in humans, considering that it has a different antigenicity from that of the seasonal human influenza viruses. Major influenza vaccine for humans is an ethel-split vaccine, but it has no effect on innate immune induction [27]. A whole-virus particle vaccine is also developed, it can enhance innate immunity [27]. Previous studies revealed that whole-virus particle vaccines prepared from the library induced effective immunity against infections with H1, H2, H5, H6, H7, H9, and H10 influenza viruses in mouse models and cynomolgus macaque models [48, 64, 59, 49, 12, 51, 63].

As nonspecific preventive measures, immunomodulatory effects of lactoferrin and vitamin D were revealed against influenza [55, 44]. Vitamin D, which is one of nonspecific preventive measure against influenza, is a fat-soluble vitamin that can be ingested and absorbed in the small intestine [5]. It is metabolized in the liver and becomes the highly stable 25-hydroxyvitamin D [25(OH)D], which circulates in the blood [39]. The association between blood revels of 25(OH)D and clinical signs of several viral infection is already clarified [15]. Reduction of the serum vitamin D metabolite 25(OH)D during the endogenous vitamin D synthesis, and poor intake of dietary vitamin D can also weaken the immune system. Influenza virus infection causes severe seasonal epidemics, especially in the elderly and in young children, partly due to a poor immune function [8]. Vitamin D has also been suggested to have immunomodulatory effects against respiratory syncytial virus [4], poliovirus [30], human immunodeficiency virus 1 [18], and influenza virus infection.

The present thesis consists of two chapters. In Chapter I, a prophylactic effect of $25(OH)D_3$ in oral supplementation against influenza virus infection was evaluated in a mouse model. After the administration of $25(OH)D_3$, anti-inflammatory effects were measured. In Chapter II, analyses of genetic and antigenic properties of H4 influenza viruses isolated from wild birds in Eurasia and North America are discussed. In addition, as an effective countermeasure for a future pandemic caused by H4 influenza viruses, the efficacy of an inactivated whole-virus particle vaccine prepared from avian H4 influenza viruses acquired from the Hokkaido University Influenza Virus Database was evaluated against a challenge with the swine H4N6 influenza virus in a mouse model.

Chapter I

Oral supplementation of the vitamin D metabolite 25(OH)D_3 against influenza virus infection in mice

Introduction

Vitamin D, primally contained in oily fishes, is a fat-soluble vitamin that promotes bone formation and calcium absorption [26]. In addition to being synthesized from cholesterol by reacting with ultraviolet rays in the epidermis, vitamin D can be ingested and is absorbed in the small intestine. It is metabolized in the liver to become highly stable 25-hydroxyvitamin D [25(OH)D], which circulates in the blood. The kidney metabolizes 25(OH)D into 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], which binds to the vitamin D receptor (VDR) expressed in various types of cells [39]. The metabolite 1,25$(OH)_2$D promotes the regeneration of respiratory epithelial cells [37] and has also been implicated in the regulation of inflammation by binding to the VDR on immune cells such as dendritic cells, macrophages, and T cells [15]. The binding of the VDR with 1,25$(OH)_2$D suppresses the production of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-5 by regulating target genes of T cells and macrophages, and reduces inflammation [7]. It also directly interacts with B cells and T cells [44]. Furthermore, 1,25$(OH)_2$D reduces inflammatory cytokines in challenge studies with the pertussis toxin [10], *Mycobacterium tuberculosis* [14], and poliovirus [30].

The influenza A virus is an enveloped, single-stranded, segmented, negative-sense RNA virus that infects the upper respiratory tract in humans, causing influenza [45]. Influenza A virus infection causes severe seasonal epidemics especially in the elderly and in young children, partly because of poor immune function. Reduction of the serum vitamin D metabolite 25(OH)D, decreases in endogenous vitamin D synthesis, and poor dietary intake of vitamin D can also weaken the immune system [8]. Furthermore, vitamin D has been suggested as having immunomodulatory effects against influenza [18]. A major form of 25(OH)D, 25-hydroxyvitamin D_3 [25$(OH)D_3$], is absorbed efficiently in the small intestine [5] and has a relatively long half-life of 15 days [57]. Therefore, 25$(OH)D_3$ is supposed to become a suitable supplementation for the preparedness against influenza. In the present study, a mouse model of influenza virus infection and direct oral ingestion of 25$(OH)D_3$ was used to evaluate the effect of this metabolite on the clinical manifestations of influenza virus infection, virus replication, and cytokine levels.

Materials and Methods

Virus and cells

Influenza virus A/Puerto Rico/8/1934 (PR8) (H1N1) was propagated in 10-day-old embryonated chicken eggs at 35 °C for 48 h, and the collected allantoic fluid was stored at −80 °C until use. Madin–Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% nonimmobilized fetal calf serum (FCS; SAFC Biosciences, Lenexa, KS, USA), 0.3 mg/mL L-glutamine (Wako Chemicals, Tokyo, Japan), 100 U/mL penicillin G (Meiji Seika Pharma, Tokyo, Japan), 0.1 mg/mL streptomycin (Meiji Seika Pharma, Tokyo, Japan), and 8 μg/mL gentamicin (Takata Pharmaceutical, Saitama, Japan) in an incubator at 37 °C with 5% CO_2.

Animals and diet

Seven-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) used in the present study were acclimatized for one week, divided into two groups (n = 18 mice/group), and fed different diets, i.e., AIN-93G standard diet (vitamin D_3 0.25 mg/100 g) or AIN-93G with 25(OH)D_3 [125 mg/kg (125 ppm)] (Oriental Yeast, Tokyo, Japan). Body weights and clinical signs were observed for 7 weeks after beginning the different diets.

Measurement of 25(OH)D_3 and 24,25(OH)$_2D_3$

Plasma concentrations of 25(OH)D_3 and 24-hydroxyvitamin D_3 [24,25(OH)$_2D_3$], metabolite of 25(OH)D_3 were measured using a modified method of liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS)/MS [66]. This method included the use of deuterated 25(OH)D_3 (d_6-25[OH]D_3) as an internal standard compound and the selection of a precursor and a product ion with an MS/MS multiple reaction monitoring (MRM) method. Measuring was conducted as previously described [50]. An API3000 LC/MS/MS System (Applied Biosystems, Foster City, CA, USA) was used. The high-performance liquid chromatography column used was a CAPCELL PAK C18 UG120, 5 μm [4.6 I.D. × 250 mm] (Shiseido, Tokyo, Japan). All MS data were collected in positive-ion mode, and quantitative analysis was carried out using MS/MS–MRM of the precursor/product ion.

Virus challenge in mice

Virus challenge in mice was conducted as previously described [47]. After 7 weeks of feeding the mice a standard or 25(OH)D_3-supplemented diet, PR8 (H1N1) was inoculated intranasally using 10 times the 50% mouse lethal dose (MLD_{50}) in 30 µL per mouse under anesthesia. The infectivity titer [10 MLD_{50} = $10^{4.6}$ 50% egg infective dose (EID_{50})] of the inoculum was adjusted by dilution with phosphate-buffered saline (PBS). For 14 days following inoculation, mice were observed daily for body weight, clinical signs, and survival.

Preparation of lung homogenates

At pre-inoculation and 3 or 5 days post-inoculation (dpi), mouse lungs were collected after euthanasia (n = 6). Lungs were homogenized with 2 mL of transport medium: MEM containing 10,000 U/mL penicillin G, 10 mg/mL streptomycin, 0.3 mg/mL gentamicin, 250 U/mL Nystatin (Sigma-Aldrich, St. Louis, MO, USA), and 0.5% bovine serum albumin fraction V (Roche, Basel, Switzerland). The homogenized lung tissue was centrifuged for 5 min at 4 °C and 8000 rpm. The supernatant was collected, and all samples were stored at −80 °C until quantification of virus titers and cytokines.

Virus titration in mouse lungs

Plaque assays were performed as previously described [25]. Briefly, tenfold dilutions of virus samples or mouse lung homogenates in MEM without FCS were inoculated onto confluent monolayers of MDCK cells and incubated at 35 °C in a 5% CO_2 incubator for 1 h. Unbound virus was removed in the supernatant, and the cells were washed with PBS. The cells were then overlaid with MEM containing 5 µg/mL acetylated trypsin (Sigma-Aldrich) and 1% Bacto Agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After incubation for 48 h at 35 °C, the cells were stained with 0.005% neutral red. After incubation for another 24 h at 35 °C, the number of plaques was counted. The number of plaque-forming units (PFU) was calculated as the product of the reciprocal value of the highest virus dilution and the number of plaques in the dilution.

Bio-Plex assay for the measurement of anti-inflammatory and proinflammatory cytokines

The Bio-Plex Pro mouse cytokine Th1/Th2 assay (Bio-Rad, Hercules, CA, USA) was used to quantify cytokines including IL-2, IL-4, IL-5, IL-10, IL-12p70, TNF-α, interferon (IFN)-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Lung homogenate samples were diluted 1:2 with the Bio-Plex sample diluent. Standard dilution

was performed using the transport medium. Beads were dispensed into wells of a 96-well plate, and the samples, standards, and blanks were added and vortexed for 30 min. After washing with Wash Buffer, a secondary antibody was added to the wells, and the plate was vortexed for 30 min. The wells were washed again, phycoerythrin-conjugated streptavidin was added, and the plate was vortexed for 10 min. After washing again, Assay Buffer was added, and measurement were carried out by Luminex 200 (Merck, Kenilworth, NJ, USA). The concentrations of cytokines (pg/mL) were determined using the Bio-Plex Manager Software (Bio-Rad).

Statistical analysis

Student's *t*-test was used to analyze differences in the concentration of vitamin D metabolites, body weights of mice, virus recovery, and cytokines between the two groups. One-way analysis of variance was used to analyze the difference among multiple groups [2]. Animal survival data were analyzed using a log rank (Mantel–Cox) test. All statistical analyses were performed by R version 3.6.3 (R Core Team, 2020).

Ethics statement

All experiments using a virus were approved by Hokkaido University Safety Management Regulations. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (17-0060), and all experiments were carried out per the guidelines of this committee. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The Faculty of Veterinary Medicine, Hokkaido University, has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International since 2007.

Results

Monitoring of adverse effects in mice

First, the adverse effects of 25(OH)D_3 supplementation were evaluated. The groups of mice were fed standard or 25(OH)D_3-supplemented diet for 7 weeks, and no significant difference in body weight and clinical signs was observed between the two groups (Figure 1); no significant difference in the intake of diet was found ($p > 0.05$). Additionally, no histopathological difference in the kidneys and hearts were detected after 7 weeks (Figure 2), indicating no adverse effects of 125 ppm 25(OH)D_3 supplementation in mice.

Blood concentrations of 25(OH)D_3 and 24,25$(OH)_2D_3$ in mice

The level of 25(OH)D_3 was significantly higher in the plasma of 25(OH)D_3-fed mice than in that of standard-fed mice after 4 and 7 weeks (Figure 3). Furthermore, the level of 25(OH)D_3 after 7 weeks was 40–50 ng/mL, significantly higher than that after 4 weeks in 25(OH)D_3-fed mice.

The compound 24,25$(OH)_2D_3$ is a metabolite of 25(OH)D_3, and in mice, its concentration increases in proportion to the uptake of 25(OH)D_3 into the body [50]. As shown in Figure 2, the level of 24,25$(OH)_2D_3$ was significantly higher in the plasma of 25(OH)D_3-fed mice than in that of standard-fed mice after 4 and 7 weeks. Additionally, the levels of 24,25$(OH)_2D_3$ in the blood were higher than those of 25(OH)D_3 after 4 and 7 weeks of dietary supplementation in the 25(OH)D_3-fed mice.

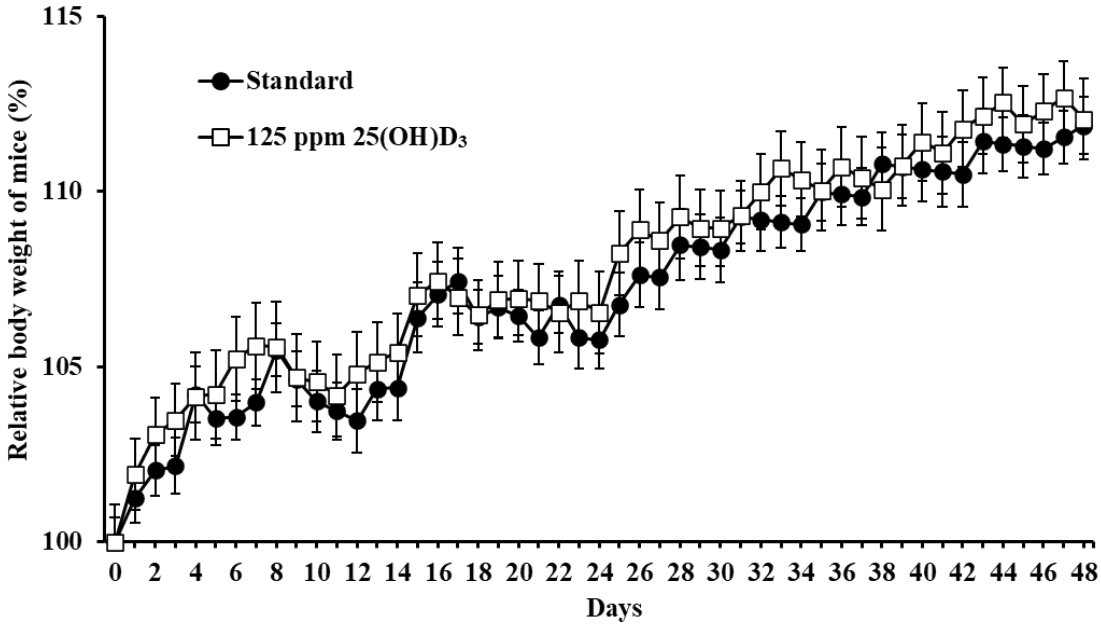

Figure 1. Changes in mouse body weight during dietary supplementation with 25(OH)D_3 for 7 weeks. Mice were divided into two groups and fed with a standard diet or a 125 ppm 25(OH)D_3-containing diet (n = 18 mice/group). The relative body weight of each mouse was measured for 7 weeks and compared with the primary body weight at day 0.

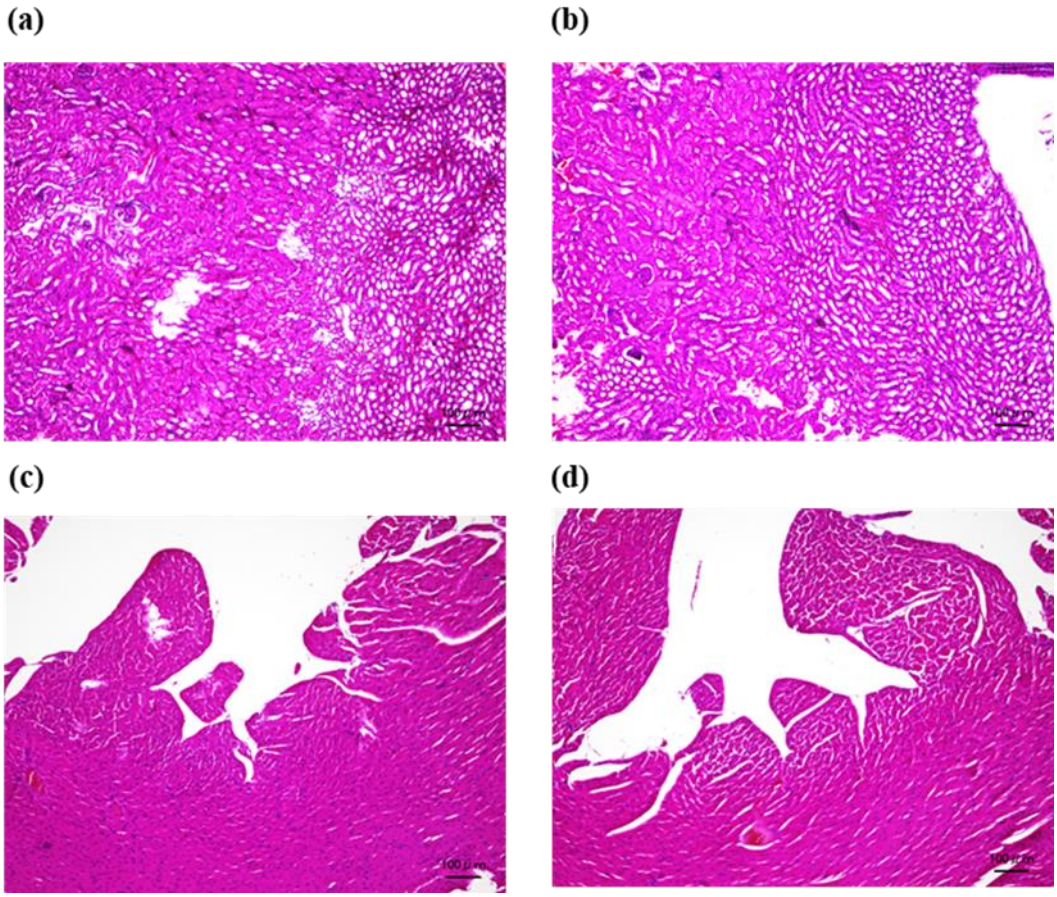

Figure 2. Histopathological assessment of mouse kidneys and hearts. Mice were fed for 7 weeks, and kidneys and hearts were sectioned and stained with hematoxylin and eosin. The kidneys of a standard-fed mouse(a) and a 25(OH)D_3-fed mouse (b). The hearts of a standard-fed mouse (c) and a 25(OH)D3-fed mouse (d).

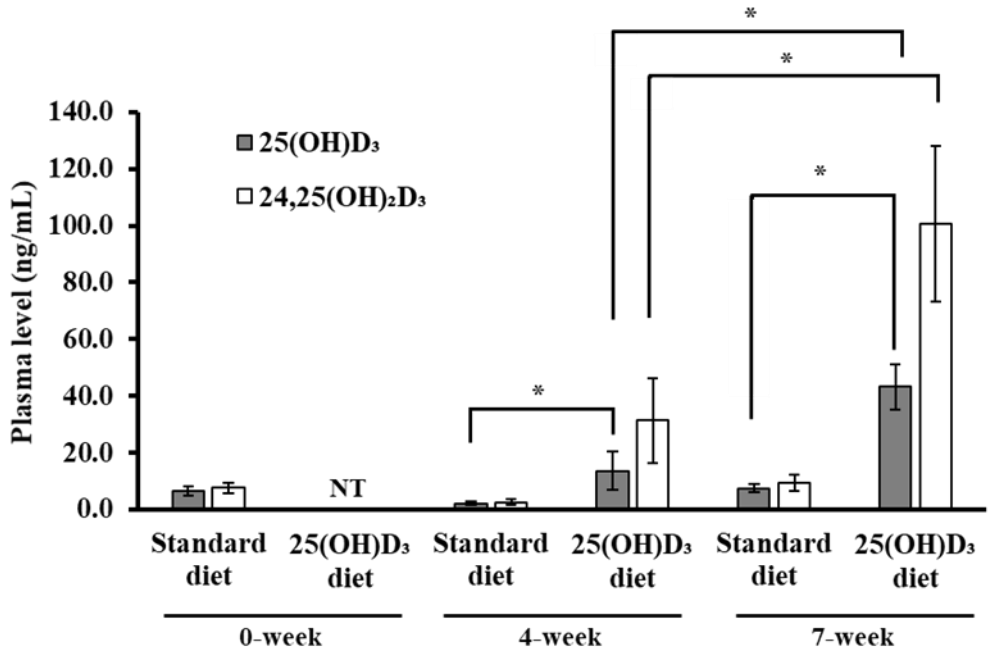

Figure 3. Plasma levels of $25(OH)D_3$ and $24,25(OH)_2D_3$ in mice. Mice were fed with a standard or a 125 ppm $25(OH)D_3$-supplemented diet for 7 weeks. The levels of $25(OH)D_3$ and $24,25(OH)_2D_3$ were measured in each group (6 mice/group) at weeks 0, 4, and 7. Blood concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$ in the $25(OH)D_3$-fed group at week 0 were not tested (NT). *, significant difference ($p < 0.05$).

Immunomodulant test for 25(OH)D_3 against influenza virus infection

The antiviral effect of the 25(OH)D_3-supplemented diet (125 ppm) against influenza virus PR8 (H1N1) infection was assessed in mice. After 7 weeks of this specialized diet, mice were challenged with PR8 (H1N1) intranasally, and clinical signs were observed until 14 dpi. Humane endpoint criterion was <70% of initial body weight. All mice in the standard diet group died by 9 dpi, and only one mouse in the 25(OH)D_3-fed group survived after 9 dpi (Figure 4a). The survival rates of the two groups were compared with the rate determined in a log rank (Mantel–Cox) test, which revealed no significant difference ($p > 0.05$). The body weight of mice in the 25(OH)D_3-fed group were significantly higher than those of mice in the standard diet group at 5 dpi (Figure 4b).

To investigate whether 25(OH)D_3 supplementation inhibits viral replication in mice at two different acute phases, the viral titer in the mouse lungs was measured by the plaque assay at 3 or 5 dpi. The viral titers in 25(OH)D_3-fed mice at 3 dpi were significantly lower than those in the standard-fed group, but no significant difference was observed between the two groups at 5 dpi (Figure 5).

Production of anti-inflammatory and proinflammatory cytokines after viral challenge in mice with 25(OH)D_3 supplementation

The amounts of anti-inflammatory and proinflammatory cytokines in each group were quantified. The levels of the anti-inflammatory cytokine IL-10 and of the proinflammatory cytokines IL-5, TNF-α, IFN-γ, and GM-CSF increased after viral infection in both groups, regardless of 25(OH)D_3 intake. In addition, the proinflammatory cytokines IL-2 and IL-12p70 decreased after infection in both groups (Figure 6a, b). The levels of anti-inflammatory IL-4 and IL-10 were not significantly different in the lungs of 25(OH)D_3-fed mice and standard-fed mice (Figure 6a). The levels of IFN-γ and IL-5, however, significantly decreased in the 25(OH)D_3-fed group after viral challenge at 3 and 5 dpi, respectively (Figure 6b). By contrast, TNF-α production increased significantly in the 25(OH)D_3-fed group at 5 dpi. In addition to these significant differences, a trend toward suppression of the levels of the proinflammatory cytokines TNF-α, GM-CSF, and IL-12p70 in the 25(OH)D_3-fed group was observed at 3 dpi, and IL-2 production tended to be higher in the 25(OH)D_3-fed group than in the standard-fed group at 3 dpi ($0.05 < p < 0.1$).

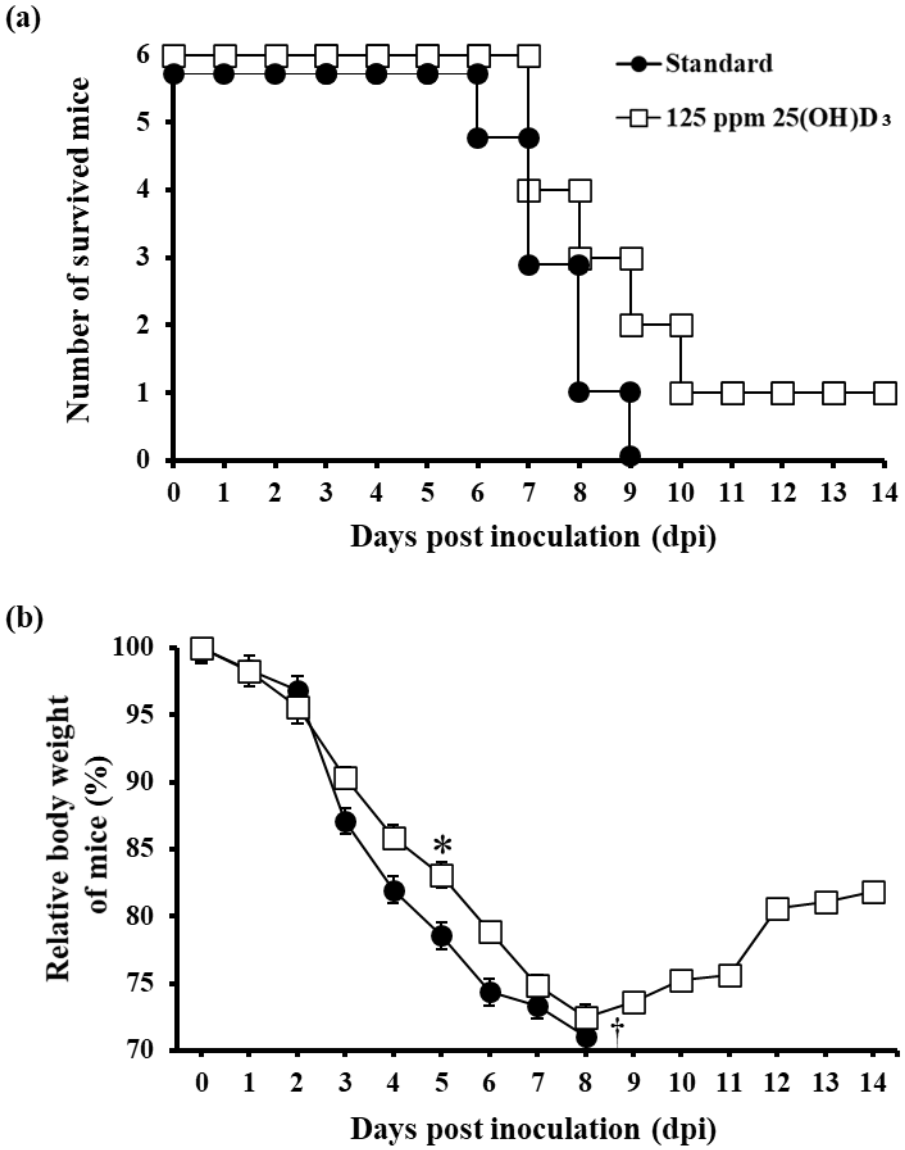

Figure 4. Survival and body weight of mice challenged with PR8 (H1N1) (n = 6 mice/group). (a) Number of surviving mice over time. (b) Relative body weight of mice after virus challenge. *, significant difference ($p < 0.05$); †, all mice deceased.

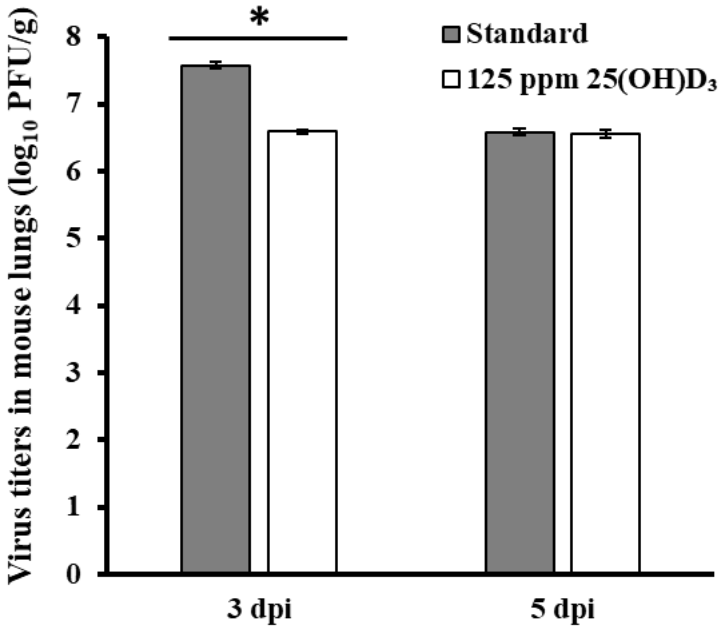

Figure 5. Virus recovery from the lungs of mice challenged with PR8 (H1N1) at 3 or 5 dpi. Mice were fed with a standard diet or a diet supplemented with 125 ppm 25(OH)D_3 for 7 weeks and then challenged with PR8 (H1N1) intranasally. The viral titers in the lung homogenates at 3 or 5 dpi were measured (PFU/g) for each group (n = 6 mice/group). *, significant difference ($p < 0.05$).

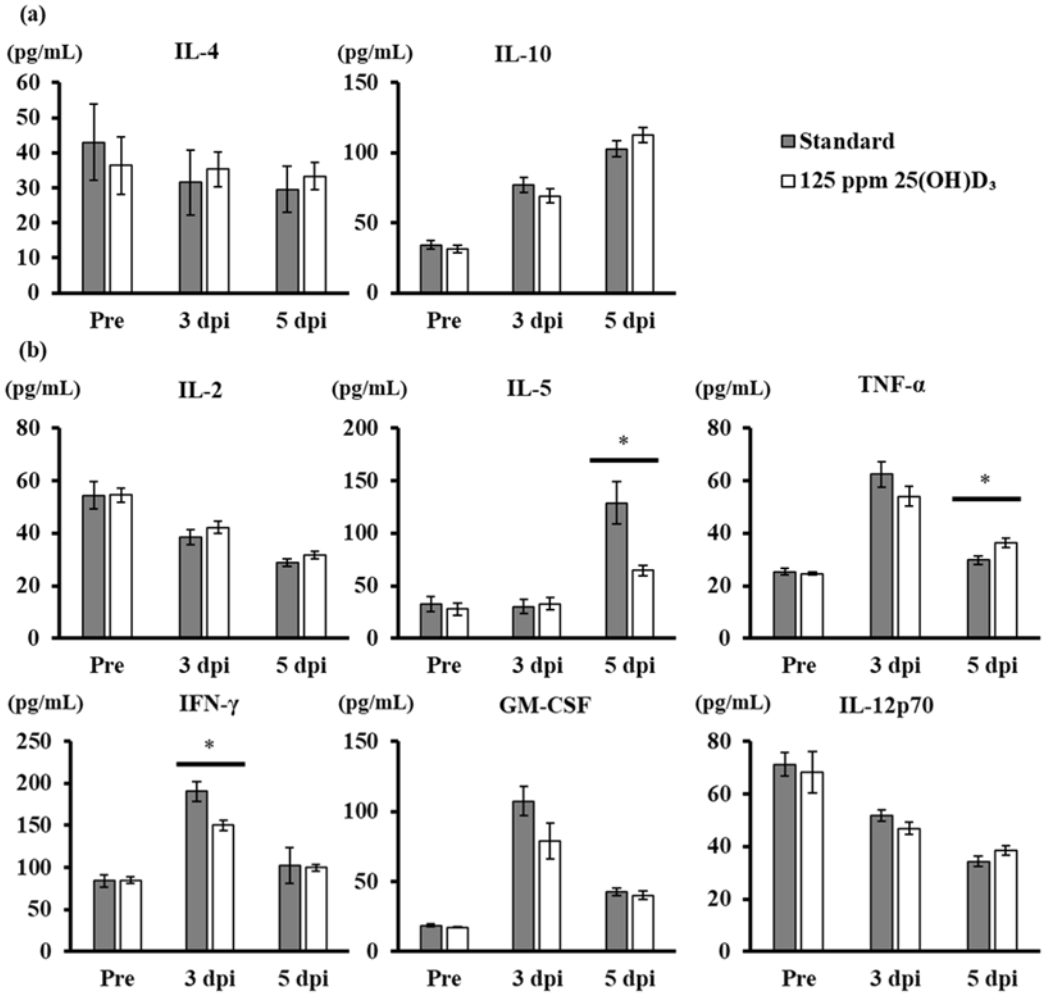

Figure 6. Levels of anti-inflammatory and proinflammatory cytokines before and after viral challenge (n = 6 mice/group). Mice were fed a standard diet or a diet supplemented with 125 ppm 25(OH)D_3 for 7 weeks and then challenged with PR8 (H1N1) intranasally. (a) Concentrations of anti-inflammatory cytokines IL-4 and IL-10. (b) Concentrations of proinflammatory cytokines IL-2, IL-5, TNF-α, IFN-γ, GM-CSF, and IL-12p70. *, significant difference ($p < 0.05$).

Discussion

Previous studies have suggested the use of dietary supplements with nonspecific immunomodulatory effects as a countermeasure against influenza virus infection [46]. Influenza is common in winter months, when the levels of 25(OH)D in the blood generally decrease [22]. A correlation between influenza virus infection and deficiency of vitamin D has been previously observed [6]. The immunomodulatory effect of orally administered vitamin D in influenza patients has been clinically tested in humans, especially in infants, and a rapid abatement of fever and influenza A virus titers has been confirmed during the administration of high doses of vitamin D [72]; however, vitamin D consumption was not effective in elderly patients [18]. Hence, animal studies that allow investigation at the time of influenza virus infection are required. The compound 25(OH)D is metabolized from vitamin D in the liver and is better absorbed than vitamin D in the body [5]. After circulating in the blood, 25(OH)D is metabolized to 1,25$(OH)_2$D in the kidney as needed [54]. In a mouse model, suppression of inflammatory cytokines induced by H9N2 influenza virus infection by the intraperitoneal administration of 1,25$(OH)_2$D after virus inoculation has been confirmed [23]. The half-life of 25$(OH)D_3$ is relatively long (15 days), but the half-life of 1,25$(OH)_2$D is only 15 h [57]. Therefore, long-term administration of 25$(OH)D_3$ may mitigate the clinical manifestation of infectious diseases prophylactically. This study is the first to indicate a prophylactic effect against influenza virus infection in mice pre-administrated with vitamin D metabolite 25$(OH)D_3$ (125 ppm for 7 weeks).

The no-observed adverse effect level of vitamin D in humans is 250 μg/day by oral administration [17], and the adverse effects resulting from excessive consumption of vitamin D are renal insufficiency, neurologic manifestations, and pathologic calcification due to hypercalcemia [43]. Levels of 25$(OH)D_3$ corresponding to 800–1000 ng/mL in mouse serum also showed adverse effects such as weight loss and hypercalcemia [16]. As shown in Figure 2, the blood level of 25$(OH)D_3$ after 7 weeks of supplemental diet was 40–50 ng/mL, a concentration recommended as the minimum effective requirement against viral respiratory infections on the basis of observational studies [67]. Indeed, no adverse effects (e.g., weight loss, neurologic manifestations, or calcification of the organs), as described previously for 25$(OH)D_3$-fed mice [42], were observed in this study (Figure 6). After 4 and 7 weeks of the supplemented diet, the plasma levels of 25$(OH)D_3$

in 25(OH)D_3-fed mice were significantly higher compared with those in standard-fed mice. In particular, the level of 24,25$(OH)_2D_3$ in 25(OH)D_3-fed mice was higher than the level of 25(OH)D_3 at 4 and 7 weeks, although the plasma level of 24,25$(OH)_2D_3$ is usually nearly equal to that of 25(OH)D_3 in mice [16]. This result suggesting that the uptake of 25(OH)D_3 into the body was accelerated, and excessive 24,25$(OH)_2D_3$, a metabolite of 25(OH)D_3, was also detected in the blood [50]. In previous study, blood levels of 25(OH)D_3 in 40–60 ng/mL could reduce the respiratory symptoms in humans [24]. These results suggested that the dosage (125 ppm) and duration (7 weeks) of 25(OH)D_3 administration secured a blood concentration of 25(OH)D_3 sufficient to reduce the clinical manifestation of infectious diseases.

The influenza A virus PR8 (H1N1) strain is lethal in mice, who succumb within 8 dpi when infected with 10 MLD_{50} of the virus [47]. Influenza onset correlates with inflammatory cytokine expression, and neuroinflammation due to influenza virus infection releases inflammatory cytokines and can cause lung tissue damage [9]. Dysregulation of inflammatory cytokines in mice early in viral infection induces the overexpression of caspases and other proteolytic enzymes and plays a role in lung injury and lethality [68]. However, appropriate suppression of the expression of inflammatory cytokines may be able to suppress lethality caused by the influenza virus [11]. Vitamin D can suppress the proinflammatory cytokines by regulating NF-κB in a mouse model [70]. In a human trial, supplementation of vitamin D reduced the production of IL-5 after influenza virus infection [22]. The effects of 1,25$(OH)_2D$ include the repression of the transcription of T cell mRNA and the suppression of the production of IFN-γ [22]. After infection, the proinflammatory cytokines IFN-γ [22] and IL-5 [33] were reduced by the administration of 25(OH)D_3. Anti-inflammatory cytokines suppress the immunopathological tissue damage caused by the production of inflammatory cytokines [31]. Consistent with previous reports, the levels of IL-5 and IFN-γ were suppressed in the 25(OH)D_3-fed group. Notably, our study showed significant suppression of virus titers in the 25(OH)D_3-fed group after 3 dpi, and this result supports a previous study that the growth of the H5N1 influenza virus in lungs was reduced by the administration of 1,25$(OH)_2D$ in mice [29]. These results suggest that long-term administration of 25(OH)D_3 prior to virus inoculation suppressed the production of proinflammatory cytokines, resulting in reduced lung damage caused by virus infection and suppressed viral replication. In a previous report, the administration of 1,25$(OH)_2D$ to mice significantly inhibited the production of TNF-α after influenza virus infection [23]. However, in our study, a trend toward the suppression of TNF-α and a significant increase

in TNF-α were observed in the 25(OH)D_3-fed group after 3 and 5 dpi, respectively. In this study, IL-6 levels was not measured, but there is a report that the administration of vitamin D could suppress the production of IL-6, which promotes the differentiation of B cells [18]. IL-33, which has an antiviral effect, was not measured, but vitamin D could enhance the production of IL-33 [65]. Therefore, the complex effects of cytokines must be elucidated in the future.

In conclusion, the present study showed that 25(OH)D_3 supplementation adequately alleviated the clinical manifestations of influenza virus infection by suppressing virus replication and inflammation in a mouse model. In this result, there was no significant difference in the mortality rate of influenza virus infection following administration of 25(OH)D_3, but we could confirm a tendency of reduced mortality rate. In other words, as a previous research showed [19], it is expected that the administration of vitamin D and its metabolites will reduce the mortality rate in an influenza pandemic. This evidence strongly supports the role of vitamin D in reducing the risk of respiratory viral diseases in humans, not only for influenza but also for the newly emerged coronavirus disease 2019 (COVID-19) pandemic [20]. Notably, however, 25(OH)D_3 supplementation had little effect on mortality against influenza virus infection. In the future, the use of 25(OH)D_3 combined with a vaccine [53] may synergistically reduce the mortality of influenza virus infection.

Brief summary

Vitamin D is a fat-soluble vitamin that is metabolized by the liver into 25(OH)D and then by the kidney into 1,25$(OH)_2$D, which activates the vitamin D receptor expressed in various cells, including immune cells, for an overall immunomodulatory effect. Here, to investigate whether oral supplementation of 25$(OH)D_3$, a major form of vitamin D metabolite 25(OH)D, has a prophylactic effect on influenza A virus infection, mice were fed a diet containing a high dose of 25$(OH)D_3$ and were challenged with the influenza virus. In the lungs of 25$(OH)D_3$-fed mice, the viral titers were significantly lower than in the lungs of standard-fed mice. Additionally, the proinflammatory cytokines IL-5 and IFN-γ were significantly downregulated after viral infection in 25$(OH)D_3$-fed mice, while anti-inflammatory cytokines were not significantly upregulated. These results indicate that 25$(OH)D_3$ suppresses the production of inflammatory cytokines and reduces virus replication and clinical manifestations of influenza virus infection in a mouse model.

Chapter II

Potency of an inactivated influenza vaccine against a challenge with A/swine/Missouri/A01727926/2015 (H4N6) in mice for pandemic preparedness

Introduction

Influenza A virus is an enveloped virus containing single-stranded, eight-segmented, negative-sense RNA; it is categorized into 18 HA and 11 NA subtypes [73]. HA binds to the glycan of the host cells, but the glycan structure differs among animal species. It is thought that an influenza pandemic may originate from influenza viruses circulating among pigs. Pigs play the role of a "mixing vessel" by expressing both human- and avian-type sialylated glycan receptors to influenza viruses in respiratory epithelial cells and cause a reassortment between two types of influenza viruses [13]. Waterfowl are natural hosts of influenza viruses, and viral antigenicity is conserved within the natural host [36]. Influenza viruses perpetuated in waterfowl, whose antigenicity is highly conserved, have been isolated. Therefore, as preparedness against an influenza pandemic, viral antigenicity and proliferation have been evaluated since 1977; thereafter, the isolates have been stored (*http://virusdb.czc.hokudai.ac.jp*). Vaccines against H1, H2, H5, H6, H7, H9, and H10 from the virus library have been previously prepared, and their preventive efficacy in mice and cynomolgus monkeys has been evaluated [48, 64, 59, 49, 12, 51, 63].

Since their first isolation in Czechoslovakia in 1954, H4 avian influenza viruses have been sporadically isolated worldwide [60]. H4 virus infection in swine has been recently reported. A/swine/Ontario/01911-1/1999 (H4N6) [32] in Canada, A/swine/Hubei/06/2009 (H4N1) [28], A/swine/Guangdong/K4/2011 (H4N8) [61] in China, and A/swine/Missouri/A01727926/2015 (H4N6) (MO/15) in 2015 were isolated [1]. Influenza viruses are classified based on the HA genes. In a previous report, the HA genes of H4 viruses were systematically divided into North American and Eurasian lineages [1]. According to the phylogenetic analysis of the nucleotide sequence of the eight gene segments of the MO/15 virus, all segments were derived from North American birds [1]. H4 viruses, including MO/15 detected in North America, showed binding to α2,6-linked sialylated glycan, which is highly expressed in the respiratory epithelium of swine and humans [11,12]. MO/15 replicated in swine lungs, causing mild lung lesions, but did not replicate in the upper respiratory tract [1]. This may indicate that H4 influenza viruses could be sustained in the pig population by continuous virus infection and might accidentally be transmitted to other species. To date, there have been no human infections with an H4 virus, although a case of human infection with A/Indiana/08/2011 (H3N2)v derived from swine influenza strain was confirmed [41], and H4 viruses could pose a risk of human infection in the future.

Therefore, the development of a vaccine against the H4 viruses, which has a different antigenicity from conventional seasonal influenza viruses, is required. No report has evaluated vaccines against H4 influenza virus infection. Therefore, this study aimed to select a candidate vaccine strain from the virus library, prepare a vaccine, and evaluate the vaccine's potency in a mouse model.

Materials and Methods

Virus and cells

MO/15 was kindly provided by Dr. Alicia Janas-Martindale (United States Department of Agriculture, Raleigh, NC, USA). A/duck/Czechoslovakia/1956 (H4N6) (Dk/Cz), A/budgerigar/Hokkaido/1/1977 (H4N6) (Budge/Hok), A/duck/Hokkaido/491003/2014 (H4N2) (Dk/Hok/491003), A/duck/Mongolia/769/2015 (H4N6) (Dk/Mon), A/duck/Hokkaido/138/2007 (H4N6) (Dk/Hok/138), A/swan/Hokkaido/481102/2017 (H4N6) (Swan/Hok), and A/mallard/Alberta/223/1979 (H4N2) (Mal/Alb) were isolated from birds. All viruses used in this study were propagated in 10-day-old embryonated chicken eggs at 35 °C for 48 h, and the allantoic fluid was collected and stored at −80 °C until use. MDCK cells were maintained in MEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% non-immobilized FCS (Sigma-Aldrich, St. Louis, MO, USA), 0.3 mg/mL L-glutamine (Nacalai Tesque, Kyoto, Japan), 100 U/mL penicillin G (Meiji Seika Pharma, Tokyo, Japan), 0.1 mg/mL streptomycin (Meiji Seika Pharma, Tokyo, Japan), and 8 μg/mL gentamicin (Takata Pharmaceutical, Saitama, Japan) in an incubator at 37 °C with 5% CO_2.

Sequencing and phylogenetic analysis

Using TRIzol LS reagent (Life Technologies, Carlsbad, CA, USA), viral RNA was extracted from the allantoic fluid of embryonated chicken eggs and reverse-transcribed with Uni12 primer (5′-AGCAAA AGCAGG-3′) and M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA, USA). The HA genome was amplified by polymerase chain reaction using Ex Taq polymerase (Takara Bio, Shiga, Japan) and a gene-specific primer set. Direct sequencing of HA gene segments was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Other nucleotide sequence data were acquired from GenBank (*https://www.ncbi.nlm.nih.gov/genbank/*) and GISAID (*https://www.gisaid.org/*). Nucleotide sequence data were processed by Genetyx version 15 (Genetyx Corporation, Tokyo, Japan). Phylogenetic analysis of the HA gene was performed by neighbor joining with 1000 bootstrap replications in MEGA 7.0 [58]. The phylogenetic tree of the H4 HA genes was rooted in A/swine/Ontario/01911-1/1999 (H4N6). The genome sequences identified in this study were registered in GenBank/EMBL/DDBJ: A/duck/Hokkaido/W195/2015 (accession no. LC498517),

A/duck/Hokkaido/W214/2006 (accession no. LC498518), and A/duck/Hokkaido/W217/2015 (accession no. LC498519). Forty-eight strains of H4 HA genes in this study were clustered into Eurasian and North American lineages based on nucleotide identities. The intragroup homology was between 96% and 99%, whereas the intergroup homology was between 81% and 92%.

Antigenic analysis

The antigenicity of H4 viruses was evaluated by the hemagglutination-inhibition (HI) test using chicken polyclonal antiserum [74]. Inactivated antigen (500 μg) was immunized to chicken twice at a 2-week interval. After confirmation of more than 2560 HI titers in the serum collected 2 weeks later from the second immunization, 500 μg of antigen was intravenously injected. One week after the last immunization, total blood was collected from the heart, and antisera were prepared. The viruses were diluted to an 8-hemagglutination unit in PBS. Then, 25 μL of the diluted virus was added to 25 μL of each antiserum serially two-fold diluted in PBS and incubated at room temperature for 30 min. Then, 50 μL of 0.5% chicken red blood cells in PBS was added and incubated at room temperature for another 30 min. The HI titer was expressed as the reciprocal of the highest serum dilution, showing complete HI.

Based on the HI test results, antigenic cartography was developed using web-based software (*http://www.antigenic-cartography.org/*) based on the previous research [14,16]. Briefly, each HI titer N_{ij} was transferred into a table antigenic distance D_{ij} between virus i and antiserum j by calculating the difference between the titer for the virus HI by each antiserum j, defined as b_j, and the measured titer for each virus N_{ij} against that antiserum: $D_{ij} = \log_2(b_j) - \log_2(N_{ij})$. To find the map distance, the Euclidean distance d_{ij} between each virus i and antiserum j was set, and the difference between the map and table distances was minimized using the error function $E = \sum_{ij} e\,(D_{ij}, d_{ij})$. The error of a serum-virus pair was defined as $e(D_{ij}, d_{ij}) = (D_{ij} - d_{ij})^2$. The ellipses of the antigenic groups were visually determined, covering all the antigens in each group with the smallest sizes. The spacing between grid lines is equivalent to an antigenic unit distance corresponding to a two-fold HI difference.

Vaccine preparation

Viruses in allantoic fluid were purified by centrifugation and sedimentation via a sucrose gradient, as described by Kida et al. [34]. The allantoic fluid was ultracentrifuged, and the resulting pellet was layered on 10–50% sucrose density gradient and

ultracentrifuged again. Fractions containing the band of virus particles were collected based on sucrose concentration, HA titer, and protein concentration. Whole-virus particles were pelleted from the sucrose fraction by ultracentrifugation and suspended in PBS. The purified virus was inactivated by incubation in 0.1% formalin at 4 °C for 7 days. The inactivation of the formalin-treated virus was confirmed by no virus growth in embryonated chicken eggs after inoculation. The purified inactivated virus was used as a whole-virus particle vaccine. The total protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The amount of vaccine in the potency test was set to 4, 20, and 100 μg based on previous research [52]. According to the ratio between HA protein (14.7 μg) and whole-particle vaccine (50 μg) for influenza A virus [51], the amount of HA proteins in each vaccine was estimated as 1.2, 5.9, and 29.4 μg, respectively.

Pathogenicity of H4 influenza virus in mice

Eight-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) were challenged with the MO/15 virus to evaluate virus pathogenicity in mice. Four mice of four groups were intranasally inoculated with MO/15 at 30 μL/mouse under anesthesia. The mixtures of tiletamine hydrochloride (20 mg/kg) (United States Pharmacopeia, Rockville, MA, USA), zolazepam hydrochloride (20 mg/kg) (United States Pharmacopeia, Rockville, MA, USA), and xylazine (20 mg/kg) (Bayer Yakuhin, Ltd., Osaka, Japan) were injected intraperitoneally into mice within 100 μl [51]. The viral titer of the challenge was $10^{5.9,}$ $10^{5.0}$, $10^{4.0}$, or $10^{3.0}$ PFU of MO/15 (30 μL). The viral titer was originally $10^{7.4}$ PFU/mL ($10^{5.9}$ PFU/30 μL), and this virus stock was used for the highest-dose group without dilution in PBS. Mice were observed daily for changes in body weight and clinical signs until 9 days post-inoculation (dpi). The humane endpoint was determined as 70% of the body weight at challenge, and if the body weight of a mouse decreased below the endpoint, the mouse was euthanized.

Potency test of the vaccine against MO/15 in mice

Swan/Hok and MO/15 vaccines with 4, 20, and 100 μg of protein were subcutaneously injected into groups of 10 4-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan). PBS was injected into negative control (NC) and non-vaccinated mice. After 2 weeks, after collecting blood from the tail vein to obtain serum, the same amount of vaccines was injected. At 2 weeks after the second immunization, blood samples were collected from the tail vein, and $10^{5.9}$ PFU/30 μL of MO/15 was intranasally inoculated

to mice under anesthesia. Three days after the challenge, five mice from each group were euthanized, and the lungs were collected for measuring virus recovery. The mice were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg) (Kyoritsu Seiyaku Corporation, Tokyo, Japan). Viral titers in the lung homogenates were measured using a plaque assay in MDCK cells. The other five mice in each group were observed for 14 days for clinical signs.

Serum neutralization test

Serum-neutralizing antibody titers in mice were measured using methods described in a previous research [52]. Briefly, mouse serum samples were heat-inactivated at 56 °C for 30 min and mixed with 100-fold median tissue culture infectious dose ($TCID_{50}$) of MO/15 or Swan/Hok virus and incubated for 1 h at room temperature. The mixture was inoculated onto confluent monolayers of MDCK cells and incubated at 35 °C for 1 h. Unbound viruses were removed, and cells were washed with PBS. Cells were incubated with MEM containing 5 μg/mL acetylated trypsin (Sigma-Aldrich, St. Louis, MO, USA). Cytopathic effects were observed after 72 h of incubation, and the neutralizing antibody titers were determined as the reciprocal of the serum dilution yielding 50% inhibition of the cytopathic effects.

Virus titration in mouse lungs

Plaque assays were performed as previously described for the measurement of viral titers [52]. Briefly, 10-fold dilutions of virus samples or mouse lung homogenates in MEM without FCS were inoculated (100 μL/well) onto confluent monolayers of MDCK cells and incubated at 35 °C in a 5% CO_2 incubator for 1 h with tilting every 15 min. The virus solution was removed, and cells were washed with PBS once. Cells were then overlaid with MEM containing 5 μg/mL acetylated trypsin (Sigma-Aldrich, St. Louis, MO, USA) and 1% Bacto Agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) heated at 42 °C. After 48 h of incubation at 35 °C, cells were stained with 0.005% neutral red with MEM and 1% Bacto Agar. After incubation at 35 °C for an additional 24 h, the number of plaques that were fewer than 100 plaques/well was counted. The number of PFU/g in the original solution was calculated as the product of the reciprocal value of the dilution and the number of plaques in that dilution.

Statistical analysis

The Student's *t*-test was used to analyze differences in the body weights of mice, virus recovery, and neutralizing antibody titer between the two groups. One-way analysis of variance was used to analyze differences among multiple groups [2]. Animal survival and body weight data were analyzed using the Mantel–Cox test.

Comparison of amino acid substitutions on the 3D structure of HA

Amino acid sequence data were acquired from GenBank (*https://www.ncbi.nlm.nih.gov/genbank/*) and data were processed by Genetyx version 15. X-ray crystallographic structural data of MO/15 (PDB ID: 6V44) [69] was acquired from Protein Data Bank (*https://www.rcsb.org/*) and visualized by Discovery Studio Visualizer (v20.1.0.19295) (*https://discover.3ds.com/discovery-studio-visualizer-download*) (Dassault Systèmes BIOVIA, San Diego, CA, USA).

Ethics statement

All experiments using viruses were approved by Hokkaido University Safety Management Regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (18-0035 and 16-0105), and all experiments were conducted per the guidelines of this committee. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The Faculty of Veterinary Medicine, Hokkaido University, has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International since 2007.

Results

Genetic analysis of H4 influenza viruses

The genetic sequence of H4 HA genes was determined and phylogenetically analyzed along with reference sequences available in the database (Figure 7). H4 viruses were roughly divided into North American and Eurasian lineages [38]. The viruses classified into the Eurasian lineage were further classified into Groups 1–4. Groups 1–3 were determined by previous research [38], and Group 4 was defined in the present research. Groups were classified based on the sequence homology of HA genes. Group 1 was composed of isolates from China, Mongolia, and Hokkaido from 2009 to 2014. Isolates from Mongolia and China from 2011 to 2015 were set as Group 2. Group 3 was composed of isolates from China, Mongolia, and Hokkaido from 2006 to 2015. Viruses isolated in Hokkaido in 2017 were set as Group 4 and are systematically close to those isolated in Mongolia and China between 2015 and 2016 with high homology. In the North American lineage, viruses were further classified into several groups [71]. They were classified into North American Groups 1 and 2, which were determined in this study for convenience. A/swine/Ontario/01911-1/1999 (H4N6) and MO/15 were included in Groups 1 and 2, respectively. MO/15 was isolated from pigs but genetically close to the viruses isolated from ducks in North America in 2015.

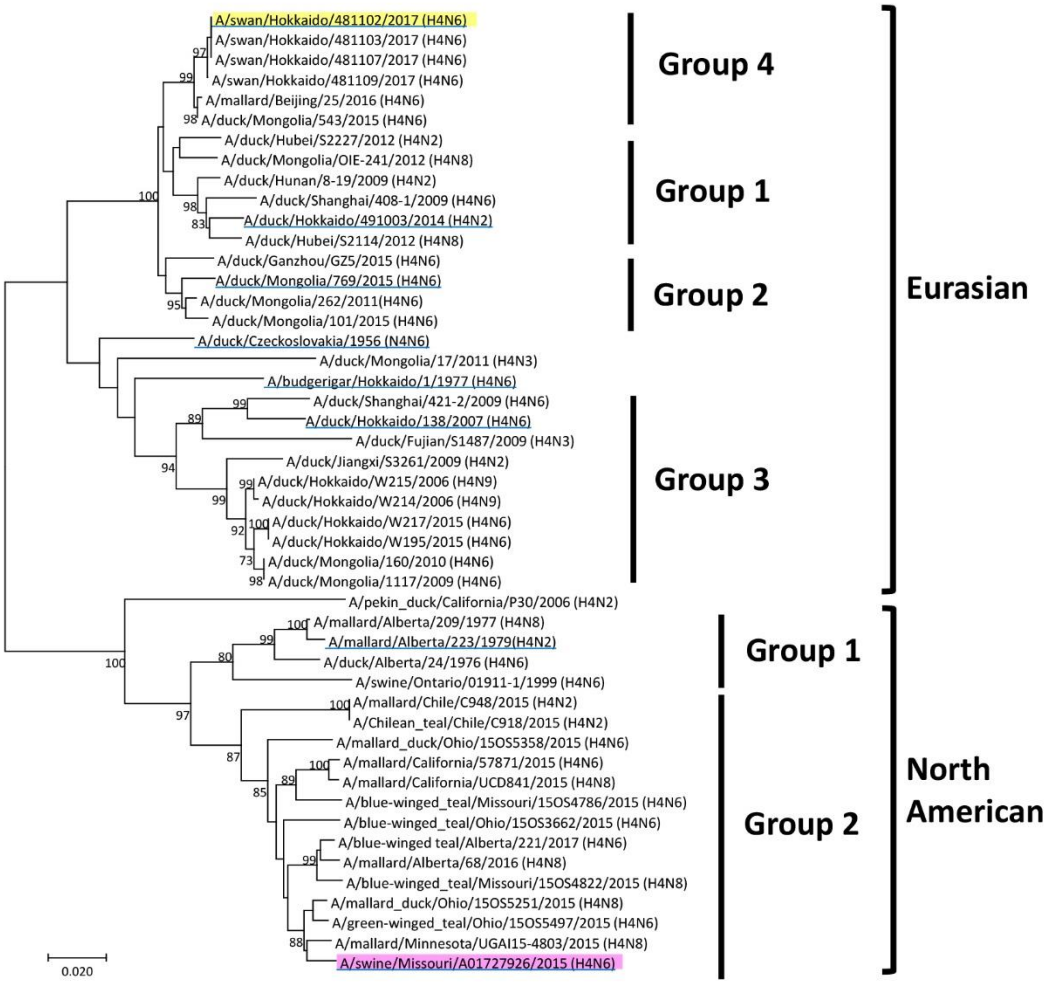

Figure 7. Phylogenetic tree of H4 hemagglutinin (HA) genes of influenza viruses. The nucleotide sequence of the HA gene segment was analyzed by neighbor joining using MEGA 7.0. The number on each node indicates the confidence level of the bootstrap analysis with 1000 replications. The Eurasian lineage was classified into Groups 1–4, and the North American lineage was classified into Groups 1 and 2. The vaccine strain is highlighted in yellow, and the challenging virus is highlighted in pink. The reference viruses used in the antigenic analysis are underlined in blue.

Antigenic analysis of H4 influenza viruses

Eight H4 viruses from the North American and Eurasian lineages were selected to represent groups based on the phylogenetic tree of the HA gene (Figure 7), and their antigenicity was compared using the cross-HI test (Table 1). Then, based on the results from the cross-HI test results, the antigenic cartography required to project the dataset onto 2D cartography was made (Figure 8). The H4 HA antigenicity was roughly divided into two groups, and all of the classification by the HA gene was not the same as the classification by antigenicity. Swan/Hok, which was isolated in Hokkaido in 2017, and Dk/Cz from the Eurasian lineage or the viruses from the North American lineage were regarded to establish a major antigenic group. Mal/Alb was also included in the major antigenic group. Viruses isolated from budgerigar in 1979 and a virus isolated from ducks in 2007 in Hokkaido were regarded to establish the unique antigenic group. MO/15 was also antigenically close to the major antigenic group. Among them, MO/15 was antigenically closest to Swan/Hok. In all strains, the HA titer of the Swan/Hok inoculum propagated in the allantoic cavity was 512 HA, which indicated that Swan/Hok grew most in embryonated chicken eggs among the viruses used in this study (Table 2). Therefore, Swan/Hok was selected as the representative vaccine strain to prepare an inactivated whole-particle vaccine.

Table 1. Cross-reactivity of H4 influenza viruses with chicken antisera to strains in the hemagglutination-inhibition (HI) test.

Group	Viruses	HI Titer of the Antiserum							
		Dk/Cz	Budge/ Hok	Dk/Hok/ 491003	Dk/Mon	Dk/Hok/ 138	Swan/ Hok	Mal/ Alb	MO/15
Eurasian	A/duck/Czeckoslovakia/1956 (H4N6)	5120	10,240	2560	10,240	5120	40,960	1280	10,240
Eurasian	A/budgerigar/Hokkaido/1/1977 (H4N6)	20,480	20,480	2560	5120	5120	2560	5120	5120
Eurasian 1	A/duck/Hokkaido/491003/2014 (H4N2)	1280	2560	560	2560	2560	20,480	1280	10,240
Eurasian 2	A/duck/Mongolia/769/2015 (H4N6)	1280	10,240	320	10,240	1280	40,960	2560	10,240
Eurasian 3	A/duck/Hokkaido/138/2007 (H4N6)	10,240	20,480	5120	10,240	10,240	20,480	10,240	10,240
Eurasian 4	A/swan/Hokkaido/481102/2017 (H4N6)	2560	10,240	640	1280	2560	40,960	640	20,480
North American 1	A/mallard/Alberta/223/1979 (H4N2)	2560	2560	640	2560	2560	20,480	10,240	5120
North American 2	A/swine/Missouri/A01727926/2015 (H4N6)	20,480	5120	640	1280	1280	20,480	2560	20,480

Homologous titers are underlined. Abbreviations: Dk/Cz, A/duck/Czeckoslovakia/1956 (N4N6); Budge/Hok, A/budgerigar/Hokkaido/1/1977 (H4N6); Dk/Hok/491003, A/duck/Hokkaido/491003/2014 (H4N2); Dk/Mon, A/duck/Mongolia/769/2015 (H4N6); Dk/Hok/138, A/duck/Hokkaido/138/2007 (H4N6); Swan/Hok, A/swan/Hokkaido/481102/2017 (H4N6); Mal/Alb, A/mallard/Alberta/223/1979 (H4N2); MO/15, A/swine/Missouri/A01727926/2015 (H4N6).

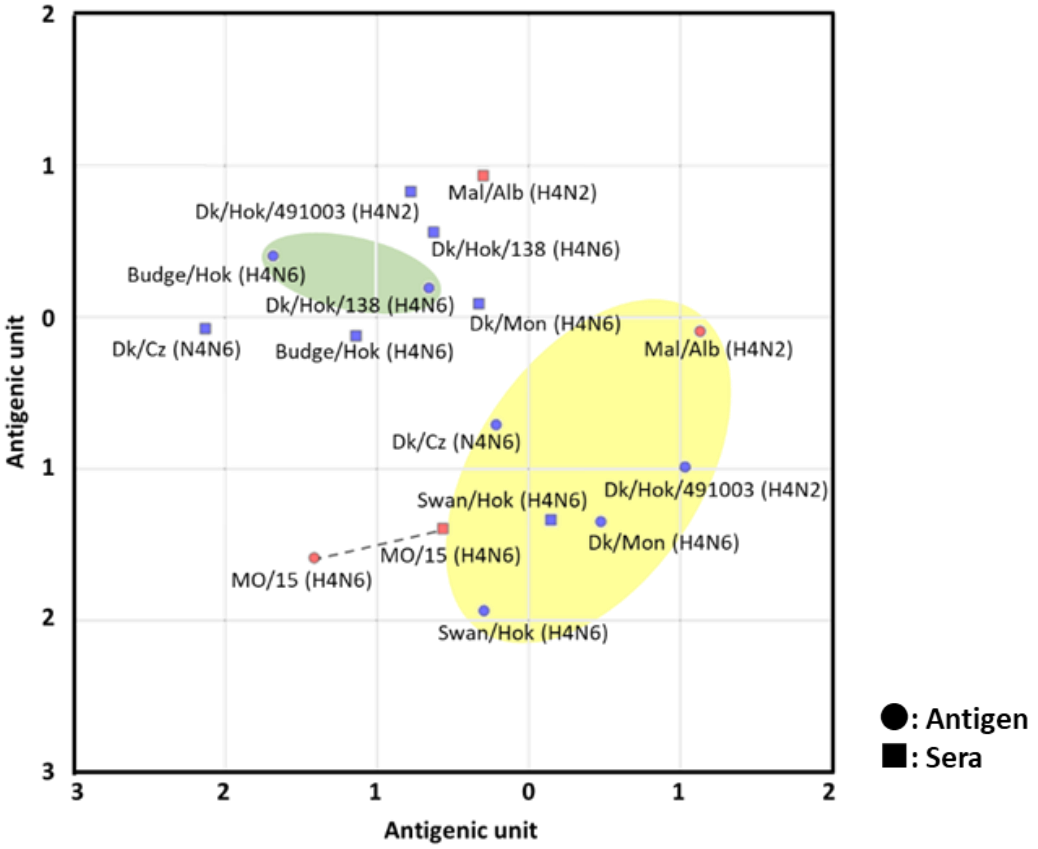

Figure 8. Antigenic cartography based on the cross-HI test of the viruses and antisera of different lineages. In antigenic cartography, vertical and horizontal axes show the distance of the antigen. Dots with round and square shapes indicate antigens and antibodies, respectively. The distance between two dots on the map represents the antigenic distance. Blue and red dots indicate the isolates belonging to the Eurasian and North American lineages, respectively. The antigenic group is shown in yellow or green. The dotted line represents homologous combination. The spacing between grid lines is equivalent to an antigenic unit distance corresponding to a two-fold HI difference.

Table 2. Growth of H4 influenza viruses in embryonated chicken eggs.

Viruses	Group	HA titer of allantoic fluid
A/duck/Czeckoslovakia/1956 (N4N6)	Eurasian	32
A/budgerigar/Hokkaido/1/1977 (H4N6)	Eurasian	128
A/duck/Hokkaido/491003/2014 (H4N2)	Eurasian 1	256
A/duck/Mongolia/769/2015 (H4N6)	Eurasian 2	128
A/duck/Hokkaido/138/2007 (H4N6)	Eurasian 3	128
A/swan/Hokkaido/481102/2017 (H4N6)	Eurasian 4	512
A/mallard/Alberta/223/1979 (H4N2)	North American 1	256
A/swine/Missouri/A01727926/2015 (H4N6)	North American 2	256

Pathogenicity of swine H4 influenza virus in mice

The dose-dependent increase in pathogenicity of MO/15 was evaluated to determine the titer at which clinical signs of mice can be clearly observed (Figure 9). After the inoculation of MO/15 from 2 dpi, compared with the NC group, the body weight of mice in the $10^{5.0}$ and $10^{5.9}$ PFU/30 μL groups was significantly reduced. From 3 dpi, the body weight of mice in the $10^{3.0}$ and $10^{4.0}$ PFU/30 μL groups was significantly reduced. Compared with the $10^{3.0}$, $10^{4.0}$, and $10^{5.0}$ PFU/30 μL groups, the $10^{5.9}$ PFU/30 μL group had more severe weight loss, and unlike the other groups, the average body weight decreased to approximately 70% of the body weight at the challenge. Based on these findings, in the potency test of the vaccine, the titer of the challenge virus was decided in sublethal dose of $10^{5.9}$ PFU/30 μL, making it easy to confirm the effect of clinical signs.

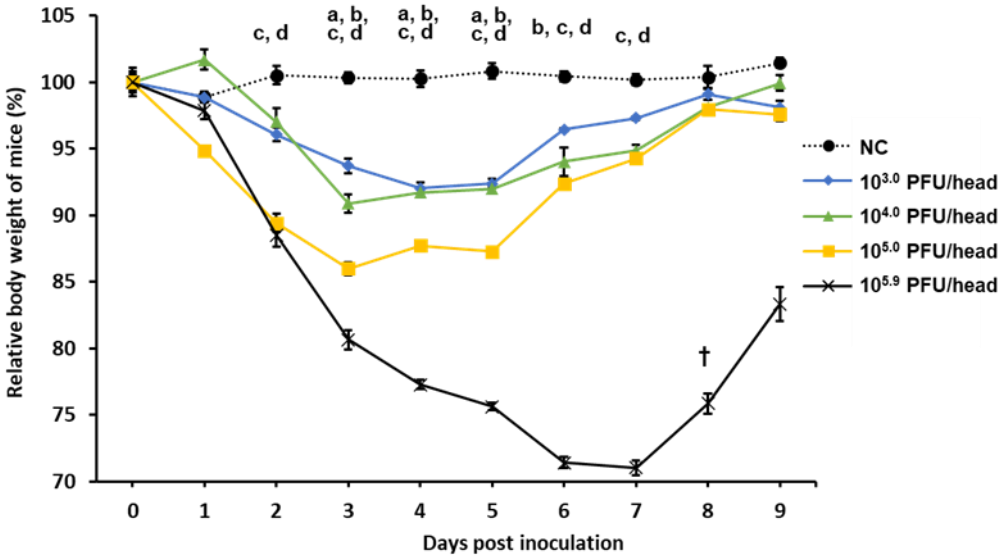

Figure 9. Changes in the body weight of mice inoculated with various doses of MO/15 influenza virus. The pathogenicity of dose-dependent viral titers was compared. Body weight was monitored for 9 days after challenge with different doses ($10^{3.0}$, $10^{4.0}$, $10^{5.0}$, or $10^{5.9}$ plaque-forming units (PFU)/30 μL) of MO/15 (n = 4 mice/group). †, euthanasia. a, significant difference between NC and $10^{3.0}$ PFU/head ($p < 0.05$); b, significant difference between negative control (NC) and $10^{4.0}$ PFU/head ($p < 0.05$); c, significant difference between NC and $10^{5.0}$ PFU/head ($p < 0.05$); d, significant difference between NC and $10^{5.9}$ PFU/head ($p < 0.05$).

Potency test of the vaccine against H4 influenza virus in mice

Based on the antigenic analysis, Swan/Hok is close to the MO/15 virus and cross-reacts with a wide range of H4 influenza viruses. The titer of the neutralizing antibody in mouse serum vaccinated twice against MO/15 and Swan/Hok was calculated (Table 3). The neutralizing antibody titer of mice immunized with 100 μg Swan/Hok against the MO/15 virus was 1:320. Compared to the 20 μg group, the antibody titers of Swan/Hok were almost 1:160 and slightly higher than the MO/15 group against the MO/15 virus. Compared to the antibody titer of the 20 μg Swan/Hok group, the titer of the serum was similar between the MO/15 virus and Swan/Hok. In these results, the Swan/Hok vaccine showed high immunogenicity and induced the cross-reactive antibody to the MO/15 virus.

Mice vaccinated with each of the two strains were then intranasally inoculated with $10^{5.9}$ PFU/30 μL MO/15. The average viral titer of the lungs in any vaccination group was significantly lower than in the non-vaccinated groups (Figure 10). The average viral titers of 4 and 100 μg Swan/Hok-vaccinated mice were not significantly different from those with a corresponding amount of MO/15. However, the average viral titer in the lungs of mice vaccinated with 20 μg Swan/Hok was significantly lower than that immunized with the same amount of homologous vaccine MO/15 at 3 dpi. The significant differences between 4 and 100 μg MO/15 groups, and 20 μg MO/15 and 100 μg Swan/Hok groups were revealed, respectively. In the Swan/Hok group, the average viral titer in the lungs immunized with 20 μg was significantly lower than that with 4 μg at 3 dpi.

Body weight change was observed until 14 dpi (Figure 11). At 3 dpi, a significant difference between all vaccinated groups and the PBS group was observed. There was no significant difference in weight loss at each observation for 14 days between the groups of mice vaccinated with the same amount of MO/15 and Swan/Hok. However, the body weights of mice in the Swan/Hok group recovered earlier than in the MO/15 group; in other words, all Swan/Hok-vaccinated mice gained their body weight to a similar level to those of the NC group, which was earlier than the recovery speed of mice vaccinated with MO/15. The body weights of mice vaccinated with 4 μg Swan/Hok showed no significant difference from those of the NC group after 11 dpi (Figure 11a). At 6 dpi, no significant difference was observed between the body weights of the NC group and those of either 20 or 100 μg Swan/Hok-vaccinated group (Figure 11b, c). Two mice in the non-vaccinated group were euthanized because their body weights reached the humane endpoint, whereas none of the vaccinated mice reached it.

Table 3. Serum-neutralizing antibody titers of vaccinated mice.

Vaccine	Dose (μg)	Neutralizing Antibody Titers Against											
		MO/15					GM	Swan/Hok					GM
MO/15	4	80	80	80	80	80	80	40	80	80	80	80	70
	20	80	160	80	160	80	106	40	80	80	80	80	70
	100	320	320	320	320	320	320	80	40	80	80	80	70
Swan/Hok	4	80	80	80	80	80	80	80	80	80	80	80	80
	20	160	160	160	160	160	160	160	160	320	160	160	184
	100	320	320	320	320	320	320	640	160	320	320	320	320
PBS	-	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40

<40: Not detected, GM: geometric mean.

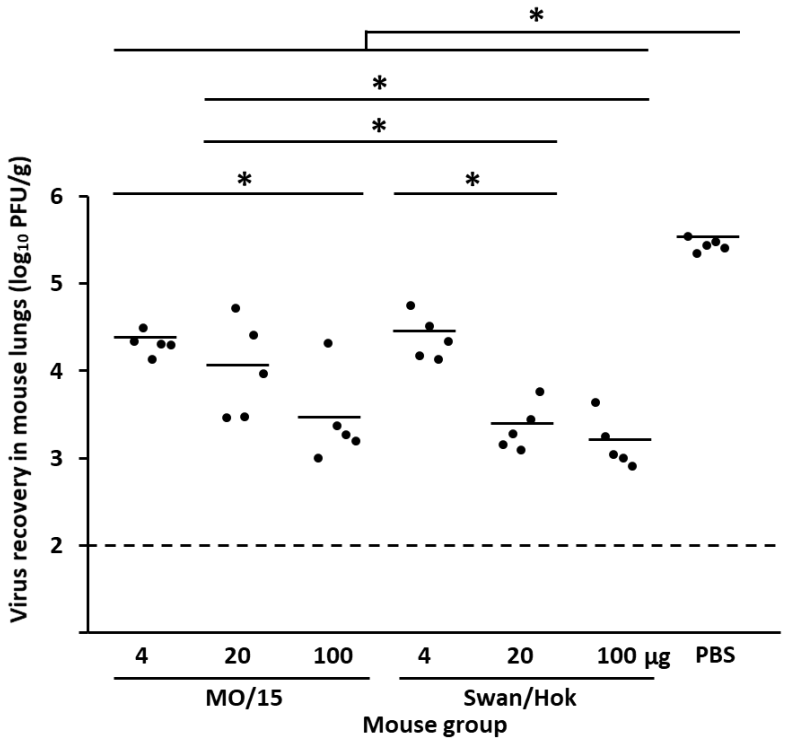

Figure 10. Virus recovery in the lungs of mice subcutaneously vaccinated twice with various amounts of the MO/15 or Swan/Hok vaccine after a challenge with $10^{5.9}$ PFU/30 µL MO/15. Viral titers were measured on day 3 after the MO/15 challenge. * $p < 0.05$.

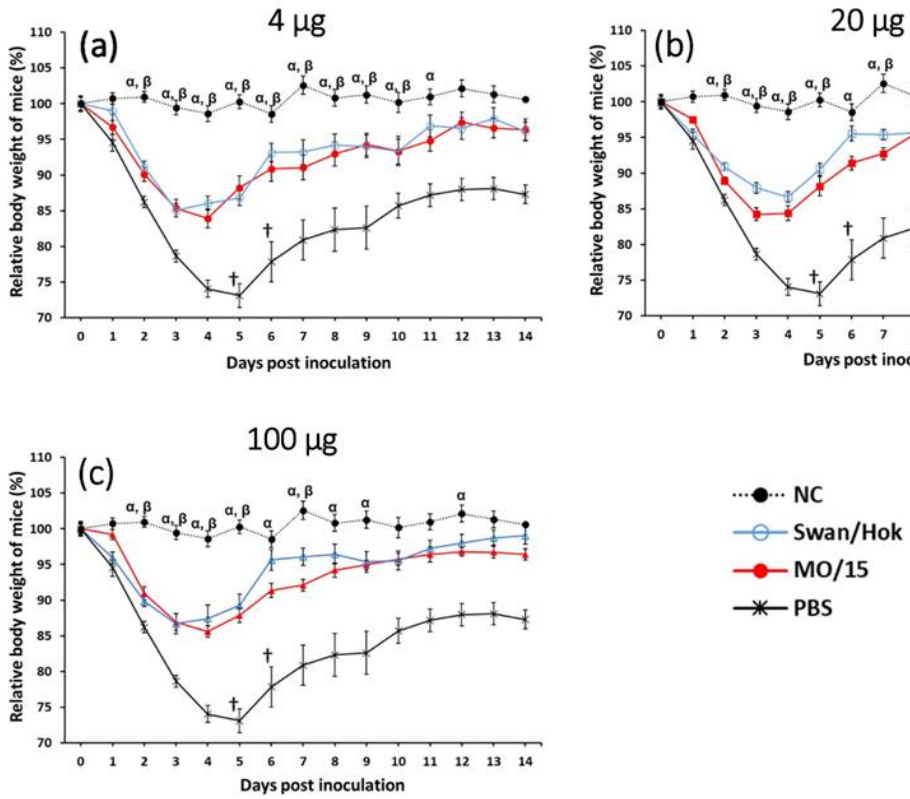

Figure 11. Changes in the body weights of mice subcutaneously vaccinated twice with Swan/Hok and homologous MO/15 after a challenge with MO/15 influenza virus. Body weights were monitored for 14 days after the MO/15 challenge (n = 5 mice/group). Mice received two immunizations: 4 μg (a), 20 μg (b), or 100 μg (c) of each vaccine subcutaneously. †, euthanasia. α, significant difference between NC and MO/15 ($p < 0.05$); β, significant difference between NC and Swan/Hok ($p < 0.05$).

Discussion

Influenza virus infection in pigs is of great interest due to the potential emergence of a new influenza pandemic strain. For example, the Eurasian avian-like H1N1 swine influenza virus was recently isolated, and its potential ability to infect humans has become a significant concern [62]. Although no human infection with the H4 virus has been confirmed to date, this virus is uniquely positioned to infect humans in the future. In 2015, the H4 virus was isolated from pigs [1]. An influenza pandemic is derived from influenza viruses circulating among pigs. Pigs are infected with both human or avian viruses [56], causing a reassortment between two types of viruses and thereby producing pandemic candidates with an antigenic shift. Because the antigenicity is conserved in waterfowl, which are natural hosts of influenza viruses [3], monitoring the viruses circulating in waterfowl to prepare for an influenza pandemic in humans via pigs is necessary. Therefore, surveillance of waterfowl-derived influenza viruses and vaccines made from influenza virus isolated from birds may be effective in preparing for future outbreaks of a human influenza pandemic. This study is the first report of creating the H4 influenza vaccine and evaluating its protective potency against the H4 influenza virus.

Based on a phylogenic tree of the HA gene, candidate vaccine strains were screened for a potential pandemic caused by H4 influenza, which has similar virus characteristics to MO/15. A trial vaccine strain was selected based on this phylogenetic tree, antigenic analysis, and proliferation in embryonated chicken eggs. Interestingly, the antigenic analysis resulted in two groups independent of genetic similarity. One of them was the major antigen group composed of Eurasian Dk/Cz, Swan/Hok, and North American Mal/Alb. Although MO/15 was classified in the North American lineage based on the HA gene phylogenetic tree, antigenic analyses placed this virus close to the major antigenic group to which the Eurasian lineage belongs. Regardless of the genetic distance from the North American lineage, the Swan/Hok influenza strain was antigenically closest to MO/15. Furthermore, Swan/Hok showed higher HA titer than other viruses and produced a high final protein concentration in chicken eggs (25.9 μg/egg; MO/15, 14.9 μg/egg). Finally, Swan/Hok was the most proliferative in embryonated chicken eggs among all the candidate strains and, therefore, was the most suitable vaccine candidate.

The vaccine prepared from Swan/Hok induced sufficient neutralizing antibody against MO/15 in mice and reduced the viral load in the lungs. In particular, the neutralizing antibody titer of Swan/Hok-vaccinated mice against a virus challenge was significantly higher than that of the MO/15-vaccinated group at 20 μg. The neutralizing

antibody titer of the MO/15-vaccinated group challenged with MO/15 virus was lower in all vaccine concentrations than that of the Swan/Hok group challenged with Swan/Hok virus, demonstrating high immunogenicity of the Swan/Hok vaccine in mice. Furthermore, the neutralizing antibody titer of the Swan/Hok-vaccinated group was similar against homologous and heterologous viruses, indicating that the Swan/Hok vaccine induced a cross-reactive antibody. Between MO/15 and Swan/Hok viruses, there were 20 amino acid differences, and 10 amino acid substitutions were located on the head domain (Figure 12). Further analysis is essential to clarify high immunogenicity of Swan/Hok vaccine in mice and cross-reactivity of the induced antibody to MO/15 because limited information is available regarding the structure and antigenic sites of H4 viruses.

MO/15 infection caused an average weight loss of 29% in mice, and a titer of $10^{5.4}$ PFU/g was measured from the lung homogenate at 3 dpi after a challenge with $10^{5.9}$ PFU/30 μL. The viral load in the lungs of mice vaccinated with 20 μg Swan/Hok was significantly lower than that of MO/15-vaccinated mice with the same amount. After the virus challenge, no significant difference was observed between the two immunized groups, but the body weight of mice immunized with Swan/Hok recovered earlier than that of the homologously challenged MO/15-vaccinated group. In particular, the body

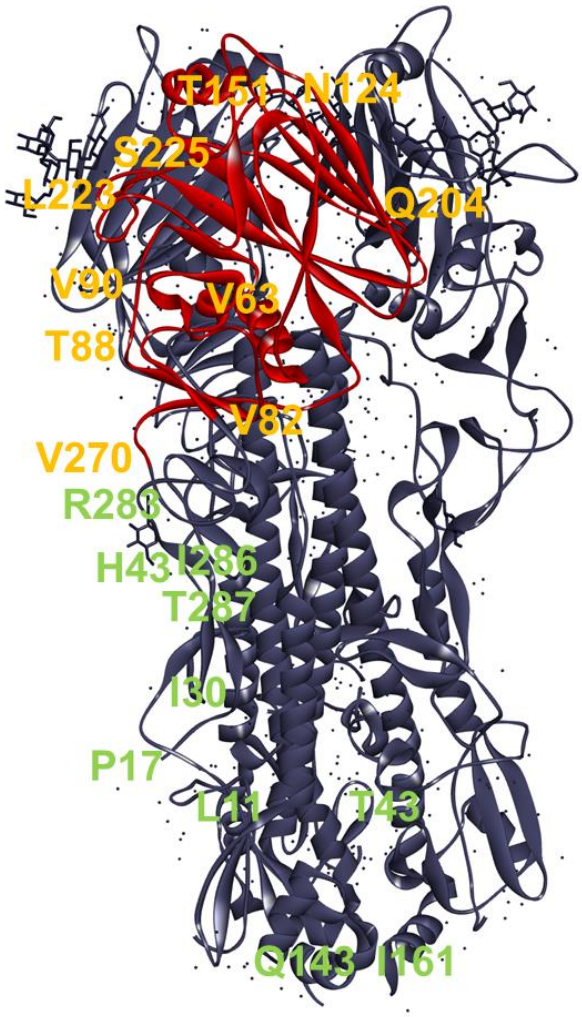

Figure 12. Three-D structure of MO/15 HA trimer and the positions of amino acid substitutions between MO/15 and Swan/Hok. Red: HA1 head domain, Yellow: Different residues at the head domain between MO/15 and Swan/Hok, Green: Different residues at the stalk domain between MO/15 and Swan/Hok.

weight of mice vaccinated with 20 μg Swan/Hok recovered at 6 dpi, and this quick recovery was also confirmed in the group receiving 100 μg Swan/Hok. In this study, an inactivated whole-particle vaccine prepared from the Swan/Hok virus selected from the virus library was used in mice after two subcutaneous injections. This vaccine induced sufficient neutralizing antibodies and protected mice from the MO/15 virus challenge. Therefore, the vaccine prepared from the Eurasian avian influenza virus in this study is effective against the H4 influenza pandemic that may occur in the future.

The H4 avian influenza virus was confirmed to be antigenically close to the virus isolated from pigs. The origin of a human influenza pandemic is primarily due to the role of pig as a mixing vessel; therefore, a virus derived from a wild waterfowl that infects pigs has a high potential for causing a future pandemic in humans. It was demonstrated that influenza viruses of both antigenicities are prevalent in waterfowl in Eurasia and North America. The genotypes of H4 viruses were divided into Eurasian or North American lineages, and the antigenicity was not determined by the genotype. A vaccine was prepared, and it produced a significant effect on mice. A virus library of nonpathogenic influenza viruses with 144 combinations of 16 HA and 9 NA subtypes to prepare for influenza pandemic (*http://virusdb.czc.hokudai.ac.jp*) has been established, and its effectiveness is revealed. Subsequently, an evaluation of a trial vaccine using the strain stocked in the library will be necessary to prepare for an influenza pandemic.

Brief summery

H4 influenza viruses have been isolated from birds across the world. In recent years, an H4 influenza virus infection has been confirmed in pigs. Pigs play an important role in the transmission of influenza viruses to human hosts. Therefore, it is important to develop a new vaccine in the case of an H4 influenza virus infection in humans, considering that this virus has a different antigenicity from seasonal human influenza viruses. In this study, after selecting vaccine candidate strain based on their antigenic relation to one of the pig isolates, A/swine/Missouri/A01727926/2015 (H4N6) (MO/15), an inactivated whole-particle vaccine was prepared from A/swan/Hokkaido/481102/2017 (H4N6). This vaccine showed high immunogenicity in mice, and the antibody induced by the vaccine showed high cross-reactivity to the MO/15 virus. The viral titers in the lungs of the mice vaccinated with the candidate vaccine were lower than those of them vaccinated with MO/15, and the vaccinated mice recovered their body weight more quickly than MO/15-vaccinated group. This vaccine induced sufficient neutralizing antibodies and mitigated the effects of an MO/15 infection in a mouse model. This study is the first to suggest that an inactivated whole-particle vaccine prepared from an influenza virus isolated from wild birds is an effective countermeasure in the case of a future influenza pandemic caused by the H4 influenza virus.

Conclusion

Influenza is caused by influenza A viruses. The natural reservoir of influenza A viruses is wild migratory waterfowls, and in the avian influenza viruses circulating in waterfowls, a virus has infectivity in humans through pig. Therefore, the virus which has a different antigenicity, and has infectivity in human-to-human may cause a pandemic influenza. For the reason that the antigenicity of the pandemic influenza virus is differing from seasonal influenza viruses, vaccines for seasonal influenza cannot induce effective antibodies against the pandemic influenza virus. Therefore, nonspecific or specific countermeasures are required for the preparedness against pandemic influenza.

In Chapter I, the immunomodulatory effect of vitamin D metabolite $25(OH)D_3$ against influenza was evaluated. Vitamin D is a fat-soluble vitamin that can be ingested and absorbed in the small intestine. It is metabolized in the liver and becomes the highly stable 25(OH)D, which circulates in the blood. The kidney metabolizes 25(OH)D into $1,25(OH)_2D$, then, $1,25(OH)_2D$ has an anti-inflammatory effect by its binding to the vitamin D receptor through the half-life of $1,25(OH)_2D$ is only 15 hours. A precursor of $1,25(OH)_2D$, $25(OH)D_3$, is absorbed efficiently in the small intestine and has a relatively long half-life of 15 days. In the present study, a mouse model of influenza virus infection and direct oral ingestion of $25(OH)D_3$ was used to evaluate the effect of this metabolite on the clinical manifestations of influenza virus infection. Oral administration of $25(OH)D_3$ for 7 weeks increased sufficient blood level of $25(OH)D_3$ and $24,25(OH)_2D_3$ [metabolite form of $25(OH)D_3$], without adverse effects in a mouse model. After the virus challenge with A/Puerto Rico/8/1934 (H1N1), virus replication was reduced in lungs, and body weight loss and mortality were mitigated in $25(OH)D_3$-supplemented group. Additionally, the production of proinflammatory cytokines was downregulated in $25(OH)D_3$-fed mice. These results showed that continuous direct oral ingestion of $25(OH)D_3$ has the potential to mitigate the clinical manifestations of influenza virus infection.

In Chapter II, a vaccine strain against pandemic H4 influenza virus was selected and its protective efficacy was evaluated. H4 avian influenza viruses have been circulating in wild waterfowls which are a natural reservoir of influenza A viruses, and H4 virus infection in swine has been sporadically reported. Therefore, the emergence of an H4 influenza pandemic in humans via pigs is concerned. In this study, after selecting vaccine candidate strain from the virus library based on their genetic and antigenic relation to one of the pig isolates, A/swine/Missouri/A01727926/2015 (H4N6) (MO/15),

an inactivated whole-particle vaccine was prepared and its protective efficacy was evaluated in a mouse model. Among them, the vaccine prepared from A/swan/Hokkaido/481102/2017 (H4N6) had high immunogenicity in mice, and the neutralizing antibodies induced by the vaccine had high cross-reactivity to the MO/15 virus. Moreover, after the MO/15 viral challenge, virus replication was reduced in the lungs of the vaccinated mice, and body weight reduction and mortality were mitigated. From those results, a whole-particle vaccine selected from the virus library is effective against H4 virus infection, and it is supposed to be an effective preventive measure against future pandemic influenza.

In conclusion, specific and nonspecific countermeasure for the future pandemic influenza was evaluated and confirmed in this study. These findings will be the effective preventive measure, therefore, further analysis for the preparedness against pandemic influenza was required.

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Summary in Japanese（和文要旨）

A 型インフルエンザウイルスはヒトや動物のインフルエンザの病原ウイルスの一つである。ウイルスの宿主レセプターへの結合特異性の違いにより鳥のインフルエンザウイルスがヒトへと直接感染することはほとんどない。一方、ブタは呼吸上皮細胞に鳥型およびヒト型レセプターを発現しており、鳥とヒトの両方のインフルエンザウイルスに感染する。そのため 2 つのウイルスの遺伝子再集合体がブタで生まれ、新型インフルエンザウイルスが出現することが過去のパンデミックから明らかになっている。新型インフルエンザウイルスはヒトの季節性インフルエンザウイルスとは抗原性が異なるので、従来のワクチンでは有効な免疫が誘導されない。そのため、将来の新型インフルエンザウイルスの出現に備えた予防法の確立が必要である。そこで、本研究ではビタミン D 代謝物による抗原非特異的な免疫の調節、およびワクチンによる抗原特異的な免疫の誘導によるインフルエンザに対する予防効果をマウスを用いて評価した。

第一章では、ビタミン D 代謝産物 $25(OH)D_3$ の抗インフルエンザウイルス効果を評価した。ビタミン D は脂溶性ビタミンの一種であり、小腸で吸収されたのち肝臓で代謝され、$25(OH)D_3$ となり、腎臓で活性型の $1,25(OH)_2D$ となる。$1,25(OH)_2D$ はビタミン D レセプターに結合し、抗炎症性作用を誘導するが、血中における半減期は 15 時間と短い。そこで $1,25(OH)_2D$ の前駆体であり、血中における半減期が 15 日と長く、さらに小腸で吸収されやすい $25(OH)D_3$ の経口給餌によるインフルエンザに対する予防効果を、マウスを用いた感染実験で評価した。その結果、$25(OH)D_3$ の 7 週間の給餌により、血中に十分な量の $25(OH)D_3$ および代謝産物の $24,25(OH)_2D_3$ が検出された。さらにこれらのマウスを A/Puerto Rico/8/1934 (H1N1) で攻撃したところ、肺から回収されるウイルス量が減少し、体重減少や死亡率の軽減も認められた。また、これらのマウスにおいて炎症性サイトカインの産生抑制が認められた。以上より、$25(OH)D_3$ の継続的な経口補給はインフルエンザに対する効果的な予防法となることが示唆された。

第二章では、H4 亜型インフルエンザウイルスによるパンデミックに備えるためのワクチン株の選抜および試製されたワクチンの評価を行った。H4 亜型インフルエンザウイルスは A 型インフルエンザウイルスの自然宿主である野生水禽内で維持されているが、ブタへの感染も散発的に報告されている。そのため H4 亜型インフルエンザウイルスがブタを介して新型インフルエンザウイルスとして出現することが危惧される。そこで、2015 年に米国でブタから分離された A/swine/Missouri/A01727926/2015 (H4N6) (MO/15) に対するワクチン株を遺伝子、抗原性をもとに当研究室のウイルスライブラリーから選抜後、全粒子不活化ワ

クチンを試製し、マウスで評価した。その結果、ワクチン株として選抜された A/swan/Hokkaido/481102/2017 (H4N6) を用いて試製されたワクチンはマウスに対して高い免疫原性を示し、誘導された抗体は MO/15 株に対して高い交差反応性を示した。さらに、これらの免疫されたマウスを MO/15 株で攻撃したところ、肺から回収されるウイルス量が減少し、体重減少や死亡率の軽減も認められた。以上より、ウイルスライブラリーから試製した不活化全粒子ワクチンが、H4 亜型ウイルスの感染に対して有用であり、将来のパンデミックインフルエンザへの効果的な予防法となることが示唆された。

以上の結果から、ビタミン D 代謝物による抗原非特異的な免疫の調節、およびワクチンによる抗原特異的な免疫の誘導によるインフルエンザに対する予防効果を確認することができた。これらの成績は、将来起こりうるインフルエンザパンデミックに対する予防法の確立に有用であることが示された。