Supplementary Data

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Limitations of this study

In this study, we tested a model of IAV/IBV survival in the gastrointestinal tract, which would explain the detection of viral RNA in feces. However, the swallowed IAV/IBV model was established only in vitro. Further studies using experimental animals are required, and we are currently developing a mouse model for the evaluation of the mechanism(s) underlying IAV/IBV protection by viscous mucus.

Methods (detailed methodology)

Cells and viruses

Madin-Darby canine kidney cells (MDCK) were purchased from the Riken BioResource Center Cell Bank (Ibaragi, Japan) and cultured in minimal essential medium (MEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Equitech-Bio, Kerrville, TX, USA) and standard antibiotics.

IAV (clinical strains H3N2 and H1N1pdm) and IBV were isolated from MDCK cells inoculated with sputum from influenza-infected patients in 2012, 2009, and 2012, respectively. These viral strains were propagated in MDCK cells. To isolate the viruses, culture supernatants were pre-cleared by centrifugation at 3,300 × g for 20 minutes, followed by filtration through 0.45-μm filters. The viruses were then purified by centrifugation at 112,500 × g for 2.5 h in phosphate-buffered saline (PBS) containing 20% sucrose. Viral pellets were resuspended in PBS, and aliquots were stored as working stocks at −80°C. Viral titers were measured with
focus-forming assays in MDCK cells and expressed as the number of focus-forming units (FFU)/ml.

**RNA extraction and quantitative PCR**

Nucleic acid was extracted from samples using the PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), and cDNA was prepared using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) [1]. IAV was detected and quantified by quantitative PCR using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO). IAV-specific PCR was performed using an IAV matrix protein gene-specific primer set (M30F: 5ʹ-TTCTAACCAGGTCGAAACG-3' and M264R2: 5ʹ-ACAAAGCGTCTACGCTGCAG-3') designed by the National Institute of Infectious Diseases, Tokyo, Japan.

**Viscosity evaluation of sputum samples**

A prospective observational study was designed to evaluate the viscoelasticity of sputum samples from individuals with acute upper respiratory infection. The study protocol was reviewed and approved by the Institutional Review Board of the Kyoto Prefectural University of Medicine (ERB-C-634). Sputum samples were obtained from individuals diagnosed with acute upper respiratory infection at the Department of General Medicine of Kyoto Prefectural University of Medicine Hospital between August 2016 and November 2016. Minors (< 20 years old), influenza-infected individuals (diagnosed by the rapid antigen
detection test), patients with chronic respiratory illness, and patients taking expectorants were excluded. Informed consent was obtained at the time of examination, and 21 patients were ultimately included in the analysis. Viscosity was measured using a Brookfield viscometer.

**Artificial gastric acid, bile/pancreatic juice, and mucus**

Gastric acid, bile/pancreatic juice, and mucus (e.g., nasal discharge and sputum) were created artificially. Simulated gastric acid was created by adjusting pH to 2 or 3 with hydrochloric acid (Nacalai Tesque, Kyoto, Japan) and supplementation with 0.35% pepsin from porcine gastric mucosa (Wako, Osaka, Japan) and 0.2% NaCl (Nacalai Tesque) [2]. The simulated bile/pancreatic juice contained PBS, 0.2% ox bile (Wako), and 0.5% pancreatin from hog pancreas (Wako) [3, 4]. Artificial mucus was prepared by dissolving guar gum (Wako), or mucin from porcine stomach (Wako) in either saline or PBS [5]. To ensure that the viscosity of the artificial mucus prepared using different solutes was constant, the viscosity was measured using a Brookfield viscometer. The artificial mucus preparations were created to exhibit viscosities of 10, 50, 100, 500, 1000, 2000, and 4000 mPa·s at 37°C. Preparations containing guar gum had viscosities of 6000 mPa·s. Although there are no clear viscosity criteria for sputum, nasal discharge, or other types of mucus, previous studies have shown that the viscosity of human sputum ranges from 100 to 4000 mPa·s or higher [6]. In our study, the mean viscosity was estimated at 995.2 mPa·s (range, 134.4–2294.1 mPa·s). This
viscosity range approximately matched the range of the previous studies, and thus the artificial mucus (guar gum or mucin from the porcine stomach) was prepared to reproduce this viscosity range (10-4000 mPa⋅s).

Detection of viral RNA after incubation with simulated digestive juices

We evaluated the resistance of IAV RNA in artificial mucus preparations (or 21 sputum samples) to digestive juices. First, 450 µl of simulated gastric acid (pH 2), simulated bile/pancreatic juice, or PBS was added to 50 µl of virus (IAV) with artificial mucus or saline (final virus titer: $1.0 \times 10^6$ FFU/ml) and incubated at 37°C for 1, 2, or 4 h. RNA extraction and quantitative PCR were then performed. In addition, 450 µl of simulated bile/pancreatic juice or PBS was added to 50 µl of virus (IAV) with sputum samples (final virus titer: $1.0 \times 10^6$ FFU/ml) and incubated at 37°C for 4 h. RNA extraction and quantitative PCR were then performed.

The RNA copy ratio was defined as the ratio of the viral RNA copy number measured after incubation with the simulated gastric acid or bile/pancreatic juice to the copy number measured after incubation with PBS alone. Measurements were made using artificial mucus preparations of different viscosities or saline alone.

Detection of infectious virions after incubation with simulated digestive juices (inactivation test)

We evaluated the resistance of infectious IAV/IBV in artificial mucus preparations to digestive juices. First, 200 µl of simulated gastric acid (pH 2 or 3) or
PBS was added to 50 µl of virus (IAV or IBV) with artificial mucus or saline (final virus titer: 2.5 × 10^6 FFU/ml) and incubated at 37°C for 1 h prior to neutralization with 750 µl of PBS. The virus was then titrated. Next, 200 µl of simulated bile/pancreatic juice or PBS was added to 200 µl of virus (IAV or IBV) with artificial mucus or saline (final virus titer: 2.5 × 10^5 FFU/ml) and incubated at 37°C for 1, 2, or 4 h. PBS (2400 µl) was then added to the mixture, and the viral titer was determined.

The titer ratio was defined as the ratio of the titer measured after incubation with the simulated gastric acid or bile/pancreatic juice to the titer measured after incubation with PBS alone. Measurements were made using artificial mucus preparations of different viscosities or saline alone. The titer ratio reflects the proportion of virus that was not inactivated by the simulated gastric acid or bile/pancreatic juices. A titer ratio closer to 1 indicates that the virus is more resistant to gastric acid or bile/pancreatic juice. Three independent experiments were performed, and the results are expressed as the mean and standard deviation.

**Inactivation test under a low- and high-titer condition.**

The inactivation test was also performed under a low- and high-titer condition. In addition, we clarified the relationship between infectious IAV titer and RNA copy number in the inactivation test. In brief, 200 µl of simulated digestive juices (simulated gastric acid or bile/pancreatic juice) or PBS was added to 50 µl of
IAV with artificial mucus or saline [final virus titer, high-titer condition: $1.0 \times 10^6$ FFU/ml; final virus titer, low-titer condition: $1.0 \times 10^4$ FFU/ml] and incubated at 37°C for 1 h prior to neutralization with 750 µl of PBS. Then, viral titer and RNA copy number were respectively determined.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 7 (GraphPad software, La Jolla, CA, USA). Categorical variables were examined using Student's t-test. All reported $P$-values were two-sided and values < 0.05 were considered significant.
References


Supplementary Fig S1. Inactivation test using simulated digestive juices under a low- and high-titer condition.

Simulated gastric acid (pH 2), simulated bile/pancreatic juice (P+B), or PBS (200 µl each) was added to 50 µl of IAV (H3N2) with artificial mucus [guar gum (G) or mucin from the porcine stomach (M)] or saline and incubated at 37°C for 1 h prior to neutralization with 750 µl of PBS [final virus titer, high-titer condition: $1.0 \times 10^6$ FFU/ml; final virus titer, low-titer condition: $1.0 \times 10^4$ FFU/ml]. The titer ratio was calculated as the ratio of the virus titer in simulated digestive juices to that in PBS. UD, undetectable. Data are expressed as the means ± S.D. based on at least three independent experiments.
Supplementary Fig S2. Relationship between infectious IAV titer and RNA copy number in the inactivation test.

Simulated gastric acid (A, C) or simulated bile/pancreatic juice (B, D) (200 µl each) was added to 50 µl of IAV (H3N2) with artificial mucus [guar gum (G) or mucin from the porcine stomach (M)] or saline and incubated at 37°C for 1 h prior to neutralization with 750 µl of PBS [final virus titer: $1.0 \times 10^6$ FFU/ml (A, B) or $1.0 \times 10^4$ FFU/ml (C, D)], and the viral titer and RNA copy number were determined. UD, undetectable. Data are expressed as the means ± S.D. based on at least three independent experiments.