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ORIGINAL ARTICLE

Is the optimal pH for membrane fusion in host cells by avian influenza viruses related to host range and pathogenicity?

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Abstract Influenza viruses isolated from wild ducks do not replicate in chickens. This fact is not explained solely by the receptor specificity of the hemagglutinin (HA) from such viruses for target host cells. To investigate this restriction in host range, the fusion activities of HA molecules from duck and chicken influenza viruses were examined. Influenza viruses A/duck/Mongolia/54/2001 (H5N2) (Dk/MNG) and A/chicken/Ibaraki/1/2005 (H5N2) (Ck/IBR), which replicate only in their primary hosts, were used. The optimal pH for membrane fusion of Ck/IBR was 5.9, higher than that of Dk/MNG at 4.9. To assess the relationship between the optimal pH for fusion and the host range of avian influenza viruses, the optimal pH for fusion of 55 influenza virus strains isolated from ducks and chickens was examined. No correlation was found between the host range and optimal pH for membrane fusion by the viruses, and this finding applied also to the H5N1 highly pathogenic avian influenza viruses. The optimal pH for membrane fusion for avian influenza viruses was shown to not necessarily be correlated with their host range or pathogenicity in ducks and chickens.

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Introduction

It is well known that interspecies transmission of influenza A viruses is infrequent in nature, and such viruses have been isolated from birds and mammals, including humans [29]. Viral and host factors affect the transmissibility and pathogenicity of influenza A viruses. One key factor affecting the receptor specificity of influenza A viruses is the viral hemagglutinin (HA). HA, a surface glycoprotein of influenza A viruses, plays functionally important roles at the early stage of infection. During viral attachment to a host cell, HA binds to sialoside receptors on the host cells. Because the structure of the sialic acid receptor on host target cells differs among host species, the host range of influenza A viruses depends primarily on the receptor specificity of HA [8, 20, 23, 26]. For example, avian influenza viruses bind preferentially to α2,3 sialic acid receptors, while human influenza viruses bind to α2,6 sialic acid receptors [13, 20]. In addition to receptor specificity, it has been reported that an optimal pH is needed for fusion of the viral envelope with the cellular membrane of the host and that this factor determines the host range of a virus [1–4, 12, 14, 16]. During the step where the virus becomes internalized in the host cell by endocytosis, influenza A viruses are exposed to low pH [11]. At an optimal pH, the flexibility of HA increases, and fusion of the viral envelope with a host cell membrane occurs [24, 31]. The optimal pH for fusion, which differs among influenza A virus strains, was recently proposed as a potential virulence factor [19].

Migratory water birds are the natural hosts of influenza A viruses [9, 29], but the molecular basis of interspecies transmission of duck influenza virus to chickens has not been completely clarified. An optimal pH for membrane fusion has been reported to contribute to the pathogenicity and transmissibility of some H5N1 highly pathogenic avian

influenza viruses (HPAIVs) isolated from chickens or ducks, thereby leading to the hypothesis that the optimal pH range for membrane fusion differs between avian influenza viruses isolated from ducks and chickens [3].

In the present study, we sought to determine whether a correlation between optimal pH and host range is generally observed by assessing the optimal pH range for membrane fusion of low pathogenic avian influenza viruses (LPAIVs) and H5N1 HPAIVs isolated from ducks and chickens.

Materials and methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were grown in minimum essential medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10 % fetal bovine serum (FBS; SAFC Biosciences, Street Lenexa, KS, USA) and antibiotics. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, CA, USA) supplemented with 10 % FBS (Cambrex, East Rutherford, NJ, USA) and antibiotics. Both cell lines were maintained at 37 °C in a 5 % CO_2 atmosphere. All viruses used in the present study were purified by plaque cloning and propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35 °C for 30–48 h. Before the infectious allantoic fluids were harvested, the eggs were chilled at 4 °C overnight, and the harvested allantoic fluids were stored at −80 °C.

Viral growth and pathogenicity of influenza viruses in chickens

Chickens (*Gallus gallus*, Boris Brown) were obtained from Hokkaido Chuo Shukeijo Corp., Hokkaido, Japan. To confirm that viral replication had occurred in the chickens, one $10^{5.0}$ plaque-forming unit (PFU) of virus was inoculated intranasally into three 4-week-old chickens. On day 3 post infection, oral and cloacal swabs were collected from the chickens, and the viral titers of the swabs were calculated by a plaque assay. For HPAIVs, four 4-week-old chickens inoculated intranasally with $10^{6.0}$ of a 50 % egg infective dose (EID_{50}) of viruses were monitored for their survival for 14 days.

Hemolysis assay

The hemolysis assay was performed as described previously [10, 28]. Virus concentrates, added to 1 ml of 1.0 % chicken red blood cells (cRBCs) in saline buffered with 0.1 M citric acid-sodium citrate at various pH values (4.7–7.0), each at a final concentration of 200 HA units, were incubated on ice for 1 h. After incubation at 37 °C for 1 h with mixing every 10 min, the cells were sedimented by centrifugation, and the optical density of the supernatants was measured for hemoglobin at 540 nm. The hemolysis status was evaluated based on the ratio of the O.D. 540 nm value of the sample to that of positive control (1 % Triton X-100 in PBS) (S/P ratio). The optimal pH for hemolysis was determined as the pH value at which highest S/P ratio was observed. A Student's t-test was applied to the data to determine whether any differences were statistically significant.

Preparation and culture of primary chicken kidney (CK) cells

Primary CK cells were prepared and cultured according to a previous report [27] with some modifications. Chicken kidneys were obtained from 4- to 6-week-old chickens. After removing the surrounding adipose and muscular tissues, the kidneys were homogenized and washed with 0.1 M citric acid buffer for 5 min with stirring. The pellets were digested with citric acid buffer containing 100 mg of trypsin (Sigma-Aldrich, St. Louis, MO, USA) per ml for 5 min at room temperature. After centrifugation at 3,000 rpm for 5 min, the cell pellets were resuspended in MEM containing 5 % FBS and incubated at 37 °C in a 5 % CO_2 atmosphere.

Reverse genetics

Influenza viruses A/duck/Mongolia/54/2001 (H5N2) (Dk/MNG), A/chicken/Ibaraki/1/2005 (H5N2) (Ck/IBR), and their reassortants were generated by reverse genetics (rg) as described previously [6] and hereafter are designated rgDk/MNG, rgCk/IBR, rgMNG/IBR-HA (the HA gene from Ck/IBR and seven genes from Dk/MNG), rgMNG/IBR-M (the M gene from Ck/IBR and seven genes from Dk/MNG), rgIBR/MNG-HA (the HA gene from Dk/MNG and seven genes from Ck/IBR), and rgIBR/MNG-M (the M gene from Dk/MNG and seven genes from Ck/IBR).

Viral growth in MDCK and CK cells

Viruses were inoculated onto MDCK or CK cell monolayers at a multiplicity of infection of 0.01. After a 1-h incubation at 35 °C, unbound viruses were washed away with PBS, and MEM containing 5 μg of acetyl trypsin per ml was added. Cells were incubated at 35 °C, and the supernatants were collected at 0, 6, 12, 24, and 48 h post infection. The supernatants were inoculated onto confluent monolayers of MDCK cells and the virus titers, calculated by the method of Reed and Muench, were

expressed as the 50 % tissue culture infective dose ($TCID_{50}$)/ml [18].

Comparison of endosomal pH

Comparison of endosomal pH between MDCK and CK cells was performed as described by Murakami et al. [14]. MDCK and CK cells grown in 12-mm glass-bottom dishes were incubated with Oregon green 488 (250 μg/ml; Life Technologies) and Alexa Fluor 647 (30 μg/ml; Life Technologies) conjugated dextran, which were endocytosed to the cells for 15 min at 37 °C. After incubation, the cells were immediately placed on ice and washed five times with ice-cold PBS and the intensities of the Oregon green 488 and Alexa Fluor 647 were measured using confocal microscopy (LSM 510; Carl Zeiss, Germany) with 10 microscopic fields for each sample. The intensity ratio between Alexa Fluor 647 and Oregon green 488 was calculated using Zen software (Carl Zeiss).

Results

Comparison of virus replication in chickens and optimal pH for membrane fusion

To examine the relationship between host specificity and optimal pH for membrane fusion of LPAIVs, viruses were first inoculated into chickens to confirm viral replication in chickens, and the optimal pH for hemolysis of cRBCs was then determined (Table 1). Viruses were recovered from the tracheal and/or cloacal swabs of chickens inoculated with viruses isolated from chickens, namely, Ck/IBR, Ck/Tainan/V156/1999 (H6N2) and Ck/Yokohama/aq55/2001 (H9N2). In contrast, no viruses were recovered from swabs from chickens inoculated with viruses isolated from ducks, namely, Dk/MNG, Dk/Pennsylvania/10218/1984 (H5N2) and Dk/Hokkaido/960/1980 (H6N2). The optimal pH values for hemolysis induced by the viruses from chickens were compared with those of ducks. Although the optimal pH for membrane fusion of chicken viruses was 5.4-5.9, and therefore higher than that of duck viruses, at 4.9-5.5, no significant difference in the pH optimum for chicken and duck viruses was found ($P > 0.05$).

Optimal pH for hemolysis of cRBCs by viruses

To assess the relationship between host range and optimal pH for viral fusion, a hemolysis assay was performed using 50 LPAIV strains isolated from ducks or chickens. The optimal pH for hemolysis in the viruses isolated from ducks or chickens varied from 4.9 to 5.8 (Table 2). Consequently, no correlation was found between the host species from which the influenza viruses were isolated and the optimal pH for fusion. In addition, the optimal pH for fusion of five H5N1 HPAIVs that showed different degrees of pathogenicity in ducks [7, 21], was also assessed. The pH for hemolysis in the five HPAIVs ranged from 5.6 to 5.8 (Table 3). Thus, the pathogenicity of HPAIVs in ducks is not correlated with the optimal pH for fusion.

Growth of rgDk/MNG, rgCk/IBR and reassortant viruses in MDCK and CK cells and comparison of endosomal pH in these cells

To assess potential differences between chicken and duck viruses, Dk/MNG and Ck/IBR were selected as representative strains; these strains showed the lowest (4.9), and the highest (5.9) optimal pH values for membrane fusion, respectively (Table 1). To identify viral factors related to optimal pH for membrane fusion, rgDk/MNG, rgCk/IBR and reassortant viruses were prepared by reverse genetics. The optimal pH for hemolysis of cRBCs induced by rgDk/MNG was 4.8, whereas that for rgCk/IBR was 5.8 (Table 4). To assess whether a relationship exists between the growth potential of each virus (rgDk/MNG and rgCk/

Table 1 Virus replication of LPAIVs in chickens and their optimal pH for hemolysis of cRBCs

Host	Virus	Replication in chickens[a)]	Optimal pH for hemolysis[b)]	Average ± SE
Chicken	Ck/IBR/1/2005 (H5N2)	3/3	5.8, 5.9, 5.9	5.67 ± 0.12
	Ck/Tainan/V156/1999 (H6N2)	3/3	5.6, 5.6, 5.7	
	Ck/Yokohama/aq55/2001 (H9N2)	3/3	5.4, 5.5, 5.5	
Duck	Dk/MNG/54/2001 (H5N2)	0/3	4.9, 4.9, 5.0	5.25 ± 0.17
	Dk/Pennsylvania/10218/1984 (H5N2)	0/3	5.4, 5.4, 5.4	
	Dk/Hokkaido/960/1980 (H6N2)	0/3	5.4, 5.4, 5.5	

Abbreviations: Ck, chicken; Dk, duck

a) Virus at $10^{5.0}$ PFU/chicken was administered intranasally

b) The data are from three independent experiments

Table 2 Optimal pH for hemolysis of cRBCs induced by viruses

Host	Subtype	Strain	pH for hemolysis[a)]	Average ± SE
Chicken	H3	Ck/Hong Kong/37/1978 (H3N2)	5.2, 5.2, 5.2	5.26 ± 0.05
		Ck/Hong Kong/526/1996 (H3N6)	5.3, 5.3, 5.3	
	H4	Ck/Alabama/1/1975 (H4N8)	5.8, 5.8, 5.8	
	H6	Ck/Chung-Hsing/4730/1990 (H6N5)	5.4, 5.4, 5.5	
		Ck/Lin-Ney/4501/1990 (H6N5)	5.5, 5.5, 5.6	
		Ck/Taiwan/4801/1990 (H6N5)	5.3, 5.4, 5.5	
	H9	Ck/Beijing/1/1995 (H9N2)	5.2, 5.2, 5.3	
		Ck/Beijing/2/1997 (H9N2)	5.5, 5.5, 5.6	
		Ck/Beijing/3/1999 (H9N2)	5.2, 5.2, 5.3	
		Ck/Guangdong/11/1997 (H9N2)	5.3, 5.4, 5.4	
		Ck/Sichuan/5/1997 (H9N2)	5.3, 5.3, 5.3	
		Ck/Hebei/1/1996 (H9N2)	4.8, 4.9, 5.1	
		Ck/Hebei/2/1998 (H9N2)	5.1, 5.2, 5.2	
		Ck/Henan/1998 (H9N2)	5.2, 5.2, 5.2	
		Ck/Hong Kong/G9/1997 (H9N2)	4.9, 5.0, 5.0	
		Ck/Hong Kong/G24/1998 (H9N2)	5.6, 5.7, 5.7	
		Ck/Hong Kong/FY20/1999 (H9N2)	4.8, 4.9, 5.1	
		Ck/Pakistan/2/1999 (H9N2)	5.4, 5.5, 5.5	
		Ck/Shijiazhuang/2/1999 (H9N2)	5.3, 5.3, 5.3	
		Ck/Liaoning/1999 (H9N2)	4.9, 4.9, 5.0	
		Ck/Vietnam/OIE-0056/2012 (H9N2)	5.3, 5.3, 5.4	
	H10	Ck/Germany/N/1956 (H10N7)	5.1, 5.2, 5.2	
Duck	H1	Dk/Miyagi/66/1997 (H1N1)	5.3, 5.3, 5.4	5.36 ± 0.04
		Dk/Mongolia/675/2010 (H1N1)	5.3, 5.3, 5.3	
	H2	Dk/Hokkaido/17/2001 (H2N3)	5.2, 5.2, 5.3	
	H3	Dk/Ukraine/1/1963 (H3N8)	5.7, 5.7, 5.8	
		Dk/Hokkaido/221/2008 (H3N6)	5.2, 5.2, 5.2	
		Dk/Hokkaido/5/1977 (H3N2)	5.2, 5.2, 5.3	
		Dk/Hokkaido/46/2010 (H3N8)	5.2, 5.2, 5.2	
	H4	Dk/Hokkaido/379/2000 (H4N6)	5.3, 5.3, 5.3	
		Dk/Mongolia/17/2011 (H4N3)	5.8, 5.8, 5.8	
	H5	Dk/Hokkaido/101/2010 (H5N2)	5.4, 5.5, 5.5	
	H6	Dk/Hokkaido/311/2009 (H6N1)	5.5, 5.5, 5.5	
		Dk/Hokkaido/262/2004 (H6N1)	5.1, 5.2, 5.2	
	H7	Dk/Hokkaido/1/2010 (H7N7)	5.4, 5.4, 5.5	
		Dk/Hokkaido/10/2010 (H7N7)	5.4, 5.4, 5.4	
	H8	Dk/Hokkaido/W285/2007 (H8N4)	5.3, 5.4, 5.4	
		Dk/Alaska/702/1991 (H8N7)	5.4, 5.5, 5.5	
	H9	Dk/Vietnam/OIE-0120/2012 (H9N2)	5.2, 5.2, 5.2	
		Dk/Hokkaido/W213/1998 (H9N2)	5.6, 5.6, 5.6	
	H10	Dk/Hokkaido/454/2000 (H10N4)	5.4, 5.5, 5.5	
		Dk/Hokkaido/131/2008 (H10N7)	5.4, 5.4, 5.5	
		Dk/Vietnam/OIE-0483/2012 (H10N7)	5.2, 5.2, 5.5	
	H11	Dk/Vietnam/OIE-0068/2012 (H11N3)	5.2, 5.3, 5.3	
		Dk/Hokkaido/302/2009 (H11N9)	4.9, 4.9, 4.9	
		Dk/England/1/1956 (H11N6)	5.8, 5.8, 5.9	
	H12	Dk/Alberta/60/1976 (H12N5)	5.0, 5.2, 5.5	
	H13	Dk/Siberia/272PF/1998 (H13N6)	5.0, 5.0, 5.0	
	H14	Mal/Astrakhan/263/1982 (H14N5)	5.3, 5.3, 5.3	

Table 2 continued

Host	Subtype	Strain	pH for hemolysis[a)]	Average ± SE
	H15	Dk/Australia/341/1983 (H15N8)	5.8, 5.8, 5.9	

Abbreviations: Ck, chicken; Dk, duck; Mal, mallard

a) The data are reported for three 3 independent experiments

Table 3 Optimal pH for hemolysis of cRBCs and pathogenicity of H5N1 HPAIVs

Virus	Optimal pH for hemolysis[a)]	Survival rate of inoculated chickens	Survival rate of inoculated ducks
Ws/Hokkaido/4/2011	5.7, 5.8, 5.8	0/4	4/4[b)]
Ws/Mongolia/3/2005	5.6, 5.6, 5.6	0/4	2/3[c)]
Dk/Vietnam/OIE-559/2011	5.6, 5.6, 5.6	0/4	3/4[b)]
Pf/Hong Kong/810/2009	5.6, 5.6, 5.7	0/4	3/4[b)]
Ws/Mongolia/6/2009	5.5, 5.6, 5.6	0/4	0/3[c)]

Abbreviations: Ws, whooper swan; Dk, duck; Pf, peregrine falcon

a) The data are reported for three independent experiments

b) Hiono et al., 2016 [7]

c) Sakoda et al., 2010 [21]

Table 4 Optimal pH for hemolysis of cRBCs induced by reassortant viruses

Virus	Optimal pH for hemolysis[a)]
rgCK/IBR	5.8, 5.8, 5.8
rgIBR/MNG-HA	4.9, 4.9, 4.9
rgIBR/MNG-M	4.8, 4.8, 4.9
rgDk/MNG	4.8, 4.8, 4.8
rgMNG/IBR-HA	5.8, 5.8, 5.9
rgMNG/IBR-M	5.7, 5.8, 5.8

a) The data are reported for three independent experiments

IBR) and the endosomal pH of the cells, the growth kinetics of the viruses in CK cells were compared with those in MDCK cells. rgDk/MNG grew better than rgCk/IBR in MDCK cells, whereas rgCk/IBR did better than rgDk/MNG in CK cells (Fig. 1). To assist with identification of the genes contributing to the replication difference of the two viruses in MDCK and CK cells, the growth kinetics of rgMNG/IBR-HA, rgMNG/IBR-M, rgIBR/MNG-HA, and rgIBR/MNG-M in MDCK and CK cells were analyzed (Fig. 1). In MDCK cells, no significant difference in growth was found between rgDk/MNG and the reassortant viruses or between rgCk/IBR and the reassortant viruses (Fig. 1). In contrast, in CK cells, rgMNG/IBR-HA and rgMNG/IBR-M grew significantly better than rgDk/MNG. In addition, rgMNG/IBR-M grew better and faster than rgMNG/IBR-HA. Moreover, rgIBR/MNG-HA and rgIBR/MNG-M showed significantly poorer growth in comparison to rgCk/IBR in CK cells. The optimal pH values for hemolysis of these reassortants also showed that the growth of the virus in CK cells is correlated to a high optimal pH value for hemolysis, 5.7-5.9 (Table 1). These results indicate that the HA and M1/M2 genes are responsible for the growth potential of the viruses in CK cells.

The endosomal pH of MDCK and CK cells was compared by introducing a dextran-conjugated fluorescent dye as a marker and measuring the intracellular intensity [14]. After a 15-min incubation, the Oregon green 488/Alexa Fluor 647 intensity ratio was higher in CK cells than in MDCK cells (Fig. 2), suggesting that the endosomal pH value at this point was higher in CK cells than in MDCK cells.

Discussion

It has been hypothesized that the host range of avian influenza viruses is correlated with the optimal pH for membrane fusion, because the cells targeted for infection differ between ducks and chickens. Duck influenza viruses replicate preferentially in the intestinal tract of ducks while chicken influenza viruses replicate in the respiratory tract of chickens [6]. It was also reported that the pathogenicity of H5N1 HPAIVs in ducks and chickens was correlated

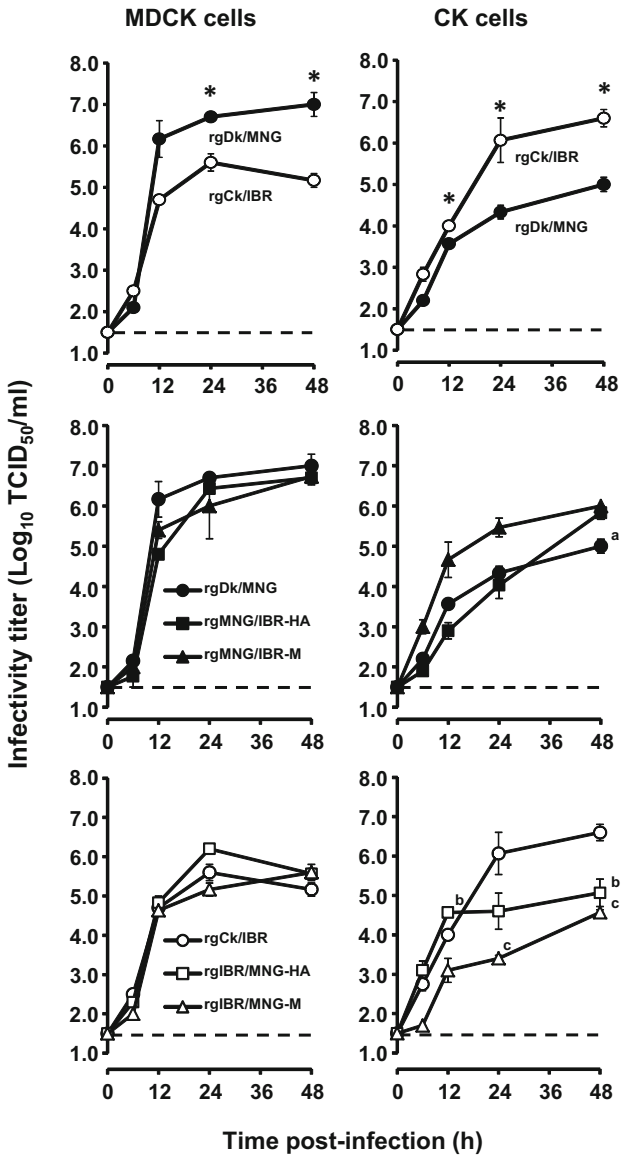

Fig. 1 Growth curves for rgDk/MNG, rgCk/IBR and their reassortant viruses in MDCK and CK cells. The viral titers were determined using the tissue culture infective dose ($TCID_{50}$) method at the indicated time points postinfection at a multiplicity of infection of 0.01 in MDCK (left) and CK (right) cells. Dashed lines indicate where the virus titer was lower than the detectable dose ($10^{1.5}$ $TCID_{50}$/ml). The data are reported as the mean titers for three independent experiments. *, $P < 0.05$ between rgDk/MNG and rgCk/IBR; a, $P < 0.05$ between rgDk/MNG and rgMNG/IBR-HA or rgMNG/IBR-M; b, $P < 0.05$ between rgCk/IBR and rgIBR/MNG-HA; c, $P < 0.05$ between rgCk/IBR and rgIBR/MNG-M

with an optimal pH for fusion: H5N1 viruses exhibiting lower fusion pH values were more virulent in ducks [3, 19]. Two H5N2 LPAIVs, Dk/MNG and Ck/IBR, grew only in their primary hosts and showed different optimal pH values for fusion: the optimal pH of Dk/MNG was 4.9, a lower value than that of Ck/IBR at 5.9. These findings support the view proposed in previous reports [3, 19].

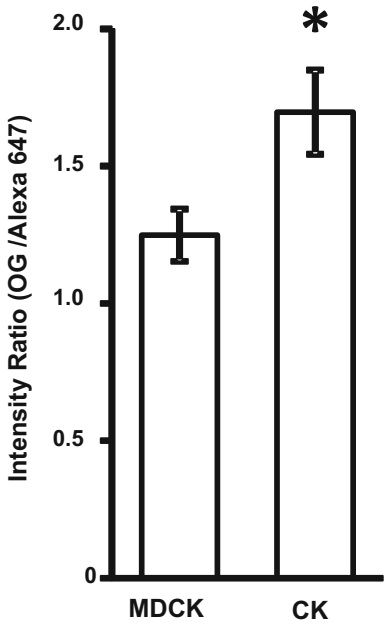

Fig. 2 Comparison of endosomal pH values in MDCK and CK cells. Oregon green 488 (OG)-conjugated dextran (250 μg/ml) and Alexa Fluor 647 (Alexa 647)-conjugated dextran (30 μg/ml) were internalized. After 15 min, the fluorescence intensity of each sample was measured and calculated as an OG/Alexa 647 ratio. The data are reported as the mean values with standard deviations obtained for 10 microscopic fields for each cell culture. *, $P < 0.05$ compared with the results for the MDCK cells

To assess the relationship between viral growth in ducks and chickens and the optimal pH for fusion, growth of the viruses in the two cell lines was evaluated. After a 15-min dextran intake period, the intensity ratio between Alexa Fluor 647 and Oregon green 488 in CK cells was higher than that in MDCK cells, indicating that the endosomal pH of CK cells was higher than that of MDCK cells. rgDk/MNG grew better than rgCk/IBR in MDCK cells; however, rgDk/MNG grew more poorly than rgCk/IBR in CK cells. In MDCK cells, influenza virus reaches the late endosome (pH 5.0) 10 min after endocytosis [11], which is much faster than in other cell lines. It has been speculated that, in MDCK cells, there is no time lag between receptor binding and membrane fusion, and thus, viral growth could not be related to the optimal pH for fusion. On the other hand, acidification of endosomes in CK cells takes longer than in MDCK cells, with the result that viral growth was affected by the optimal pH. Similarly, it has been reported that vesicular stomatitis virus shows higher growth in Vero cells than in MDCK cells because of its high optimal pH for fusion (pH 6.0) and the higher endosomal pH of Vero cells [14]. In this study, we found that viral growth in CK cells depended on HA, which mediates membrane fusion (Fig. 1). Additionally, viruses carrying the M gene from rgCk/IBR showed more efficient growth than those

carrying the M gene from rgDk/MNG in CK cells, indicating that M1, M2, or both are also responsible for the difference in viral growth in CK cells. M1 forms a shell under the membrane envelope that stabilizes the architecture of the virion [17]. M2 functions as an ion channel, transferring protons to the virus in the endosome [22, 25]. Before HA-mediated fusion, the M2 ion channel is activated by low endosomal pH to allow proton influx from the acidic endosome into the virion, and the acidified virion induces dissociation of M1 from the viral nucleoproteins and other structural components [5, 16]. Therefore, the stability of M1 and the activity of the M2 ion channel are closely related to endosomal pH, which may lead to the difference in viral growth we observed in the CK cells. Based on the present observations, together with previous findings [14, 15, 30], the difference in endosomal pH affected viral growth in MDCK and CK cells, and was related to the optimal fusion pH and the function of M1 and/or M2 of rgDk/MNG and rgCk/IBR. Furthermore, we suggest that the difference observed in viral replication in ducks and chickens between these viruses (Dk/MNG and Ck/IBR) may also be affected by the optimal pH for fusion and the function of M1 and/or M2.

In the present study, a correlation between the host range and the optimal pH for fusion of Dk/MNG and Ck/IBR was observed; however, no correlation was observed among the 50 LPAIV isolates from ducks and chickens. Since field isolates are a mixed population, we performed plaque purification of viruses in cell culture to assess the optimal pH for fusion of a single population. It is possible that these viruses have mutations compared with the virus replicating in the host tissues not only due to plaque cloning but also due to virus isolation step using eggs. Further study is necessary to analyze virus populations and their tissue tropism in the host, including the optimal pH for fusion. Based on a comparison of the optimal pH for hemolysis induced by the two HPAIVs, namely, Ws/Mongolia/6/2009 (H5N1), which is highly pathogenic in ducks, and Ws/Hokkaido/4/2011 (H5N1), there are indications that HPAIVs with lower fusion pH are highly pathogenic in ducks (Table 3). However, the optimal pH for hemolysis induced by the other three HPAIVs, which all showed mild pathogenicity to ducks, was the same as for Ws/Mongolia/6/2009 (H5N1) (Table 3). Thus, for membrane fusion, a correlation between host range and optimal pH was found only with certain strains.

Host range and pathogenicity in avian influenza viruses are modulated by a combination of viral and host factors. As the optimal pH for membrane fusion was assumed in previous reports to be a factor in determining the pathogenicity of H5N1 influenza viruses [3, 19], it was found in the present study that the optimal pH for fusion of rgDk/MNG and rgCk/IBR reflected their host range and that HA and M1 and/or M2 were responsible for their susceptibility to endosomal pH. Nonetheless, we also revealed that not only the optimal pH for fusion but also a combination of other factors affects the host range. The present study shows that the optimal pH for membrane fusion is not sufficient to determine the host range and pathogenicity of a virus. The optimal pH for fusion has much less influence on the host range of viruses than other factors such as the insertion of a polybasic amino acid sequence at the cleavage site, receptor-binding specificity, and polymerase activity. Further studies should help to reveal the factors involved in determining the host range and pathogenicities of avian influenza viruses.

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