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ORIGINAL ARTICLE

Attenuation of an influenza A virus due to alteration of its hemagglutinin-neuraminidase functional balance in mice

Fumihiro Gen · Shinya Yamada · Kentaro Kato · Hiroomi Akashi · Yoshihiro Kawaoka · Taisuke Horimoto

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Abstract Influenza A viruses possess two surface glycoproteins, hemagglutinin (HA), which binds to sialic-acid-containing receptors, and neuraminidase (NA), which removes sialic acid from host cells. It is well established that the HA-NA functional balance regulates the efficiency of virus replication. Here, we selected a plaque variant of the WSN (H1N1) strain that grew better than the wild-type virus in NA-expressing MDCK cell culture. A reverse genetics study revealed that the single mutation HA E190K, which occurs infrequently in naturally isolated H1N1 viruses, was responsible for the phenotype of this variant. Receptor assays indicated that this mutation did not affect the receptor specificity of HA but enhanced its receptor-binding affinity, resulting in altered HA-NA functional balance relative to that of the wild-type virus. We also found that this variant replicated in nasal turbinates at an equivalent level but in lungs at a lower level compared with wild-type virus, demonstrating its attenuation in mice. Together, our data demonstrated the importance of the HA-NA functional balance for influenza virus replication in an *in vivo* biological setting.

F. Gen · K. Kato · H. Akashi · T. Horimoto (✉)
Department of Veterinary Microbiology,
Graduate School of Agricultural and Life Sciences,
The University of Tokyo, Tokyo 113-8657, Japan
e-mail: ahorimo@mail.ecc.u-tokyo.ac.jp

S. Yamada · Y. Kawaoka
Division of Virology, Department of Microbiology
and Immunology, Institute of Medical Science,
The University of Tokyo, Tokyo 108-8639, Japan

Y. Kawaoka
ERATO Infection-Induced Host Responses Project,
Saitama 332-0012, Japan

Y. Kawaoka
Department of Pathobiological Sciences, School of Veterinary
Medicine, University of Wisconsin-Madison,
Madison, WI 53706, USA

Y. Kawaoka
International Research Center for Infectious Diseases,
Institute of Medical Science, The University of Tokyo,
Tokyo 108-8639, Japan

Introduction

Influenza A viruses are enveloped viruses that contain a segmented genome of eight different negative-strand RNA molecules. The envelope carries two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Both glycoproteins recognize the same host-cell molecule, sialic acid. HA binds to sialic-acid-containing receptors on target cells to initiate virus infection, whereas NA cleaves sialic acids from cell receptors to facilitate progeny virus release from cells. NA also cleaves extracellular mucus inhibitors containing sialic acids and prevents HA-mediated self-aggregation of virions by desialylation of virus glycoconjugates, promoting the spread of the infection to neighboring cells [3].

It is well established that the interplay between HA receptor-binding and NA receptor-destroying activities affects virus replication in eggs and cell culture; an optimal HA-NA functional balance is required for efficient virus replication [18]. In addition, a recent study showed that the HA-NA functional balance is critical for efficient respiratory droplet transmission of a pandemic H1N1 virus in ferrets, thereby contributing, at least in part, to virus pathogenicity in host animals [20]. Functional imbalance between HA and NA could be caused by mutations around the HA receptor-binding site, the NA enzymatic active site

or both, under various selective pressures of *in vitro* and *in vivo* biological environments [5, 6, 13, 19].

Here, we selected a plaque variant of the influenza A virus laboratory strain WSN (H1N1) in cell culture stably expressing viral NA and found that its HA-NA functional balance differed from that of the wild-type WSN virus, leading to a change in its plaque phenotype and growth properties in cell culture. To gain further insight into the responsibility of the HA-NA functional balance for the virus phenotype, we performed *in vivo* experiments showing that this variant exhibited reduced pathogenicity in mice.

Materials and methods

Cells and virus

Madin-Darby canine kidney (MDCK) and human embryonic kidney 293T cells were maintained in minimal essential medium (MEM) supplemented with 5 % newborn calf serum (NCS) and in Dulbecco's modified Eagle's medium with 10 % fetal calf serum, respectively. Cells were maintained at 37 °C in 5 % CO_2. We used A/WSN/33(H1N1; WSN) virus maintained in our laboratory. The WSN virus was propagated in MDCK cells in serum-free medium with 0.3 % bovine serum albumin (BSA) and 1 μg/ml TPCK-trypsin and was stored at −80 °C until use.

Reverse genetics

To generate a WSN mutant, we used our previously produced series of PolI constructs, derived from the WSN strain, for plasmid-based reverse genetics [15]. We also used pCAGGS plasmids expressing WSN NP, PA, PB1, or PB2 under the control of the chicken β-actin promoter for the system. Briefly, PolI plasmids generating viral RNAs and protein expression plasmids were mixed with a transfection reagent, Trans-IT 293T (Panvera), incubated at room temperature for 15 min, and then added to 293T cells. Transfected cells were incubated in Opti-MEM I (GIBCO-BRL) for 48 h. Supernatants containing infectious viruses were harvested, biologically cloned by limiting dilution in MDCK cells, and used for further experiments.

Cells stably expressing NA

MDCK cells were co-transfected with plasmids expressing WSN NA (pCAGGS-NA) and puromycin N-acetyltransferase, using an electroporator (Amaxa) according to the manufacturer's instructions. A cell clone that stably expressed NA was then selected in MEM containing 5 % NCS and puromycin dihydrochloride (5 μg/ml). NA expression was confirmed by immunostaining with an anti-NA mouse monoclonal antibody (WS5-29; a gift from Dr. Takashita, Yamagata University, Japan) after confluent cells were washed with phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde in PBS for 30 min at room temperature. NA enzyme activity of the cells was measured using a commercial assay kit with fluorescent methylumbelliferone N-acetylneuraminic acid (MUNAN-A) substrate (NA-Fluor™ kit; Applied Biosystems).

Viral replication in cell culture

The plaque assay was conducted with MDCK cells and TPCK-trypsin (1 μg/ml). The growth kinetics of viruses was assessed in MDCK cells inoculated at a multiplicity of infection (MOI) of 0.01 PFU/cell. After adsorption for 1 h, the cells were overlaid with MEM containing 0.3 % BSA and TPCK-trypsin and were incubated at 37 °C. At various times post-infection, virus titers in the culture supernatant were determined using plaque assays.

Receptor-specificity analysis with sialylglycopolymers

Glycopolymers composed of poly-α-L-glutamic acid backbones with 5-*N*-acetylneuraminic acid linked to galactose through either α2,3 or α2,6 bonds (Neu5Acα2-3LacNAcb-pAP and Neu5Acα2-6LacNAcb-pAP) were chemoenzymatically synthesized as described elsewhere [17]. Virus suspension (200 HA units/ml) diluted in ice-cold PBS was used to coat 96-well polystyrene microplates (F96 Cert. MaxiSorp Nunc-Immuno Plate; Nunc), which were then incubated for 5 h at 4 °C (on ice). As a control, wells without virus were also incubated. Unbound virus was removed by washing the wells three times with ice-cold PBS. The wells were then blocked by incubating them at 4 °C overnight with 300 μl of PBS containing 0.001 % Tween 20 (TPBS). The virus-coated wells were then washed three more times with ice-cold PBS before 25 μl of horseradish peroxidase (HRP)-conjugated bovine fetuin (which possesses both Neu5Acα2-3Gal and Neu5Acα2-6Gal) diluted in TPBS (1:2,000) was added. Then, 25 μl of serially diluted sialylglycoconjugated polymers was added, and the plates were incubated at 4 °C for 2 h. After being washed five times with ice-cold PBS, the plates were incubated with 100 μl of substrate solution (0.4 mg/ml of *o*-phenylenediamine, 0.01 % H_2O_2 in 50 mM citrate-phosphate buffer, pH 5.5) at room temperature for 10 to 20 min. To stop the reaction, 50 μl of 0.1 N H_2SO_4 was added to each well. The extent of inhibition of fetuin binding to virions with sialylglycoconjugate polymers was determined by measuring the absorbance at 490 nm.

Virus elution assay from erythrocytes

Fifty μl of a twofold dilution of virus with an HA titer of 1:64 was incubated with 50 μl of 0.5 % chicken or 0.8 % guinea pig erythrocytes in a microtiter plate at 4 °C for 1 h. The plate was then stored at 37 °C, and the reduction in HA titer was recorded periodically. Opti-MEM was used as a diluent.

Viral pathogenicity in mice

Five-week-old female BALB/c mice (n = 10/group) were infected intranasally with 50 μl of viral suspension containing diluted viruses in sterile 0.9 % sodium chloride. Animals (n = 4/group) were monitored daily for survival over the next 14 days. On days 3 and 6 post-inoculation, three mice per group were euthanized, and their lungs, trachea, and nasal turbinates were harvested and titrated for the presence of virus.

Vaccine studies

Five-week-old female BALB/c mice (n = 4/group) were infected intranasally with the variant virus; control mice (n = 4) were inoculated with PBS. At day 22 post-vaccination (challenge day), all of the mice were similarly infected with 100 median mouse lethal doses (MLD_{50}) of wild-type WSN virus. Survival was monitored daily for the next 14 days.

Results

Establishment of cells stably expressing NA

Previous reports indicated that mutant viruses with an altered HA-NA functional balance could be obtained by using MDCK cells that had been treated with exogenous bacterial NA or lectin [5, 6]. Here, we established NA-expressing cells to obtain such a virus, and selected a cell clone designated as NA-MDCK cells. Immunostaining with an anti-NA antibody confirmed NA expression on the cell surface, although the reactivity appeared to be weak (Fig. 1a), possibly suggesting a low level of NA expression. Therefore, we performed fluorescent-activated cell sorting (FACS) analysis with the anti-NA antibody, which clearly showed that the NA-MDCK cells expressed NA on their surface (Fig. 1b). To assess the enzyme activity of NA expressed on cell surface, we used an MUNANA-based assay, which showed positive NA activity of NA-MDCK cells with cell numbers of more than 2.5×10^3, unlike MDCK cells.

To confirm that the expressed NA was functional, we used FACS analysis with two lectins, MALII, which is specific for sialic-acid-linked galactose with an α2,3 linkage (SAα2,3Gal), and SNA, which is specific for sialic-acid-linked galactose with an α2,6 linkage (SAα2,6Gal). The assay demonstrated that the parent MDCK cells possessed both types of SA, as reported previously [8]. By contrast, NA-MDCK cells retained strong binding to MALII lectin but showed weaker SNA lectin binding than that of the MDCK parent (Fig. 1c), indicating a reduction in the amount of SAα2,6Gal at the cell surface. This observation suggests that WSN NA, when expressed on the cell surface, cleaves SAα2,6Gal but not SAα2,3Gal.

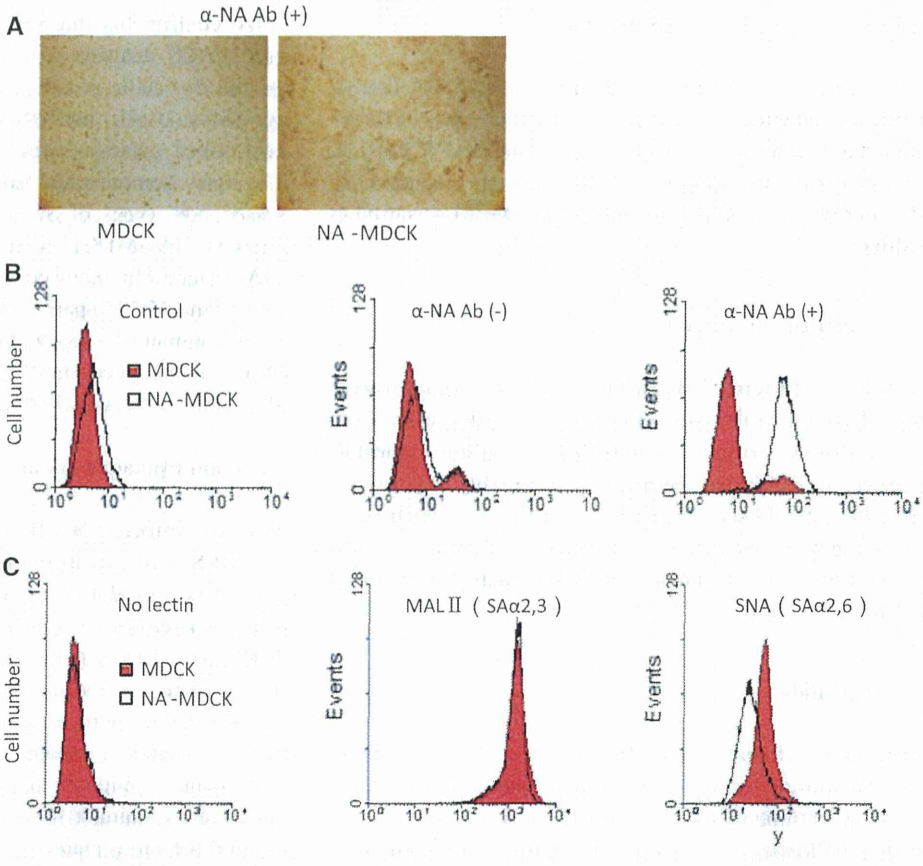
Selection of plaque variants on NA-expressing cells

We next infected NA-MDCK and parental MDCK cells with WSN virus. Inoculation with a 10^{-4} dilution of the virus stock produced no visible plaques on NA-MDCK cells, whereas large plaques were clearly observed on the MDCK parent cells (Fig. 2). However, inoculation with a 10^{-2} dilution of virus produced large plaques on NA-MDCK cells, whereas on the MDCK parent, all of the cells were damaged by cytopathic effects (data not shown). Of note, many pinpoint plaques, which were not clearly detected by staining with crystal violet, were also present around the large plaques on NA-MDCK cells. These data indicate that WSN virus forms pinpoint plaques on NA-MDCK cells and that the viruses forming large plaques on this cell clone are probably variants. To further analyze the plaque variants, we purified them by three plaque-to-plaque passages on NA-MDCK cells and designated them as WSN-LPv.

A mutation determines the plaque phenotype of the variant

We hypothesized that the HA-NA functional balance of the WSN-LPv variant differed from that of parent WSN and that it matched the receptor properties or distribution on NA-MDCK cells. To test this concept at the molecular level, we examined the HA and NA gene sequences of WSN-LPv and found only one amino acid difference at position 190 (H3 numbering) of the HA; the parent WSN HA possessed Glu (E), whereas the variant HA possessed Lys (K). We observed no differences between amino acid sequences of the two NAs.

To determine the role of this HA E190K mutation in the large-plaque phenotype on NA-MDCK cells, we used reverse genetics to generate a mutant WSN virus with the HA E190K substitution, which was designated the WSN-E190K(HA) virus. We found that this virus formed large plagues on NA-MDCK cells, as did WSN-LPv (Fig. 3),



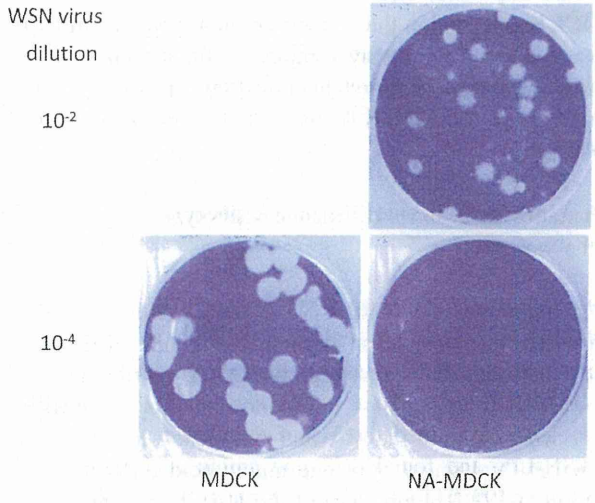


Fig. 1 Characterization of NA-expressing MDCK cells (NA-MDCK). **a** Immunostaining with an anti-NA antibody (α-NA Ab). A commercial ABC kit (Vector Laboratories) was used for detection. **b** FACS analysis of NA expression on the MDCK and NA-MDCK cell surface. An anti-mouse IgG FITC conjugate was used as the secondary antibody. **c** FACS analysis of the sialic acid content on the MDCK and NA-MDCK cell surface. MALII lectin, specific for SAα2,3Gal, and SNA lectin, specific for SAα2,6Gal, were used for the analysis

Fig. 2 Plaque morphology of the parent WSN virus on MDCK and NA-MDCK cells. Crystal violet staining was used to visualize plaques 2 days post-infection. All MDCK cells were detached from the well surface due to the cytopathic effect of the WSN virus at a dilution of 10^{-2} (not shown)

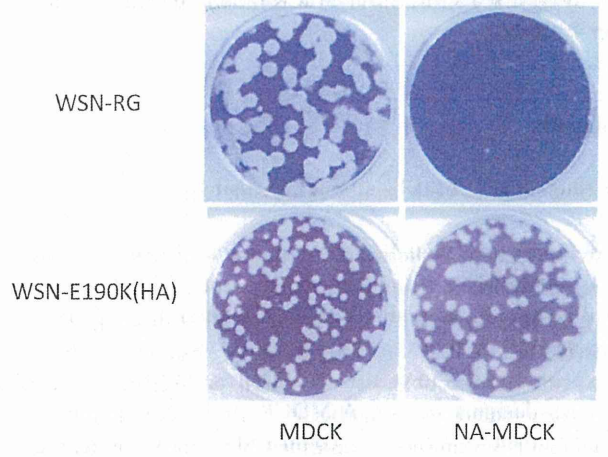


Fig. 3 Plaque morphology of parent WSN and mutant WSN-E190K(HA) viruses on MDCK and NA-MDCK cells. Both viruses were generated by using reverse genetics

demonstrating that the single mutation of E190K in HA is responsible for the large-plaque phenotype of WSN-LPv on NA-MDCK cells.

Growth properties of the HA mutant

To test the growth properties of the mutant in detail, we first compared the plaque phenotype of the reverse-

genetics-generated parent WSN (WSN-RG) virus with the plaque phenotypes of the mutant viruses (Fig. 3). We confirmed that the WSN-RG virus formed large plaques on MDCK cells and pinpoint plaques on NA-MDCK cells, whereas WSN E190K(HA) virus formed large plaques on NA-MDCK cells. Interestingly, this mutant also formed large plaques on MDCK cells, but their sizes were significantly smaller ($P < 0.05$, Student t-test) than those on NA-MDCK cells. In addition, the plaque sizes of the mutant on NA-MDCK cells were significantly smaller ($P < 0.05$) than those of WSN-RG virus on MDCK cells.

To determine whether the plaque phenotypes correlated with the growth efficiencies of these two viruses, we infected MDCK cells with the viruses at an identical MOI of 0.01 and compared the growth kinetics of the parent and mutant viruses. We found that the growth rate of the mutant was lower than that of the parent WSN (Fig. 4), indicating a positive correlation between plaque phenotype and growth on MDCK cells. On the other hand, although the mutant tended to grow better than the parent virus, the difference in growth rates between the mutant and parent viruses on NA-MDCK cells was not large. This finding is in contrast to that of the plaque sizes of the two viruses, suggesting that, unlike MDCK cells, there is no correlation between the plaque phenotype and growth rate on NA-MDCK cells. Heterogeneous receptor distribution on NA-MDCK cells may be responsible for this discrepancy.

Receptor specificity of the HA mutant

To determine whether the differences in growth properties observed between the parent and mutant viruses are responsible for changes in receptor specificity, we measured the receptor specificity of both viruses by means of a receptor assay using sialylglycopolymers. Interestingly, both viruses had the same receptor specificities; they both recognized both SAα2,3Gal and SAα2,6Gal (Fig. 5).

Since this receptor assay is not quantitative, we next used virus elution from erythrocytes as an assay to compare the receptor binding affinities of the two viruses. The elution assay provides a quantitative assessment of virus binding and release. When we used chicken erythrocytes, the mutant virus was not easily eluted, unlike the parent virus, which was easily eluted for up to 5 h after the temperature shift. By contrast, with guinea pig erythrocytes, we did not observe a marked difference in the elution kinetics between the mutant and parent viruses (Fig. 6). Previous studies [7, 12] as well as our own unpublished observations have shown that chicken and guinea pig erythrocytes contain both SAα2,3Gal and SAα2,6Gal, but quantitative differences between these sialic acids in these species have not been defined. Nonetheless, these data, together with those from the virus elution assay, strongly suggest that the mutant and parent viruses differ in their receptor-binding affinities.

Pathogenicity of the mutant virus in mice

Our *in vitro* assays demonstrated that the HA-NA functional balance of the mutant differs from that of the parent virus. We then asked whether this alteration affects virus pathogenicity in mice. We infected mice with various dilutions of virus and determined the median mouse lethal dose (MLD_{50}), and the results indicated that the mutant was attenuated in these animals ($10^{5.25}$ PFU for WSN-RG vs. $>10^{6.5}$ PFU for WSN-E190K(HA)). To confirm the attenuation phenotype of the mutant, we compared virus titers in organs following intranasal infection with the same amount of the two viruses. We observed no differences in the virus titers in the nasal turbinates but observed significant ($P < 0.05$) differences in the virus titers in the lungs (Fig. 7), indicating that the mutant virus was attenuated in mice and that its pathogenicity may be determined by its growth rate in the lung.

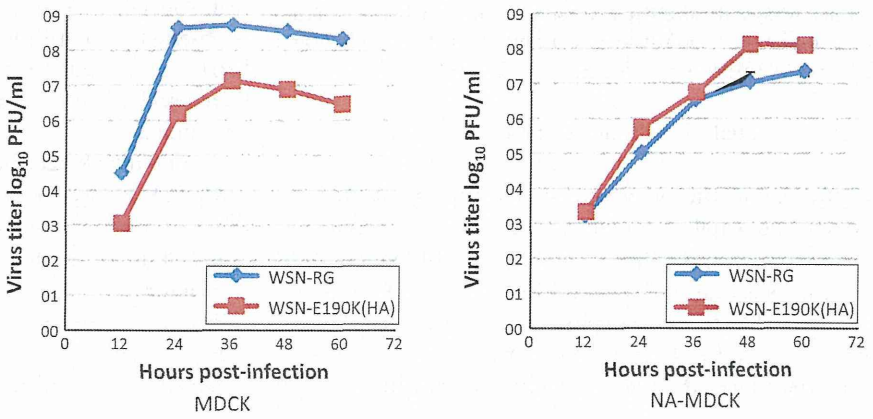


Fig. 4 Growth kinetics of parent WSN and mutant WSN-E190K(HA) viruses on MDCK and NA-MDCK cells. Following infection of cells with each virus at an MOI of 0.01, virus titers were determined by plaque titration with MDCK cells. Experiments were performed three times independently

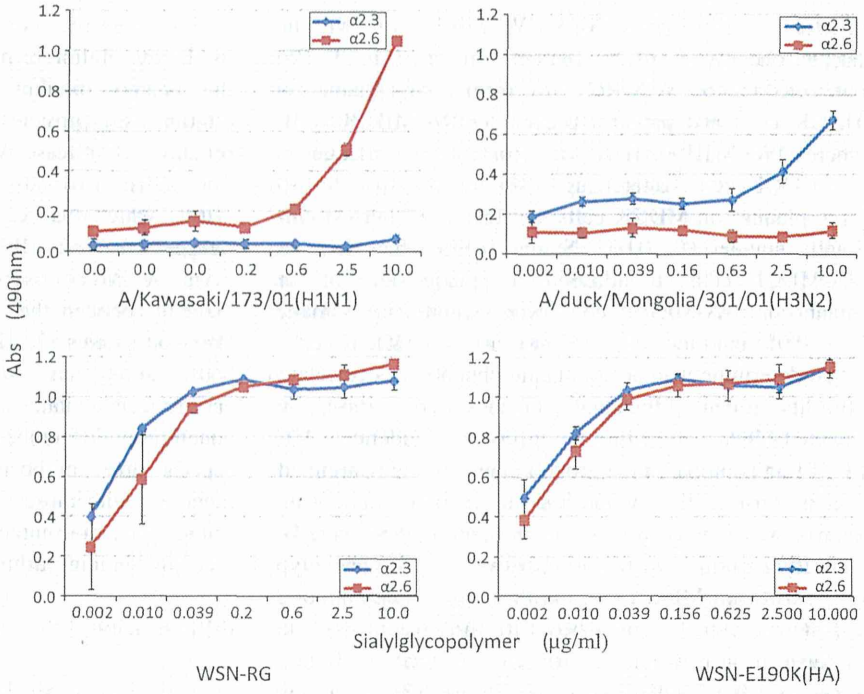


Fig. 5 Receptor binding assay of parent WSN and mutant WSN-E190K(HA) viruses. Each virus was purified through a 25 % sucrose cushion from the supernatant of the infected cell culture and used for the assay. A/Kawasaki/173/01(H1N1) and A/duck/Mongolia/301/01(H3N2) served as controls because they specifically recognize SAα2,6- and SAα2,3-linked receptors, respectively. Experiments were performed three times independently

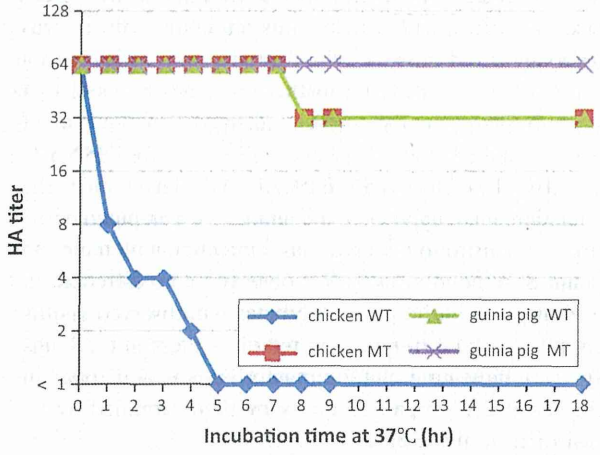


Fig. 6 Virus elution assay using chicken and guinea pig erythrocytes for parent WSN (WT) and mutant WSN-E190K(HA) (MT) virus, respectively. Virus preparations with identical HA titers (64) were incubated with erythrocytes at 4 °C for 1 h, and the plates were then shifted to 37 °C. The HA titers were recorded up to 18 h. Identical results were obtained in two independent experiments

Vaccine potential of the mutant virus

The attenuation phenotype of this mutant suggests that it may have potential as a live vaccine. To assess this possibility, we inoculated mice with a series of dilutions of the mutant virus and then challenged them with 100 MLD_{50} of wild-type WSN at 3 weeks post-immunization. The mice were observed for lethality for 2 weeks. None of the immunized mice died during this observation period, although the body weights of the mice immunized with low titers decrease following challenge with wild-type virus (Fig. 8a). When we attempted to isolate viruses from mouse organs at 3 days post-challenge, no virus was detected from mutant-immunized mice, unlike mock-immunized control mice (Fig. 8b). These data indicate that the mutant virus has potential as a live vaccine against the wild-type virus.

Discussion

In this study, we selected a plaque variant virus, WSN-LPv, derived from the laboratory H1N1 influenza virus, by using NA-expressing MDCK cells. We found that this variant was attenuated in mice when compared with the parent virus, due to an alteration in its HA-NA functional balance that resulted from a single mutation (E190K) in HA. This observation supports the notion that the HA-NA functional balance is responsible for efficient virus growth and can, therefore, be a determinant factor for virus pathogenicity in host animals.

To our knowledge, the HA E190K mutation observed in WSN-LPv has not been found in any naturally occurring H1N1 viruses, although it is well documented that the nature of the amino acid at position 190, in addition to that at position 225, of the HA receptor-binding pocket determines the receptor specificity of H1 viruses [11]. Indeed, Asp (D) at positions 190 and 225 (found in human viruses) confers binding to SAα2,6Gal oligosaccharides, whereas D

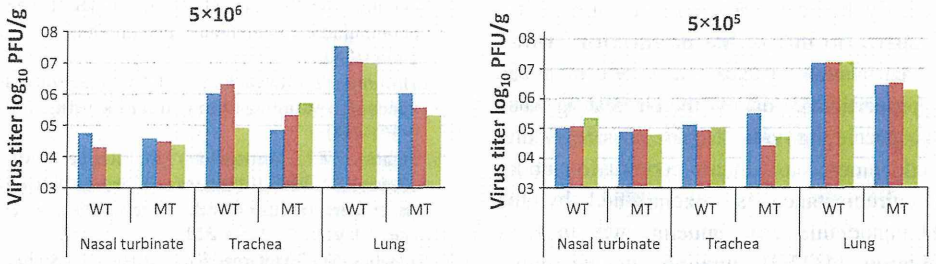


Fig. 7 Virus growth in the organs of mice infected with parent WSN (WT) and mutant WSN-E190K(HA) (MT) viruses. Mice were infected intranasally with two different concentrations of the viruses (5×10^6 or 5×10^5 PFU), and respiratory organs were collected at 3 days post-infection. Virus titers in each organ were determined by plaque titration. Each *bar* indicates the result from one infected mouse

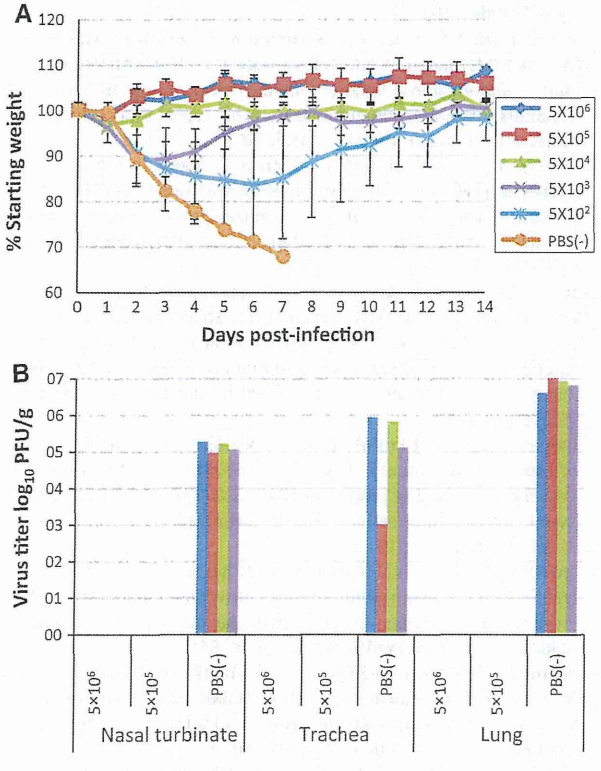


Fig. 8 Protective effects of mutant WSN-E190K(HA) virus against wild-type WSN virus challenge. **a** Mice were immunized intranasally with the indicated titers of mutant virus and were then infected with 100 MLD_{50} of wild-type WSN-RG at 3 weeks post-immunization. Body weights of mice (n = 4/group) were monitored for 14 days. **b** At 3 days post-challenge, four mice in each group were sacrificed for titration of virus present in their organs. Each *bar* indicates the result from one infected mouse. Mock-immunized mice (PBS) were also challenged and examined as controls

and Gly (G) at these positions (found in swine viruses) allow binding to both α2,6 and α2,3 linkages; Glu (E) and G at positions 190 and 225, respectively, (found in avian viruses) permit binding to α2,3-linked sialic acids. Interestingly, WSN-LPv possesses K and D at positions 190 and 225, respectively, whereas the parent WSN virus and even its original human isolate (WS virus; GenBank accession no. CY009604) possess E and D at these positions, which is an unusual combination of amino acids among natural H1N1 viruses. The WSN virus was obtained experimentally through successive passages of the WS virus in ferrets, mice and cell culture and has been maintained by further passages in various laboratories [2, 16]. We do not know the passage history of the WS virus that was used for the database assignment. Nonetheless, these findings support the notion that the amino acids at positions 190 and 225 are "hot spots" for HA mutation in H1N1 viruses that may determine organ tropism as well as host specificity of the virus by altering HA receptor-binding properties.

WSN-LPv with the HA E190K mutation was successfully selected by using our established NA-MDCK cells. Interestingly, SAα2,6Gal, but not SAα2,3Gal, levels were reduced on the surface of NA-MDCK cells when compared with MDCK parent cells. This observation stands in contrast to a previous report describing the preferential substrate specificity of H1N1 virus NAs for SAα2,3Gal over SAα2,6Gal [14]. We do not know the reason for this discrepancy because there is no information about the substrate specificity of WSN NA when expressed on the cell surface. One possible explanation is that a low level of NA expression on NA-MDCK cells may result in different substrate specificity from that measured with other assays in previous studies.

We demonstrated that the HA-NA functional balance of WSN-LPv was altered relative to that of the parent virus, although the receptor specificity of the viruses was not changed. The virus elution assay indicated slower elution from erythrocytes of the variant than of the parent viruses, indicating that the variant had a higher affinity for the receptor molecules. This observation may explain why the variant can replicate efficiently and form large plaques on NA-MDCK cells that have fewer receptor molecules with SAα2,6Gal.

Here, we selected an HA variant with altered receptor-binding activity by using NA-MDCK cells. This kind of

variant may not easily be selected in nature, due to its inferior growth relative to that of the parent virus. However, the idea that mutant viruses with heterologous receptor-binding properties are part of the circulating virus population as quasispecies provides for the possibility that such mutants could emerge as a major population under some biological circumstance, as exemplified by the isolation of 2009 pandemic virus mutants with an HA D225G (H1 numbering: D222G) mutation that are more pathogenic than the original pandemic strain and have enhanced binding to SAα2,3Gal avian-type receptors [1, 10]. Additionally, accidental infection of other animal hosts may lead to the emergence of HA receptor mutants with pandemic potential. Indeed, previous pandemics suggest that pigs are the likely intermediate host animal in which HA receptor mutants are generated [4].

Attenuation of the variant in mice suggests that the HA-NA functional balance of the parent virus was superior to that of the variant for efficient virus replication in lungs, but not in nasal turbinates of mice, revealing that the organ specificity of the virus could be affected by the altered HA-NA functional balance. This finding supports exploring this variant as a potential live vaccine virus because its replication is substantially hampered in the lower respiratory tract. However, attenuation of the variant through an HA single mutation may lead to considerable potential for reversion. Therefore, artificial HA mutations with enhanced receptor-binding affinity should be included in one of the genetic loci for the attenuation phenotype, in addition to cold adaptation with current live vaccines [9], which in turn could lead to the construction of safe live vaccines with high attenuation phenotypes and reduced potential for reversion.

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Conflict of interest The authors declare that they have no conflicts of interest

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ヘパリンによる侵入阻害作用に基づく抗原虫薬およびワクチン開発へのアプローチ

小林　郷介[1,2]、竹前　　等[1]、杉　　達紀[1]、龔　　海燕[1]、F. レクエンコ[1]
岩永　達也[1]、堀本　泰介[1]、明石　博臣[1]、加藤健太郎[1]
[1]東京大学大学院農学生命科学研究科獣医微生物学研究室
[2]東京大学医科学研究所付属幹細胞治療研究センター幹細胞プロセシング分野

Approach for the development of antiprotozoal agents and vaccines on the basis of invasion inhibitory effect of heparin

Kyousuke Kobayashi[1,2], Hitoshi Takemae[1], Tatsuki Sugi[1], Haiyan Gong[1], Recuenco C. Frances[1]
Tatsuya Iwanaga[1], Taisuke Horimoto[1], Hiroomi Akashi[1], Kentaro Kato[1]
[1]Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, University of Tokyo
[2]Division of Stem Cell Processing, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo

我々は、原虫独特の生活環を遮断する新たな薬剤やワクチンのターゲットとして「宿主細胞侵入」に注目して研究を行っている。中でもヒトに深刻な健康危害をもたらす熱帯熱マラリア原虫は、細胞侵入機構の研究において優れたモデルといえる。我々が着目する「ヘパリン」が、マラリア原虫に対して増殖阻害活性を示すことは過去にも報告があるが、阻害のメカニズムは明らかではない。そこで我々は、ヘパリンによる細胞侵入阻害機序の解明を試みた。

解析の結果、いくつかの侵入阻害モデルが浮かび上がってきた。ひとつは、侵入を媒介する複数のレセプター・リガンド結合が同時に阻害される可能性。あるいは、メロゾイト先端部がヘパリンで覆われることで赤血球表面との接近が物理的に障害される可能性である（図1）。

我々は、この結果を薬剤やワクチンの開発に応用することを目指したアプローチとして以下の二点を現在検討している。(1) ヘパリンが阻害活性を示す上で重要となる構造を特定することで、実用的な薬剤が開発できるか。(2) 分離したヘパリン結合タンパク質を有効なワクチン抗原として用いることができるか。

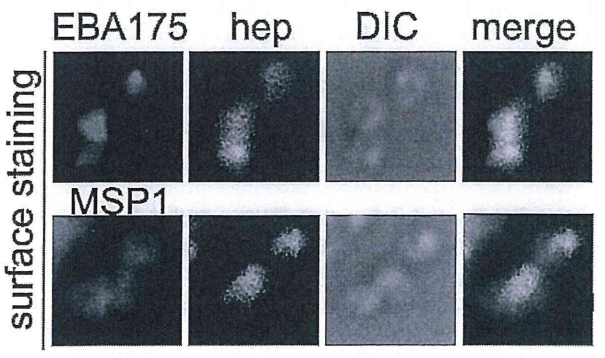

図1．メロゾイト表面におけるビオチン標識ヘパリンの結合部位

マラリア原虫以外にもヘパリンによる細胞侵入阻害を示す原虫が報告されているため、本研究で得られた知見は、抗原虫感染症戦略における一つのモデルとなることが期待できる。

Key words：malaria, merozoite, invasion, heparin

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連絡先責任者：加藤健太郎、東京大学大学院農学生命科学研究科獣医微生物学研究室、〒113-8657　東京都文京区弥生 1-1-1、E-mail：akkato@mail.ecc.u-tokyo.ac.jp
Correspondence：K. Kato, Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657